Characterisation of bovine testicular hyaluronidase and a hyaluronate lyase from *Streptococcus agalactiae*

Investigations on the effect of pH on hyaluronan degradation and preclinical studies on the adjuvant administration of the enzymes in cancer chemotherapy

Dissertation

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Julia Hoechstetter
aus Pappenheim

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Prüfungsausschuß:	Prof. Dr. W. Wiegrebe	(Vorsitzender)
	Prof. Dr. A. Buschauer	(Erstgutachter)
	PD Dr. G. Bernhardt	(Zweitgutachter)
	Prof. Dr. A. Göpferich	(Prüfer)

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Contents

1 Introduction

1.1 Hyaluronic acid

1.1.1 Structure and physicochemical properties

Hyaluronic acid (hyaluronan, HA) is a high-molecular mass polysaccharide found in the extracellular matrix, especially of soft connective tissues. In 1934 it was first isolated from the vitreous humor of bovine eyes by Meyer and Palmer (1934). They showed that the substance contained uronic acid and hexosamine, and thus, coined the term hyaluronic acid from hyaloid (vitreous) + uronic acid. About 20 years later the precise chemical structure of HA was solved (Weissmann and Meyer, 1954). By chemical and enzymatic methods Meyer and co-workers could establish that hyaluronan is a linear polymer built from repeating disaccharide units with the structure …[D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)]$_n$... (Fig. 1-1).

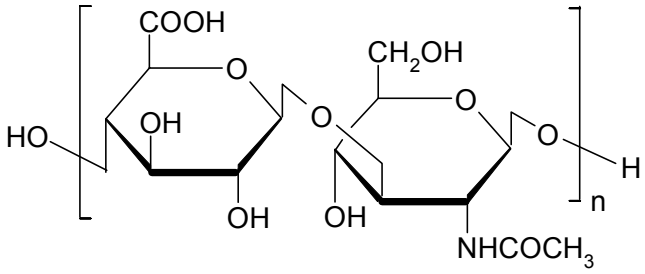

Fig. 1-1: The chemical structure of hyaluronan. The polymer is built from alternating units of glucuronic acid (GlcUA) and N-acetylglucosamine (NAG). n = 20-25 000.

Depending on the tissue source, the polymer usually consists of 2000 to 25000 disaccharides, giving rise to molecular masses ranging from 10^6 to 10^7 Da with extended lengths of 2-25 μm. Under in vivo conditions hyaluronic acid exists as a polyanion as the carboxyl groups of the glucuronic acid residues (pK_a = 3-4, depending on ionic conditions (Hascall and Laurent, 1997)) are predominantly charged.

Chemically, hyaluronan is a member of the familiy of glycosaminoglycans (GAG), including chondroitin-, dermatan- and keratan sulphate, heparin and heparan sulphate. The GAG are

linear polysaccharides made of repeating disaccharides, which consist of uronic acid (or galactose) and hexosamines. In contrast to the other glycosaminoglycans, HA is not sulphated. In addition, it is distinguished from the other GAG as it is not covalently attached to a polypeptide core, but exists as an independent polysaccharide that associates non-covalently with proteoglycans in the extracellular matrix.

The conformation of hyaluronan in aqueous solution is a gently undulating, tape-like, two-fold helix which forms as a result of 180° rotations between alternating disaccharides and is stabilised via internal hydrogen bonds and interactions with the solvent (Scott et al., 1984; Heatley and Scott, 1988) (Fig. 1-2 (A/B)).

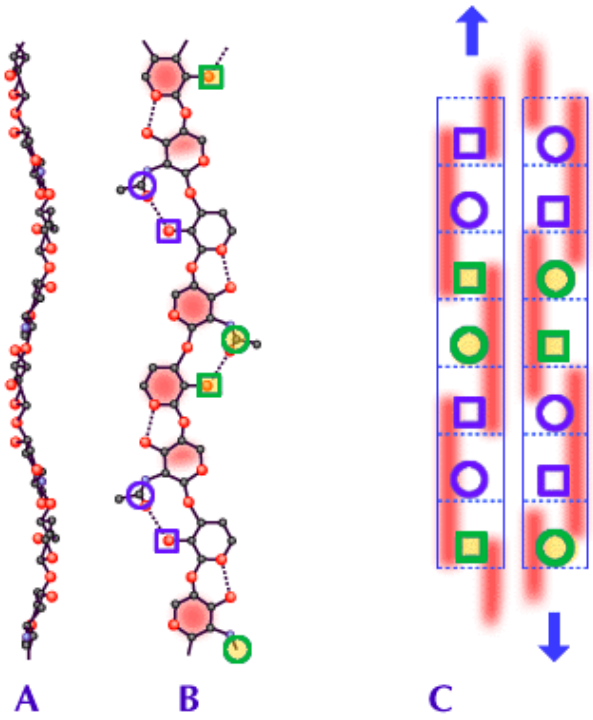

Fig. 1-2: Models depicting the polymeric structure and interaction of HA molecules. (A/B): projections, at right angles to each other, showing the two-fold helix that a HA molecule adopts in aqueous solution. The dotted lines in (B) represent hydrogen bonds; the circle-square pairs joined by dotted lines represent water bridges between acetamido and carboxyl groups. (C): proposed mode of interaction between two antiparallel HA molecules in which hydrophobic patches are opposed and acetamido and carboxyl groups are within hydrogen bonding distance. Red shading: hydrophobic patches; circles: acetamido groups; squares: carboxyl groups. (From Scott (1998) and Toole (2000)).

The two-fold helical conformation of the HA molecule is characterised by repeating hydrophobic patches (8 CH-groups, extended over approximately three carbohydrate units), which are arrayed along the two flat sides of the tape-like polymer, with sequential patches

alternating between the two sides. As indicated in Fig. 1-2 C, the described secondary structure of HA allows for a further organization of the macromolecule in an aqueous environment. Rotary shadowing-electron microscopy showed that HA did self-aggregate into strands of a honeycomb meshwork in aqueous solution already at very low HA concentrations (1 μg/ml), and that the thickness of the strands increased with HA concentration (Scott et al., 1991). Nuclear magnetic resonance studies (Scott and Heatley, 1999) then revealed that aggregation of HA chains in solution is stabilised both by hydrophobic interactions between the hydrophobic patches and by H-bonding between acetamido and carboxylate groups of neighbouring HA chains, which are aligned in an antiparallel fashion (Fig. 1-2 C). As these hydrophobic and hydrophilic bonds can form on both sides of the hyaluronan polymer, higher order aggregates can assemble, giving rise to an increased thickness of the strands in a HA meshwork.

In a recent publication Scott and Heatley (2002) demonstrated that the tertiary structures (aggregation of antiparallel HA chains) are specifically and reversibly disaggregated by mild physicochemical methods (raising temperature or pH, respectively). Under physiological conditions the supramolecular organization of HA was found to be on the edge of stability, suggesting that reversible formation and breakdown of tertiary structures control important biological properties. Interestingly, NMR studies showed that HA oligosaccharides do not form tertiary structures, since they cannot form sufficient intermolecular bonds to produce stable aggregates. This may account for the size dependent biological effects of hyaluronan fragments.

When HA is present at a very dilute concentration in saline solution, a HA molecule assumes an expanded random coil structure, which occupies a large domain, wherein the mass of hyaluronan itself is very low (0.1% (w/v) or less) due to the high water binding capacity of the macromolecule. Within the occupied domain the HA chains are forming pores, which are continuously changing in size, as the HA chains are constantly moving in solution. Thus, in principle all molecules can pass through the HA network, however, with different degrees of retardation depending on their hydrodynamic volumes. As the concentration is increased, high molecular mass hyaluronan is organized in the aforementioned tertiary structure. At concentrations of 0.5 mg/ml or more, as found in many tissues such as synovial fluid, umbilical cord and skin, the organized structure of HA leads to highly viscous solutions with viscoelastic properties (Hascall and Laurent, 1997; Toole, 2000).

1.1.2 Occurrence and physiological importance

Hyaluronan is present in tissues and body fluids of all vertebrates as well as in the capsule of some strains of streptococci. As a component of the extracellular matrix hyaluronan is a major constituent in some tissues. The concentration is particularly high in rooster comb (up to 7.5 mg/ml), in umbilical cord (~ 4 mg/ml), in the synovial joint fluid (3 - 4 mg/ml), in the vitreous body of the eye (0.1 – 0.4 mg/g wet weight), in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~ 0.5 mg/ml) or in the pathological matrix that occludes the artery in coronary restenosis. The largest amount of hyaluronan (7 - 8 g, ca. 50 % of the total in the body) resides in skin tissue where it is present in both the dermis (~ 0.5 mg/g wet tissue) and the epidermis (~ 0.1 mg/g wet tissue). In cartilage hyaluronan serves as an essential structural component of the matrix, as it retains aggrecan (the large chondroitin sulphate proteoglycan) molecules in the matrix by specific protein-hyaluronan interactions. In proliferating cells hyaluronan has been detected also intracellularly (Hascall and Laurent, 1997; Prehm, 2002).

Hyaluronan fulfils several distinct physiological functions that contribute both to structural properties of tissues and to cell behaviour during formation or remodelling of tissues. One of these functions is the direct contribution of hyaluronan to tissue homeostasis and biomechanics due to its unique physicochemical properties. In synovial joint fluid and also between soft tissue surfaces that slide along each other (e.g. fibrils in skeletal muscle) hyaluronan serves as a lubricant due to its viscoelastic behaviour in solution. The rheological and network-forming properties of hyaluronan also contribute to water homeostasis, tissue hydration and transport of macromolecules within tissues. That hyaluronan forms a diffusion barrier for other molecules was first demonstrated by Duran-Reynals, who showed that spreading of intra- or subcutaneously injected substances was facilitated by a ‘spreading factor’ (Duran-Reynals, 1928), which was later found to be a hyaluronidase (Chain and Duthie, 1939).

In addition to the functions arising directly from the physicochemical properties of the polymer, hyaluronan also exerts biological effects via specific interactions with hyaluronan binding proteins (hyaladherins). The great number of hyaladherins known so far can be grouped into (i) the structural hyaluronan-binding proteins of the extracellular matrix, such as link protein and the aggregating proteoglycans, (ii) cell surface hyaluronan receptors and (iii) intracellular hyaluronan binding proteins.

Interactions between hyaluronan, link protein and the proteoglycan aggrecan account for the structural integrity and distinctive biomechanical properties of the extracellular matrix of cartilages. Similar proteoglycans with the potential to form aggregates with hyaluronan have been identified in various connective tissues (versican) and the brain (neurocan and brevican). By the interaction with cell surface receptors hyaluronan affects cell behaviour during morphogenesis, tissue remodelling, inflammation and diseases such as cancer and atherosclerosis. The most studied hyaluronan receptor to date is CD44 (lymphocyte homing receptor), which is known to participate in a wide variety of cellular functions, e.g. receptor mediated internalization/degradation of hyaluronan, cell migration and cell proliferation. Motility and proliferation of cells are also stimulated by the interaction of hyaluronan with RHAMM (receptor for hyaluronic acid mediated motility). Several other cellular receptors for hyaluronan have been identified including, ICAM-1 (intercellular adhesion molecule-1), the LEC receptor (Liver Endothelial Cell clearance receptor) and LYVE-1 (Lymphatic endothelial hyaluronan receptor) (Knudson and Knudson, 1999; Toole, 2000; Jackson, 2004).

1.1.3 The role of hyaluronan during morphogenesis, tissue regeneration and tumorigenesis

Migrating and proliferating cells, during morphogenesis of embryonic organs, during tissue regeneration and also during tumorigenesis are surrounded by pericellular matrices, which are enriched in hyaluronan. Hyaluronan can be tethered to the cells by binding to specific cell surface hyaluronan receptors (e.g. CD44), or the layer can be built from newly synthesized hyaluronan, which is formed on the inside of the plasma membrane and is translocated to the pericellular space. In the latter case the pericellular hyaluronan appears to be tethered to the cells by sustained attachment to hyaluronan synthase or associated proteins on the cytoplasmatic face of the plasma membrane (Laurent et al., 1996; Toole, 2000).

The hyaluronan enriched pericellular matrix can affect cell proliferation and migration. One important way in which the pericellular “coat” may promote cell proliferation is by provision of a hydrated pericellular zone that facilitates cell rounding during mitosis. Migration and invasion of cells are facilitated by the hyaluronan enriched matrices, as they create hydrated pathways that separate cellular or fibrous barriers to penetration by the invading cells (Toole, 2000). In addition, this pericellular “coat” is a structural component that isolates cells from contact with other cells and matrix compounds and protects cells against cytotoxic lymphocytes and viruses (Laurent and Fraser, 1992).

On the other hand hyaluronan can mediate aggregation of cells depending on the concentration and the molecular mass of the hyaluronan molecules. If present at low concentration, cell surface hyaluronan of high molecular mass can interact multivalently with receptors on adjacent cells, thus leading to cross-bridges between the cells. However, excess of high molecular mass hyaluronan inhibits cell aggregation due to saturation of the hyaluronan receptors. Hyaluronan fragments of low molecular mass, as they may be generated by degradation of hyaluronan with hyaluronidase, show the same effect, since they are too small for multivalent binding at receptors on adjacent cells.

In addition to providing a suitable hydrated milieu or to cross-bridging cells, interaction of hyaluronan with its cell surface receptors initiates signaling pathways that promote cell movement, proliferation or differentiation. The function of hyaluronan varies depending on the size of the hyaluronan fragments also with respect to the receptor mediated effects on cell behaviour (Turley et al., 2002). For example it was shown in several in vitro and in vivo models that small hyaluronan fragments stimulate angiogenesis, whereas high molecular mass hyaluronan exerts inhibitory effects (West and Chen, 2000; Slevin et al., 2002).

Hyaluronan and its degradation products of low molecular mass thus play a crucial role in tumor growth and metastasis. Elevated levels of hyaluronan are found in most malignant solid tumors (Knudson et al., 1989) and experimental evidence has been obtained which demonstrates that hyaluronan promotes tumor progression (Toole, 2002). For example it was shown that perturbation of the endogenous hyaluronan-protein interactions by overexpression of soluble hyaladherins, which competitively displace hyaluronan from its cell surface receptors (Peterson et al., 2000; Ahrens et al., 2001), or by treatment with antibodies that block hyaluronan-CD44 binding (Guo et al., 1994) leads to inhibition of tumor growth and invasion. Likewise, treatment with hyaluronan oligosaccharides was found to inhibit growth of several tumor types in vivo, presumably due to competitive inhibition of high molecular mass hyaluronan-CD44 binding (Zeng et al., 1998; Ghatak et al., 2002). However, the involvement of hyaluronan and its degradation products in tumor progression appears to be quite complex, considering that hyaluronan oligosaccharides may directly induce biological effects, which promote tumor growth and invasion, e.g. increased angiogenesis (Slevin et al., 2002) or enhanced tumor cell motility (Sugahara et al., 2003).

Tumor cells often exhibit not only elevated levels of hyaluronan but also increased hyaluronidase activity, and studies on transplantable tumors and cultured cell lines suggest that hyaluronidase-mediated hyaluronan degradation is related to increased angiogenesis and

metastasis (West and Chen, 2000). However, the finding that administration of hyaluronan oligosaccharides in vivo was found to inhibit tumor growth and that at least one of the several hyaluronidase genes corresponds to a previously mapped tumor suppressor (Csoka et al., 1998), shows that both hyaluronan and hyaluronidases are involved in tumor promotion as well as in tumor suppression in a complex way, which is actively investigated at present.

1.2 Hyaluronidases

1.2.1 History and occurrence

The term hyaluronidase was introduced by Karl Meyer in 1940 to denote enzymes that degrade HA (Meyer et al., 1940). The discovery of hyaluronidases resulted from two independent lines of investigation. In 1928 Duran-Reynals observed that extracts from mammalian testes and other tissues contained a "spreading factor", a substance that facilitated the diffusion of antiviral vaccines, dyes and toxins injected subcutaneously (Duran-Reynals, 1928). After the first isolation of HA from vitreous humor (Meyer and Palmer, 1934) a HA degrading enzyme was identified in autolysates of pneumococci by Meyer et al. (1937). In 1939 it was shown that the spreading factor present in extracts from mammalian testes was also an enzyme, degrading HA (Chain and Duthie, 1939).

In subsequent years hyaluronidases were identified from a large number of tissues and organisms, e.g. from some bacteria (bacteriophage-associated and bacterial hyaluronidase), pathogenic fungi (*Candida*, *Streptomyces*) and invertebrate animals (crustaceans, insects). In vertebrates hyaluronidases were found in the venom of snakes and lizards, in testes and in various somatic tissues, e.g. liver, kidney, lymphatic system and skin. The isolated enzymes differ in their molecular mass, substrate specificity, pH optimum and the mechanism of substrate degradation (Meyer, 1971; Kreil, 1995; Frost et al., 1996; Csoka et al., 1997).

1.2.2 Classification of hyaluronidases

The first classification scheme for hyaluronidases was established in 1971 by Karl Meyer. According to their catalytic mechanism the different types of hyaluronidase are grouped into three main families (Fig. 1-3) (Meyer, 1971).

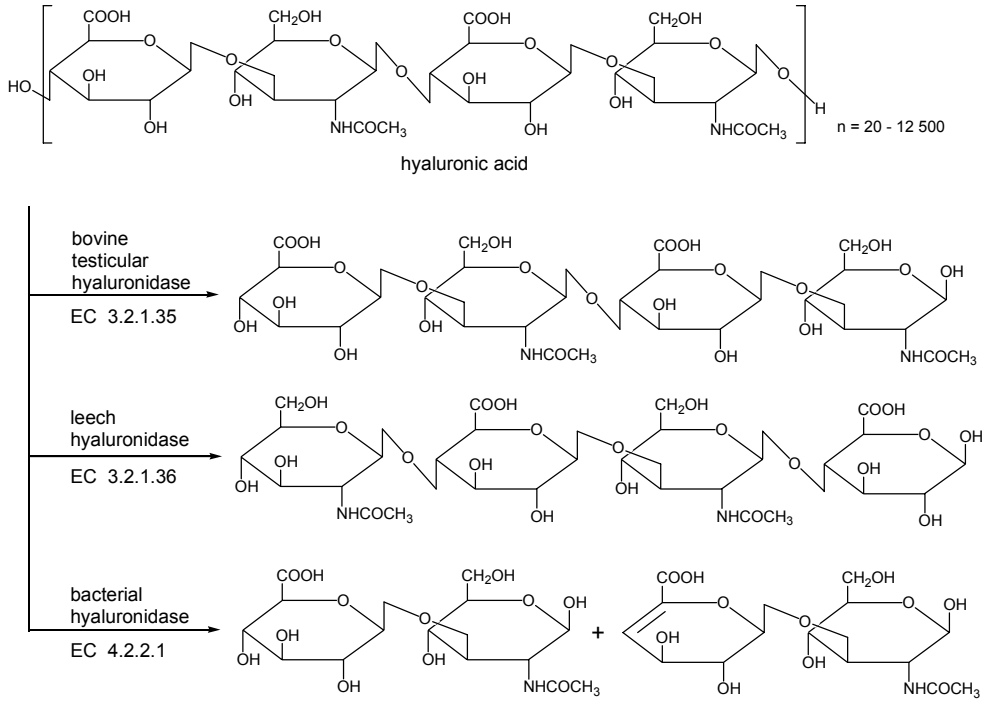

Fig. 1-3: Classification of hyaluronidases according to Meyer (1971).

The first group of hyaluronidases are the hyaluronate 4-glycanohydrolases (EC 3.2.1.35) degrading HA by cleavage of the β-1,4-glycosidic bond to the tetrasaccharide as the main product. These enzymes are glycosidases with both hydrolytic and transglycosidase activity and degrade HA, chondroitin, chondroitin-4- and -6-sulphate and, to a small extent, dermatan sulphate. The best known enzymes are the testicular, the lysosomal and the bee venom hyaluronidase.

The second type is represented by hyaluronidases occurring in the salivary glands of leeches and hookworms. These enzymes are hyaluronate 3-glycanohydrolases (EC 3.2.1.36) which degrade HA by cleavage of the β-1,3-glycosidic bond, thus, yielding sugar fragments having glucuronic acid at the reducing end. The main degradation product is the tetrasaccharide, too.

The third group, the microbial hyaluronidases (4.2.2.1) are hyaluronate lyases. They degrade HA by a β-elimination reaction to yield the unsaturated disaccharide 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose as the main product. The hyaluronate lyases, isolated from various microorgansims, including e.g. strains of *Clostridium, Micrococcus, Streptococcus* and *Streptomyces*, differ in substrate specificity (Suzuki, 2000).

The classification of hyaluronidases established by Meyer was based on biochemical analysis of the enzymes and their reaction products. Molecular genetic analysis has shown that, as an alternative, hyaluronidases can be grouped into two main families - the hyaluronidases from eukaryotes and from prokaryotes - according to amino acid sequence homology (Csoka et al., 1997; Csoka et al., 2001).

1.2.3 Hyaluronidases from eukaryotes

1.2.3.1 Mammalian hyaluronidases

In the last years six hyaluronidase sequences with about 40 % identity to each other were identified in the human genome. Three genes (*HYAL1*, *HYAL2* and *HYAL3*) are clustered on chromosome 3p21.3, coding for Hyal-1, Hyal-2 and Hyal-3. Another two genes (*HYAL4* and *PH20* (*SPAM1*)), coding for Hyal-4 and PH-20, and one expressed pseudogene (*HYALP1*) are similarly clustered on chromosome 7q31.3 (Csoka et al., 2001).

Hyal-1:

Hyal-1, first isolated from human plasma, is the predominant hyaluronidase in mammalian plasma and urine, and is also found at high levels in the liver, kidney, spleen and heart. It is localised in lysosomes and active at acidic pH (Frost et al., 1997). Mutations in the gene *HYAL1* cause a newly described lysosomal disorder, mucopoysaccharidosis IX (Natowicz et al., 1996; Triggs-Raine et al., 1999). Furthermore, Hyal-1 appears to play a role in tumor formation. *HYAL1* (also known as *LUCA1*) is a candidate tumor suppressor gene, which is inactivated in many tobacco-related tumors (Csoka et al., 1998; Frost et al., 2000). In addition, Hyal-1 was found to promote tumor cell cycling (Lin and Stern, 2001).

Hyal-2:

Also the Hyal-2 protein was found in many tissues, except the adult brain (Lepperdinger et al., 1998). It is localised either in lysosomes or anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) link. Like Hyal-1, the Hyal-2 enzyme has a pronounced activity optimum at pH 4, however, as shown for Hyal-2 from *Xenopus laevis*, low activity can be also detected under physiological conditions. Hyal-2 has an unusual substrate specificity cleaving high molecular mass HA to intermediate size fragments of approximately 20 kDa (50-60 disaccharide units) (Lepperdinger et al., 2001). As the major hyaluronidases in mammalian somatic tissues Hyal-1 and Hyal-2 are believed to act in succession on degrading

HA: HA fragments of approx. 20 kDa are generated at the cell surface by GPI-anchored Hyal-2, internalised and then further digested to tetrasaccharides by the lysosomal Hyal-1 (Csoka et al., 2001; Stern, 2004). Like Hyal-1, also the Hyal-2 protein is involved in tumor formation: it can function as either an oncogene or a tumor suppressor gene product. At the one hand, overexpression of Hyal-2 is reported to accelerate the formation of murine astrocytoma cells (Novak et al., 1999) and on the other hand, Hyal-2 was found to accelerate apoptosis (Chang, 2002).

Hyal-3:

Very little is known about the Hyal-3 protein. It is widely expressed, e.g. in testes and bone marrow, but no activity can be identified using the available hyaluronidase assays (Stern, 2003).

Hyal-4:

Hyal-4 appears to be a chondroitinase having absolute specificity for chondroitin and chondroitin sulphate with no ability to degrade HA. This substrate specificity is in marked contrast to the Hyal-1 and PH-20 enzymes, which can cleave both HA and - at a slower rate - chondroitin sulphate (Stern, 2003).

PH-20:

The PH-20 protein (also known as SPAM 1 (sperm adhesion molecule 1)) appears to be a multifunctional protein. The GPI-anchored protein is located on the surface of mammalian sperm and in the lysosome derived acrosome, where it is linked to the inner acrosomal membrane. The hyaluronidase activity of PH-20 is essential for penetration of the HA-rich cumulus ECM that surrounds the egg. In addition, plasma membrane PH-20 mediates HA-induced cell signalling (increase in intracellular calcium) via a HA binding domain that is separate from the hyaluronidase domains. The inner acrosomal membrane PH-20 is a receptor for the zona pellucida surrounding the egg and plays a key role in sperm interaction with and penetration of the zona pellucida.

While plasma membrane PH-20 shows hyaluronidase activity only at neutral pH, inner acrosomal membrane PH-20 was found to be active both at neutral and acidic pH. Recent results (Yudin et al., 2001) suggest that the enzyme activities at different pH involve two different domains in the protein: PH-20 may attain the capability of acid active hyaluronidase activity after the acrosome reaction, where the enzyme is endoproteolytically cleaved but held together by a disulfide bond. This endoproteolysis may alter the three-dimensional structure

of PH-20, enabling the domain, which is responsible for activity at acidic pH, to become active and thus imparting both neutral and acid-active activities to PH-20 (Cherr et al., 2001). PH-20 is relatively specific for testes, however, by sensitive techniques, it can also be detected in the epididymis, the female genital tract, breast, placenta and fetal tissues. Also in certain malignancies the expression of PH-20 is reported (Stern, 2003).

Bovine testicular hyaluronidase (BTH)

It has been known for a long time that extracts from mammalian testes contain hyaluronidase activity (Chain and Duthie, 1939), and preparations of bovine and ovine testicular hyaluronidase were therapeutically applied as a sprading factor in several medical fields for many years (Baumgartner and Moritz, 1988; Menzel and Farr, 1998). In 1997 it was shown that the major soluble hyaluronidase present in bull testes extracts is a fragment of the membrane bound PH-20 enzyme (Meyer et al., 1997).

BTH is an endo-glycanohydrolase (EC 3.2.1.35) degrading HA by cleavage of the β-1,4-glycosidic bond. In addition to HA, BTH also degrades chondroitin, chondroitin-4- and -6-sulphate and, to a small extent, dermatan sulphate. By using ion-spray mass spectrometry, tetrasaccharide and saturated disaccharide fragments were identified as major and as smallest hydrolysis products, respectively (Takagaki et al., 1994).

The mammalian PH-20 was found to be homologous to bee venom hyaluronidase (BVH) showing ca. 30 % sequence similarity and conservation of the active site residues (Gmachl and Kreil, 1993; Markovic-Housley et al., 2000). The hydrolysis of HA by BTH is thus supposed to occur according to the double-displacement substrate assisted mechanism described for BVH (cf. to 1.2.3.2, Fig. 1-5). Sequence alignment of BTH and BVH revealed that Glu149 of BTH corresponds to the acid-base catalyst Glu113 of BVH (Botzki et al., 2004).

Preparations of BTH typically show hyaluronidase activity at neutral and acidic pH. However, the pH activity profiles reported in the literature for various BTH preparations vary widely, depending on the mostly heterogeneous composition of the BTH preparations (Oettl, 2000), the source of the substrate (HA), the hyaluronidase assay (Hoechstetter et al., 2001) and the incubation conditions (Gorham et al., 1975; Gacesa et al., 1981; Oettl, 2000). Optimum activity of BTH was found at acidic pH (pH 3.5 - 4.0) (Bonner and Cantey, 1966; Muckenschnabel et al., 1998a; Seaton et al., 2000) as well as at weakly acidic pH (pH 5.0 –

6.0) (Dorfman and Ott, 1948; Allalouf et al., 1975; Highsmith et al., 1975) and at neutral pH (Tung et al., 1994; Csoka et al., 1998).

In addition to hydrolysis, BTH also catalyses the reverse reaction, the transglycosylation reaction (Fig. 1-4). The transglycosylase activity of BTH is dependent on the pH value and the salt content of the incubation buffer. Investigations of Saitoh et al. (1995) revealed that the optimal pH for the transglycosylation reaction is about pH 7.0, whereas hydrolysis is favoured at pH < 5.0. The highest transglycosylase activity was found in the absence of NaCl, whereas transglycosylation was nearly completely inhibited at concentrations of NaCl higher than 0.5 M (Saitoh et al., 1995).

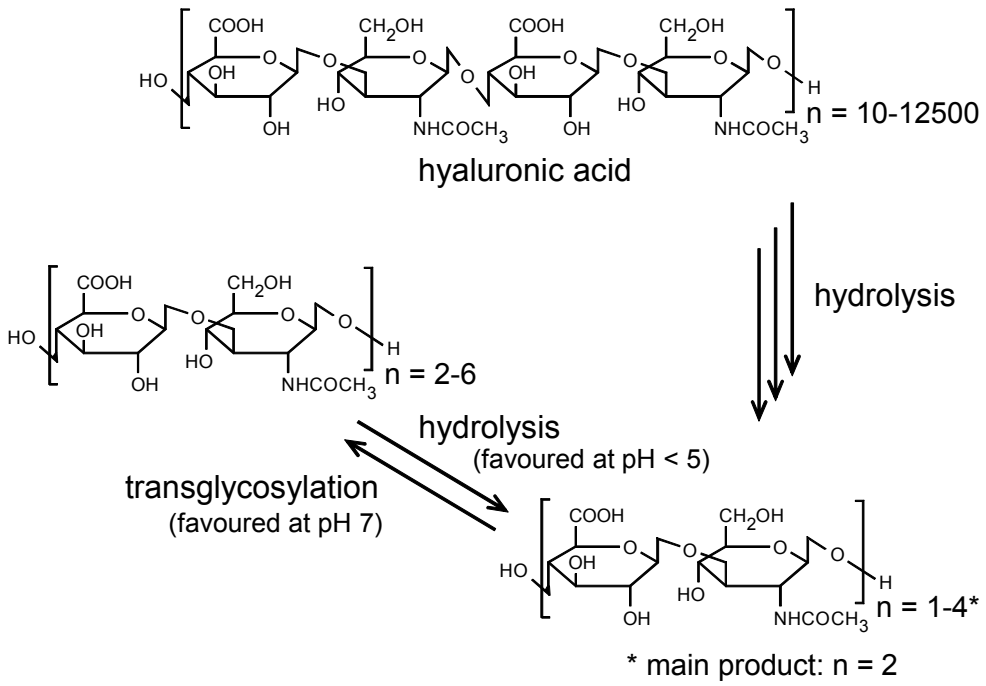

Fig. 1-4: Mechanism of hyaluronan degradation by bovine testicular hyaluronidase (BTH).

1.2.3.2 Hyaluronidase from bee venom

Bee venom hyaluronidase (BVH), a major allergen of bee venom, is a hyaluronate 4-glycanohydrolase (E.C. 3.2.1.35) sharing ca. 30 % sequence similarity with the mammalian hyaluronidases. On the basis of sequence similarity mammalian hyaluronidases and BVH are assigned to family 56 of glycosyl hydrolases (Henrissat and Bairoch, 1996). Compared to the

human and bovine enzymes BVH lacks a C-terminal domain of ca. 120-150 amino acids (Gmachl and Kreil, 1993). Like BTH, in addition to HA the hyaluronidase from bee venom also degrades chondroitin sulphate. The pH activity profile of BVH reported by Allalouf et al. (1975) shows maximum activity at pH 4.5 and remarkable activity at neutral pH.

In 2000, the crystal structure of BVH in complex with a HA-based tetrasaccharide was determined by Markovic-Housley et al. (2000). Due to the co-crystallised HA tetrasaccharide fragment, the catalytic mechanism of HA degradation could be elucidated. In general, glycosidases act via a double or a single nucleophilic displacement mechanism, which results in either retention or inversion of the configuration at the anomeric carbon atom. In both cases, the glycosidic bond to be cleaved is positioned between the carboxylates of two catalytic acids, one acting as acid/base and the other as nucleophile (Withers and Aebersold, 1995; Markovic-Housley and Schirmer, 2002). In the crystal structure of BVH Glu113 appears to be the acid/base catalyst, since it forms a hydrogen bond with the glycosidic oxygen of N-acetylglucosamine in subsite -1*. However, a catalytic residue being in the right position to act as a nucleophile is missing. Therefore a substrate-assisted mechanism is proposed with Glu113 acting as the proton donor and the N-acetyl carbonyl group of the substrate acting as the nucleophile (Fig. 1-5). This double displacement mechanism involves the binding of the saccharide in subsite -1* in a boat conformation and the formation and subsequent hydrolysis of a covalent oxazolinium ion intermediate.

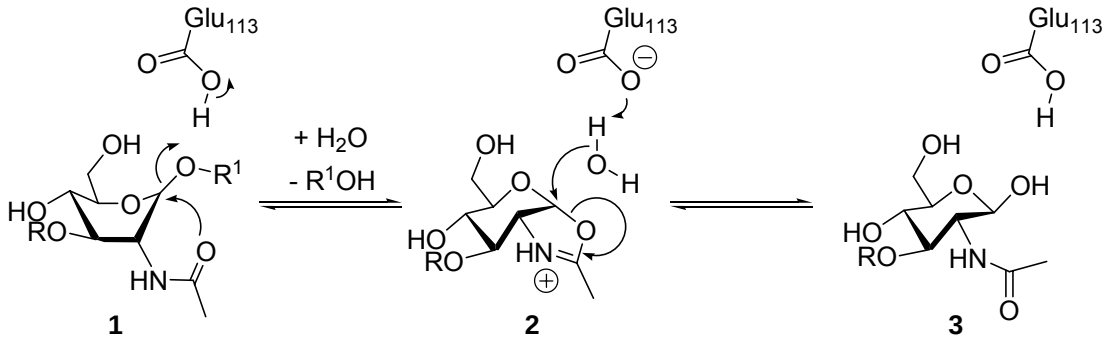

Fig. 1-5: Double-displacement substrate assisted mechanism of bee venom hyaluronidase. The saccharide in subsite -1* (**1**) binds in boat conformation, and catalysis is proposed to occur via a formation of a covalent oxazolinium ion intermediate **2** to the product **3**. Adapted from Marcovic-Housley and Schirmer (2002).

* By convention, the sugar residue subsites are labelled from –n to +n, with –n at the non-reducing end and +n at the reducing end of the substrate. Cleavage occurs between the -1 and +1 subsites (Davies et al., 1997).

A similar mechanism has been postulated for chitinolytic enzymes of the glycosyl hydrolase families 18 and 20 (Terwisscha van Scheltinga et al., 1995; Drouillard et al., 1997).

1.2.4 Hyaluronidases from prokaryotes

A wide variety of microorganisms produce enzymes capable of degrading hyaluronate. Detailed overviews over the microbial hyaluronidases are given by Suzuki (2000) and Hynes and Walton (2000).

To date, the amino acid sequences of a variety of hyaluronidases from prokaryotes have been decoded (Coutinho and Henrissat, 1999). The best known and characterised bacterial hyaluronidases are the hyaluronate lyases from *Streptococcus pneumoniae* and *Streptococcus agalactiae* (group B streptococcus, GBS), respectively (Jedrzejas and Chantalat, 2000; Pritchard et al., 2000; Jedrzejas, 2002). Both enzymes degrade HA by a β-elimination reaction to yield the unsaturated disaccharide 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-ene-pyranosyluronic acid)-D-glucose (△DiHA) as the main product (Pritchard et al., 1994; Li et al., 2000).

Recently, the 3D structures of the hyaluronate lyases from *S. pneumoniae* and from *S. agalactiae* strain 3502 were elucidated by X-ray analyses (Li et al., 2000; Li and Jedrzejas, 2001). The active site of the hyaluronate lyase from *S. agalactiae* is composed of two main parts, a catalytic triad responsible for the substrate degradation and an aromatic patch responsible for the selection and the positioning of cleavage sites on the polymeric substrate. The residues that form the aromatic patch of the enzyme are Trp371, Trp372 and Phe423, those of the catalytic triad are His479, Tyr488 and Asn429 (Li and Jedrzejas, 2001).

The catalytic mechanism of *S. agalactiae* hyaluronate lyase, termed proton acceptance and donation (see Fig. 1-6), was revealed, based on the crystal structure of the native enzyme, the structures of the enzyme in complex with the disaccharide product of degradation (△DiHA) (Li and Jedrzejas, 2001) and in complex with hyaluronate hexasaccharide (Mello et al., 2002), the modelled complex with hyaluronate tetrasaccharide, site-directed mutagenesis studies (Pritchard et al., 2000) and comparison to the hyaluronate lyase from *S. pneumoniae* (Li et al., 2000; Jedrzejas et al., 2002). In the first step, the positively charged cleft of the enzyme attracts and binds the negatively charged substrate chain. Three disaccharide units can be accommodated into the cleft (see Fig 1-6, only two, HA1 and HA2, of the three units are shown). In the second step, the aromatic patch of the active site interacts with the substrate

chain and anchors it in optimal position. In the third step, the glucuronic acid of HA1 is deprotonated at C5 by His479. At the same time, Tyr488 donates a proton to the glycosidic oxygen connecting D-glucuronic acid of HA1 and N-acetyl-D-glucosamine of HA2. The glycosidic bond is cleaved by 1,2-elimination forming the double bond of the unsaturated final product (fourth step). Finally (fifth step), the catalytic triad is regenerated: His479 is deprotonated and Tyr488 is protonated by a water molecule.

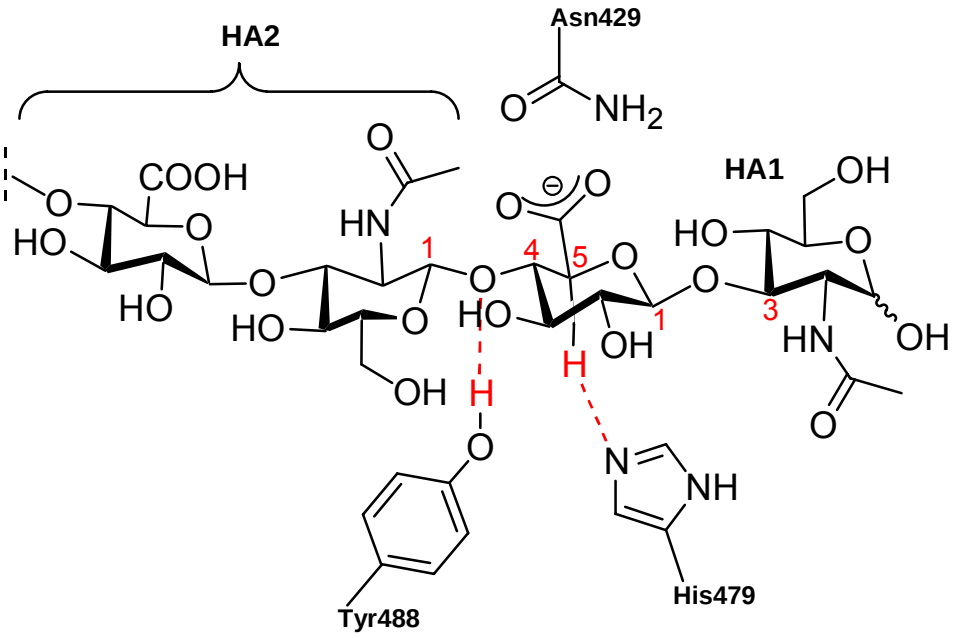

Fig. 1-6: Mechanism of hyaluronan degradation by hyaluronate lyase from *S. agalactiae* according to Li and Jedrzejas (2001). Schematic representation of hyaluronic acid with HA1 and HA2 as disaccharide units and the position of the side chains of Tyr488, His 479 and Asn429 relative to the substrate.

The hyaluronate lyases from *S. pneumoniae* and *S. agalactiae*, respectively, are supposed to degrade the polymeric substrate via a processive mode of action (Li et al., 2000; Li and Jedrzejas, 2001). In this processive mechanism of action the enzyme probably binds randomly to a HA molecule and cleaves it into two pieces by the aforementioned β-elimination process (initial endolytic cleavage). The resulting unsaturated fragment, containing the reducing end of the primary HA molecule, leaves the catalytic cleft of the enzyme, whereas the other fragment remains in the cleft, where it is translocated by one disaccharide unit toward the reducing end. By this translocation process the truncated HA chain is positioned in the active site of the enzyme, so that an unsaturated disaccharide product (△DiHA) is generated by the following catalytic cleavage. The produced △DiHA is released from the enzyme, and the

remaining HA chain is again translocated in the cleft by one disaccharide unit for the next round of catalysis (exolytic processive degradation).

The processive degradation mechanism was initially suggested, based on the analysis of the products of HA degradation (Baker and Pritchard, 2000), and could be supported by elucidation of the 3D structures of the enzymes in complex with hyaluronan fragments of varying length (Jedrzejas et al., 2002; Mello et al., 2002). With respect to the direction of hyaluronan degradation the data reported in the literature are contradictory. Analysis of the structural data of the enzyme in complex with substrate suggests that the degradation takes place from the reducing to the nonreducing end of a HA chain, as described above (Jedrzejas et al., 2002). However, based on the analysis of the degradation products Baker and Pritchard (2000) proposed that the processive degradation proceeds from the nonreducing to the reducing end of the substrate chain. Therefore the mechanism proposed by Baker and Pritchard (2000) differs from that suggested by Jedrzejas et al. (2002) insofar as - after the initial endolytic cleavage - not the saturated fragment but the unsaturated fragment remains at the enzyme to be further degraded to disaccharides.

1.3 Methods for the determination of hyaluronidase activity

1.3.1 Classical methods

A variety of assays has been devised for the determination of hyaluronidase activity over the years. The classical methods can be grouped into biological, physicochemical and chemical methods according to Meyer and Rapport (1952). Since in this thesis hyaluronidase preparations were investigated by several of the classical physicochemical and chemical methods, a short introduction into the classical assays for hyaluronidase is given in the following.

1.3.1.1 Biological methods

Spreading effect

Biological assays measuring the effect of hyaluronidase co-administration on the spreading of an indicator dye injected into the skin of animals were described by a number of authors (for complete listing of references see Gibian (1959)). Although the spreading assay cannot be used as an accurate quantitative assay of hyaluronidase (Meyer and Rapport, 1952), it is the

most direct method for the determination of the therapeutical efficacy of hyaluronidase preparations, which are intended to be applied as spreading factors to improve the absorption of drugs. In this context it is noteworthy that a spreading assay developed by Jaques (1953) showed results comparable to those obtained by viscosimetric and turbidimetric in vitro methods (Humphrey and Jaques, 1953).

Decapsulation of mucoid strains of streptococci

Another biological method is based on the ability of hyaluronidase to degrade the mucoid capsule of group A and group C streptococci (Fulton et al., 1950; Meyer and Rapport, 1952).

1.3.1.2 Physicochemical methods

Mucin clot prevention method

This method is based on the observation that native hyaluronate in acid solution precipitates with protein in a fibrous clot. After incubation of hyaluronate with hyaluronidase the character of the precipitate changes from fibrous to flocculent, finally no precipitate is obtained (Robertson et al., 1940; McClean, 1943). One unit of hyaluronidase was defined as the amount of enzyme, which prevents the clotting of 0.4 mg of crude hyaluronate (Harris and Harris, 1950).

Spinnability method

The spinnability of dialised bovine synovial fluid is destroyed by hyaluronidases. The spinnability is determined by a special apparatus which measures the length to which a filament of the substrate solution can be drawn at a standard velocity. One unit of enzyme was defined as the amount of enzyme, which reduced the spinnability to 50% of the initial value in 20 min under standard conditions (Gunter, 1949).

Viscosity reduction method

In this method the reduction in viscosity of a solution of hyaluronic acid, induced by the action of hyaluronidase, is measured. The viscosimetric method, originally elaborated by Madinaveitia and Quibell (1940), has been extensively employed in various modifications (Meyer and Rapport, 1952). Viscosimetric units of hyaluronidase have been established by defining 1 unit as the amount of enzyme required to reduce the viscosity of a HA solution to half the initial viscosity under specified conditions (Madinaveitia and Quibell, 1940; McClean and Hale, 1941; Meyer, 1947). However, although the viscosimetric method proved to be

sensitive and accurate, comparison of the results obtained by different research groups turned out to be difficult, since the half viscosity reduction times were found to be highly dependent on the initial viscosity of the hyaluronate solution, which varies depending on the molecular mass of the respective HA preparation (Hadidian and Pirie, 1948; Alburn and Whitley, 1951).

In the viscosimetric assay, which is used for the standardisation of hyaluronidase preparations according to the European Pharmacopoeia (2002), the activity of hyaluronidase is quantified in terms of International Units (IU) by comparing the rate of viscosity reduction induced by the hyaluronidase preparation to be quantified with the rate obtained with the "International Standard for Hyaluronidase" (Humphrey, 1957) or a reference preparation calibrated in International Units.

A viscosimetric approach to determine the activity of hyaluronidase expressed as mol of bonds broken per unit time was reported by Vercruysse et al. (1995).

Turbidimetric method

The turbidimetric assay relies on the observation by Kass and Seastone (1944) that hyaluronate of high molecular mass forms precipitates with diluted acidified serum, whereas depolymerised hyaluronate remains clear under the same conditions. The average molecular mass at which turbidity formation disappears is reported to lie between 6 and 8 kDa (Rapport et al., 1950). Several modifications of the method have been described. In addition to horse serum, rabbit serum, human serum or human plasma also purified protein fractions as horse serum albumin or bovine plasma albumin (Fraction V) are reported to serve as precipitating protein reagents (Meyer, 1947; Dorfman and Ott, 1948; Tolksdorf et al., 1949; Schmith and Faber, 1950).

Other turbidimetric methods are based on the formation of insoluble complexes between high molecular mass HA and quarternary ammonium salts (Scott, 1955). Hyaluronidase assays using cetyltrimethylammonium bromide or cetylpyridinium chloride as precipitating agents are described by Di Ferrante (1956) and Bohn et al. (1969), respectively.

In the turbidimetric assay hyaluronidase activity is expressed in turbidity reduction units (TRU): 1 TRU is defined as the amount of enzyme which will reduce the turbidity produced by 0.2 mg of HA to that produced by 0.1 mg of HA within 30 min under specified conditions (Kass and Seastone, 1944; Meyer, 1947). However, by some authors, employing the turbidimetric method, slightly modified arbitrary units were defined: (1) to overcome the

problem that different preparations of hyaluronate may produce different turbidities at a given concentration, Tolksdorf et al. introduced a new unit based on a standard initial turbidity instead of the standard HA concentration (0.2 mg) used in the aforementioned definition: 1 unit was defined "as the amount of enzyme, which will hydrolise one half of a quantity of substrate sufficient to cause a turbidity corresponding to 50 ± 5 per cent light transmission" (Tolksdorf et al., 1949). (2) By Gerlach and Köhler (1972) 1 unit was defined as the amount of enzyme, which produces a 50 per cent reduction of the turbidity given by the initial quantity (0.1 mg) of hyaluronate.

In 1957 an "International Standard for Hyaluronidase" was established: tablets were prepared from lyophilised bovine testicular hyaluronidase blended with lactose, and their activity was assayed turbidimetrically. On the basis of this examination the International Unit (IU) of hyaluronidase was defined as the activity of 0.1 mg of the international standard preparation, which is almost equal to one TRU (Humphrey, 1957).

The turbidimetric method, using horse serum as precipitating agent, is used for the standardisation of hyaluronidase preparations according to the United States Pharmacopeia (USP) (2002). Enzyme activity is quantified in terms of "USP Hyaluronidase Units" by comparing the turbidity reduction induced by the hyaluronidase preparation to be quantified with the turbidity reduction obtained with the "USP Hyaluronidase Reference Standard".

1.3.1.3 Chemical methods

Quantification of reducing sugars

The determination of the increase in reducing sugars resulting from the cleavage of the glucosidic bonds of hyaluronan molecules has been widely used for the quantification of hyaluronidase activity (Meyer, 1947). The reductimetric procedure provides a method to measure product formation. However, it is more sensitive than the physicochemical methods and requires highly purified substrate (Rapport et al., 1950).

Determination of reducing N-acetylglucosamine (NAG)

As the hyaluronidases of the enzyme classes EC 3.2.35 (hyaluronate 4-glycanohydrolases) and EC 4.2.2.1 (hyaluronate lyases) liberate N-acetylglucosamine end groups from hyaluronan (cf. to Fig. 1-3), these enzymes can be assayed by a colorimetric method based on the Morgan-Elson reaction for the determination of carbohydrates containing terminal N-acetylhexosamine moieties (Morgan and Elson, 1934). This colorimetric method (Morgan-

Elson assay), which was optimised by Reissig et al. (1955) and further modified for the estimation of hyaluronidase acitivity in human plasma (Gacesa et al., 1981; Muckenschnabel et al., 1998b), represents one of the most frequently used hyaluronidase assays. Recently new modifications of this colorimetric assay were published (Asteriou et al., 2001; Takahashi et al., 2003).

The principle of the Morgan-Elson assay is illustrated in Fig.1-7. NAG moieties at the reducing ends of HA and its fragments generated by the hydrolase (and transglycosylase) activity of hyaluronidase are determined after derivatisation to a red-colored product with p-dimethylaminobenzaldehyde (Ehrlich's reagent).

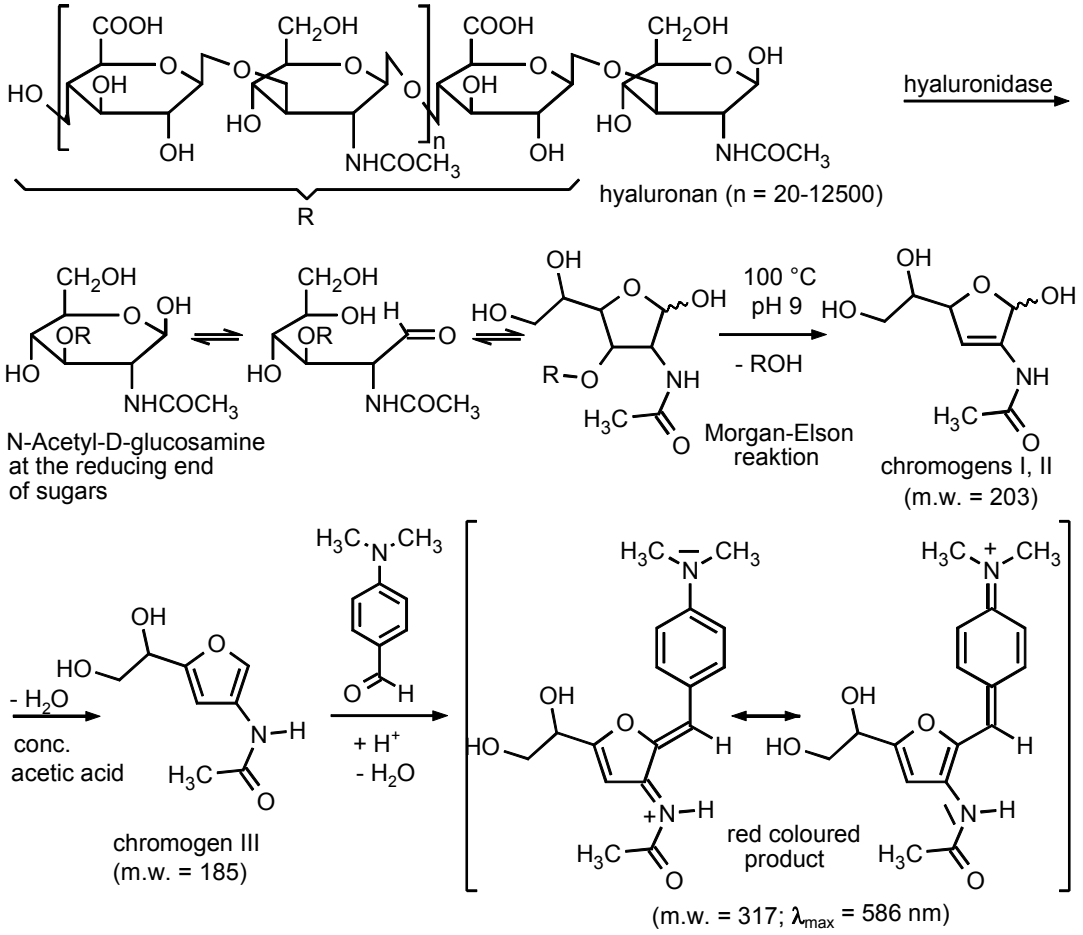

Fig. 1-7: Mechanism of the Morgan-Elson reaction as proposed by Muckenschnabel et al. (1998b).

In contrast to the physicochemical methods, which are based on the detection of residual high molecular mass substrate and measure the hyaluronidase induced changes in the

physicochemical properties of the substrate solution, by the colorimetric method product formation is detected. Therefore, in the Morgan-Elson assay hyaluronidase activity can be quantified according to the definition of the International Union of Biochemistry by defining 1 unit (U) as the amount of enzyme that catalyses the liberation of 1 µmol of NAG at the reducing ends of sugars per min under specified conditions.

For bovine testicular hyaluronidase it was shown that, according to the aforementioned definition, 0.1 mU (0.1 nmol NAG/min) is equivalent to approximately 1 TRU and 1 IU, respectively, when using HA from rooster comb as substrate (Oettl, 2000). However, this relation may not be generalised, since it may depend on various factors like the molecular mass of the substrate or the pH of the incubation medium (Hoechstetter et al., 2001). In addition, it has to be pointed out that an entirely different relation may be observed in case of bacterial hyaluronidases due to their different mechanism of HA degradation.

1.3.2 Other methods

In addition to the classical methods quantitative hyaluronidase assays are also performed by spectrophotometry of complexes between hyaluronan and dyes (Benchetrit et al., 1977; Turner and Cowman, 1985; Homer et al., 1993), chromatography (Cramer and Bailey, 1991; Cramer et al., 1994; Vercruysse et al., 1994), capillary zone electrophoresis (Pattanaargson and Roboz, 1996) or polyacrylamide gel electrophoresis (Ikegami-Kawai and Takahashi, 2002). Also a variety of assays using fluorogenic hyaluronate as substrate have been reported (Nakamura et al., 1990; Calabro et al., 2000; Krupa et al., 2003; Nagata et al., 2004). An ELISA-like assay for hyaluronidase and hyaluronidase inhibitors was developed by the group of Robert Stern (Stern and Stern, 1992; Frost and Stern, 1997; Nawy et al., 2001). A new method combining agarose gel electrophoresis and enhanced chemoluminescence-assisted detection was recently described by Mülleger et al. (2001) as a sensitive assay for the Hyal-2 enzyme, which is difficult to assay by most other methods due to its unusual substrate specificity (see 2.3.1.). Furthermore zymographic methods are used for the detection of hyaluronidases (Liefländer and Stegemann, 1968; Steiner and Cruce, 1992; Miura et al., 1995) and hyaluronidase inhibitors (Mio et al., 2001).

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2 Scope and objectives

Hyaluronidases represent a heterogeneous group of enzymes from different sources. They catalyse the degradation of hyaluronate (HA) and other acidic glycosaminoglycans with varying substrate specificities by different reaction mechanisms. Bovine testicular hyaluronidase (BTH) has been therapeutically applied as a spreading factor degrading the glycosaminoglycans of the extracellular matrix for many years. However, in the context with the risk of BSE, several companies stopped the production of BTH preparations (e.g. Hylase® "Dessau", Neopermease®, Wydase®) which led to a shortage of this drug, characterised by a unique mechanism of action.

A major goal of this thesis was to find out if hyaluronate lyase from *Streptococcus agalactiae*, which can be produced biotechnologically, may serve as a substitute for BTH as a spreading factor and activity enhancer in cancer chemotherapy. In the first part of this thesis a preparation of hyaluronate lyase from *S. agalactiae* and two BTH preparations (Hylase® "Dessau", Neopermease®) were compared with respect to purity (composition) and enzyme activity. In subsequent preclinical in vitro and in vivo studies it was investigated if the adjuvant administration of *S. agalactiae* hyaluronate lyase results in an improved intratumoral accumulation of coinjected chemotherapeutics and, thus, enhances the efficacy of cancer chemotherapy. Due to the different mechanisms of hyaluronan degradation by bovine and bacterial hyaluronidase enzyme concentrations being equiactive in the frequently used colorimetric assay (Morgan-Elson assay) are not necessarily pharmacologically equieffective. The Morgan-Elson assay detects the formation of N-acetylglucosamine end groups liberated from hyaluronan during enzymatic cleavage and not the reduction of the molecular mass of the substrate chains, the latter being responsible for the pharmacological spreading effect. Therefore, the activities of the bacterial and bovine enzyme preparations were investigated by turbidimetry and viscosimetry. The results obtained by these physicochemical methods, by which hyaluronidase acitivity is quantified according to the reduction of the size of high molecular mass HA, were supposed to correlate with the spreading effect.

Another goal of this thesis was to get more detailed information about the influence of pH on hyaluronan degradation by BTH and hyaluronate lyase from *S. agalactiae*, respectively. As several methods, differing with respect to the incubation conditions (ionic strength, type of

salt and buffer) and the type of assay, are used for the determination of hyaluronidase activity, the pH activity profiles reported in the literature for BTH are inconsistent in part or even contradictory. To understand the influence of the type of assay, the two most frequently used techniques, the colorimetric assay and the turbidimetric assay, were compared under identical conditions. In addition, anion exchange HPLC and CE analysis of the oligosaccharide mixtures produced by the digestion of HA with BTH at varying pH was performed to detect pH-dependent differences in the mechanism of HA degradation by BTH. Additionally, the degradation of hyaluronan by hyaluronate lyase from *S. agalactiae* was investigated with respect to the effect of pH on the kinetic parameters V_{max} and K_m in order to find out if the pH dependency observed for the activity of inhibitors of *S. agalactiae* hyaluronate lyase may be explained by a different affinity of the enzyme to the substrate depending on pH.

3 Comparative characterisation of bovine testicular hyaluronidase (BTH) and a hyaluronate lyase from *Streptococcus agalactiae* in pharmaceutical preparations

3.1 Introduction

Preparations of bovine testicular hyaluronidase (BTH) have been applied therapeutically in the fields of orthopaedia, ophthalmology and internal medicine for many years (Baumgartner and Moritz, 1988; Menzel and Farr, 1998). A common field of application of BTH is its addition to local anesthetic agents for ophthalmic anesthesia, as it is known to improve the rapidity of onset, dispersion, depth and duration of the local anesthesia (Kallio et al., 2000). In the course of systemic oncological therapies with vinca alkaloids hyaluronidase is the only highly effective antidote to the extravasation of these antineoplastic agents, especially vinblastine, as it prevents local necrosis by improving the absorption of the paravasate (Bertelli et al., 1994; Albanell and Baselga, 2000). As, in context with the risk of BSE, companies have stopped the production of BTH preparations (e.g. Hylase® "Dessau", Neopermease®, Wydase®) there has been a shortage of this drug having an unique mechanism of action. As a consequence of the depleted supplies of pharmaceutical BTH preparations in surgical facilities a number of cases of postoperative complications, such as strabismus, diplopia and ptosis are reported in the context with cataract surgeries carried out after periocular anesthesia without hyaluronidase (Brown et al., 2001; Jehan et al., 2001).

Considering the various methods used for the isolation and purification of BTH (Borders and Raftery, 1968; Yang and Srivastava, 1975; Lyon and Phelps, 1981) an identical composition of the different commercially available hyaluronidase preparations seems unlikely. However, according to different pharmacopoeia, the enzyme activity of hyaluronidase preparations from mammalian testes is the only test criterion, regardless whether the individual preparation is a single enzyme or a complex mixture of proteins.

In continuation of our preclinical studies on hyaluronidase as an adjuvant in cancer chemotherapy (Spruß et al., 1995; Muckenschnabel et al., 1996), we compared two BTH preparations (Hylase® "Dessau", Neopermease®) with respect to purity (composition) and enzyme activity. To find a substitute for the bovine enzyme preparation, the enzymatically

active constituents of BTH preparations were identified by size exclusion chromatography and characterised with respect to molecular mass, glycosylation and specific activity.

A hyaluronate lyase from *Streptococcus agalactiae*, strain 4755, which can be produced biotechnologically, and therefore in higher purity compared to the testicular extracts, proved to retain enzyme activity after limited proteolysis with trypsin. In view of a potential therapeutic application, such enzymatically active fragments of low molecular mass are of particular interest as they are expected to have more favourable pharmacokinetic properties compared to the parent protein.

3.2 Materials and methods

3.2.1 Chemicals

Hyaluronic acid from *Streptococcus zooepidemicus* was purchased from Aqua Biochem GmbH (Dessau, Germany). Hyaluronic acid from rooster comb and bovine serum albumin (BSA) were from Serva (Heidelberg, Germany). Chondroitin 6-sulphate was a preparation from shark cartilage and purchased from Fluka (Buchs, Switzerland). Chondroitin 4-sulphate from bovine trachea was purchased from Sigma (Deisenhofen, Germany). The investigated hyaluronidases were either approved drugs or enzyme preparations from different sources.

Neopermease®, containing 200 000 units[#] of lyophilised bovine testicular hyaluronidase (4 mg) plus 25 mg gelatin per vial and the stabiliser (25 mg gelatin per vial) were gifts from Sanabo (Vienna, Austria), whereas lyophilised bovine testicular hyaluronidase (50 000 units[#]·mg^{-1}), the basic material of Neopermease®, was kindly provided by Dr. R. Domanig (Biochemie GmbH, Kufstein, Austria).

Hylase® "Dessau", containing 1 500 units[#] of lyophilised bovine testicular hyaluronidase per vial, stabilised with gelafusal (partially hydrolysed gelatin), the dialysed basic material of Hylase® "Dessau" and gelafusal were gifts from Pharma Dessau (Dessau, Germany).

Stabilised hyaluronate lyase, i.e. 200 000 units[#] of lyophilised hyaluronidase (0.572 mg) from *Streptococcus agalactiae,* strain 4755, plus 2.2 mg BSA and 37 mg Tris-HCl per vial, and an

[#] according to the declaration of the supplier

ammonium sulphate precipitate of the hyaluronate lyase from *S. agalactiae* were kindly provided by id-pharma GmbH (Jena, Germany).

All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Eschborn, Germany).

3.2.2 Determination of hyaluronidase activity

3.2.2.1 Colorimetric method

Hyaluronidase activity was measured by colorimetric (Morgan-Elson reaction) determination of N-acetyl-D-glucosamine (NAG) at the reducing ends of sugars liberated from hyaluronic acid according to the method of Reissig et al. (1955). Enzyme activity was quantified by comparing the absorbance of the samples with the absorbance of standard solutions, containing known amounts of NAG (Sigma, Deisenhofen, Germany). The assay was performed as described elsewhere in detail (Muckenschnabel et al., 1998b). The composition of the different incubation mixtures used in the various experiments is given in the following:

incubation mixture 3-I: 100 µl of formate buffer (0.2 M sodium formate, 0.1 M NaCl, pH 3.7), 150 µl of H_2O, 100 µl of BSA (0.2 mg BSA per ml of H_2O: solution 1), 50 µl of substrate (5 mg hyaluronic acid from rooster comb per ml of H_2O: solution 2);

incubation mixture 3-II: 100 µl of citrate-phosphate buffer (solution A: 0.2 M Na_2HPO_4/0.1M NaCl, solution B: 0.1 M citric acid/0.1 M NaCl; solutions A and B were mixed in appropriate portions to adjust the required pH), 150 µl of H_2O, 100 µl of BSA (solution 1), 50 µl of substrate (solution 2);

incubation mixture 3-III: 200 µl of citrate-phosphate buffer, 100 µl of BSA (solution 1), 50 µl of substrate (solution 2);

incubation mixture 3-IV: 150 µl of citrate-phosphate buffer, 150 µl of H_2O, 50 µl of BSA (solution 1), 50 µl of substrate (solution 2).

3.2.2.2 UV difference spectroscopy

The activity of hyaluronate lyase from *S. agalactiae* was additionally determined by quantifying the unsaturated degradation product 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-ene-pyranosyluronic acid)-D-glucose (△DiHA) photometrically at 232 nm according to Greiling (1957). The incubation mixture consisted of 300 µl of citrate-phosphate buffer (see 3.2.2.1.),

150 µl of H_2O, 100 µl of BSA (solution 1, see 3.2.2.1.) and 250 µl of substrate (2 mg hyaluronic acid (HA) from rooster comb per ml of H_2O: solution 3).

To quantify product (△DiHA) formation, a set of matched tandem cuvettes (Hellma®, No. 230-QS, Müllheim, Germany), containing two compartments of equal pathlength (l = 4.375 mm) was used. The described mixture (800 µl) was filled into the left compartment of the sample cuvette, whereas the right compartment of the cell was filled with 300 µl of citrate-phosphate buffer, 400 µl of H_2O and 200 µl of BSA (solution 1). The left compartment of the reference cuvette contained 300 µl of citrate-phosphate buffer, 150 µl of H_2O, 200 µl of BSA solution and 250 µl of substrate (solution 3), the right compartment contained 300 µl of citrate-phosphate buffer, 400 µl of H_2O, 100 µl of BSA (solution 1), and 100 µl of the enzyme (5 units[#], dissolved in BSA (solution 1)). The cuvettes were placed in the sample and the reference beam of the spectrophotometer (Uvikon 930, Kontron Instruments, Eching, Germany) and equilibrated at 37 °C for 10 minutes. After addition of 100 µl of the enzyme into the left compartment of the sample cell and thorough mixing, the increase in absorbance was monitored at 232 nm as a function of time at 37 °C.

From the increase in absorbance (△A) at 232 nm per time (△t) the velocity of the enzymatic reaction was calculated according to the following equation (eq. 3-1):

$$v\ [\mu mol \cdot l^{-1} \cdot min^{-1}] = \triangle A / (\triangle t \cdot \varepsilon \cdot l) \qquad \text{(eq. 3-1)}$$

where $\varepsilon = 4550\ l \cdot mol^{-1} \cdot cm^{-1}$. The molar absorptivity (ε_{232}) of unsaturated hyaluronate disaccharide (△DiHA) was determined at 37 °C by measuring the absorbance of a known concentration of △DiHA (sodium salt, Calbiochem®, Bad Soden, Germany) at 232 nm under the conditions prevalent in the incubation mixture as described above.

3.2.3 Separation of the basic material of Neopermease® by size exclusion chromatography

Size exclusion chromatography was performed on a TSK 2000 SW column (7.5 mm x 600 mm, LKB, Bromma, Sweden) with a model 420 pump, a 430 UV-VIS detector (Kontron Instruments, Eching, Germany) and a Rheodyne 7725 injector, equipped with a 20 µl loop. For separation 0.2 mg of the Neopermease® basic material were dissolved in 20 µl of mobile

[#] according to the declaration of the supplier

phase (25 mM KH_2PO_4, 25 mM K_2HPO_4, 0.2 M NaCl, pH 6.7) and applied onto the column, which was equilibrated with mobile phase for at least 2 h. Intensity was monitored at 210 nm at a flow rate of 0.2 ml/min.

3.2.4 Dialysis of the ammonium sulphate precipitate of the hyaluronate lyase

The ammonium sulphate precipitate of the hyaluronate lyase from *S. agalactiae* was desalted by dialysis against 10 mM Tris-HCl, pH 7.5, using a dialysis bag with a pore size of 5000 Da (Zellu Trans, Roth, Karlsruhe). After dialysis the enzyme preparation was aliquoted and stored at –20 °C until use.

3.2.5 Determination of protein content

The amount of protein in the investigated enzyme preparations and the gel filtration fractions was determined by the method of Bradford (1976) using the Bio-Rad protein assay with BSA as standard. The assay was carried out according to the supplier's instructions. In case of Neopermease® the concentration of the stabiliser was taken into account by preparing the BSA standards in the presence of identical gelatin concentrations. As the amount of gelafusal in Hylase® "Dessau" is not declared by the manufacturer, it was proven that a concentrated gelafusal solution does not interfere with the Bradford protein assay.

3.2.6 Molecular mass determination

The molecular mass of the proteins in the enzyme preparations and the gel filtration fractions of Neopermease® basic material was determined by SDS-PAGE under reducing conditions. Electrophoresis was performed in a Mini-Protean II Electrophoresis System (Bio-Rad, München, Germany) following the protocol of Laemmli (1970). Molecular masses were assigned by a set of molecular mass standards (Dalton Mark VII-L in case of the bovine enzymes, and a high molecular mass marker in case of the bacterial enzyme, respectively (Sigma, Deisenhofen, Germany)).

3.2.7 Identification of glycosylated proteins

After SDS-PAGE glycoproteins were stained by the periodic acid-Schiff (PAS) technique according to the procedure of Zacharius et al. (1969).

3.2.8 Identification of IgG

IgG was identified by precipitation with anti bovine IgG antiserum (whole molecule, developed in goat, Sigma, Deisenhofen, Germany) according to the Ouchterlony method as described by Drews (1983).

3.2.9 Isoelectric focussing (IEF)

For IEF, carried out with a PhastSystem apparatus (Pharmacia Biotech, Freiburg, Germany), 0.6 µg of the dialysed bacterial enzyme were applied on a PhastGel of broad pH range (pH 3.5 – 9.5, Pharmacia Biotech, Freiburg, Germany) at 1570 V, 4.5 mA (7.0 W), at 15 °C for 30 min. Gels were stained with Coomassie/$CuSO_4$ (0.5 g of Coomassie Brilliant Blue R-250 were dissolved in 25 ml of methanol and mixed with a solution of 1 g of $CuSO_4$ in 650 ml of H_2O and 190 ml of acetic acid) for 2 hours prior to treatment with a destaining solution (30 % methanol/7 % acetic acid). The pI values of the enzyme proteins were determined by using a set of pI marker proteins (Broad pI Kit (pH 3.5 - 9.3), Pharmacia Biotech, Freiburg, Germany).

3.2.10 Zymography

Substrate gel electrophoresis of the dialysed bacterial enzyme was essentially performed according to the method described by Cherr et al. (1996). SDS polyacrylamide gels were prepared with the Laemmli buffer system (1970), containing 66.6 µg of hyaluronic acid from *S. zooepidemicus* per ml in the separating gel. The stacking gel did not contain hyaluronic acid. After electrophoresis SDS was removed from the gels by washing with a solution of 2.5 % Triton-X-100 (Sigma, St. Louis, USA), and the gels were incubated in NaCl-containing citrate-phosphate buffer, pH 5.0 (c.f. 2.2.1.), for 30 minutes. To visualize regions of hyaluronan digestion the gels were stained with 0.5 % alcian blue in 7 % acetic acid (pH 2.5) for 1 h, destained in 7 % acetic acid and counterstained with Coomassie Brilliant Blue G-250.

3.2.11 Densitometric analysis

SDS, IEF and substrate gels were analysed with a Bio-Rad gel detection system (GS-710 Imaging Densitometer) using Quantity One quantitation software, version 4.0.3 (Bio-Rad, München, Germany).

3.2.12 Limited proteolysis of the bacterial enzyme

10 µl of the dialysed bacterial enzyme (350 µg/ml) were mixed with 10 µl of trypsin (0.35 µg/ml, dissolved in 25 mM Tris-HCl, pH 7.5) and incubated at 37 °C. After defined incubation times (0 – 7 h, at intervals of 10 min within the first hour, and 30 min, respectively) the proteolytic digestion of the bacterial enzyme was stopped by addition of SDS reducing sample buffer (Laemmli, 1970) and boiling for 2 min prior to separation by SDS-PAGE and SDS substrate-PAGE, respectively.

3.3 Results and discussion

3.3.1 Comparison of the pharmaceutical preparations Neopermease® and Hylase® "Dessau"

3.3.1.1 Quantification of enzyme activity

Enzyme activity was quantified according to the definition of the International Union of Biochemistry, i.e. 1 unit (U) of hyaluronidase catalyses the liberation of 1 µmol N-acetyl-D-glucosamine (NAG) at the reducing ends of sugars per minute under specified conditions. The hydrolysis of hyaluronan was measured under optimised conditions (pH, substrate, NaCl and BSA concentration) after varying incubation periods by the colorimetric method of Reissig et al. (1955). Enzyme activity was calculated from the formation of the red-colored product per unit time, using standards with known NAG concentration (Muckenschnabel et al., 1998b).

One designated unit of Neopermease® amounted to $5.0 \cdot 10^{-5}$ µmol NAG per minute, i.e. 0.050 mU according to the aforementioned definition, whereas in case of Hylase® "Dessau" one unit (as declared by the manufacturer) corresponds to $8.6 \cdot 10^{-5}$ µmol NAG per minute (0.086 mU) in the Morgan-Elson assay. Thus, the equieffective concentrations differ by a factor of 1.7 for the two pharmaceutical preparations.

Comparing the specific activities of these preparations, it becomes obvious that the enzyme activity per mg of protein of Neopermease® is about 12.5 times higher than that of Hylase® "Dessau" (cf. to Table 3-1).

Table 3-1: Comparison of the enzyme activities of Neopermease® and Hylase® "Dessau"

50 µl of enzyme solution (7.5 units[a]) were incubated with incubation mixture 3-I (400 µl) and NAG at the reducing ends was determined as a function of time at intervals of 5 min over a period of 1 h.

Hyaluronidase Preparation	Liberation of NAG per Unit[a] [µmol·min^{-1}]	Specific Activity[b] [µmol·min^{-1}·mg^{-1}]
Neopermease®	$5.0 \cdot 10^{-5}$	2.5
Hylase® "Dessau"	$8.6 \cdot 10^{-5}$	0.23

[a] according to the declaration of the supplier
[b] for calculation the protein content, determined by the method of Bradford, was used

The data are consistent with the results published for mammalian hyaluronidases depending on the degree of purification. Zaneveld et al. (1973) reported in acrosomal extracts from bull spermatozoa a specific hyaluronidase activity of 0.221 µmol·min^{-1}·mg^{-1}, which increased to 3.54 µmol·min^{-1}·mg^{-1} by further purification. Likewise, in purified bull seminal plasma, a specific activity of 3.63 µmol·min^{-1}·mg^{-1} was reported by Srivastava and Farooqui (1979).

3.3.1.2 Effect of pH on enzyme activity

To determine the activity of Neopermease® and Hylase® "Dessau" as a function of pH aliquots of each enzyme preparation were incubated in NaCl-containing citrate-phosphate buffer of varying pH for 1 h at 37 °C, and NAG at the reducing end of the produced hyaluronan fragments was measured (Fig. 3-1).

Within the limits of experimental error both pharmaceutical BTH preparations are characterised by identical pH profiles. Interestingly, the complex curves are asymmetric with a steep increase in activity at low pH and a maximum around pH 3.5. As shown in Fig. 3-1, enzyme activity was measured over a broad range up to pH 8, with a second maximum at pH 5. These pH profiles are similar to those reported by Zaneveld et al. (1973), Yang and Srivastava (1975) and Muckenschnabel et al. (1998a). However, there are reports on maximum BTH activity at weak acidic and neutral pH (Allalouf et al., 1975; Highsmith et al., 1975; Tung et al., 1994) or on the existence of two distinct activity maxima at pH 4 and pH 7, respectively (Frost and Stern, 1997).

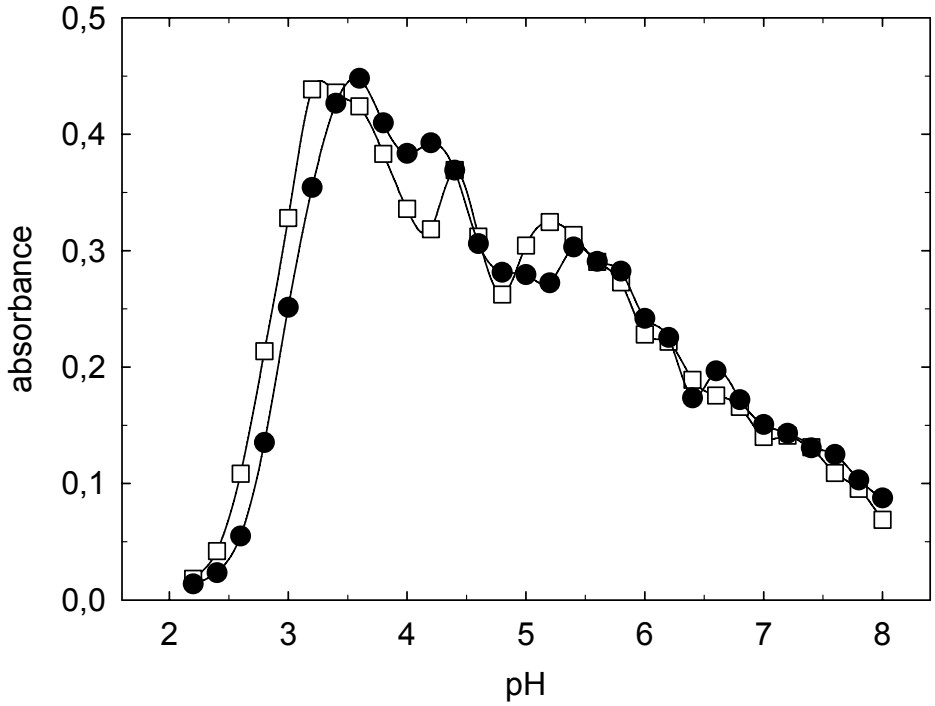


Fig. 3-1: Effect of pH on the activities of Neopermease® (—●—) and Hylase® "Dessau" (—□—). To determine the pH-profiles, aliquots of the enzyme were incubated in the Morgan-Elson assay at 37 °C for 1 h. 50 µl of enzyme solution (10 units, as declared by the supplier) were incubated with incubation mixture 3-II (400 µl).

These discrepancies may result from differences in the enzyme assay (Hoechstetter et al., 2001), especially with respect to the composition of the buffer, which is known to diversely affect the activity of BTH (Gorham et al., 1975; Gacesa et al., 1981). It can also be speculated that the complex pH profiles of Neopermease® and Hylase® "Dessau" may reflect the presence of different enzymatically active proteins.

3.3.1.3 Effect of buffer composition on enzyme activity

Neopermease® and Hylase® "Dessau" were examined with respect to the effect of BSA and NaCl concentration on enzyme activity. For this purpose the activities of the two preparations were determined as a function of the BSA and the NaCl concentration in the incubation mixture. The activities of both preparations were higher in the presence of BSA and NaCl. Optimum enzyme activities were measured at 0.05 - 0.2 M NaCl and around 0.8 mg of BSA per ml, respectively. However, higher concentrations of the investigated additives were shown to exert an inhibitory effect on BTH activity (data not shown).

3.3.1.4 SDS-PAGE

As the enzymatic properties (specific activity, pH profile, activation by NaCl and BSA) of the basic materials of Neopermease® and Hylase® "Dessau", which are devoid of the stabiliser gelatin, proved to be essentially the same as those of the corresponding drugs (data not shown), subsequent studies were performed with the basic materials to avoid a potential interference of the gelatin with electrophoresis. The BTH preparations were separated by SDS-PAGE in order to determine the purity and the molecular mass of the individual proteins. SDS-PAGE revealed that Neopermease® is an inhomogeneous preparation, containing a 58 kDa and a 33 kDa protein as the main constituents after Coomassie staining. Additional bands of very weak intensity correspond to a 77 kDa and a 22 kDa protein, respectively. Compared to Neopermease®, Hylase® "Dessau" appeared to be much more heterogeneous. In addition to two prominent bands of comparable intensity, corresponding to an apparent molecular mass of 54 and 24 kDa, respectively, several weakly stained bands were detected on the gel (Fig. 3-2, right lanes).

Glycoproteins were identified by the periodic acid-Schiff (PAS) technique. The 58 kDa protein in Neopermease® shows the highest staining intensity, whereas the 77 kDa band appears only faint. Likewise, the 93 kDa and 54 kDa components of Hylase® "Dessau" are stained very weakly (Fig. 3-2, left lanes). The fact that in both preparations only the high molecular mass proteins are glycosylated suggests that deglycosylation processes might be involved in the formation of the smaller proteins (Meyer et al., 1997), present in the investigated hyaluronidase preparations.

Densitometric analysis revealed that, although Neopermease® and Hylase® "Dessau" are enzyme preparations with comparable hyaluronidase activity (including activation by NaCl and BSA) and similar pH profiles, they do not contain identical constituents, except for the weakly stained 22 kDa protein (Fig. 3-3).

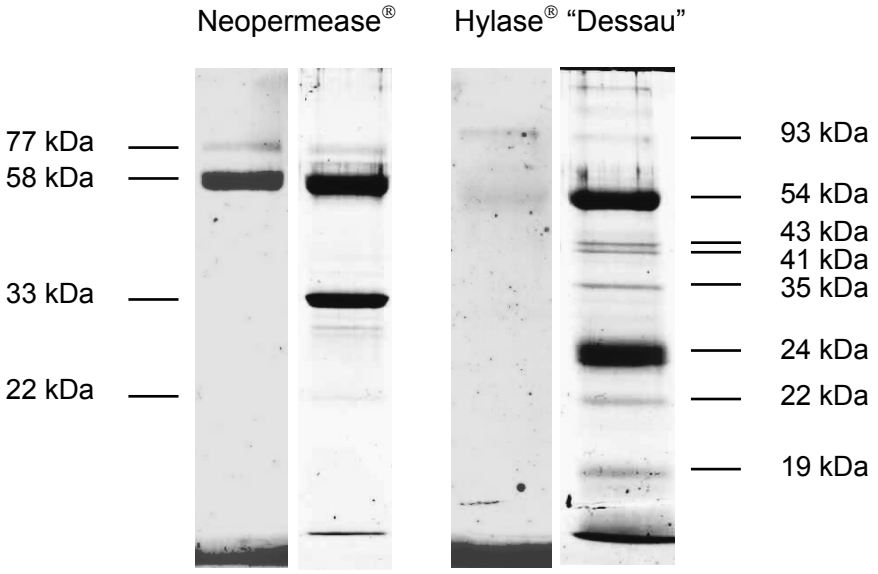


Fig. 3-2: SDS-PAGE of the basic materials of Neopermease® and Hylase® "Dessau". The basic materials of Neopermease® (6.8 µg) and Hylase® "Dessau" (6.5 µg) were separated on a 12.0 % gel. The gel was stained according to the periodic acid-Schiff (PAS) technique (left lane) prior to staining with Coomassie Brilliant Blue G-250 (right lane). Molecular masses were assigned by the use of marker proteins.

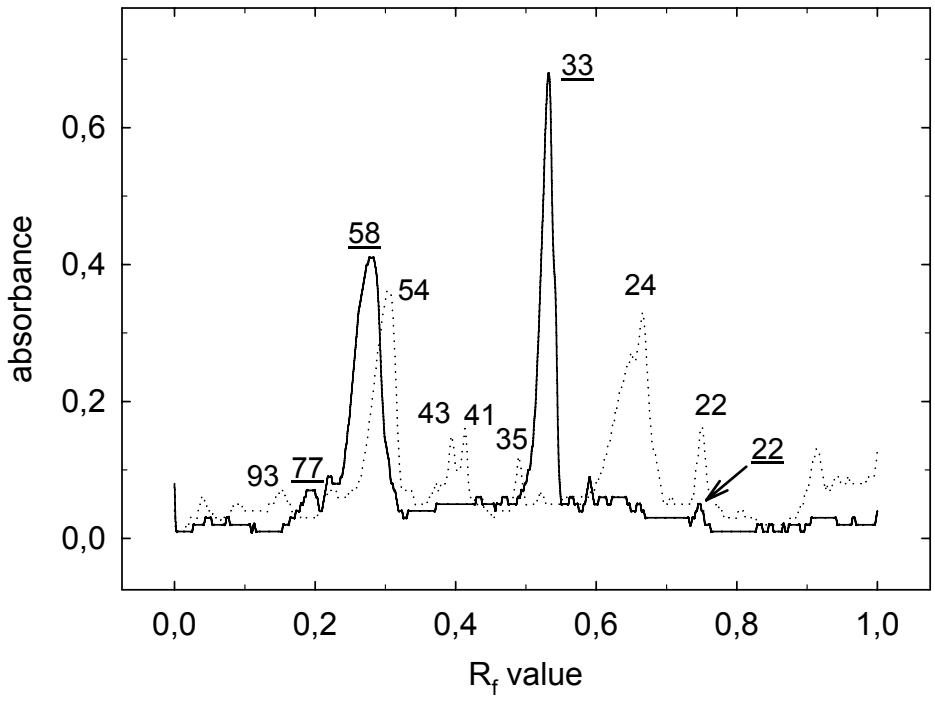


Fig. 3-3: Densitograms of the basic materials of Neopermease® (solid line) and Hylase® "Dessau" (dotted line) after separation by SDS-PAGE (see Fig. 2, right lanes). After staining with Coomassie Brilliant Blue G-250 the gels were subjected to densitometric analysis using the Quantity One quantitation software. The numbers given with the individual peaks indicate the apparent molecular masses (kDa) of the respective proteins.

3.3.1.5 Identification of IgG by immunodiffusion

IgG was identified in both preparations by precipitation with anti-bovine IgG antiserum according to the Ouchterlony method (results not shown). Compared to Neopermease® basic material, the basic material of Hylase® "Dessau" was found to contain an approximately 5 times higher concentration of bovine IgG. Merging of the precipitation lines indicated serological identity.

3.3.2 Characterisation of the individual proteins of Neopermease®

3.3.2.1 Separation of the basic materials of Neopermease® and Hylase® "Dessau" by size exclusion chromatography

To investigate, if the basic material of Neopermease® contained the two enzymatically active proteins described by Meyer et al. (1997), the preparation was separated on a LKB TSK 2000 SW column. The chromatogram (Fig. 3-4) showed three peaks, consistant with the results published by Meyer et al. (1997).

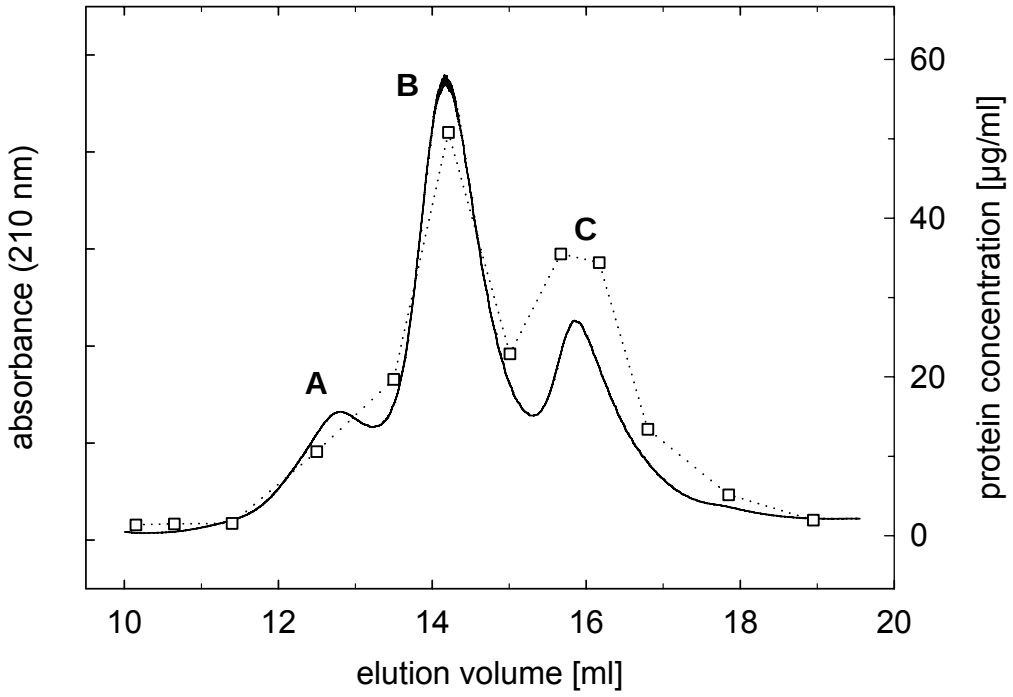


Fig. 3-4: Size exclusion chromatography of Neopermease® basic material. Chromatogram (solid line): 0.2 mg of Neopermease® basic material were applied to a TSK 2000 SW column; intensity was monitored at 210 nm at a flow rate of 0.2 ml/min. The eluate was collected and the protein content of the fractions was determined by the method of Bradford (□, dotted line).

On the contrary, the elution profile for the basic material of Hylase® "Dessau" was more complex showing 5 peaks corresponding to elution volumes of 16, 19, 21, 23.5 and 26.5 ml. Enzyme acitvity was only detected in the 16 and 19 ml fraction with the main activity in the first eluate (data not shown).

Due to the high specific activity of the Neopermease® basic material compared with the basic material of Hylase® "Dessau" three fractions of the former were collected for subsequent characterisation, i.e. the ascending range of peak A, the central range of peak B and the descending range of peak C.

3.3.2.2 SDS-PAGE of fractions from size exclusion chromatography

The molecular mass of the individual proteins present in the different fractions obtained by size exclusion chromatography was determined, i.e. 77 kDa in A, 60 and 58 kDa in B and 33 kDa in C (results not shown). The fact that fraction B contained two proteins of nearly identical molecular mass became obvious by the densitometric analysis of a gel with an acrylamide/bisacrylamide content of 8.1 %.

3.3.2.3 Determination of enzymatic activity in fractions from size exclusion chromatography

Subsequently, enzyme activity of the three fractions was determined by the Morgan-Elson reaction. In Fig. 3-5 activities are plotted as a function of pH. The high and the low molecular mass fractions A and C show comparable pH profiles with a maximum at pH 6 and a shoulder around pH 4, probably due to traces of fraction B (Table 3-2). Fraction B is characterised by two maxima at pH 3.6 and pH 4.8.

The specific activity of the three fractions was determined as described in 3.3.1.1.The highest activity per mg of protein was found in fraction B, whereas the high (A) and low (C) molecular mass fractions showed markedly lower specific activities (Table 3-2). These results can explain the complex effect of pH on the hydrolytic activity of Neopermease® (Fig. 3-1) as the sum of the pH profiles of several proteins, differing in specific activity. With 15.1 µmol·min-1·mg-1 the specific activity of the 58 kDa fraction appears to be considerably high compared to the specific activity (3.36 µmol·min-1·mg-1), which is reported for a 62 kDa bull seminal plasma hyaluronidase (Srivastava and Farooqui, 1979). However, it is in good agreement with the results of Lyon and Phelps (1981), who determined a specific activity of

19.1 µmol·min-1·mg-1 for a purified BTH preparation consisting of two components with molecular weights of 61 kDa and 67.2 kDa, respectively.

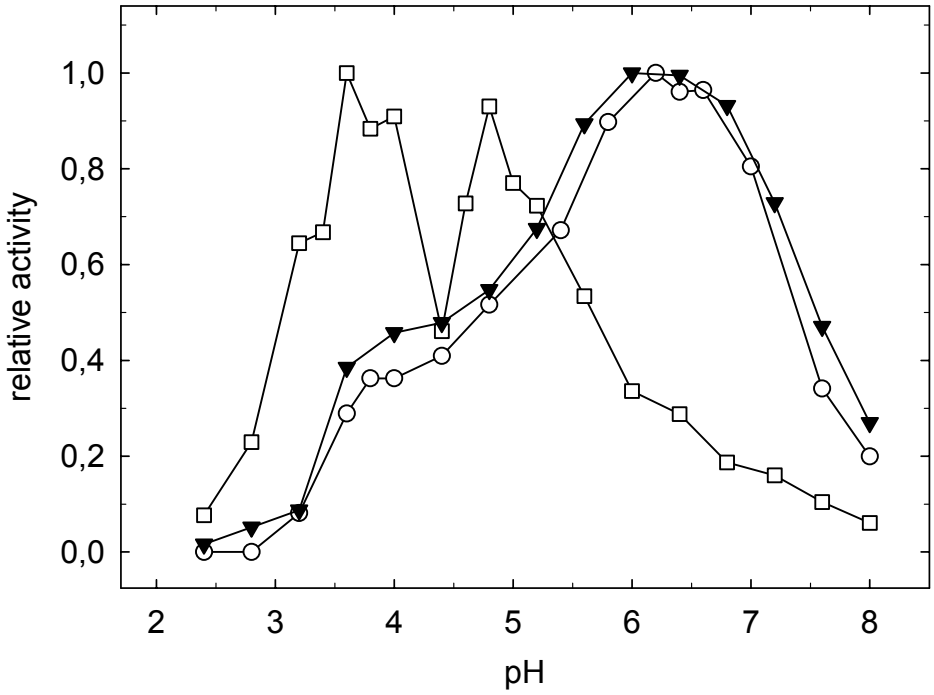


Fig. 3-5: Activity of the size exclusion chromatography fractions as a function of pH. Samples of each fraction were tested for hyaluronidase activity by the Morgan-Elson reaction. 100 µl of eluate were incubated with incubation mixture 3-III (350 µl). (fraction A: —○—; fraction B: —□—; fraction C: —▼—).

Table 3-2: Characterisation of the various eluate fractions after gel filtration of Neopermease® basic material

NAG at the reducing ends was measured as a function of time at optimal pH in each fraction, and the protein content was determined. 100 µl of eluate were incubated with incubation mixture 3-III (350 µl).

Fraction	Apparent molecular mass[a] (kDa)	Optimum pH	Specific activity [µmol·min^{-1}·mg^{-1}]
A	77	6.2	3.6
B	58	3.6 4.8	15.1 8.8
C	33	6.2	0.5

[a] determined by SDS-PAGE

3.3.3 Characterisation of a hyaluronate lyase from S. agalactiae

3.3.3.1 Quantification of enzyme activity

As hyaluronan degradation by hyaluronate lyase from group B streptococci yields an unsaturated disaccharide (△DiHA) as product (Pritchard et al., 2000), enzymatic activity was measured at 232 nm. When measured under similar conditions as used for the bovine enzyme one designated unit of the stabilised bacterial hyaluronidase catalysed the formation of $2.8 \cdot 10^{-3}$ µmol △DiHA per minute, which is equivalent to 2.8 mU according to the definition of the International Union of Biochemistry (cf. section 3.3.1.1). The specific activity was 980 U per mg for the stabilised enzyme preparation and 1080 U per mg for the ammonium sulphate precipitate (table 3-3). To compare the activities of the bacterial hyaluronidase preparation and the two BTH preparations, the activity of the bacterial enzyme was also determined by the Morgan-Elson reaction.

Table 3-3: Activity of hyaluronate lyase from *S. agalactiae*

Enzyme activity was determined by UV difference spectroscopy and by the colorimetric method at optimum pH (pH 5) under identical conditions, which are described in the materials and methods section. In case of the colorimetric method 50 µl of enzyme (1 unit[a] dissolved in BSA (solution 1)) were incubated with incubation mixture 3-IV (400 µl).

Method	Product formation per unit[a] of the stabilised hyaluronate lyase [µmol·min^{-1}]	Specific activity [µmol·min^{-1}·mg^{-1}]	
		Stabilised hyaluronate lyase[b]	Ammonium sulphate precipitate[c]
UV difference spectroscopy	$2.8 \cdot 10^{-3}$	980	1080
Colorimetric method	$2.9 \cdot 10^{-3}$	1015	1150

[a] according to the declaration of the supplier
[b] for calculation the amount of enzyme protein per vial, as indicated by the supplier, was used
[c] for calculation the protein content, determined by the method of Bradford, was used

As shown in table 3-3 the results obtained by the different methods are in good agreement. As one designated unit of the stabilised bacterial hyaluronidase catalysed the formation of $2.9 \cdot 10^{-3}$ µmol of product per minute in the colorimetric assay, the equiactive concentration of

the bacterial enzyme preparation was by a factor of 60 and 35 lower, when compared with the two pharmaceutical preparations Neopermease® and Hylase® "Dessau", respectively. However, equiactive concentrations with respect to product formation are not necessarily pharmacologically equieffective considering the different hyaluronan degradation mechanisms of the bovine and the bacterial enzyme. While hyaluronan degradation by hyaluronate lyase from group B streptococci involves an initial random endolytic cleavage followed by rapid exolytic and processive release of unsaturated disaccharide (Baker and Pritchard, 2000; Li and Jedrzejas, 2001), the bovine enzyme acts as an endolytic enzyme (Vercruysse et al., 1994) with tetra- and hexasaccharides as the main degradation products (Cramer et al., 1994; Takagaki et al., 1994). Thus, every cleavage of hyaluronic acid chains by BTH results in the formation of two shorter chains, whereas in case of the bacterial enzyme the chain is shortened by only one disaccharide per cleavage step. In view of the different catalytic mechanisms, the administration of equiactive amounts of the two hyaluronidases as a spreading factor should result in a faster reduction of the viscosity of the extracellular matrix in case of BTH.

When comparing the preparations with respect to their specific activities, the bacterial hyaluronidase preparation was 410 times more active than Neopermease® and 5100 times more active than Hylase® "Dessau".

3.3.3.2 Effect of pH on enzyme activity

By analogy with the BTH preparations the activity of the hyaluronidase from *S. agalactiae* was determined as a function of pH. Aliquots of the enzyme were incubated in NaCl-containing citrate-phosphate buffer of varying pH for 1 h at 37 °C, and NAG at the reducing end of the produced hyaluronan fragments was measured. Under identical incubation conditions the influence of pH on the initial velocity of the enzyme reaction was investigated by monitoring the product formation at 232 nm. The pH profiles obtained with both methods were identical as shown in Fig. 3-6.

The highest hydrolytic activity was determined at pH 5.0. At acidic pH a marked decrease in enzyme activity was observed within 1 pH unit, resulting in approx. 10 % of the maximum activity at pH 4.0, whereas considerable activity was detected at neutral pH with 45 % of the maximum activity at pH 7.0. In this context it is noteworthy that Ozegowski et al. (1994) reported maximal activity at pH 6.3 for a 116 kDa hyaluronate lyase from *S. agalactiae*.

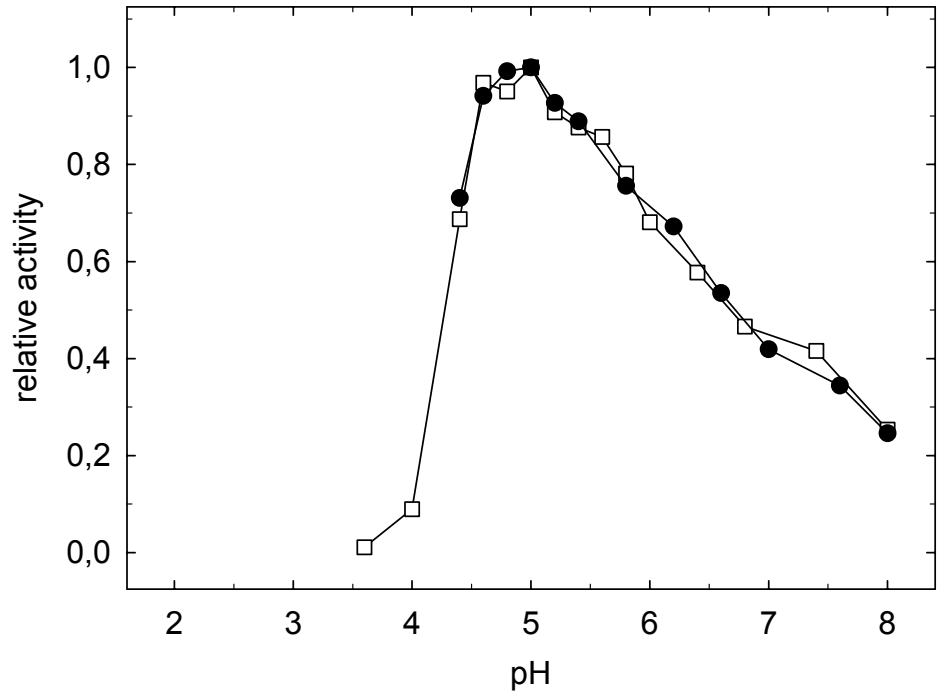


Fig. 3-6: Effect of pH on the activity of *S. agalactiae* hyaluronate lyase. Activity of the stabilised hyaluronate lyase was determined as a function of pH by UV difference spectroscopy (—●—) and by the colorimetric method (—□—) using the incubation conditions which are described in the materials and methods section. In case of the colorimetric method 50 µl of enzyme (1 unit (as declared by the supplier) dissolved in BSA (solution 1)) were incubated with incubation mixture 3-IV (400 µl).

3.3.3.3 SDS-PAGE

To obtain information about the purity of the bacterial hyaluronidase preparations and the molecular mass of the contained proteins the stabilised hyaluronate lyase and the ammonium sulphate precipitate were separated by SDS-PAGE.

In addition to the intense broad band of BSA (66 kDa), which is present in the stabilised preparation, the Coomassie stained gel shows two prominent bands corresponding to a protein with an apparent molecular mass of 85 kDa and 100 kDa, respectively (Fig. 3-7A, left lane). Bands of relatively poor staining intensity were assigned to 46 kDa and 55 kDa proteins. The weakly stained high molecular mass proteins (215 kDa, 255 kDa) may represent dimers and trimers of the two main components. SDS-PAGE of the ammonium sulphate precipitate revealed the existence of only two proteins of an apparent molecular mass of 100 and 85 kDa, respectively, corresponding to the two main components of the stabilised enzyme (Fig. 3-7A, right lane).

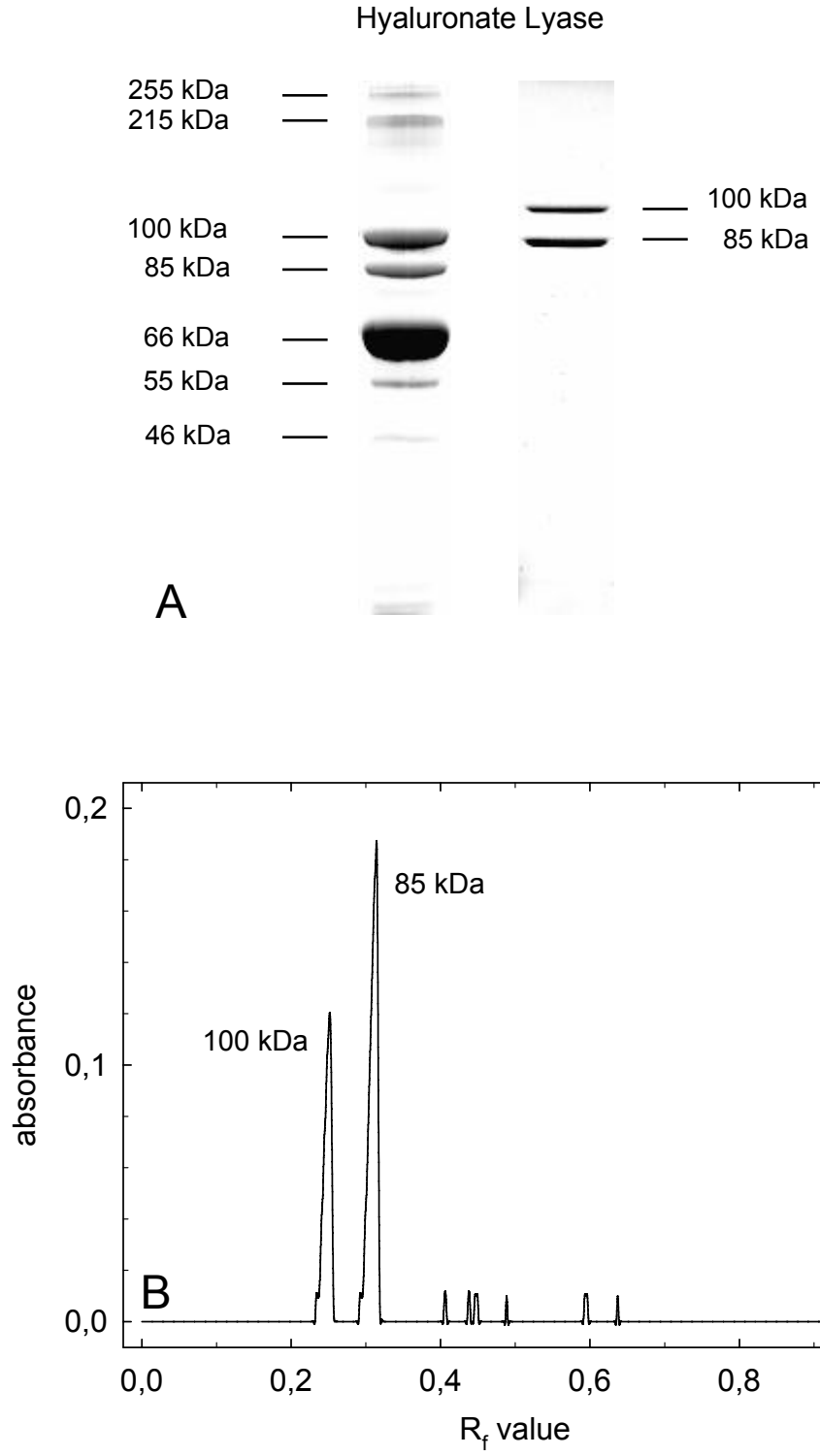


Fig. 3-7: SDS-PAGE of the *S. agalactiae* hyaluronate lyase preparations. A: stabilised hyaluronate lyase (2.15 µg, left lane) and the ammonium sulphate precipitate (0.9 µg, right lane) were separated on a 7.5 % gel (left lane) and a 8% gel (right lane), respectively. Both gels were stained with Coomassie Brilliant Blue G-250. B: Densitogram of the right lane (ammonium sulphate precipitate).

No additional protein components were detected on the gels, neither by Coomassie nor by the more sensitive silver staining technique (results not shown). Densitometric analysis of the Coomassie stained gel (Fig. 3-7B) shows that the amount of the 85 kDa protein in the ammonium sulphate precipitate is about 60 % higher compared with the 100 kDa protein. The symmetry of the peaks indicates a high purity of the two proteins. The fact that hyaluronate lyase preparations from *S. agalactiae* consist of two different proteins, has also been reported by Gase et al. (1998) and Jedrzejas and Chantalat (2000), although the assigned molecular masses differ for the individual proteins.

3.3.3.4 Determination of pI

By means of isoelectric focussing (IEF) the ammonium sulphate precipitate was separated into two proteins corresponding to pI values of 9.2 and 8.9 (results not shown). Densitometric analysis of the IEF gel revealed that the band at pH 9.2 showed a considerably higher staining intensity than the band at pH 8.9. Combining the IEF results with the results of SDS-PAGE (Fig. 3-7) the polypeptide with an apparent pI of 9.2 corresponds to the 85 kDa protein, whereas the band with an apparent pI of 8.9 corresponds to the 100 kDa protein. Interestingly, the densitometric analysis revealed that the band at pI = 9.2 consists of at least three proteins with slightly different pI values, which were not resolved by SDS-PAGE. For the 116 kDa hyaluronate lyase from *S. agalactiae* Ozegowski et al. (1994) determined a pI value of 8.75.

3.3.3.5 Detection of enzymatically active proteins by zymography

As the ammonium sulphate precipitate was found to contain two proteins, which differ with respect to molecular mass and pI, it was of interest to find out if both proteins were enzymatically active. For this reason the capability of the two proteins to degrade hyaluronic acid was investigated by zymography. SDS substrate-PAGE, using hyaluronic acid immobilised in the gel, demonstrated that both the 85 kDa and the 100 kDa protein exhibit enzymatic activity (Fig. 3-8). From the densitogram it can be estimated that the enzyme activity, present in the 85 kDa band, was 25 % higher than that associated with the 100 kDa band, a finding consistent with the different amounts of protein, determined by SDS-PAGE (Fig. 3-7B). In principle, these results are in agreement with the investigations of Gase et al. (1998) and Jedrzejas and Chantalat (2000), who identified two different enzymatically active proteins in hyaluronidase preparations from *S. agalactiae*. However, the apparent molecular masses of the proteins reported by these authors were 94 kDa and 110 kDa (Gase et al., 1998) and 92 kDa and 111 kDa (Jedrzejas and Chantalat, 2000), respectively.

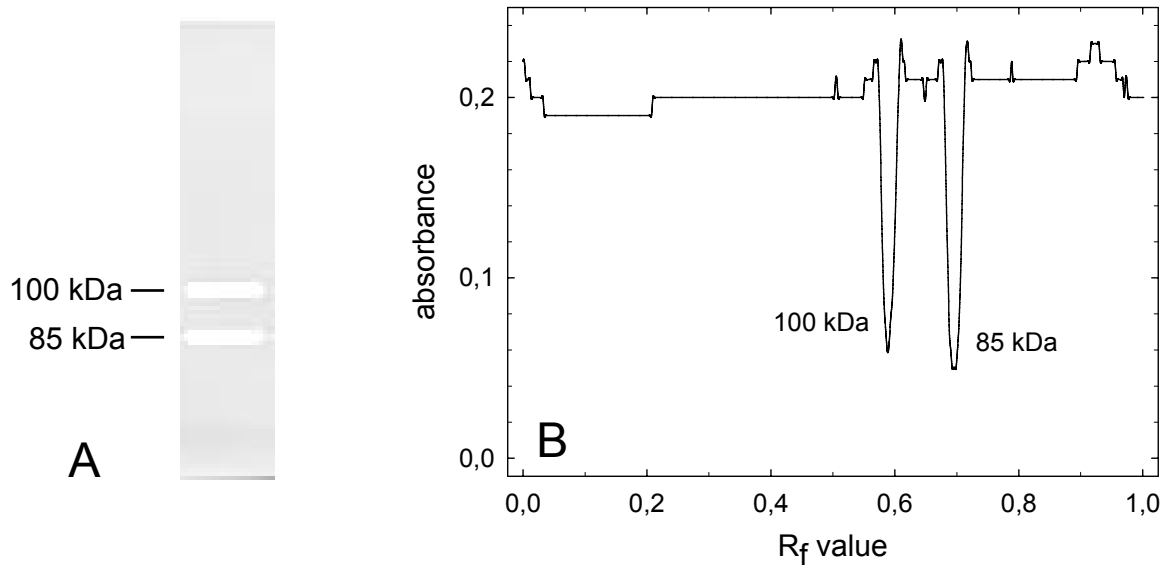


Fig. 3-8: Zymography of the *S. agalactiae* hyaluronate lyase. A: 3.5 ng of the ammonium sulphate precipitate were separated by SDS substrate-PAGE on a 5 % gel. The gel was stained with alcian blue/acetic acid, pH 2.5. B: densitogram of A.

3.3.3.6 Limited proteolysis of S. agalactiae hyaluronate lyase

S. agalactiae hyaluronate lyase is composed of three structural domains which are connected by two peptide linkers: the N-terminal β-sheet domain (βI-domain), the middle α-helical domain (α-domain) and the C-terminal β-sheet domain (βII-domain) (Li and Jedrzejas, 2001). The catalytic region, where the substrate is bound, is located in the α-domain of the enzyme, while the two β-domains are not involved in the hyaluronan degradation process (Li and Jedrzejas, 2001). It can be assumed that removal of one or both of the β-domains by proteolytic cleavage retains the hyaluronidase activity of the residual protein fragment. Such fragments with an expected molecular mass of about 50 to 75 kDa might have more favourable pharmacokinetic properties compared to the holoenzyme. Therefore, limited proteolysis was performed with the ammonium sulphate precipitate, and the products were analysed by SDS-PAGE and zymography. Densitometric analysis of Coomassie stained gels, loaded with samples after various incubation times from 0 to 120 min (trypsin/hyaluronate lyase ratio of 0.001), showed decreasing amounts of the 85 kDa and the 100 kDa protein with increasing incubation time. While the 100 kDa protein was hardly detectable after an incubation time of 120 min, considerable amounts of the 85 kDa protein were present,

reflecting the higher stability of the latter against proteolytic cleavage by trypsin (results not shown). Although Coomassie staining demonstrated a time-dependent decrease in the 100 kDa and the 85 kDa protein, no cleavage products were detected, except for a 20 kDa polypeptide, which became obvious after 90 min. With the more sensitive silver staining method several degradation products with apparent molecular masses between 20 and 75 kDa were detected in samples which had been digested with trypsin for 2 h (results not shown).

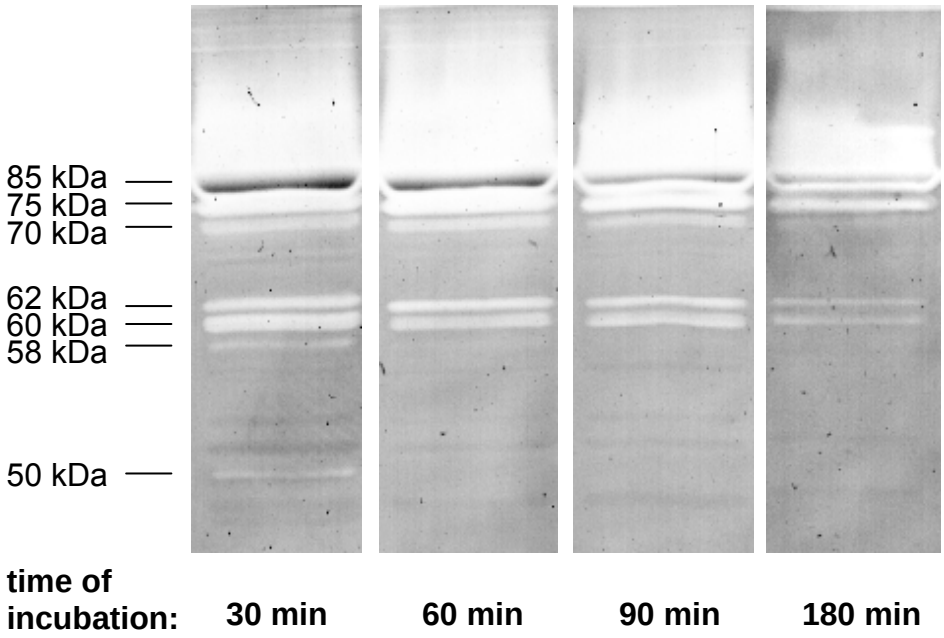


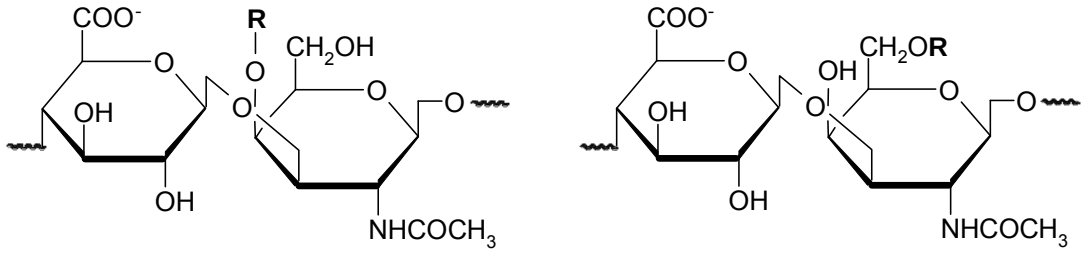
Fig. 3-9: Zymography of the *S. agalactiae* hyaluronate lyase after proteolytic digestion with trypsin. 3.5 µg of the ammonium sulphate precipitate were digested with trypsin (trypsin-hyaluronidase ratio of 0.001) for varying times prior to separation by SDS substrate-PAGE on a 10 % gel. The gel was stained with alcian blue/acetic acid, pH 2.5, and counterstained with Coomassie Brilliant Blue G-250. The unstained areas on the gels are indicative for enzymatic activity, whereas the dark bands are proportional to the protein content.

The respective zymograms (Fig. 3-9) demonstrate that enzyme activity is present in the fragments with molecular masses of 75 kDa, 70 kDa, 62 kDa, 60 kDa and 50 kDa. A comparison of the zymograms of the samples, which had been incubated with trypsin at varying times, indicates that with increasing incubation time the enzymatically active fragments are further degraded to smaller proteins which are devoid of the capabiliy to degrade hyaluronic acid. Interestingly, as the 85 kDa parent protein, the 75 kDa degradation product appears to be quite stable against trypsin cleavage.

3.3.3.7 Activiy against chondroitin 4-sulphate and chondroitin 6-sulphate

For hyaluronidases from mammalian testes it is known that their substrate specificity is not limited to hyaluronan. Other glycosaminoglycans, i.e. chondroitin, chondroitin 4- and 6-sulphate and, to a limited extent, dermatan sulphate, are also degraded by BTH, albeit at a much slower rate (Meyer, 1971; Saitoh et al., 1995).

To find out, if the *S. agalactiae* hyaluronate lyase acts also on other glycosaminoglycans of the extracellular matrix, the capability of the enzyme to degrade preparations of chondroitin 4- and 6-sulphate was investigated. The structure of chondroitin differs from that of hyaluronic acid only in the epimeric configuration at the C-4 carbon of the amino sugar, i.e. the N-acetyl-D-glucosamine of hyaluronic acid is replaced by N-acetyl-D-galactosamine. 4- and 6-sulphation of chondroitin occur at the C-4 and C-6 position of the N-acetyl-D-galactosamine (Fig. 3-10). Chains of chondroitin sulphate also contain unsulphated regions. The degree of sulphation and the arrangement of sulphated and unsulphated disaccharide repeats vary depending on the tissue source of the respective chondroitin sulphate (Baker et al., 1997).



chondroitin 4-sulphate

Di4S (R = SO_3^-)
DiOH (R = H)

chondroitin 6-sulphate

Di6S (R = SO_3^-)
DiOH (R = H)

Fig. 3-10: Structures of the repeating disaccharide units of chondroitin 4-sulphate (Di4S/DiOH) and chondroitin 6-sulphate (Di6S/DiOH), respectively.

Enzyme activity was determined by UV difference spectroscopy at 232 nm under similar conditions as used for the substrate hyaluronan (standard conditions, see 3.2.2.2). Measurements were performed at pH 5.0 and hyaluronic acid was replaced with preparations of chondroitin 4-sulphate (from bovine trachea) and chondroitin 6-sulphate (from shark cartilage), respectively.

In case of chondroitin 6-sulphate product formation was very slow when using enzyme and substrate concentrations equivalent to the standard conditions (5.56 units[#] of enzyme per ml; 0.56 mg of substrate per ml, results not shown). Even when the concentrations of enzyme and substrate were increased to 55.6 units[#] per ml and 33.3 mg per ml, respectively, the increase in absorbance at 232 nm per unit time was about tenfold less compared to the measurements with hyaluronic acid as substrate under standard conditions (Fig. 3-11A).

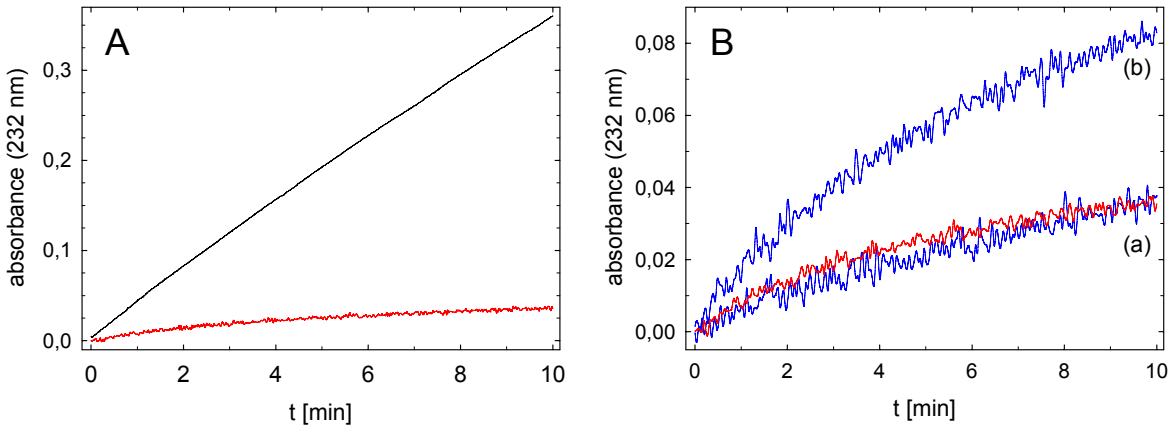


Fig. 3-11: Activity of the *S. agalactiae* hyaluronate lyase against various substrates determined by UV difference spectroscopy (cf. Section 2.2.2.) using various incubation conditions with respect to the concentration of the stabilised hyaluronate lyase (units[#]/ml) and the respective substrate (mg/ml). A: black line (—): HA from rooster comb (0.56 mg/ml), 5.56 units[#]/ml; red line (—): chondroitin 6-sulphate (33.3 mg/ml), 55.6 units[#]/ml; B: red line (—): chondroitin 6-sulphate (33.3 mg/ml), 55.6 units[#]/ml; blue lines (—): chondroitin 4- sulphate (33.3 mg/ml), (a) 55.6 and (b) 111 units[#]/ml.

Similar results were obtained with chondroitin 4-sulphate. As shown in Fig. 3-11B the absorbance at 232 nm increased at the same rate in the presence of chondroitin 4-sulphate or chondroitin 6-sulphate, when equal concentrations of the respective substrate were applied. Doubling of the enzyme concentration in the incubation mixture resulted in a twofold increase in the velocity of product formation.

The fact that the investigated hyaluronate lyase from *S. agalactiae*, strain 4755, is capable of degrading preparations of chondroitin sulphate at a slow rate is in agreement with the results described in the literature for a hyluronate lyase from *S. agalactiae*, strain 3502. Pritchard et al. (1994) showed that *S. agalactiae* hyaluronate lyase cleaves chondroitin sulphate preferentially at unsulphated regions, and found that the extent of digestion was remarkably increased by prior chemical desulphation of the polysaccharide.

[#] as declared by the supplier

By Baker et al. (1997) it was shown that tetrasaccharides consisting of unsulphated (DiOH) and/or 4-sulphated (Di4S) disaccharide units of chondroitin were cleaved by the hyaluronate lyase from *S. agalactiae*, strain 3502, only when there was no sulphation of the disaccharide unit at the reducing end, i.e. the tetrasaccharides DiOH(1→4)DiOH and Di4S(1→4)DiOH were substrates, whereas DiOH(1→4)Di4S and Di4S(1→4)Di4S were not cleaved. In addition, it is reported by Baker and Pritchard (2000) that tetrasaccharides consisting of 4-sulphated (Di4S) and/or 6-sulphated (Di6S) disaccharide units of chondroitin were cleaved only when there was a 6-sulphated disaccharide unit at the reducing end, i.e. the tetrasaccharides Di6S(1→4)Di6S and Di4S(1→4)Di6S were cleaved, whereas Di6S(1→4)Di4S and Di4S(1→4)Di4S were not.

These results suggest that *S. agalactiae* hyaluronate lyase cleaves the β1,4 linkages of macromolecular chondroitin 4-sulphate (containing unsulphated regions) only when there is no sulphation at the nonreducing side of the β1,4 linkage (see Fig. 3-12A), whereas in polymers of chondroitin 6-sulphate every β1,4 linkage is susceptible to cleavage (see Fig. 3-12B).

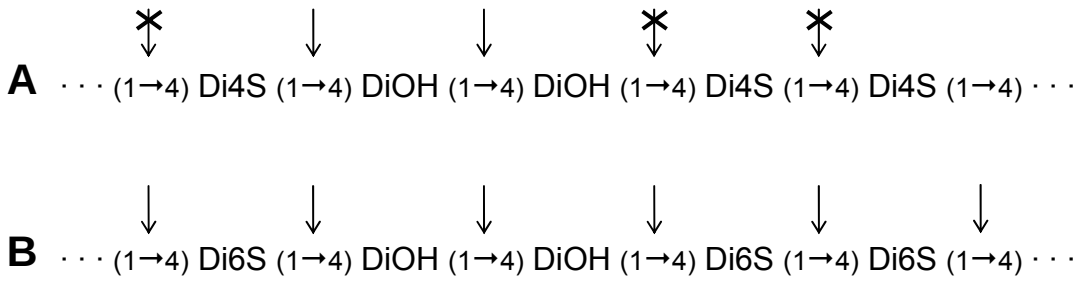


Fig. 3-12: Schematic representation of macromolecular chondroitin 4-sulphate (A) and chondroitin 6-sulphate (B), respectively. The arrows point at the β1,4 linkages susceptible to cleavage by *S. agalactiae* hyaluronate lyase, as suggested by Baker et al. (1997; 2000).

According to the aforementioned considerations chondroitin 6-sulphate is expected to be a better substrate for the hyaluronate lyase from *S. agalactiae* compared to chondroitin 4-sulphate. However, in our experiments no difference in the extent and velocity of product formation was observed during the action of *S. agalactiae* hyaluronate lyase on preparations of chondroitin 4-sulphate and chondroitin 6-sulphate, respectively (Fig. 3-11B).

3.3.3.8 Stability of hyaluronate lyase from S. agalactiae

The effect of different storage conditions on the activity of the stabilised hyaluronate lyase, dissolved in BSA [50 units[#]/ml, i.e. the concentration of the enzyme solution, used for UV difference spectroscopy under standard conditions (cf. Section 3.2.2.2)], was investigated by UV difference spectroscopy.

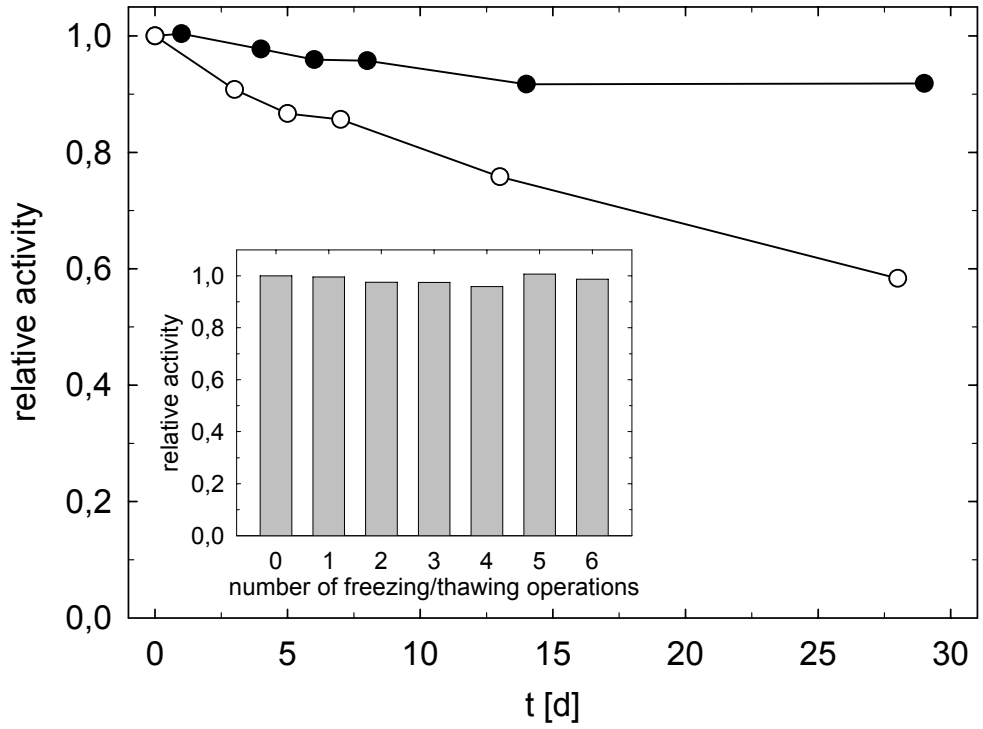


Fig. 3-13: Stability of hyaluronate lyase from *S. agalactiae* under different storage conditions. Hyaluronidase activiy was determined by UV difference spectroscopy (cf. Section 3.2.2.2.) at optimum pH (pH 5.0) after storage of the enzyme solution in portions of 2 ml [50 units[#] per ml of BSA (solution 1)] at 4 °C (—●—) and room temperature (—○—), respectively. *Inset:* Activity of the *S. agalactiae* hyaluronate lyase after repeated freezing and thawing of the enzyme solution (50 units[#] per ml, see above). Immediately after the determination of the enzyme activity, the solution was stored at -26 °C for at least 1 day, before activity was redetermined after thawing of the solution at room temperature.

As shown in Fig. 3-13, 92 % of the activity is retained after 4 weeks, when the enzyme solution is kept at 4 °C, whereas storage at room temperature over the same period results in a continuous decrease of hyaluronidase activity down to 58 % (day 28) compared to the activity measured at the beginning of the experiment (day 0). In addition, it was shown that hyaluronidase activity was not affected by multiple freezing and thawing of the investigated enzyme solution (Fig. 3-13, inset).

[#] as declared by the supplier

These results indicate that the investigated solution of *S. agalactiae* hyaluronate lyase (working solution for UV difference spectroscopy) is quite stable, i.e. almost 100 % of the activity is retained after storage for one day at 4 °C. If necessary, working solutions can readily be stored at -26 °C and used after thawing without loss of activity. However, it should be emphasized that these results are not valid for any hyaluronate lyase solution, as the stability of enzymes in solution generally depends on the concentration of the protein.

3.4 Conclusion

Our results show that Neopermease® and Hylase Dessau® are hyaluronidase preparations with nearly the same enzymatic properties, though identical enzymatically active proteins were not detected. Although the existence of isoenzymes can not be definitely ruled out, the pattern of proteins appears mainly to depend on different partial proteolytic cleavage, e.g. during the isolation and purification procedure.

Bacterial hyaluronidases such as the enzyme from *S. agalactiae* seem to meet the requirements for a pharmaceutical preparation with respect to purity and specific activity, being an appropriate substitute (Ozegowski et al., 2001) for the outdated bovine testicular hyaluronidase.

Moreover, the bacterial enzyme may be advantageous over the bovine enzyme especially as an adjuvant in cancer chemotherapy as hyaluronan degradation by the former does not yield a heterogenous mixture of oligosaccharides and hyaluronan fragments of different size, to which certain biological functions have been ascribed (Noble, 2002), e.g. induction of irreversible phenotypic and functional maturation of dendritic cells during inflammation (Termeer et al., 2000), induction of angiogenesis (West and Chen, 2000) by stimulation of the proliferation and migration of vascular endothelial cells via multple signaling pathways (Slevin et al., 2002). The generation of the aforementioned degradation products of hyaluronan may harbour the risk of increased tumor metastasis and angiogenesis when BTH is applied in combination with antitumor agents.

Furthermore, since enzyme activity is retained in fragments of lower molecular mass, these polypeptides are supposed to have an advantage over the holoenzyme with respect to faster tissue penetration.

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4 Preclinical investigations on the adjuvant administration of a hyaluronate lyase from *S. agalactiae* in cancer chemotherapy

4.1 Introduction

Sufficient penetration of cytostatics into solid tumors is essential for the satisfactory outcome of cancer chemotherapy. In the treatment of solid tumors a major problem results from the fact that a uniform spreading of the chemotherapeutics within the neoplasm is impaired by the elevated intratumoral pressure, which counteracts drug penetration, and by natural barriers such as the basal laminae and the tumor stroma (Jain, 1994).

As a major component of the extracellular matrix hyaluronan (HA) plays a crucial role in controlling diffusion processes (Levick, 1987). Although the prevalence of hyaluronan is not a universal feature of all tumors, numerous cancers, both of epithelial and mesenchymal origins, have been shown to exhibit elevated levels of hyaluronan relative to normal tissues. Highly increased levels of hyaluronan are reported e.g. for human Wilms` tumor and mesothelioma, as well as for malignant breast carcinoma and malignant melanoma (Knudson and Knudson, 1995). The tumor-associated hyaluronan can be synthesised either by fibroblasts, which are induced to increase their HA synthesis following direct cell-to-cell contact with the tumor cells or by a paracrine mechanism, or by the tumor cells themselves (Decker et al., 1989; Knudson and Knudson, 1995). The increased synthesis and deposition of hyaluronan in tumor tissues results in an expansion and hydration of the extracellular matrix and, thus, provides an extracellular milieu, which is supposed to be conducive to cellular invasion and migration (Knudson and Knudson, 1995).

A number of clinical pilot studies are reported, in which hyaluronidase was added to conventional chemotherapy regimens (Baumgartner, 1987; Baumgartner and Moritz, 1988; Smith et al., 1997; Klocker et al., 1998) or combined with radiochemotherapy (Schumer et al., 1990). The adjuvant application of hyaluronidase improved the prognosis of most patients and induced responses in previously chemoresistant patients. The clinically observed recoveries are generally explained by the assumption that degradation of the hyaluronate-enriched extracellular matrix by hyaluronidase reduces the edemas around and within the tumors, thus providing higher effective concentrations of the co-injected chemotherapeutics in the tumor area (Baumgartner and Moritz, 1988; Beckenlehner et al., 1992; Spruß et al., 1995). Indeed,

this assumption could be confirmed by the work of Muckenschnabel et al. (1996; 1998) and Gürtler (2001), who showed that s.c. injected chemotherapeutics were accumulated (3-8fold) by human malignant melanoma, implanted in nude mice, after combined administration of bovine testicular hyaluronidase (BTH).

The reported preclinical and clinical trials using hyaluronidase as an additive in cancer chemotherapy were all performed with preparations of bovine testicular haluronidase, which had been commercially available for therapeutic use at that time (e.g. Neopermease®, Permease®, Hylase® "Dessau"). However, in the context with the risk of BSE, several companies have stopped the production of BTH preparations, which led to a shortage of this drug. To find a substitute for BTH a hyaluronate lyase from *Streptococcus agalactiae*, which can be produced biotechnologically, was recently characterised with respect to purity and specific activity and was found to meet the requirements for a pharmaceutical preparation (Oettl et al., 2003). Furthermore, Ozegowski et al. (2001) reported that hyaluronate lyase from *S. agalactiae* may be useful to improve the tissue absorption of drugs.

In the present work it was therefore investigated in preclinical in vitro and in vivo studies, if bacterial hyaluronate lyase may serve as an appropriate substitute for BTH as an additive in cancer chemotherapy. Especially as an adjuvant in cancer chemotherapy the hyaluronate lyase from *S. agalactiae* may be of advantage over the bovine enzyme, since - in contrast to the hydrolysis of HA by BTH - the degradation of HA by bacterial hyaluronidase does not yield a heterogenous mixture of low molecular mass HA fragments, which are discussed to induce biological effects, which may promote tumor growth and invasion, e.g. increased angiogenesis (Slevin et al., 2002) or enhanced tumor cell motility (Sugahara et al., 2003).

4.2 Materials and methods

4.2.1 Chemicals

Eagle's minimum essential medium (EMEM) and Mc Coy's medium were from Sigma (Deisenhofen, Germany). Fetal calf serum (FCS) was purchased from Gibco (Eggenstein, Germany). Trypsin/EDTA solution was obtained from Boehringer (Mannheim, Germany). Potassium sorbate was from Merck (Darmstadt, Germany), chloramphenicol was from Sigma (München, Germany). N-phenyl-2,2´-iminodiethanol was purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) was purchased from Serva (Heidelberg,

Germany). Methanol was of HPLC grade (Baker, Groß-Gerau, Germany) and water was purified by a Milli-Q system (Millipore, Eschborn, Germany). All other chemicals were of analytical or HPLC grade and were from Merck.

4.2.2 Drugs

Alkeran® (one vial contained melphalan-HCl equivalent to 50 mg of melphalan and 20 mg of povidone as lyopilized powder) was from Glaxo Wellcome (Bad Oldesloe, Germany).

Melphalan-HCl (ca. 95 %) was purchased from Sigma (Deisenhofen, Germany).

Velbe® (10 mg vinblastine sulphate per vial) was obtained from Lilly (Giessen, Germany).

Stabilised hyaluronate lyase, i.e. 200 000 units[#] of lyophilised hyaluronidase (0.572 mg) from *Streptococcus agalactiae,* strain 4755, plus 2.2 mg BSA and 37 mg Tris-HCl per vial, and the pure stabiliser BSA were kindly provided by id-pharma GmbH (Jena, Germany).

Neopermease®, containing 200 000 units[#] of lyophilised bovine testicular hyaluronidase (4 mg) plus 25 mg of gelatin per vial, was a gift from Sanabo (Vienna, Austria),

4.2.3 Tumor cell lines and culture conditions

The human malignant melanoma cell lines (SK-MEL-3 and SK-MEL-24) and the human glioblastoma/astrocytoma cell line U-87 MG (HTB 14) were purchased from the American Type Culture Collection (ATCC; Rockeville, Md., USA). Cell banking and quality control were performed according to the seed stock concept reviewed by Hay (1988). SK-MEL-24 and U-87 MG cell were grown in EMEM containing L-glutamine, 2.2 g/l $NaHCO_3$ and 110 mg/l sodium pyruvate, whereas SK-MEL-3 cells were grown in McCoy's medium containing 2.2 g/l $NaHCO_3$. FCS was added to the culture media to a final concentration of 10 %. The cells were cultured in a water-saturated atmosphere of 95 % air and 5 % CO_2 at 37 °C in 75 cm^2 culture flasks (Costar, Tecnomara, Fernwald, Germany) and were serially passaged following trypsination using trypsin (0.05 %)/EDTA (0.02 %) solution. Detailed characterisation of the melanoma cell lines with respect to morphology, karyology and proliferation kinetics is described in the work of Christl (1992), whereas the U-87 MG cells were characterised by Altenschöpfer (1998).

[#] according to the declaration of the supplier

For tumor cell transplantation (SK-MEL-3 and -24) into nude mice cells were harvested mechanically, spun down at 100 *g* for 10 min and washed twice with serum-free medium. The cell concentration was adjusted to approx. 10^7 cells per ml of serum-free medium.

4.2.4 Animals and housing conditions

NMRI *nu/nu* mice were randomly bred in the nude mouse laboratory of the university of Regensburg under specified pathogen-free conditions at 25 ± 1 °C, 65 ± 5 % relative humidity, and a 12-h/12-h light/dark cycle (Spruß et al., 1996). Nude mice were fed ad libitum with combined breeding-maintenance diet 1434 (Altromin, Lage, Germany) and water containing 1.3 g/l potassium sorbate and 2g/l chloramphenicol; the pH was adjusted to pH 2.5 with HCl (Fortmeyer, 1981). The animals were housed in macrolon cages of size III (Ehret, Emmendingen, Germany) in groups of 7 animals per cage.

4.2.5 Tumor transplantation

Solid tumors were established in NMRI *nu/nu* mice by injection of about 2 - 3 · 10^6 melanoma cells s.c. into the right flank of the thorax (passage 0). For serial passage the tumors were cut into 2-mm^3 pieces and were transplanted s.c. into the right thoracic flank with a trocar (gauge 13) (passage 1). A detailed description of the procedure is given by Gürtler (2001).

4.2.6 Chemosensitivity assay

A standardised kinetic microassay based on crystal violet staining was used. The technical details of the procedure and the method of evaluating drug action have been described in detail by Bernhardt et al. (1992). In brief: cells were seeded (100 µl/well) in 96-well flat-bottomed microtitration plates (Greiner Bio-One GmbH, Kremsmünster, Austria) at an appropriate density of about 15 cells/ microscopic field (magnification 320 x). The plates were incubated at 37 °C (water-saturated atmosphere; 5 % CO_2). After 42-48 h the culture medium was carefully removed by suction and replaced by fresh medium (200 µl/well) containing drug or pure solvent. Drugs were added as a 1000-fold concentrated stock solution. The hyaluronate lyase preparation and the stabiliser BSA were dissolved in water, whereas Doxorubicin and Vinblastin were dissolved in 70 % ethanol. No influence on the growth characteristics caused by the properties of the organic solvent was observed. On every plate two rows (n = 16) served as controls and two rows (n = 16) were used per drug concentration.

After various times of incubation the culture medium was shaken off and the cells were fixed with glutardialdehyde and stored at 4 °C. At the end of the experiment all plates were stained with crystal violet simultaneously. Absorbance was measured at 578 nm using a Biotek 309 Autoreader (Tecnomara, Fernwald, Germany). Processing procedure, data analysis and evaluation were performed as described previously (Reile et al., 1990; Bernhardt et al., 1992).

Results are presented as plots of absorbance or corrected T/C values versus time of incubation according to T/C_{corr} (%) = 100 · (T – C_0)/(C – C_0), where T is the absorbance of the treated cells, C the absorbance of the controls, and C_0 the absorbance of the cells at the time when drug was added (t = 0). The proliferation curves were fitted with a polynom (3^{rd}), using SigmaPlot (Version 8.0).

4.2.7 In vivo experiments

4.2.7.1 Influence of hyaluronidase on melphalan enrichment in solid melanoma

The influence of hyaluronidase on melphalan enrichment in solid melanomas was investigated according to the experimental design described by Muckenschnabel et al. (1996). The experiments were carried out with NMRI *nu/nu* mice (male and female; mean body weight approx. 25 g) bearing solid human SK-MEL-3 and SK-MEL-24 melanoma, respectively. The average tumor burden was approximately 200 mg. The number of the passage of the tumors used in the different experiments is given in the context with the respective experiments in the results section.

Melphalan (Alkeran® or melphalan-HCl from Sigma) was dissolved in 0.01 N HCl containing 0.9% NaCl immediately before use. The drug was injected in a volume of 0.1 ml/20 g body weight, yielding a dose of 50 µmol/kg. Hyaluronidase (*S. agalactiae* hyaluronate lyase and Neopermease®, respectively) was dissolved in 0.9 % NaCl and also applied in a volume of 0.1 ml/20 g body weight to provide an enzyme activity of 250 000 units[#]/kg and 500 000 units[#]/kg in case of *S. agalactiae* hyaluronate lyase and of 100 000 units[#]/kg in case of Neopermease®.

Melphalan alone or both, melphalan and hyluronidase, were given peritumorally (s.c.) to 5-8 animals per treatment group. In the treatment group of the hyaluronidase/melphalan combination therapy hyaluronidase was injected 1 h prior to melphalan application, whereas

[#] according to the declaration of the supplier

in the control group (melphalan alone) 0.9 % NaCl was injected 1 h prior to melphalan application in a volume of 0.1 ml/ 20 g body weight. Injection of the drugs and the vehicle 0.9 % NaCl, respectively, was carried out in the vicinity of the melanoma ca. 5 mm away from the neoplasms with care being taken to avoid mechanical irritation of the tumor. Exactly 1 h after melphalan administration the tumors were resected, instantly shock-frozen in liquid nitrogen and stored at -78 °C until analysis (cf. 4.2.8).

4.2.7.2 Co-application of hyaluronidase in the regional chemotherapy of malignant melanoma

The chemotherapeutic experiments to study the effect of hyaluronidase on the efficacy of regional chemotherapy of malignant melanoma (SK-MEL-3) were performed according to Spruß et al. (1995) and Gürtler (2001). Solid tumor pieces (approx. 2 mm^3) were implanted subcutaneously on day 0 into the right flank of NMRI *nu/nu* mice (only male animals; 6-8 weeks old in case of the melphalan-experiment; 3-4 weeks old in case of the vinblastine experiment). The treatment was started when the solid tumors were visible, measuring at least 2 mm in diameter (day 8 in both experiments). The regional therapy with either melphalan (vinblastine) alone or in combination with hyaluronidase was carried out weekly (4 therapy cycles) by injecting the agents s.c. in the vicinity of the tumors in a volume of 0.1 ml/20 g body weight. Hyaluronidase was administered 1 h before melphalan (vinblastine). Each treatment group consisted of 12 animals. In the control group (also 12 animals) instead of melphalan (vinblastine) the respective solvents were injected in a volume of 0.1 ml/20 g body weight.

Melphalan (Alkeran®) was dissolved in 0.01 N HCl containing 0.9 % NaCl immediately before use. The drug was injected in a volume of 0.1 ml/20 g of body weight, yielding a dose of 6 mg Alkeran® (4 mg melphalan) /kg. Vinblastine (Velbe®) was dissolved in 0.9 % NaCl and injected in a volume of 0.1 ml/20 g of body weight, yielding a dose of 0.3 mg/kg. Hyaluronidase (*S. agalactiae* hyaluronate lyase) was dissolved in 0.9 % NaCl and also applied in a volume of 0.1 ml/20 g body weight to provide an enzyme activity of 250 000 units[#]/kg (melphalan experiment) and 500 000 units[#]/kg (vinblastine experiment), respectively.

Tumor growth kinetics was recorded by measuring tumor diameters with an electronic caliper twice a week. Tumor areas were calculated as the product of two perpendicular diameters, one

[#] according to the declaration of the supplier

measured across the greatest width of the tumor. To evaluate the tolerance of the therapy the body weight of the animals was documented twice a week. The tumor growth and change of body weight were registered until slight cachexia appeared in several animals, otherwise the experiment was terminated on day 71 (melphalan experiment) or day 66 (vinblastine experiment).

T/C values were calculated according to the following equation:

$$\text{T/C (\%)} = \frac{\text{median tumor area of therapy group (mm}^2\text{)}}{\text{median tumor area of control group (mm}^2\text{)}} \cdot 100 \qquad \text{(eq. 4 - 1)}$$

4.2.8 Quantitative analysis of melphalan in solid tumor samples

Quantification of melphalan accumulation in solid tumor samples was performed according to the HPLC method, which was developed by Muckenschnabel et al. (1996; 1997) and slightly modified by Gürtler (2001).

4.2.8.1 Instrumentation

Melphalan analysis was performed on a stainless-steel column (250 mm x 4 mm I.D.) with a guard column (25 mm x 4 mm i.d.), both packed with 5 µm LiChrosorb RP-18 material (Merck, Darmstadt, Germany). Measurements were carried out on a TSP (tsp Thermo Separation Products GmbH, Egelsbach, Germany) liquid chromatograph consisting of a SN 4000 controller, a P 4000 pump, an SCM 400 degasser, an AS 3000 autosampler and a FL 3000 fluorescence detector. The chromatograms were recorded with the TSP software on a microcomputer running on OS2/Warp.

4.2.8.2 HPLC conditions

The mobile phase consisted of methanol buffer (50:50, v/v) and was degassed with helium prior to use. The mobile phase buffer (MPB) contained 10 mM $NaH_2PO_4 \cdot H_2O$ and 2.3 mM SDS as ion pairing reagent; the pH was adjusted to 3.0 by the dropwise addition of concentrated phosphoric acid. Isocratic analyses were performed at a flow rate of 1 ml/min at 40 °C. As internal standard (I.S.) N-phenyl-2,2´-iminodiethanol was used. Detection of the analytes was carried out fluorimetrically using an excitation wavelength of 250 nm and an emission wavelength of 350 nm.

4.2.8.3 Standards and calibration

Standard mixtures containing melphalan (L-PAM; (S)-3-{4-[bis(2-chloroethyl)amino]-phenyl}alanine), "dihydroxymelphalan" (L-DOH; (S)-3-{4-[bis(2-hydroxyethyl)amino]-phenyl}alanine) and N-phenyl-2,2´-iminodiethanol (I.S.) were used for calibration.

The preparation of the calibration mixtures was carried out as described in detail by Gürtler (2001). In brief: stock solutions were prepared for each of the 3 components: L-PAM: 4 mM, dissolved in methanol; L-DOH: 0.4 mM, prepared by treating melphalan with H_2O at 60 °C for 4 h; I.S.: 0.4 mM, dissolved in H_2O. To preclude hydrolysis reactions, calibration mixtures were freshly prepared by adding certain volumina of the respective stock solutions (L-PAM: 20 µl; L-DOH: 40 µl; I.S.: 110 µl) to 630 µl of mobile phase, adjusted to pH 1.6 with HCl. Appropriate dilution of this calibration mixture with mobile phase (pH 1.6) produced standard concentrations ranging from 100 µM to 1 µM for L-PAM, from 20 µM to 0.2 µM for L-DOH and from 55 µM to 0.55 µM for I.S.

To quantify the melphalan concentration in the tumor samples calibration curves were run daily by injecting 30 µl of each calibration mixture.

4.2.8.4 Sample preparation

The frozen tumor samples (cf. 4.2.7.1) were cut up in an agate mortar with a scalpel, covered with liquid nitrogen and crumbled. The homogenised tumor samples were weighed (approx. 80 - 100 mg) and transferred to glass centrifugation tubes to guarantee efficient cooling during subsequent sonication. After the addition of 1 ml of MPB (pH 3) the crude homogenates were sonicated with a type G 15 sonifier equipped with a microtip (Branson Ultraschall GmbH, Heusenstamm, Germany) at step 4 for 10 min. Sonication was applied in an ice bath and the procedure was interrupted when the temperature of the sample rose above 5 °C. The lysates were centrifuged at 4300 g and 4 °C for 10 min.

200 µl aliquots of the supernatants were diluted with 300 µl of MPB (pH 3) and 25 µl of I.S. solution (50 µM) were added. This mixture was passed through an activated 200 mg LiChrolut reversed-phase RP-18 cartridge (Merck), where the analytes and the I.S. were adsorbed. After washing with 2 ml of MPB (pH 3), the cartridge was left at room temperature for exactly 10 min. Then the compounds of interest were eluted with 1.5 ml methanol and collected in 2-ml polyethylene reaction vessels (Eppendorf, Hamburg, Germany). The solvent was evaporated in a vacuum concentrator (Bachofer, Reutlingen, Germany) at 40 °C. The

solid residue was dissolved in 200 µl of mobile phase, which was adjusted to pH 1.6 with HCl, and aliquots of 30 µl were injected for HPLC analysis. For a more detailed description of the sample preparation procedure see the work of Gürtler (2001).

4.3 Results and discussion

4.3.1 In vitro experiments

As a first approach to characterise the potential efficacy of the adjuvant administration of *S. agalactiae* hyaluronate lyase in cancer chemotherapy, we systematically assayed the effect of hyaluronate lyase on the in vitro chemosensitivity of two different human cancer cell lines (SK-MEL-3 and U-87 MG) against the chemotherapeutics doxorubicin and vinblastine.

4.3.1.1 Effect of hyaluronidase on the cell proliferation of tumor cells in vitro

In a preliminary study the effect of hyaluronate lyase on the cell proliferation of the human SK-MEL-3 malignant melanoma cells and human glioblastoma/astrocytoma cells (U-87 MG) was investigated. Proliferation curves were determined with the kinetic crystal violett assay (cf. 4.2.6) in the presence of increasing concentrations of hyaluronate lyase.

As shown in figure 4-1 (left side), monotherapy with 2700, 27 000 and 80 000 units[#]/l of hyaluronate lyase did not affect the growth kinetics of the investigated cells. Also a solution of pure BSA, which is contained in the hyaluronate lyase preparation as a stabiliser, was found to have no effect on the proliferation of the cells, when applied at a concentration contained in the enzyme preparation at 80 000 units[#]/l (Fig. 4-1, right side).

[#] according to the declaration of the supplier

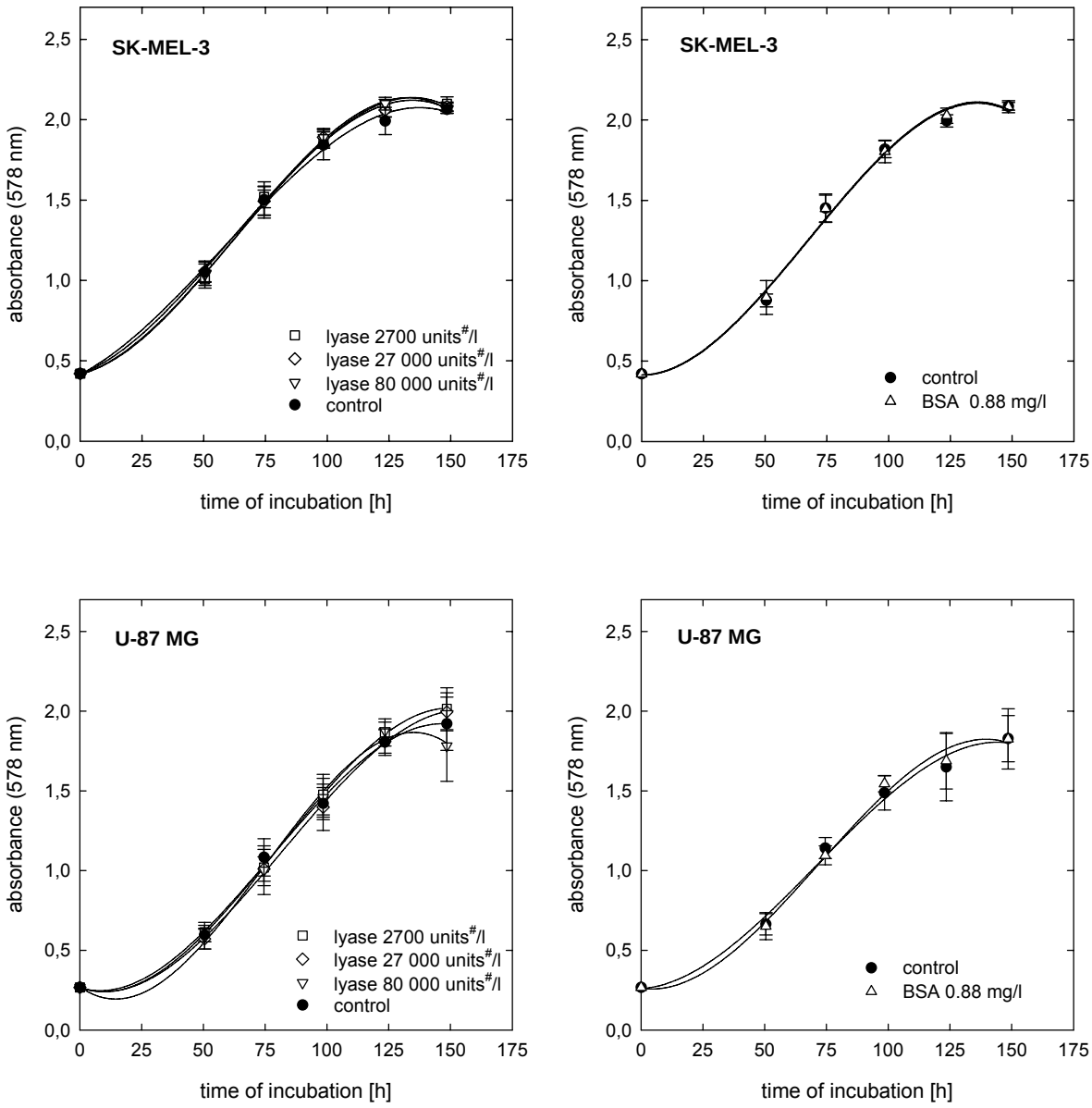


Fig. 4-1: Proliferation curves of SK-MEL-3 cells (passage 7) and U-87 MG cells (passage 137) in the presence of increasing concentrations of *S. agalactiae* hyaluronate lyase (left side) and in the presence of BSA, which is contained in the hyaluronate lyase preparation as a stabiliser (right side). Growth curves were determined with a kinetic microassay based on crystal violet staining (cf. 4.2.6.).

[#] according to the declaration of the supplier

4.3.1.2 Combination therapy with hyaluronidase and doxorubicin in vitro

SK-MEL-3 cells

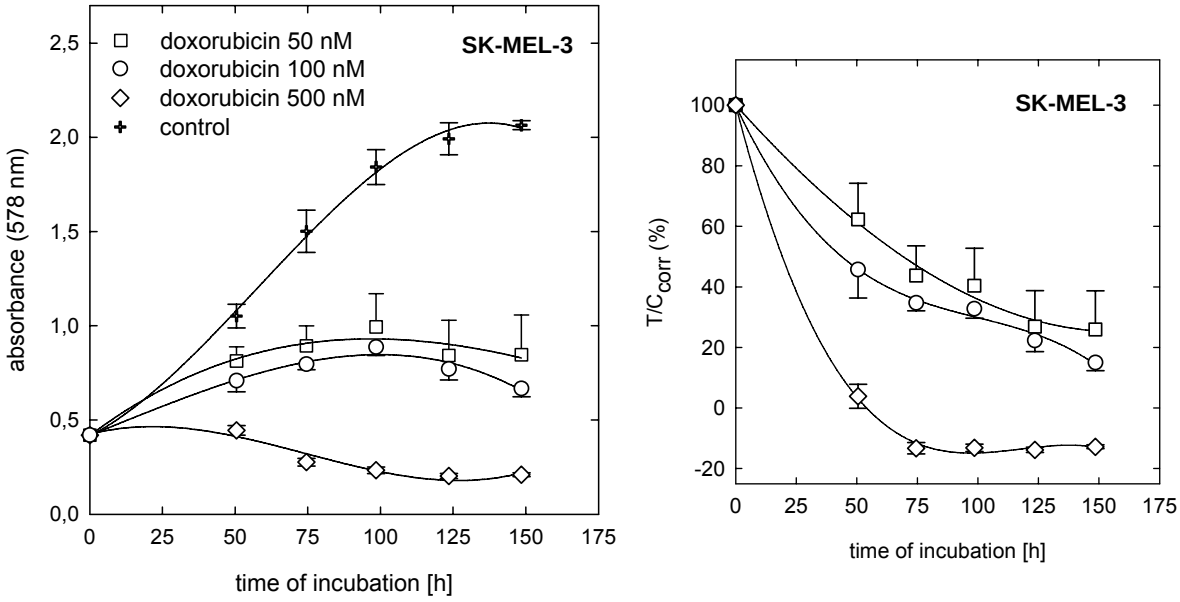
The chemosensitivity of the SK-MEL-3 cell line against different antitumor drugs (doxorubicin, vinblastine, melphalan, methotrexate, cisplatin) was investigated in a previous study by Christl (1992). For doxorubicin a cytocidal effect was determined at a concentration of 1 µM by applicaton of long-term exposure (300 h), whereas at a concentration of 100 nM only cytotoxicity was observed.

In the following experiment it was investigated, if the treatment of SK-MEL-3 cells with a combination of doxorubicin, at suboptimal concentrations, and hyaluronidase leads to an enhancement of the antineoplastic activity of doxorubicin - as it was observed by Beckenlehner et al. (1992), who report on a significant enhancement of doxorubicin activiy by combination with high dose hyaluronidase (Neopermease®) in the therapy of MXT^+ breast cancer cells in vitro and in vivo. In principle, the experiment with the SK-MEL-3 cells was carried out according to Beckenlehner et al. (1992), however, instead of the bovine testicular hyaluronidase (BTH) preparation Neopermease® the bacterial hyaluronate lyase preparation from *S. agalactiae* was used. The hyaluronidase activity applied in our experiment (80 000 units[#] of hyaluronate lyase per liter) was by a factor of 1.6 higher compared to the experiment of Beckenlehner et al. (50 000 units[#] of Neopermease® per liter) according to the activity declaration of the respective supplier. However, taking the results of the investigations concerning the activity of the different enzyme preparations into account (cf. Table 5-3), it turned out, that in the present experiment the hyaluronidase activity was higher compared to the experiment of Beckenlehner et al. by a factor of 6 according to the results of a turbidimetric hyaluronidase assay and even by a factor of 96 according to the results of a colorimetric hyaluronidase assay.

Using the kinetic crystal violet chemosensitivity assay the effects of doxorubicin monotherapy (50 nM, 100 nM, 500 nM) and combination therapy (50 or 100 nM doxorubicin plus 80 000 units[#]/l of hyaluronate lyase) on cell proliferation were compared. The results are summarised in Fig. 4-2.

[#] according to the declaration of the supplier

Doxorubicin monotherapy:



Combination therapy (doxorubicin + hyaluronidase):

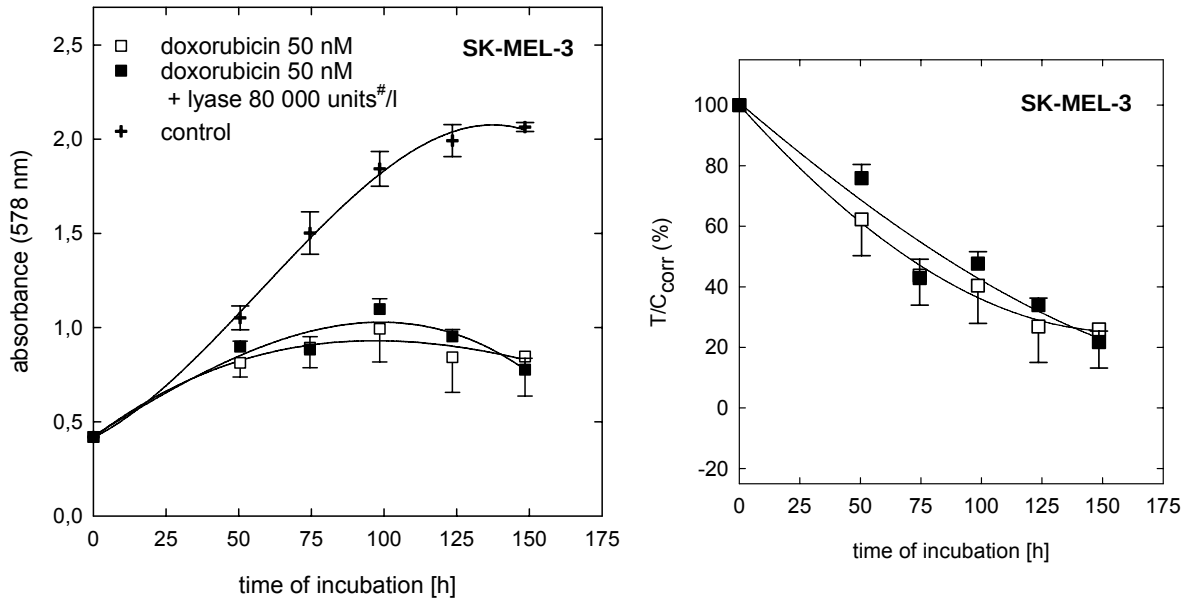


Fig. 4-2: Treatment of SK-MEL-3 cells (passage 7) with doxorubicin alone and with doxorubicin in combination with *S. agalactiae* hyaluronate lyase. Left side: proliferation curves, determined with the crystal violet assay (cf.. 4.2.6); right side: plots of corrected T/C values (cf. 4.2.6) versus time.

[#] according to the declaration of the supplier

As expected from the investigations of Christl (1992), by doxorubicin monotherapy a cytocidal effect was observed at a drug concentration of 500 nM, whereas cytotoxic effects were observed at the lower concentrations of the drug. However, neither the cytostatic effect of 50 nM doxorubicin nor that of 100 nM doxorubicin (results not shown) was enhanced by the combination therapy with high dose hyaluronidase.

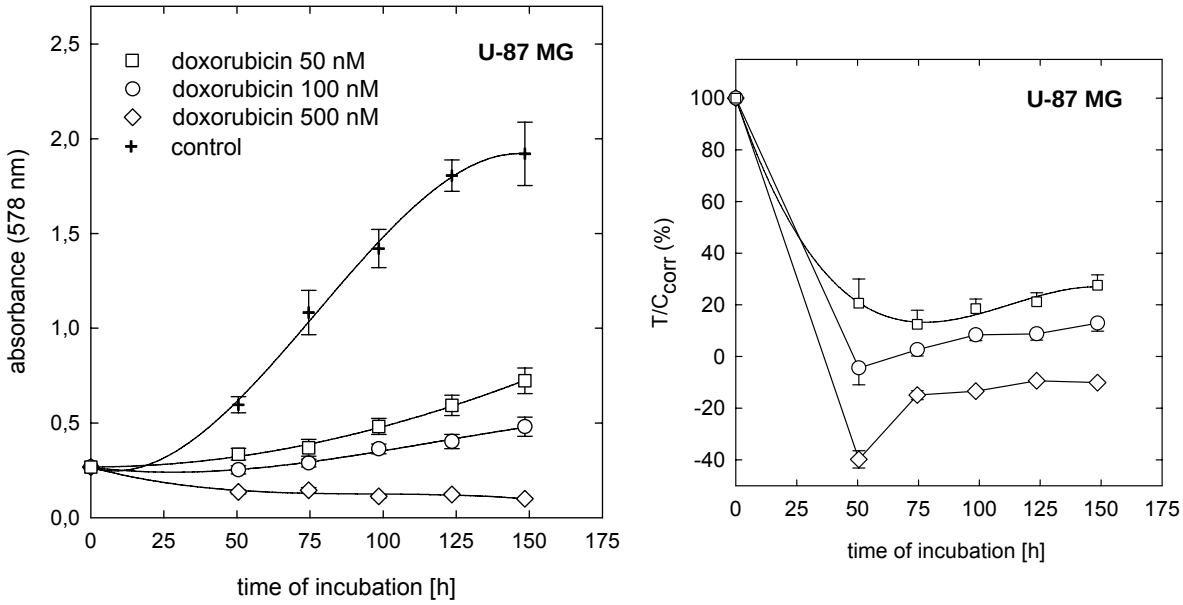
U-87 MG cells

As described for the SK-MEL-3 cell line the human glioblastoma cell line U-87 MG was investigated with respect to the effect of *S. agalactiae* hyaluronate lyase on the chemosensitivity of the cells against doxorubicin. The chemosensitivity of U-87 MG cells against doxorubicin monotherapy had been investigated previously by Altenschöpfer (1998), who reported on a cytocidal effect at a doxorubicin concentration of 1 µM and a cytotoxic effect for 100 nM doxorubicin, respectively.

In the following experiment the effects of doxorubicin monotherapy (50 nM, 100 nM, 500 nM) and combination therapy (50 or 100 nM doxorubicin plus 80 000 units[#]/l of hyaluronate lyase) on the cell proliferation were compared. The results are presented in figure 3. The growth curves obtained with doxorubicin monotherapy are in agreement with the results of Altenschöpfer, i.e. the 500 nM concentration of doxorubicin was cytocidal, whereas at drug concentration of 50 nM and 100 nM, after initial inhibition of proliferation, the cells showed a slight tendency to regain proliferative activity with prolonged incubation time.The cytotoxic effects observed at the 50 nM and 100 nM concentration of doxorubicin were not enhanced by the combination therapy with high dose hyaluronidase. A comparison of the plots of corrected T/C values versus incubation time shows that in the hyaluronidase/doxorubicin (50 nM) combination therapy the initial inhibitory effect on cell proliferation was even less pronounced compared to the doxorubicin (50 nM) monotherapy.

[#] according to the declaration of the supplier

Doxorubicin monotherapy:



Combination therapy (doxorubicin + hyaluronidase):

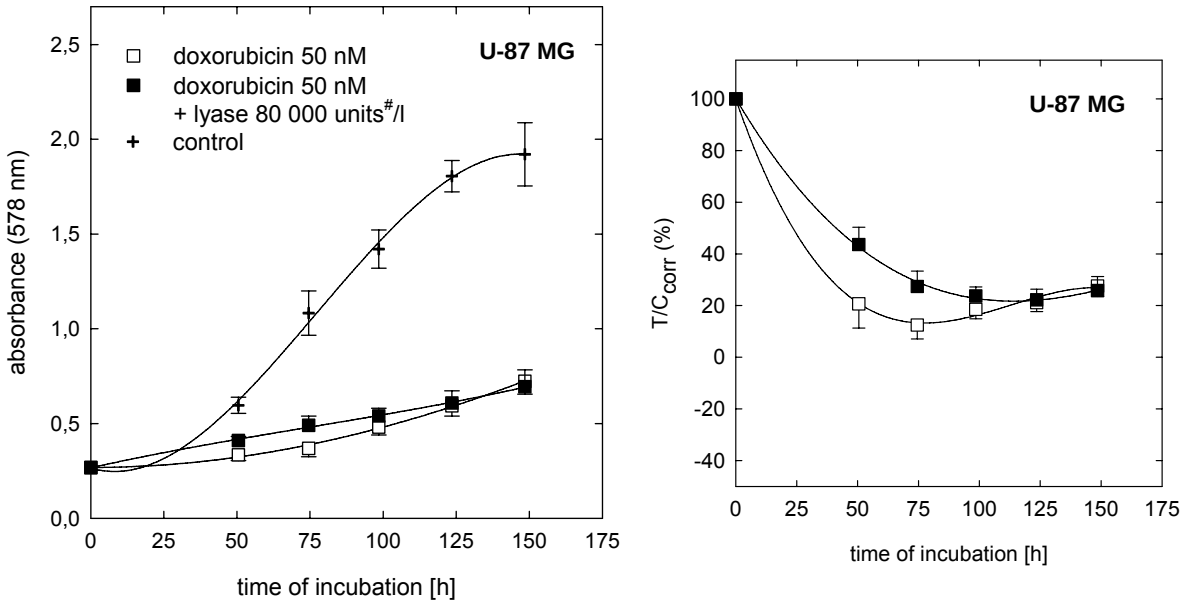


Fig. 4-3: Treatment of U-87 MG cells (passage 137) with doxorubicin alone and with doxorubicin in combination with *S. agalactiae* hyaluronate lyase. Left side: proliferation curves, determined with the crystal violet assay (cf.. 4.2.6); right side: plots of corrected T/C values (cf. 4.2.6) versus time.

[#] according to the declaration of the supplier

4.3.1.3 Combination therapy with hyaluronidase and vinblastine in vitro

SK-MEL-3 cells

In a previous study it was demonstrated on SK-MEL-3 melanoma that the adjuvant administration of hyaluronidase (Neopermease®) enhanced the efficacy of regional vinblastine chemotherapy in vivo (Spruß et al., 1995). Further investigations showed that the concentration of the drug in the SK-MEL-3 tumor, obtained after s.c. peritumoral injection, was enhanced by a factor of 2.5 by the adjuvant s.c. administration of Neopermease® (Muckenschnabel et al., 1998).

In the following experiment it was investigated if the treatment of SK-MEL-3 cells with a combination of vinblastine, at suboptimal concentrations, and hyaluronidase from *S. agalactiae* leads to an enhancement of the antiproliferative activity of vinblastine in vitro. In figure 4-4 the results of vinblastine monotherapy (10 nM, 30 nM, 100 nM) are presented in comparison with those obtained by the vinblastine/hyaluronidase combination therapy (10 nM vinblastine, 80 000 units[#]/l of hyaluronate lyase). In monotherapy vinblastine was cytocidal against the SK-MEL-3 cells at a concentration of 100 nM, whereas at vinblastine concentrations of 30 nM or 10 nM, respectively, after an initial inhibition of cell growth, a slight tendency of the cells to regain proliferative activity was observed. Under combination therapy (vinblastine (10 nM)/ hyaluronidase) the tendency of the culture to recover was slightly lower compared to the 10 nM and 30 nM vinblastine monotherapies, indicating that the chemosensitivity of the SK-MEL-3 cells against vinblastine was slightly increased by hyaluronidase. However, due to the relatively high antiproliferative activity of the 10 nM vinblastine monotherapy per se, only a marginal activity enhancing effect of hyaluronidase could be detected. Therefore, further experiments, using less effective concentrations of vinblastine, would be necessary to confirm an activity enhancing effect of hyaluronidase.

U-87 MG cells

In case of the U-87 MG cell line vinblastine proved to be cytocidal at each of the investigated concentrations (10 nM; 30 nM; 100 nM). It was therefore impossible to detect a potential activity enhancing effect in the combination therapy with hyaluronidase (cf. Fig. 4-4).

[#] according to the declaration of the supplier

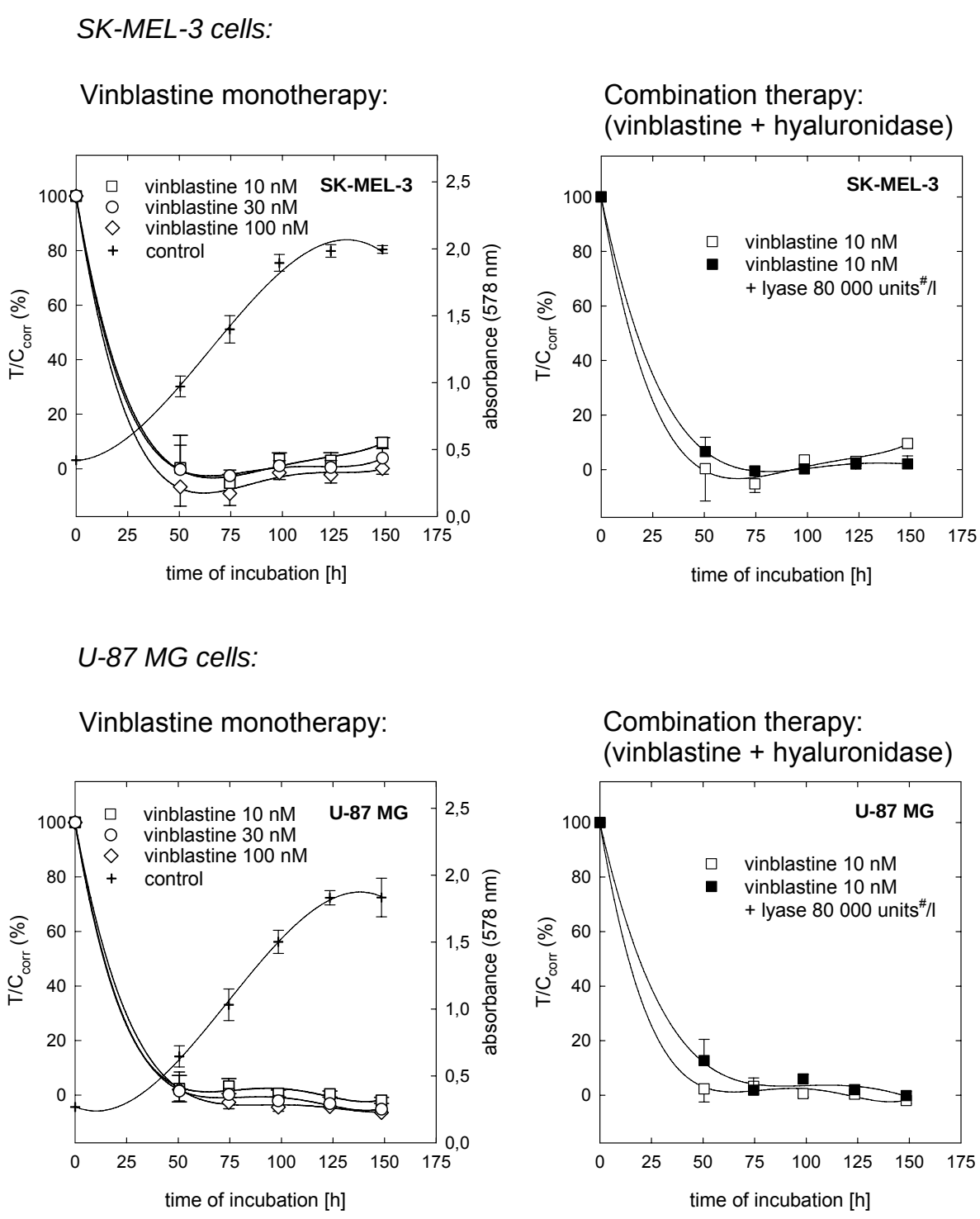


Fig. 4-4: Treatment of SK-MEL-3 cells (passage 7) and U-87 MG cells (passage 137) with vinblastine alone and with vinblastine in combination with *S. agalactiae* hyaluronate lyase. Proliferation curves were determined with the crystal violet assay (cf. 4.2.6). Proliferation curve of the untreated control (**+**); the results of the treatment groups are presented as plots of corrected T/C values (cf. 4.2.6) versus time.

[#] according to the declaration of the supplier

4.3.1.4 Resume of the in vitro experiments

The effect of *S. agalactiae* hyaluronate lyase on the in vitro chemosensitivity of the human cancer cell lines SK-MEL-3 and U-87 MG against the chemotherapeutics doxorubicin and vinblastine was systematically investigated.

From the literature it is known that hyaluronan can influence the processes of mitosis, cell differentiation and cell proliferation in cultured cells in a complex way (Decker et al., 1989; Laurent and Fraser, 1992). Degradation of hyaluronate by treatment of cells with hyaluronidase can thus directly affect proliferation kinetics. However, in case of the cell lines investigated in this work hyaluronidase monotherapy was found to have no effect on the growth kinetics of the cells (cf. Fig. 4-1), suggesting that a potential enhancement of the chemosensitivity of cells against chemotherapeutics by the combination with hyaluronidase can be ascribed to an indirect effect of hyaluronidase, i.e. improved availability of the chemotherapeutics due to degradation of the cell associated hyaluronan.

To compare the monotherapy (doxorubicin, vinblastine) with the combination therapy (hyaluronidase and chemotherapeutic) concentrations of the chemotherapeutic drug, which were not cytocidal per se were used. In case of doxorubicin no enhancement of the antineoplastic activity was observed in the combination therapy compared to the monotherapy - neither against SK-MEL-3 cells nor against U-87 MG cells (cf. figures 4-2 and 4-3). For vinblastine a slight tendency to improved activity was observed against SK-MEL-3 cells, when the drug was combined with hyaluronate lyase, whereas in case of the U-87 MG cells already the lowest concentration of vinblastine showed a cytocidal effect in the monotherapy, which was not improved in the combination therapy (cf. Fig. 4-4).

Summarising the results of the in vitro experiments it was shown that the coapplication of hyaluronidase did not result in an improved antiproliferative activity of the chemotherapeutics - except for the slight but statistically insignificant activity enhancing effect that was observed in case of the vinblastine therapy of the SK-MEL-3 cell line. Compared to the amount of hyaluronan produced in cell culture, the amount of hyaluronan found in solid tumors is often much higher due to overproduction of hyaluronan in the tumor stroma (Knudson and Knudson, 1995). Despite the results obtained in the in vitro experiments it can thus not be ruled out that the adjuvant administration of *S. agalactiae* hyaluronate lyase can significantly enhance the efficacy of the regional chemotherapy of tumors of the investigated cell lines in vivo.

4.3.2 Regional therapy of human malignant melanoma implanted in nude mice

4.3.2.1 Regional therapy with hyaluronidase and vinblastine

In the following in vivo experiment it was investigated if the adjuvant administration of *S. agalactiae* hyaluronate lyase enhances the efficacy of the regional vinblastine chemotherapy of malignant melanoma implanted in nude mice. The experiment was based on the results of Spruß et al. (1995) who showed that in the regional chemotherapy of malignant melanoma a peritumoral pre-treatment with bovine testicular hyaluronidase drastically increased the activity of low-dose vinblastine, administered in the vicinity of the tumor.

Using a vinblastine dose of 0.3 mg/kg body weight (s.c.), which had been shown to be ineffective per se against the SK-MEL-3 tumor by Spruß et al. (1995), the efficacy of vinblastine monotherapy was compared with that of a hyaluronidase/vinblastine combination therapy, in which *S. agalactiae* hyaluronate lyase was administered peritumorally (s.c.) 1 h before vinblastine application. According to the activity declaration of the respective supplier, the hyaluronidase activity applied in our experiment (500 000 units[#] of hyaluronate lyase per kg) was by a factor of 5 higher compared to the experiment of Spruß et al., who used 100 000 units[#] of Neopermease® per kg. Substance administration began on day 8 after tumor transplantation and was repeated weekly. A total of four treatment cycles was performed from day 8 to day 29 and the observation of tumor growth and change of body weight was continued until day 66.

The results of the different therapy regimens are presented in figure 4-5. Other than expected from the results of Spruß et al. (1995), in this experiment in the treatment group which received vinblastine monotherapy a moderate tumor growth delay was observed in comparison to the untreated control (T/C = 37 % (day 28) and 43 % (day 38)). However, the moderate antitumoral activity of vinblastine was not enhanced by pre-treatment with S. agalactiae hyaluronate lyase (T/C = 36 % (day 28) and 29 % (day 38)). Figure 4-5 shows that in the course of the therapy up to day 28 the tumor growth curves were identical for the vinblastine monotherapy and the combination therapy with hyaluronate lyase. Further registration of the median tumor area revealed a slightly delayed tumor growth in the combination therapy compared to the vinblastine monotherapy until day 59. However, in this context it has to be considered that, starting at day 35, animals had to be removed from the experiment due to the occurrence of a slight cachexia or ulcerated tumors.

[#] according to the declaration of the supplier

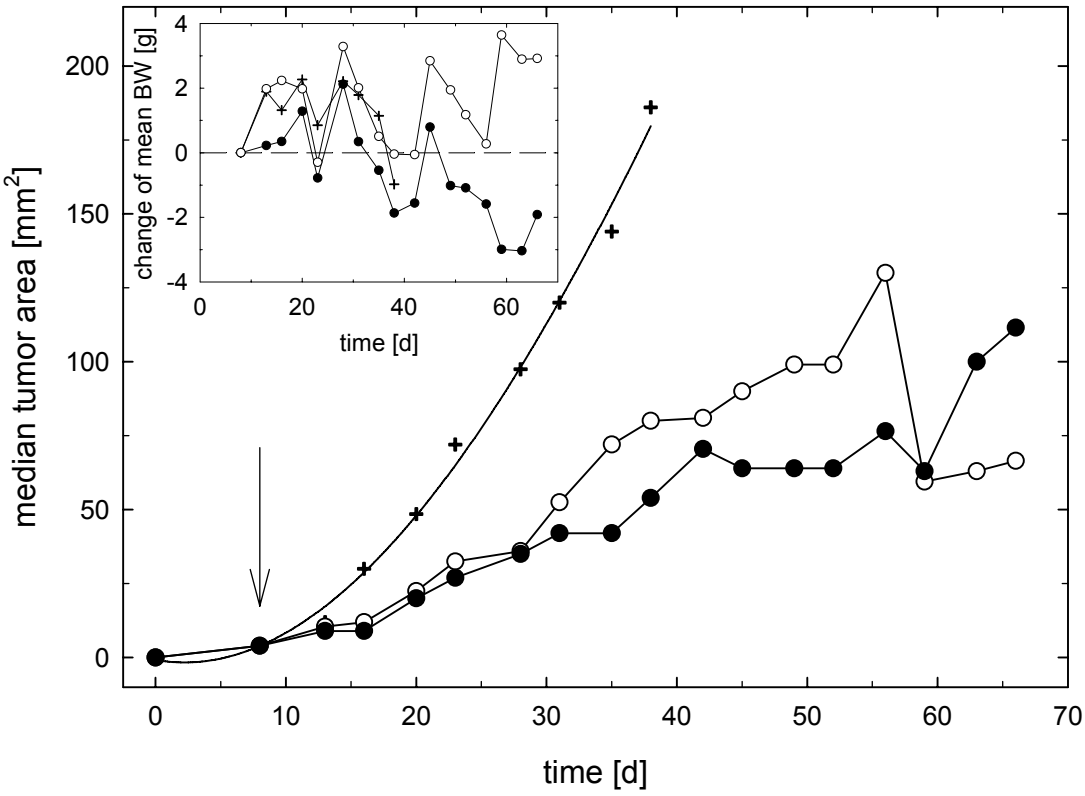


Fig. 4-5: Regional chemotherapy of the human SK-MEL-3 melanoma (passage 34) implanted into NMRI *nu/nu* mice (all drugs administered perimtumorally (s.c.); arrow: start of therapy). Untreated control (—✚—): 0.9 % NaCl (n = 12); vinblastine monotherapy (—○—): vinblastine 0.3 mg/kg (n = 12); combination therapy (—●—): *S. agalactiae* hyaluronate lyase (500 000 units[#]/kg) 1 h prior to vinblastine (0.3 mg/kg) treatment (n = 12). Therapy was carried out weekly (4 treatment cycles). *Inset:* change of mean body weight (BW) during the course of the experiment.

In the group of the combination therapy 6 animals were removed in the period from day 35 to 49, whereas in the vinblastine monotherapy group only 3 animals showed cachexia or ulcerated tumors from day 38 to 45. As these animals were not taken into account for the calculation of the median tumor area of the respective treatment group, beginning at day 35, the growth curves of figure 4-5 do not reflect the efficacy of the therapy without fail.

As indicated in figure 4-6, which shows the tumor growth curves of the individual animals, the slightly delayed increase in the median tumor area of the group receiving combination therapy compared to the monotherapy group (cf. Fig. 4-5) results from the fact that in the combination therapy group two animals bearing large tumors were removed from the experiment. In general, comparison of the tumor growth in the individual animals revealed high interindividual variations in both therapy groups.

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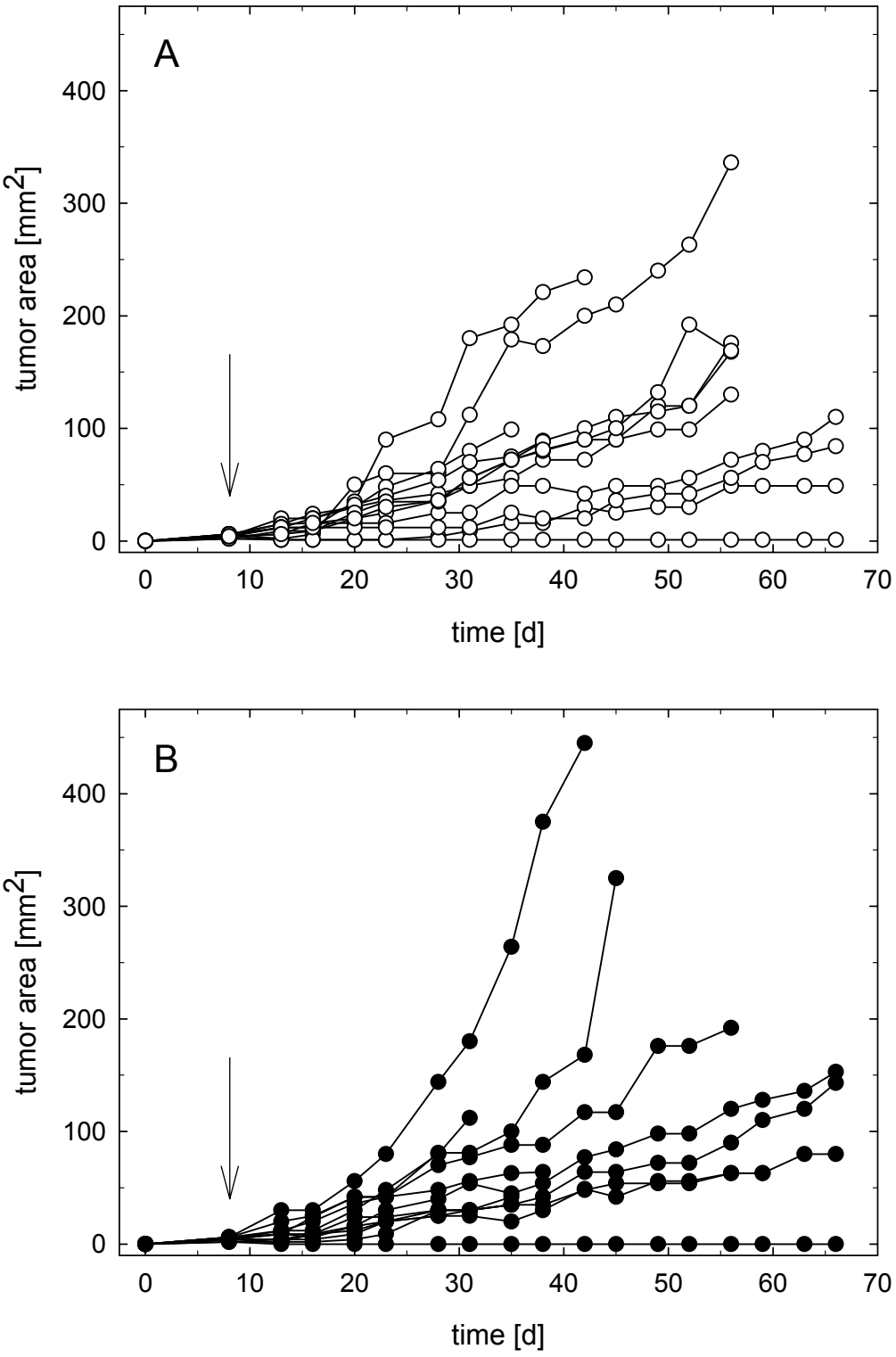

Fig. 4-6: Tumor growth curves of individual animals treated with vinblastine (A) and a combination of vinblastine and *S. agalactiae* hyaluronate lyase (B). For the experimental conditions confer to Fig. 4-5. The arrow indicates the start of therapy.

Comparison of the change of the mean body weight of the animals in the different treatment groups (cf. inset of Fig. 4-5) revealed a similar development in all three groups. Referring to the mean body weight at the start of the therapy the highest loss of body weight was observed in the group receiving the hyaluronidase combination therapy (- 5.7 % (day 59)).

Summarising the results of the regional chemotherapy of the SK-MEL-3 melanoma, it was found that pre-treatment with *S. agalactiae* hyaluronate lyase did neither increase the antitumoral activity of peritumorally applied vinblastine nor exhibit a positive effect on the physical condition of the animals. The finding, that cachexia occurred earlier in animals of the group receiving the combination therapy than in animals of the monotherapy group, indicates that *S. agalactiae* hyaluronate lyase was not well tolerated by the animals - maybe due to the reactivity of the unsaturated disaccharides resulting from HA degradation by the bacterial hyaluronidase. However, since the results obtained so far indicate that the enzyme is lacking the desired therapeutic effect, no further efforts were made to investigate the reason for the observed intolerance to the bacterial enzyme preparation so far.

4.3.2.2 Regional therapy with hyaluronidase and melphalan

4.3.2.2.1 Effect of hyaluronidase on melphalan enrichment in solid melanoma

The preclinical and clinical observations, showing that the efficacy of anticancer drugs is increased by the adjuvant administration of hyaluronidase, are generally explained by the assumption that hyaluronidase facilitates the diffusion of the chemotherapeutics into the tumors by degradation of the hyaluronate-enriched extracellular matrix around and within the tumors (Baumgartner et al., 1988; Baumgartner and Moritz, 1988; Beckenlehner et al., 1992; Spruß et al., 1995). Indeed, in a previous study it was shown that s.c. injected melphalan was dramatically accumulated by SK-MEL-2 tumors, implanted in nude mice, after both i.p. and s.c. administration of bovine testicular hyaluronidase (BTH, Neopermease®) 1 h before melphalan application. Compared with s.c. administration of melphalan alone the melphalan concentration in the tumor was found to be increased by a factor of 6.6 (hyaluronidase i.p.) and 7.8 (hyaluronidase s.c.), respectively (Muckenschnabel et al., 1996). Analogous studies on the SK-MEL-3 melanoma also revealed accumulation of the chemotherapeutics in the tumors after co-administration of Neopermease®, however, to a lesser extent: Muckenschnabel et al. (1998) report that combined administration of Neopermease® (s.c.) with vinblastine (s.c.) enhanced the intratumoral vinblastine concentration by a factor of 2.4,

and Gürtler (2001) observed a 3fold increase of the intratumoral melphalan concentration after s.c. administration of Neopermease® 1 h prior to melphalan (s.c.).

In the following experiments it was investigated if an increased intratumoral accumulation of chemotherapeutics is also obtained by the combined administration of *S. agalactiae* hyaluronate lyase. Following the experimental design of Gürtler (2001) the effect of hyaluronidase co-administration on melphalan enrichment in tumors was determined in nude mice bearing the SK-MEL-3 melanoma. Melphalan alone (50 µmol/kg) or a combination of melphalan (50 µmol/kg) and hyaluronate lyase (500 000 units[#]/kg) was injected s.c. in the vicinity of the tumors. The administration of hyaluronate lyase was carried out 1 h before that of melphalan. Exactly 1 h after melphalan administration the tumors were resected and melphalan concentrations were analysed by HPLC according to the method which was characterised in detail by Muckenschnabel et al. (1996; 1997). The melphalan concentration of the tumors was calculated on a molar basis assuming a density of 1.0 g/cm^3 for the tumors. The results obtained in two independent test series are presented in figure 4-7.

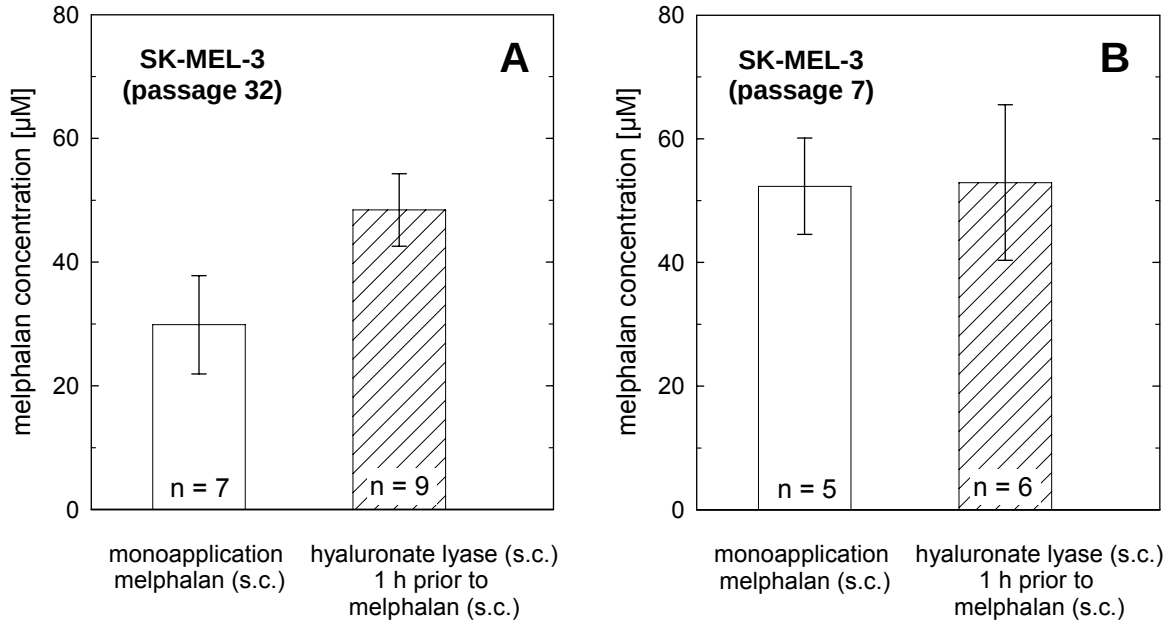

Fig. 4-7: Effect of hyaluronidase application on the accumulation of melphalan in the human SK-MEL-3 melanoma implanted into NMRI *nu/nu* mice (all drugs administered s.c. in the vicinity of the tumors). *S. agalactiae* hyaluronate lyase (500 000 units[#]/kg) was injected 1 h before melphalan application (50 µmol/kg; (A): Alkeran®, (B): melphalan-HCl from Sigma). Exactly 1 h after melphalan administration the tumors were resected and melphalan was analysed by HPLC (n = 6-9; mean ± SEM).

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The SK-MEL-3 melanoma in passage 32 was a serial passage of the SK-MEL-3 tumor (passage 25) used by Gürtler (2001) for the analogous experiment with Neopermease®, where he observed a 3fold increase in the intratumoral melphalan concentration after hyaluronidase pre-treatment. As shown in Fig. 4-7A, the pre-treatment with bacterial hyaluronate lyase produced only a minor augmentation (1.6fold) of the intratumoral melphalan concentration, compared to melphalan monoapplication. But even this slight increase in melphalan accumulation, induced by hyaluronate lyase, was not reproducible in further experiments.

Considering that the investigated SK-MEL-3 melanoma were tumors of an advanced serial passage, the low effect of hyaluronate lyase co-application and the lacking reproducibility might have been caused by the genetical instability of the tumors, e.g. with respect to the HA content, over the course of the different passages. Therefore, SK-MEL-3 tumors were newly established (cf. 4.2.5) in nude mice, and two more experiments were performed on tumors of the freshly established melanoma (passage 2 (results not shown) and passage 7 (cf. Fig. 4-7B), respectively). However, in both experiments, enhancement of melphalan accumulation in the tumors was not observed by pre-treatment with hyaluronate lyase.

By contrast, an additional experiment with Neopermease® on the freshly established melanoma (passage 6) revealed a 3fold increase in melphalan enrichment in the tumors by co-administration of the bovine hyaluronidase (Fig. 4-8). Although the treatment groups were small in this experiment (n = 2/3), the results indicate that the assumption the experiment is based on, i.e. that the uptake of melphalan into the tumors is facilitated by hyaluronidase induced degradation of the extracellular matrix, was valid in the investigated tumor model.

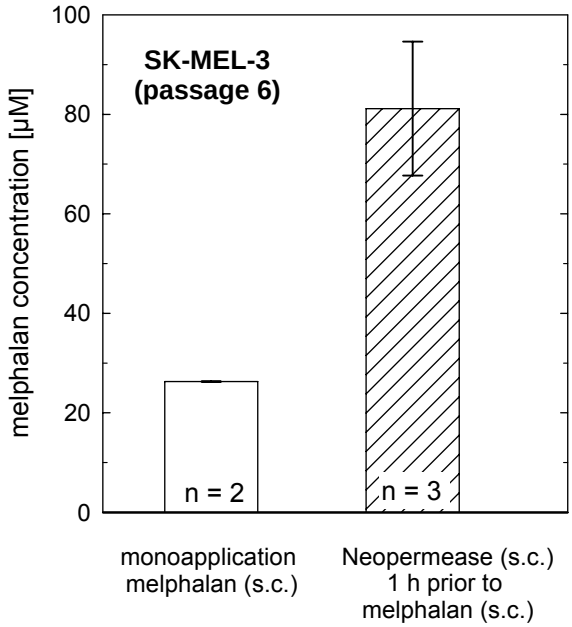

Fig. 4-8: Effect of hyaluronidase application on the enrichment of melphalan in the human SK-MEL-3 melanoma implanted into NMRI *nu/nu* mice (all drugs administered s.c. in the vicinity of the tumors). Neopermease® (100 000 units[#]/kg) was injected 1 h before melphalan application (50 µmol/kg; melphalan-HCl from Sigma). Exactly 1 h after melphalan administration the tumors were resected and melphalan was analysed by HPLC (n = 2/3; mean ± SEM).

It is thus surprising that melphalan accumulation was not observed by co-application of the bacterial hyaluronate lyase, although the hyaluronidase activity applied in the experiments with the bacterial enzyme (500 000 units[#]/kg) was – according to the declaration of the supplier – by a factor of 5 higher compared to the activity used in the experiments with Neopermease® (100 000 units[#]/kg). Comparison of the enzyme preparations with respect to their activity in a colorimetric hyaluronidase assay (cf. Table 3-3) revealed that the preparations differed in the activity measured for 1 designated unit: in the colorimetric assay, which detects product formation by derivatization of N-acetylglucosamine residues at the reducing end of hyaluronan fragments, 1 designated unit of the bacterial enzyme showed 60 times higher activity compared to 1 designated unit of Neopermease®. According to the results of the colorimetric assay the hyaluronidase activity applied in the above described experiments was thus higher in the experiments with the bacterial enzyme, compared to those with Neopermease®, even by a factor of 300.

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An explanation for the discrepancy between the enzyme activity applied in the experiments and the observed pharmacological effect may be found when considering that the bacterial and the bovine hyaluronidase differ in their mechanism of hyaluronan degradation. While hyaluronan degradation by group B streptococcal hyaluronate lyase involves an initial random endolytic cleavage followed by rapid exolytic and processive release of unsaturated disaccharide (Baker and Pritchard, 2000; Li and Jedrzejas, 2001), the bovine enzyme acts as an endolytic enzyme (Vercruysse et al., 1994) with tetra- and hexasaccharides as the main degradation products (Cramer et al., 1994; Takagaki et al., 1994). Thus, every cleavage of hyaluronic acid chains by BTH results in the formation of two shorter chains, whereas in case of the bacterial enzyme the chain is shortened by only one disaccharide per cleavage step. Equiactive concentrations with respect to product formation, as determined in the colorimetric assay, are therefore not necessarily equieffective in reducing the molecular mass of the substrate chains and thus in reducing the viscosity of the hyaluronate-enriched extracellular matrix.

Indeed, comparison of the enzyme preparations with respect to their activity in a turbidimetric hyaluronidase assay, where hyaluronidase activity is quantified according to the ability to reduce the molecular mass of substrate chains (residual high molecular mass substrate is detected by precipitation), revealed that 1 designated unit of the bacterial enzyme showed only 3.86 times higher activity compared to 1 designated unit of Neopermease® (cf. Table 5-3). In addition, the activity in the turbidimetric assay was found to be well correlated with the activity in a viscosimetric hyaluronidase assay, which quantifies the activity of hyaluronidases according to their ability to reduce the viscosity of hyaluronate solutions (cf. chapter 5).

However, although - according to the results of the turbidimetric and viscosimetric assay - the difference in the hyaluronidase activities applied for the investigation of the effect on melphalan enrichment was less pronounced than indicated by the results of the colorimetric assay, the activity applied in the experiments with the bacterial hyaluronate lyase was still by a factor of 19.3 higher compared to the experiments with Neopermease®. The different mechanisms of hyaluronate degradation by the bacterial and bovine hyaluronidase do thus not provide a satisfactory explanation for the observation that, by contrast to Neopermease®, the hyaluronate lyase from *S. agalactiae* did not show the desired therapeutic effect.

Another possible explanation for the lacking efficacy of hyaluronate lyase co-application may be that under in vivo conditions enzyme activity may be inhibited by compounds of the extracellular matrix. However, concerning this, no systematic investigations have been carried out so far.

4.3.2.2.2 Regional chemotherapy

The tumor growth curves which were obtained by the regional chemotherapy of the SK-MEL-3 melanoma, implanted in nude mice, with melphalan alone and with a combination of *S. agalactiae* hyaluronate lyase and melphalan, respectively, are presented in figure 4-9.

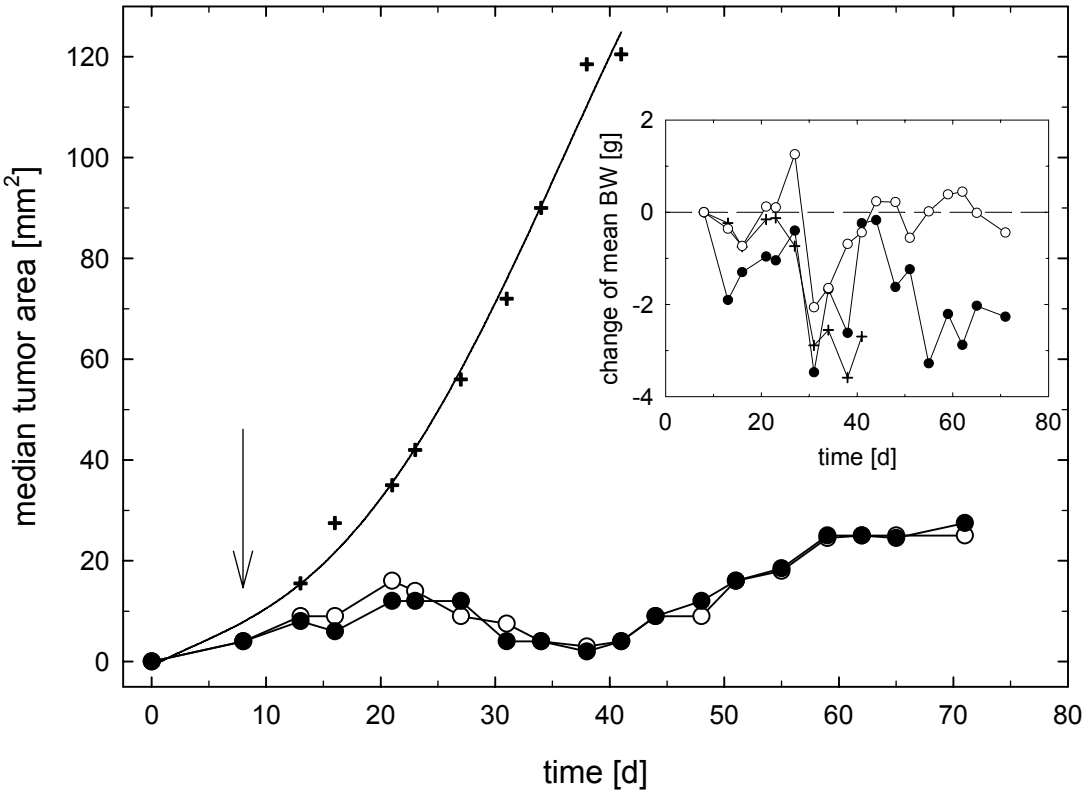

Fig. 4-9: Regional chemotherapy of the human SK-MEL-3 melanoma (passage 31) implanted into NMRI *nu/nu* mice (all drugs administered peritumorally (s.c.); arrow: start of therapy). Control (—✚—): 0.01 N HCl containing 0.9 % NaCl (n = 12); melphalan monotherapy (—○—): melphalan 4 mg/kg (n = 12); combination therapy (—●—): *S. agalactiae* hyaluronate lyase (250 000 units[#]/kg) 1 h prior to melphalan (4 mg/kg) treatment (n = 12). Therapy was carried out weekly (4 therapy cycles). *Inset:* change of mean body weight (BW) during the course of the experiment.

The almost identical tumor growth curves of the different therapy regimens clearly demonstrate that the efficacy of melphalan monotherapy was not increased by the co-

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administration of *S. agalactiae* hyaluronate lyase. In both treatment groups (monotherapy (MT) and combination therapy (CT)) the median tumor area was reduced after the third (day 22) and forth (day 29) treatment cycle, resulting in T/C values ≤ 10 % (day 31: T/C = 10.4 % (MT) and 5.6 % (CT); day 38: T/C = 2.5 % (MT) and 1.7 % (CT)), before a recovery of tumor growth was observed after day 40 of the experiment.

Observation of the change of the mean body weight of the animals revealed that in the final stage of the experiment (after day 40) the physical condition of the animals was worse in the group receiving the combination therapy compared to the monotherapy group (cf. inset of Fig. 4-9). In addition, cachexia was observed earlier and on more animals in the group receiving the combination therapy (5 animals; on days 16, 44, 51, 55, 59 and 65) than in the monotherapy group (3 animals; on days 55 and 71). These findings indicate that *S. agalactiae* hyaluronate lyase was not well tolerated by the animals, as it was also observed in case of the hyaluronate lyase/vinblastine combination therapy (cf. section 4.3.2.1).

Comparison of the results with those obtained by Gürtler (2001) in the corresponding experiments with Neopermease® revealed, that the tumor growth curves of the untreated controls were in good agreement, whereas the antitumoral effect of the melphalan monotherapy was slightly more pronounced in the present experiment, since in the experiment of Gürtler a reduction of the mean tumor area was not observed at any stage of the experiment. However, also in the present experiment tumor growth was not fully inhibited by melphalan alone and thus allowed for the detection of a potential activity enhancing effect of hyaluronate lyase co-administration.

The finding that an increased inhibition of tumor growth, as it is reported for the adjuvant administration of Neopermease® (Gürtler, 2001), was not observed after hyaluronate lyase pre-treatment, is in agreement with the results of the preceding section, considering, that the activity enhancing effect of hyaluronidase is ascribed to an increased accumulation of the chemotherapeutic into the tumor, which was observed in case of pre-treatment with the bovine enzyme but not in case of co-application of the bacterial enzyme (cf. 4.3.2.2.1).

4.4 Conclusion

Systematic investigation of the effect of hyaluronate lyase from *S. agalactiae* on the in vitro chemosensitivity of the human cancer cell lines SK-MEL-3 and U-87 MG against the chemotherapeutics doxorubicin and vinblastine revealed that the coapplication of the bacterial hyaluronidase did not result in an improved antiproliferative activity of the drugs.

Also in the regional chemotherapy of SK-MEL-3 tumors, implanted in nude mice, the coapplication of hyaluronate lyase from *S. agalactiae* did not enhance the efficacy of the monotherapy with vinblastine or melphalan, respectively. In fact, it turned out that the bacterial hyaluronidase preparation was not well tolerated by the animals, which may be ascribed to the reactivity of the unsaturated disaccharides produced during the degradation of hyaluronate by the bacterial enzyme.

Determination of the effect of hyaluronidase co-administration on the accumulation of peritumorally (s.c.) injected melphalan in SK-MEL-3 melanoma revealed that pre-treatment with bacterial hyaluronate lyase did not increase the intratumoral melphalan concentration, whereas the adjuvant application of Neopermease® resulted in a 3fold increase in melphalan enrichment in the tumors, as it was also observed by Gürtler (2001).

Taken together, the results of the reported preclinical investigations indicate that the bacterial hyaluronate lyase preparation does not represent an appropriate substitute for bovine testicular hyaluronidase as a spreading factor and activity enhancer in cancer chemotherapy.

The hyaluronate lyase from *S. agalactiae* did not show the desired spreading effect, although it was administered at concentrations showing - according to the results of the colorimetric assay - 300 times higher hyaluronidase activity compared to the activity applied in the corresponding experiments with Neopermease®. As outlined in the results section (cf. 4.3.2.2.1), the apparent discrepancy between the activities determined in the colorimetric assay and the observed pharmacological effect may in part be ascribed to the different mechanims of HA degradation by the bacterial and the bovine hyaluronidase. The investigations which have been carried out to test this hypothesis are subject of the following chapter.

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5 Comparative analysis of the apparent enzymatic activities of bovine testicular hyaluronidase, a hyaluronate lyase from *S. agalactiae* and bee venom hyaluronidase by turbidimetric and viscosimetric methods

5.1 Introduction

Preclinical investigations on the adjuvant administration of a hyaluronate lyase from *S. agalactiae* in cancer chemotherapy, which are reported in the preceding chapter, revealed that the bacterial enzyme preparation did not show the therapeutic effect that was observed in analogous experiments with a preparation of bovine testicular hyaluronidase (BTH, Neopermease®). In contrast to Neopermease®, which is reported to serve as a spreading factor increasing the access and, thus, the effectiveness of anticancer drugs in tumors due to degradation of the hyaluronate (HA) of the extracellular matrix (Spruß et al., 1995; Muckenschnabel et al., 1996), the bacterial hyaluronate lyase was found to lack the desired spreading effect, as its administration shortly before that of melphalan did not lead to enhanced concentrations of the chemotherapeutic in malignant melanoma.

The spreading effect of BTH is based on the ability of the enzyme to degrade HA of the extracellular matrix (ECM), which results in a reduced viscosity of the ECM and facilitates the diffusion of substances through tissues. Quantification of HA degradation by bovine and bacterial hyaluronidase using a colorimetric assay, which detects product formation by derivatisation of liberated NAG residues, revealed that 1 unit[#] of hyaluronate lyase from *S. agalactiae* showed a 60-times higher activity than 1 unit[#] of Neopermease® (cf. chapter 3). The apparent discrepancy between the activities determined in the colorimetric assay and the observed pharmacological effect may result from the different HA degradation mechanisms of the bacterial and the bovine hyaluronidase. In case of BTH, which acts as an endolytic enzyme (Vercruysse et al., 1994), every cleavage of HA chains results in the formation of two shorter chains and, thus, effectively reduces the viscosity of the ECM. By contrast, the degradation of HA by the bacterial enzyme occurs via an initial endolytic attack followed by exolytic and processive release of unsaturated disaccharides (Baker and Pritchard, 2000; Li

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and Jedrzejas, 2001), i.e. HA chains are shortened by only one disaccharide per cleavage step resulting in a minimal reduction of the molecular mass of the HA chains and, thus, in a much slower reduction of the viscosity of the ECM compared to the action of BTH.

Humphrey and Jaques (1953) investigated a number of hyaluronidase preparations with respect to their activity in a biological assay measuring the effect of hyaluronidase co-administration on the spreading of an indicator dye injected into the skin of animals. By comparison of the spreading activity observed in the biological assay with the activities of the respective enzyme preparations in a turbidimetric and a viscosimetric in vitro assay the authors found a quite good correlation of the results obtained by the three methods for preparations of both testicular hyaluronidase and bacterial hyaluronate lyase.

Both, the turbidimetric and the viscosimetric hyaluronidase assay, are physico-chemical in vitro methods in which the activity of hyaluronidase is quantified according to the reduction of the size of high molecular mass HA molecules. The turbidimetric assay relies on the principle that high molecular mass HA forms precipitates with acidified serum albumine reagent or quartery ammonium salts (e.g. cetyltrimethylammonium bromide, cetylpyridinium chloride), whereas precipitation does not occur after degradation of HA with hyaluronidase to HA fragments < 6-8 kDa. In the viscosimetric assay the depolymerisation of HA by the action of hyaluronidase is detected by measuring the reduction in the viscosity of HA solutions.

In the present study Neopermease® and hyaluronate lyase from *S. agalactiae* were compared with respect to their activity in a turbidimetric and a viscosimetric assay. Since, according to Humphrey and Jaques (1953) the activities obtained by turbidimetry and viscosimetry are well correlated to the pharmacological acitivity of the enzymes when applied as a spreading factor, the results of the following turbidimetric and viscosimetric investigations were expected to provide a plausible explanation for the lacking spreading activity of *S. agalactiae* hyaluronate lyase described in the preceding chapter.

In addition, a recombinant bee venom hyaluronidase (BVH), which belongs to the same family of glycosyl hydrolases (EC 3.2.1.35) as BTH, was investigated with respect to its activity in the different assays, and the results were compared with those obtained with the bovine testicular enzyme.

5.2 Materials and methods

5.2.1 Chemicals

The preparations of bovine testicular hyaluronidase (BTH) and the *S. agalactiae* hyaluronate lyase are characterised in chapter 3. Bee venom hyaluronidase (BVH) (Gmachl and Kreil, 1993; Soldatova et al., 1998) (2.4 mg/ml dissolved in 5 mM sodium acetate, pH 5.4) was a gift from Dr. Zora Markovic-Housley, University of Basel (Switzerland).

Hyaluronic acid (HA) from *Streptococcus zooepidemicus* was purchased from Aqua Biochem GmbH (Dessau, Germany). HA from rooster comb and bovine serum albumin (BSA) were purchased from Serva (Heidelberg, Germany). Cetyltrimethylammonium bromide (CTAB) was from Roth (Karlsruhe, Germany). Deuteriumoxide (D_2O, isotopic enrichment 99.98 %) was purchased from Deutero GmbH (Kastellaun, Germany). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Eschborn, Germany).

5.2.2 Determination of hyaluronidase activity

5.2.2.1 Turbidimetric method

The turbidimetric determination of hyaluronidase activity was carried out according to the method described by Di Ferrante (Di Ferrante, 1956), i.e. cetyltrimethylammonium bromide (CTAB) was used as a precipitating agent for the residual high molecular weight hyaluronic acid (HA) after incubation with hyaluronidase. Experiments were performed under different incubation conditions concerning the composition of the incubation mixture, the enzyme concentration and the time of incubation:

incubation mixture 5-I: 0.2 mg HA from rooster comb per ml of 0.1 M sodium acetate buffer, pH 6.0, containing 0.01 M $MgCl_2$;

incubation mixture 5-II: 200 µl of substrate (0.2 mg HA from rooster comb per ml of water), 380 µl of phosphate buffer (solution A: 0.01M KH_2PO_4/0.01M NaCl, solution B: 0.01 M Na_2HPO_4/0.01M NaCl; solutions A and B were mixed in appropriate portions to adjust the required pH);

incubation mixture 5-III: 200 µl of substrate (0.5 mg HA from rooster comb per ml of water), 150 µl of citrate-phosphate buffer (solution A: 0.2 M Na_2HPO_4/0.1M NaCl, solution B: 0.1 M

citric acid/0.1 M NaCl; solutions A and B were mixed in appropriate portions to adjust the required pH), 50 µl of BSA (0.2 mg BSA per ml of water, i.e. solution 1);

incubation mixture 5-IV: 100 µl of substrate (1 mg HA from rooster comb per ml of water), 200 µl of citrate-phosphate buffer, 50 µl of BSA (solution 1), 50 µl of water.

Incubation was carried out at 37 °C using varying incubation times and enzyme concentrations, specified in the results section. In all experiments the required dilutions of the investigated enzyme preparations were prepared with solution 1 (0.2 mg/ml BSA). Reference samples without substrate and enzyme, respectively, were prepared by using the respective solvents.

Unless otherwise noted, the enzyme reaction was stopped by addition of 1200 µl of CTAB (2.5 g CTAB dissolved in 100 ml of 0.5 N NaOH) and turbidity was allowed to develop for 20 min at 25 °C. The mixtures were then transferred to cuvettes (l = 1 cm) and optical density was measured at 600 nm using an Uvikon 930 spectrophotometer (Kontron Instruments, Eching, Germany).

Calculation of relative activities:

For the determination of enzyme activity as a function of pH, relative activities were calculated according to the following equation (eq. 5-1):

$$\text{relative activity} = \frac{(OD_{substrate} - OD_{sample})}{(OD_{substrate} - OD_{sample\,at\,pH\,optimum})} \qquad \text{(eq. 5-1)}$$

where $OD_{substrate}$ is the OD of the reference sample without enzyme.

5.2.2.2 Viscosimetric method

The viscosimetric determination of hyaluronidase activity was performed according to the official method for the standardisation of hyaluronidase preparations as described in the European Pharmacopoeia (2002), i.e. the decrease in viscosity of the substrate solution induced by the action of hyaluronidase was determined as a function of time by the use of an Ubbelohde capillary viscosimeter.

The instrumentation was from SCHOTT-GERÄTE (Mainz, Germany): the viscosimeter (Ref. No. 53210, capillary diameter 0.63 mm) was clamped into an AVS measurement tripod (AVS/ST), which was connected to an AVS automated measuring device (AVS/N). The

viscosimeter was immersed in a water bath together with the tripod and maintained at 37 °C. In a reaction vessel 18 ml of substrate solution were equilibrated at 37 °C for at least 15 min. The experiment was started by the addition of 100 µl of enzyme solution. After thorough but careful mixing (to avoid the formation of air bubbles) the reaction mixture was transferred to the viscosimeter, drawn into the capillary tube, and the viscosity was determined by automated measuring of the outflow time t, i.e. the time required for the liquid to pass between two light barriers along the capillary tube. The time period between addition of the enzyme and triggering the upper light barrier is termed incubation time (T). The determination of the outflow time t was repeated after increasing incubation times T. Outflow times for reference mixtures were determined in triplicate in the absence of enzyme ($t_{(substrate)}$) and in the absence of substrate ($t_{(buffer)}$), respectively.

The relative viscosity (η_r) was calculated according to the following equation:

$$\eta_r = (k \cdot t) / (k \cdot t_{(buffer)}) \qquad \text{(eq. 5-2)}$$

with k being the viscosimeter constant (0.00991 mm^2/s^2). η_r or $(\ln \eta_r)^{-1}$ were plotted as a function of the reaction time. Since the enzymatic reaction continues during the measurement of the outflow time the reaction time equals T+t/2.

Calculation of relative activities:

For the determination of enzyme activity as a function of pH relative activities were calculated according to the following equation:

$$\text{rel. activity} = \frac{\eta_r(\textit{substrate}) - \eta_r(\textit{sample after T})}{\eta_r(\textit{substrate at pH optimum}) - \eta_r(\textit{sample after T at pH optimum})} \qquad \text{(eq. 5-3)}$$

The composition of the incubation mixtures used in the various experiments is given in the following:

incubation mixture 5-V: 6 ml of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 12 ml of citrate-phosphate buffer (cf. 5.2.2.1), 120 µl of BSA (10 mg BSA per ml of water);

incubation mixture 5-VI: 6 ml of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 12 ml of phosphate buffer, pH 6.0 (2.114 g NaH_2PO_4, 0.3409 g Na_2HPO_4 and 6.67 mg BSA were dissolved in 100 ml of water);

incubation mixture 5-VII: 6 ml of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 12 ml of NaCl containing phosphate buffer, pH 6.0 (2.114 g NaH_2PO_4, 0.3409 g Na_2HPO_4, 6.67 mg BSA and 0.584 g NaCl were dissolved in 100 ml of water);

incubation mixture 5-VIII: 66.67 mg of substrate (HA from *S. zooepidemicus*) were dissolved in 40 ml of NaCl containing phosphate buffer, pH 6 (cf. incubation mixture 5-VII, instead of water D_2O was used as solvent).

In all experiments the required dilutions of the investigated enzyme preparations were prepared with solution 1 (0.2 mg/ml BSA).

5.2.2.3 Colorimetric method

The colorimetric determination of the activity of the bee venom hyaluronidase (BVH) was based on the method of Reissig et al. (1955) and essentially performed as described in the material and methods section of chapter 3. Briefly, 50 µl of enzyme solution (24 ng; the stock solution (cf. 5.2.1) was diluted 1:5000 with solution 1 (0.2 mg/ml BSA)) were incubated with incubation mixture 5-IX: 50 µl of substrate (5 mg HA from rooster comb per ml of water), 250 µl of citrate-phosphate buffer, pH 3.6 (cf. 5.2.2.1), 50 µl of BSA (solution 1), 50 µl of water. The specific activity was calculated using the enzyme concentration of the stock solution as indicated by the supplier.

5.2.3 Molecular mass determination

The molecular mass of the bee venom hyaluronidase was determined by SDS-PAGE under reducing conditions as described in the material and methods section of chapter 3. A low molecular mass marker (Sigma, Deisenhofen, Germany) was used for the assignment of the molecular masses.

5.2.4 Identification of glycosylated proteins

After SDS-PAGE glycoproteins were stained by the periodic acid-Schiff (PAS) technique according to the procedure of Zacharius et al. (1969).

5.3 Results and discussion

5.3.1 Optimisation of the test conditions of the turbidimetric method

5.3.1.1 Incubation in sodium acetate buffer containing $MgCl_2$

Using the turbidimetric hyaluronidase assay according to Di Ferrante (1956) Ozegowski et al. (1994) obtained maximum hydrolytic activity of a *S. agalactiae* hyaluronate lyase at pH 6.3 and found that activity was stimulated in the presence of Mg^{2+}. With respect to these results the turbidimetric determination of hyaluronidase activity was performed in sodium acetate buffer, pH 6, containing $MgCl_2$, in a subsequent publication (Rodig et al., 2000).

To compare the hydrolytic activity of our stabilised hyaluronate lyase with the results reported by Ozegowski et al. (1994) and Rodig et al. (2000) for the hyaluronate lyase from *S. agalactiae*, the turbidimetric assay was performed according to the method described by these authors with respect to the incubation conditions and the type of precipitating agent.

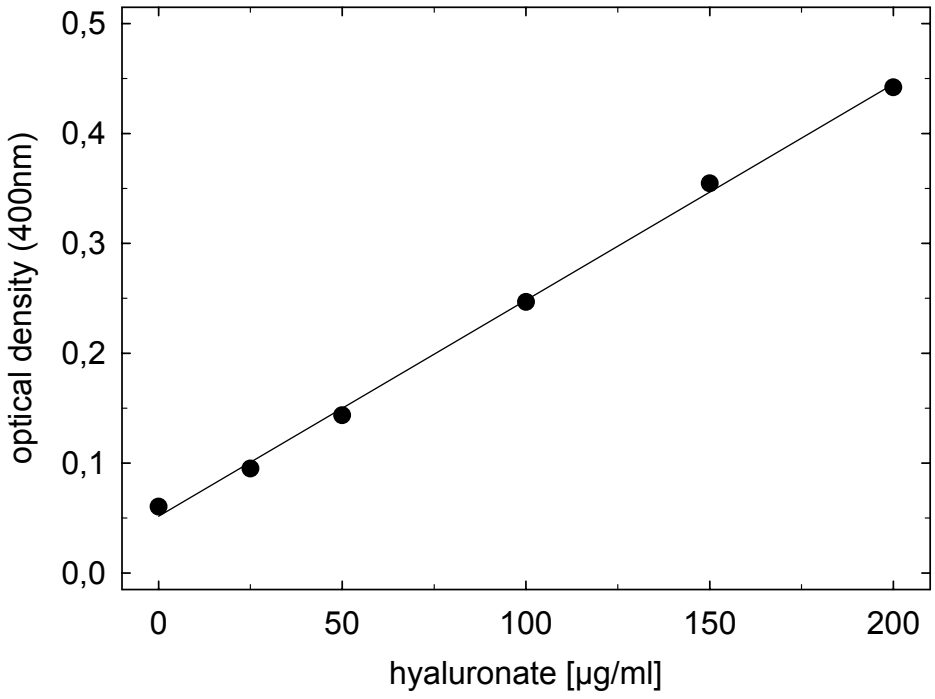

Fig. 5-1: Relation between the turbidity, measured at 400 nm, and the hyaluronate concentration in the incubation mixture. 500 µl of hyaluronate (0-200 µg per ml of 0.1 M sodium acetate buffer, pH 6.0, containing 0.01 M $MgCl_2$) were equilibrated at 37 °C for 10 min. before 1 ml of CTAB was added. Turbidity was allowed to develop for 10 min at 37 °C.

In a preliminary study, it was shown that the turbidity developed by the addition of CTAB was proportional to the hyaluronate concentration in the incubation mixture (Fig. 5-1). However, it is noteworthy that considerable turbidity (OD = 0.06) was measured even in the absence of hyaluronate, which is likely due to the formation of insoluble $Mg(OH)_2$ under the alkaline conditions prevalent in the sample after addition of the CTAB reagent.

In a subsequent experiment the effect of different concentrations of hyaluronate lyase incubated in the presence of a constant concentration of hyaluronate (200µg/ml) was examined.

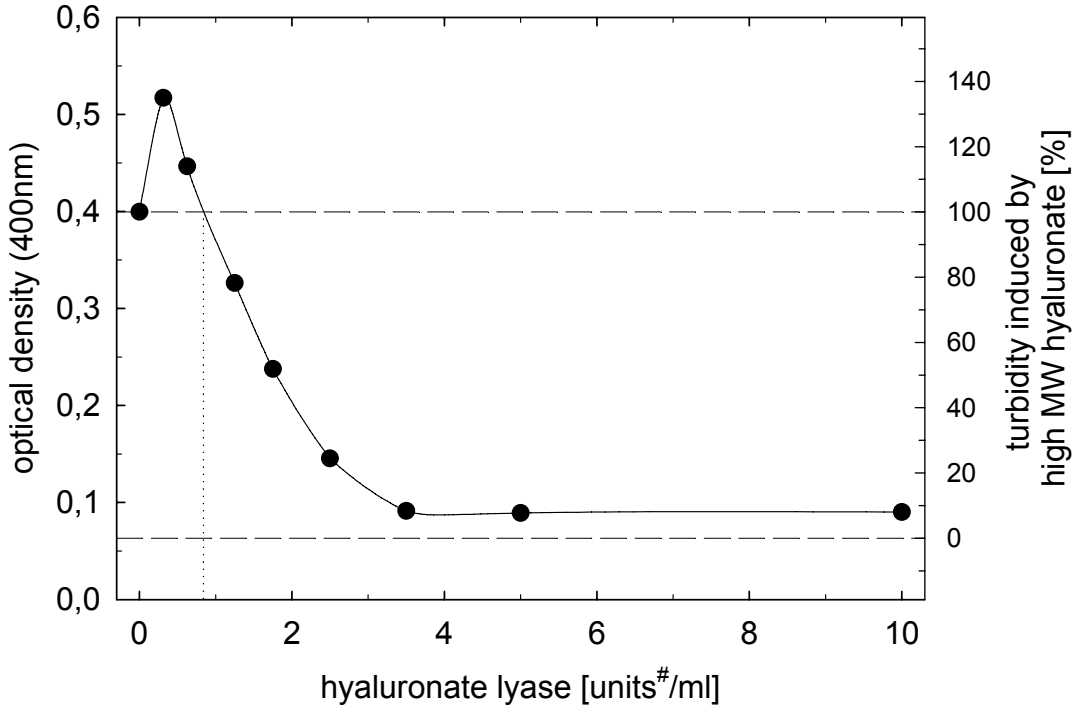

Fig. 5-2: Turbidity as a function of hyaluronate lyase concentration in the incubation mixture. 20 µl of enzyme solution (appropriately diluted to achieve the concentrations indicated) were incubated with incubation mixture 5-I (480 µl) for 15 min. at 37 °C. After addition of 1 ml of CTAB, turbidity was allowed to develop for 10 min at 37 °C. The dashed lines indicate the optical densities measured in the absence of enzyme (upper line) and in the absence of hyaluronate (lower line), respectively.

Interestingly, this experiment revealed that incubation with low concentrations of hyaluronate lyase caused an increase in turbidity (Fig. 5-2). This phenomenon has also been observed by Alburn and Whitley, when assaying a hyaluronidase from bull testes by the turbidimetric method (Alburn and Whitley, 1951) and might be explained by the occurrence of endolytic cleavage of the high MW hyaluronate chains, leading to an increasing concentration of

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hyaluronate molecules, which are still large enough to produce turbidity with the precipitating agent.

Turbidity reduction was observed at enzyme concentrations > 0.8 units[#]/ml. However, even at high enzyme concentrations turbidity was not decreased to the turbidity, measured in the absence of hyaluronate (OD = 0.6). Considering the fact that in this test system the measured turbidity can not exclusively be ascribed to the insoluble complexes formed between high MW hyaluronate and CTAB, but is also partly caused by insoluble $Mg(OH)_2$, this system was considered inappropriate for an exact determination of hyaluronidase activity.

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5.3.1.2 Incubation in phosphate buffer

Concerning the effect of pH on the hydrolytic activity of *S. agalactiae* hyaluronate lyase, the results, i.e. optimum activity at pH 5.0 (cf. chapter 3), obtained by the colorimetric hyaluronidase assay and UV difference spectroscopy disagree with those reported by Ozegowski et al (1994), who found optimum activity at pH 6.3 in the turbidimetic assay. To rule out the possibility that this discrepancy is due to the different assays or incubation buffers used, the activity of the investigated hyaluronate lyase preparation was determined as a function of pH by the procedure described by Ozegowski et al. (1994), too.

To assure that the selected test conditions give rise to reliable results a series of preliminary experiments were carried out. As depicted in Fig. 5-3, the development of turbidity proved to be directly proportional to the concentration of hyaluronate in the incubation mixture (for hyaluronate concentrations ranging from 10 µg/ml and 66.7 µg/ml) and no turbidity was measured in the absence of hyaluronate. In addition, it was verified that the turbidity induced by a certain hyaluronate concentration was not affected by the pH of the incubation mixture (results not shown).

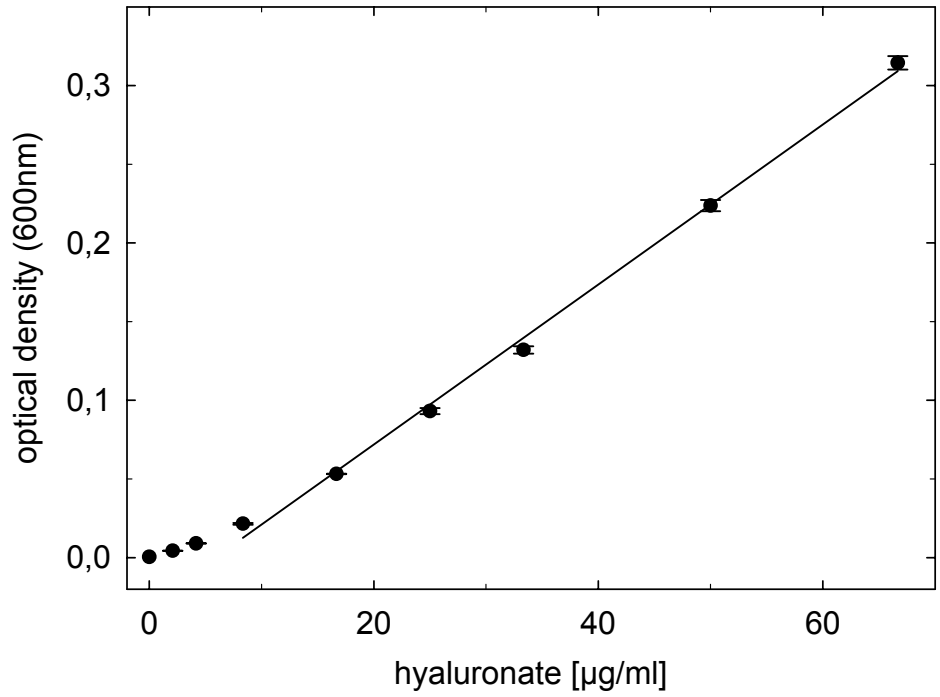

Fig. 5-3: Relation between the turbidity, measured at 600 nm, and the hyaluronate concentration in the incubation mixture. 200 µl of hyaluronate (0-200 µg per ml of water) were mixed with 400 µl of phosphate buffer, pH 4.65 (cf. section 5.2.2.1). The precipitation of hyaluronate was then performed as described in the materials and methods section.

Incubation of a certain hyaluronate concentration (66.7µg/ml) with increasing amounts of hyaluronate lyase revealed a constant decrease in turbidity down to the value, obtained in the absence of hyaluronate (Fig. 5-4), which is in contrast to the corresponding experiment using acetate buffer containing $MgCl_2$ (cf. 5.3.1.2.1), where turbidity only decreased to the value corresponding to 10 % of the initial turbidity (Fig. 5-2). Furthermore, an increase in turbidity at low enzyme concentrations (Fig. 5-2) was not observed under these incubation conditions. In fact, the relationship between the concentration of hyaluronate lyase (0 – 0.52 units[#]/ml) and turbidity was found to be approximately linear (indirectly proportional). However, it was not investigated if enzyme concentrations lower than 0.10 units[#]/ml would cause an increase in turbidity compared to the value obtained in the absence of enzyme (initial turbidity).

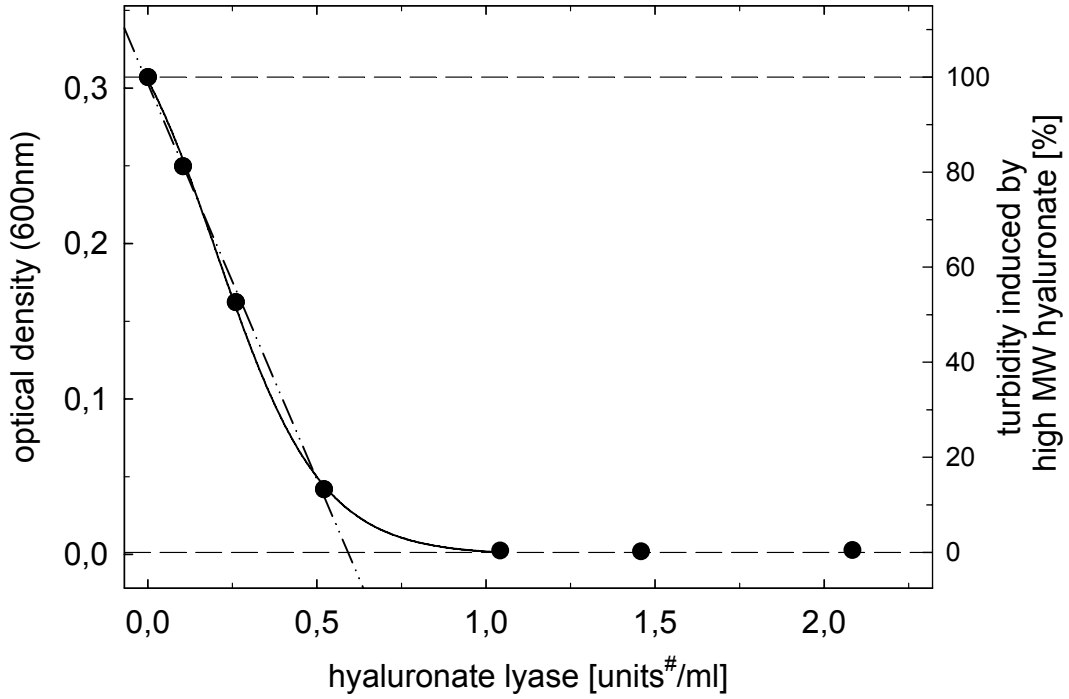

Fig. 5-4: Turbidity as a function of the hyaluronate lyase concentration in the incubation mixture. 20 µl of enzyme solution (appropriately diluted to achieve the indicated concentrations) were incubated with incubation mixture 5-II (580 µl; pH 4.65) for 15 min at 37 °C. The dashed lines indicate the optical densities measured in the absence of enzyme (upper line) and in the absence of hyaluronate (lower line), respectively.

To determine the activity of *S. agalactiae* hyaluronate lyase as a function of pH, aliquots of the enzyme were incubated in NaCl-containing phosphate buffer of varying pH for 15 min at 37 °C. With 0.42 units[#]/ml an enzyme concentration, that has been shown to reduce turbidity

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by about 75 % at pH 4.65 under the given test conditions (cf. Fig. 5-4), was used. The obtained pH activity profile is shown in Fig. 5-5.

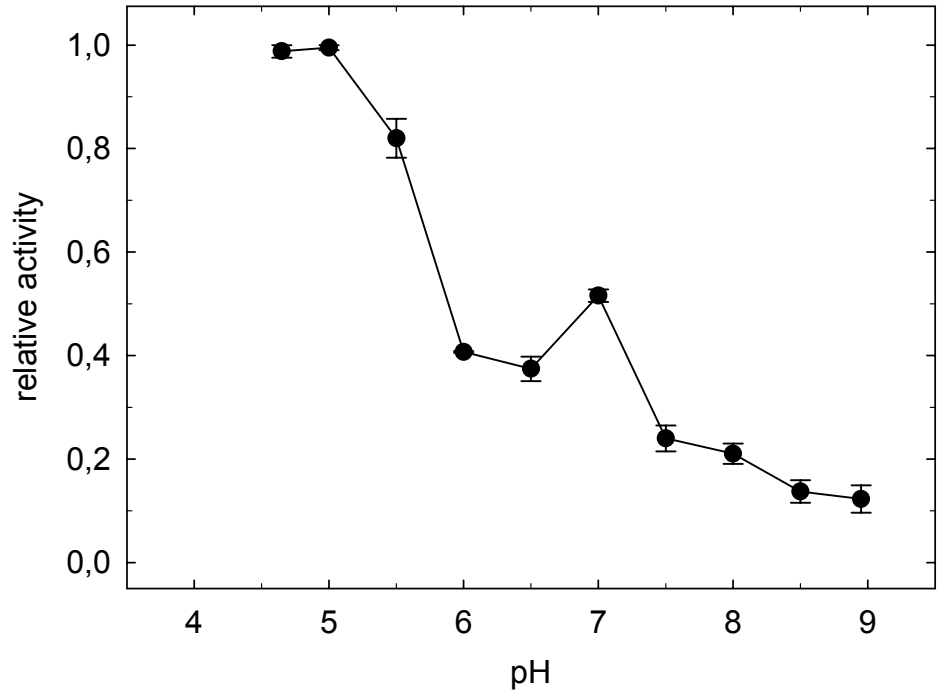

Fig. 5-5: Effect of pH on the activity of *S. agalactiae* hyaluronate lyase. 20 µl of enzyme solution (appropriately diluted to achieve a concentration of 0.42 units[#]/ml in the incubation mixture) were incubated with incubation mixture 5-II (580 µl) for 15 min at 37 °C.

Maximum activity was measured at pH 5, being consistent with the results obtained by the colorimetric assay and UV difference spectroscopy (cf. chapter 3). Based on this result it can be ruled out that the discrepancies in the pH activity profiles of our hyaluronate lyase preparation and the enzyme investigated by Ozegowski et al. (1994), who found maximum activity at pH 6.3, are due to different test conditions.

Interestingly, at neutral pH, after an initial decrease resulting in about 40 % of maximum activity at pH 6, the curve shows an additional small maximum with around 50 % of the maximal activity at pH 7. This second pH optimum might be explained by the presence of two enzymatically active proteins in the investigated hyaluronate lyase preparation (cf. chapter 3). However, the fact that the second maximum was not observed in our former experiments using the colorimetric assay and UV difference spectroscopy caused us to systematically compare the different assays, i.e. to perform turbidimetric determination of enzyme activity after incubation under identical conditions as used in case of the colorimetric

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assay and UV difference spectroscopy. Moreover, it was of interest to study the effect of pH on enzyme activity in the turbidimetric assay also at the acidic side of the maximum obtained so far.

5.3.1.3 Incubation in citrate-phosphate buffer containing BSA

The turbidimetric determination of enzyme activity after incubation under identical conditions as used in the colorimeric assay and in UV difference spectroscopy allows for a comparison of the results obtained by the different methods and assures that possible discrepancies in the apparent activity are due to the different principles of the assays and can not be ascribed to differences in the composition of the incubation mixture, which is known to affect the hydrolytic activity of hyaluronidases significantly.

By analogy to the previously used incubation buffers the selected incubation mixture of the colorimetric assay (i.e. standard incubation mixture) was examined with respect to its suitability for the use in the turbidimetric assay in preliminary studies.

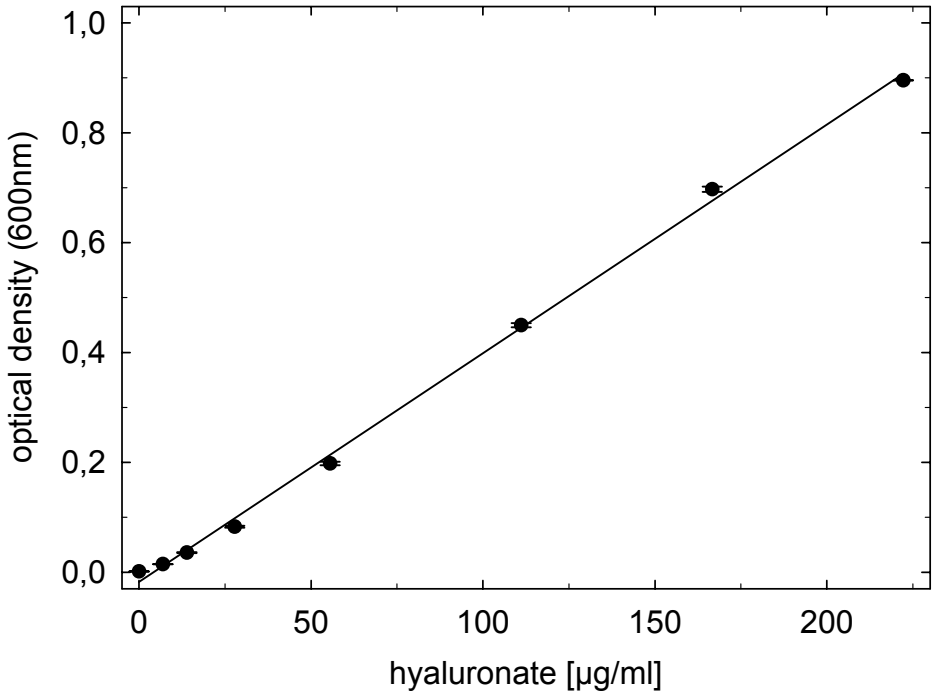

Fig. 5-6: Relation between the turbidity, measured at 600 nm, and the hyaluronate concentration in the incubation mixture. 200 µl of hyaluronate (0-500 µg per ml of water) were mixed with 100 µl of BSA (solution 1) and 150 µl of citrate-phosphate buffer, pH 5.0 (cf. section 5.2.2.1). The precipitation of hyaluronate was then performed as described in the materials and methods section.

A linear relationship between turbidity and hyaluronate concentration in the incubation mixture was observed for hyaluronate concentrations up to 222 µg/ml (Fig. 5-6). Although the substrate concentration of the standard incubation mixture is higher (555 µg/ml), a concentration of 222 µg/ml was used for the following turbidimetric experiments to guarantee a linear relationship between the concentration of high molecular weight hyaluronate in the incubation mixture and the measured optical density. Furthermore, it was assured that the turbidity induced by a certain substrate concentration was not affected by the pH of the investigated incubation mixture (results not shown).

Fig. 5-7 shows that turbidity is constantly reduced to the value, obtained in the absence of substrate by the action of increasing amounts of hyaluronate lyase, i.e. no interfering components are present in the buffer system. The incubation conditions prevailing in the colorimetric assay proved to be applicable for the turbidimetric determination of hyaluronidase activity, too, and they were used for the subsequent investigations.

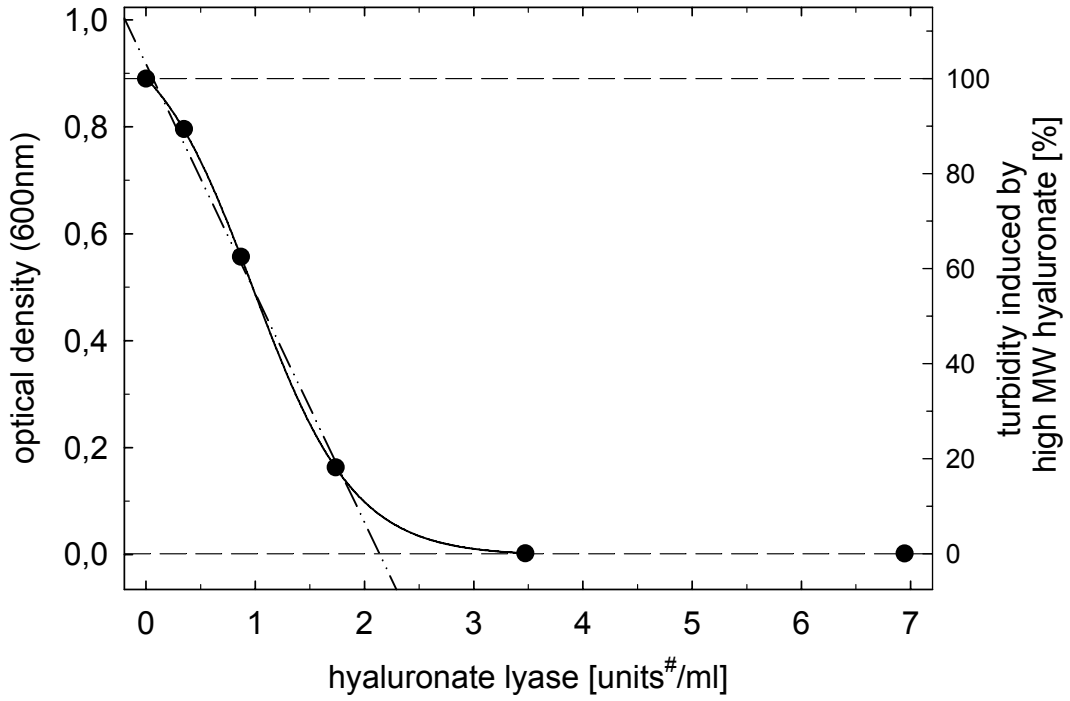

Fig. 5-7: Turbidity as a function of the hyaluronate lyase concentration in the incubation mixture. 50 µl of enzyme solution (appropriately diluted to achieve the indicated concentrations) were incubated with incubation mixture 5-III (400 µl; pH 5.0) for 30 min at 37 °C. The dashed lines indicate the optical densities measured in the absence of enzyme (upper line) and in the absence of hyaluronate (lower line), respectively.

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5.3.2 Characterisation of S. agalactiae hyaluronate lyase in the turbidimetric assay

5.3.2.1 Effect of pH on enzyme activity

To determine the activity of *S. agalactiae hyaluronate* lyase as a function of pH, aliquots of the enzyme were incubated in NaCl-containing citrate-phosphate buffer of varying pH for 30 min at 37 °C and the residual high molecular weight substrate was turbidimetrically detected by precipitation with CTAB. An appropriate enzyme concentration of 1.67 units[#]/ml inducing a decrease in turbidity by about 80 % at pH 5.0, i.e. at the expected pH optimum, was selected from the turbidity reduction curve in Fig. 5-7.

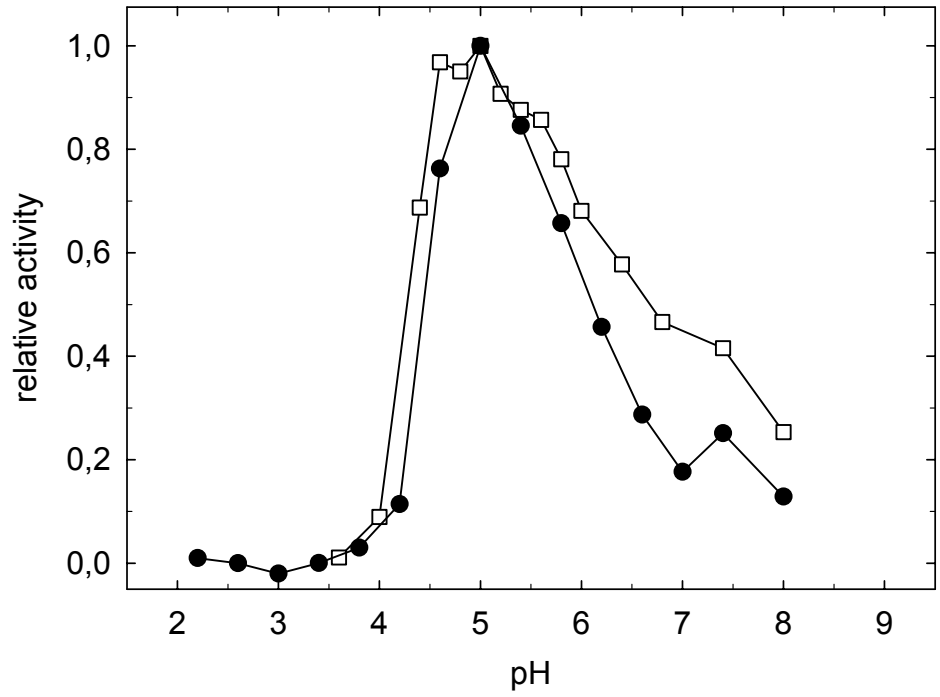

Fig. 5-8: Effect of pH on the activity of *S. agalactiae* hyaluronate lyase, determined by the turbidimetric method (—●—). 50 µl of enzyme solution (appropriately diluted to achieve a concentration of 1.67 units[#]/ml in the incubation mixture) was incubated with incubation mixture 5-III (400 µl) for 30 min at 37 °C. pH activity profile, obtained by the colorimetric method (—□—; cf. chapter 3)

As illustrated in Fig. 5-8, the pH activity profile obtained by turbidimetry is in good agreement with the results of the colorimetric assay with respect to the determined activity optimum at pH 5 and the steep decrease in activity at acidic pH. However, at neutral pH it is noteworthy that the turbidimetrically determined relative activity (20 % of the maximum activity at pH 7) is considerably lower compared to the relative activity detected in the

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colorimetric assay (45 % of the maximum activity at pH 7). Interestingly, as observed in former experiments with phosphate buffer as incubation medium (cf. section 5.3.1.2.2), the pH activity curve obtained with the turbidimetric assay shows an additional small maximum at neutral pH (pH 7.4), which is not existent in the pH activity profile, determined in the Morgan Elson assay using the identical incubation buffer.

5.3.2.2 Determination of the turbidity reducing unit (TRU)

According to other authors (Gerlach and Köhler, 1972; Ozegowski et al., 1994) one turbidity reduction unit was defined as the amount of enzyme, which will reduce the turbidity produced by 100 µg of hyaluronate to the turbidity produced by 50 µg of hyaluronate within 30 min under specified conditions.

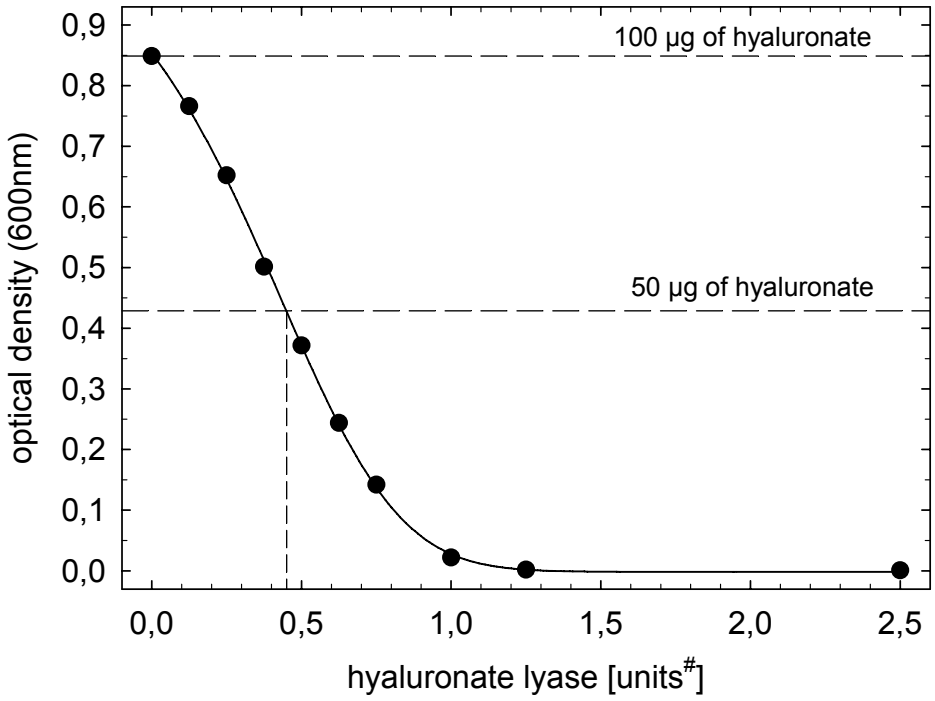

Fig. 5-9: Turbidity as a function of the amount of hyaluronate lyase in the incubation mixture. 50 µl of enzyme solution (appropriately diluted to achieve the required concentrations in the incubation mixture) were assayed with incubation mixture 5-IV (400 µl; pH 5.0) for 30 min at 37 °C. The dashed lines indicate the turbidity induced by incubation mixtures containing 100 µg and 50 µg of hyaluronate, respectively, in the absence of enzyme.

For the determination of hyaluronidase activity in terms of TRU, increasing amounts of enzyme were added to incubation mixtures containing 100 µg of hyaluronate in citrate-phosphate buffer of optimal pH (pH 5.0) and incubated for 30 min at 37 °C. In addition

[#] as declared by the supplier

reference samples containing 100 µg and 50 µg of hyaluronate in the absence of enzyme were prepared in triplicate. Plotting the measured turbidities as a function of the amount of enzyme in the incubation mixture allows for graphical determination of the TRU, i.e. the turbidity produced by 50 µg of hyaluronate can be assigned to a certain amount of enzyme (Fig. 5-9).

As shown in Fig. 5-9, 1 TRU of the investigated *S. agalactiae* hyaluronate lyase was found to correspond to 0.45 units, according to the declaration of the supplier. With reference to the results of the colorimeric assay and UV difference spectroscopy (cf. chapter 3) a correlation can be made between the turbidity reducing unit and the unit (U) as defined according to the International union of Biochemistry (cf. chapter 3). Thus, 1 TRU of hyaluronate lyase amounts to 1.3 mU, i.e. it will catalyse the formation of $1.3 \cdot 10^{-3}$ µmol of N-acetyl-D-glucosamine (NAG) at the reducing ends of sugars per minute under the conditions used in the performed experiments (citrate-phosphate buffer, pH 5.0). The relation between the different units used for the declaration of the activity of *S. agalactiae* hyaluronate lyase is summarised in table 5-1.

Table 5-1: Correlation between different units used for the declaration of the activity of *S. agalactiae* hyaluronate lyase

Turbidity reducing units (TRU)	Units (U) according to the definition of the International union of Biochemistry	Units according to the declaration of the supplier
1 TRU	1.3 mU	0.45

5.3.3 Characterisation of BTH in the turbidimetric assay

5.3.3.1 Neopermease®

5.3.3.1.1 Effect of pH on enzyme activity

By analogy with the bacterial hyaluronate lyase the activity of the BTH preparation Neopermease® was determined as a function of pH by the turbidimetric method under identical incubation conditions as used for the colorimetric determination of the pH activity profile of the enzyme (cf. chapter 3). In contrast to the investigations on the bacterial enzyme, where the results from both assays were in good agreement, in case of Neopermease® almost inverted pH activity profiles were obtained by the two methods (Fig. 5-10).

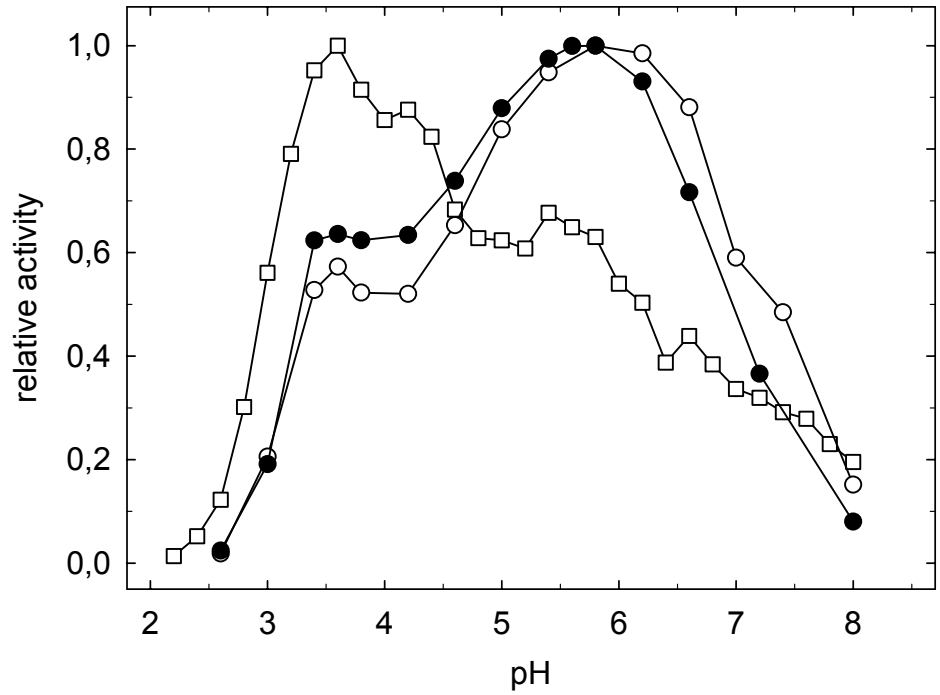

Fig. 5-10: Effect of pH on the activities of Neopermease® (—●—) and the basic material of Neopermease® (—○—) as determined by the turbidimetric method. 50 µl of enzyme solution (Neopermease®: 1.5 units#; basic material: 1.75 units#) were incubated with incubation mixture 5-IV (400 µl) for 1h at 37 °C. pH activity profile of Neopermease®, obtained by the colorimetric method (—□—; cf. chapter 3).

Like the colorimetric assay the turbidimetric determination yielded an asymmetric curve when enzyme activity was measured over a broad range from pH 3 up to pH 8. However, maximum activity was found at pH 5.8, whereas at pH 3.5, which is the optimum pH in the colorimetric assay, a second small maximum with 60 % of the maximal activity was detected. For the colorimetric assay it has been shown that the complex effect of pH on the activity of Neopermease® can be explained as the sum of the pH profiles of the three different enzymatically active proteins contained in the enzyme preparation (cf. chapter 3). Interestingly, two of the three fractions - the 33 kDa and 77 kDa fraction - were found to exhibit maximum activity at pH 6 in the colorimetric assay, while the activity maximum of Neopermease® at pH 3.6 results from the high specific activity of the main 58 kDa fraction, which is characterised by two maxima at pH 3.6 and pH 4.8.

Based on these results one can assume that the maximum at pH 5.8 in the turbidimetrically determined pH activity profile might result from the fact that the turbidimetric assay detects

as declared by the supplier

the activities of the 33 kDa and 77 kDa fractions more sensitively compared to the main 58 kDa fraction. However, this can be excluded, since, like in the colorimetric assay, by the turbidimetric method the highest activity was detected in the 58 kDa fraction (results not shown). A comparison of the isolated components of Neopermease® with respect to their pH profiles obtained by both methods revealed that the 58 kDa fraction gave the inverse profiles typical for the whole preparation (Fig. 5-11), whereas the pH profiles of the high and low molecular mass fraction were not affected by the type of assay (Hoechstetter et al., 2001).

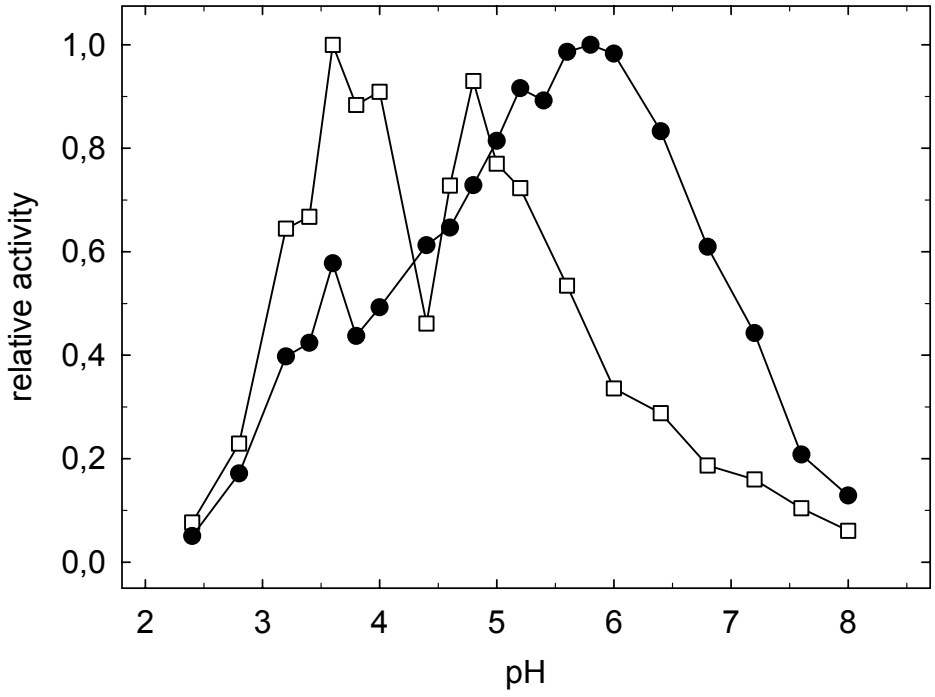

Fig. 5-11: Effect of pH on the activity of the 58 kDa fraction of Neopermease®, determined by the turbidimetric method (—●—). 50 µl of enzyme solution (14.0 ng) were incubated with incubation mixture 5-IV (400 µl) for 1h at 37 °C. pH activity profile of the 58 kDa fraction as obtained by the colorimetric method (—□—; cf. chapter 3).

Assuming, that the 58 kDa fraction contains two enzymatically active proteins with different pH optima (pH 3.6 and 4.8) the turbidimetrically obtained pH profile may be explained by a higher sensitivity of the turbidimetric assay towards the isoform, which is active at weak acidic pH (pH 5 – 6) compared to the isoform exhibiting activity at pH 3.6.

The observed phenomenon reflects the fundamentally different principals of the colorimetric and the turbidimetric assay (cf. chapter 1), i.e. the discrepancies in the apparent activity of the investigated BTH preparation may result from the fact that hydrolysis and transglycosylation taking place on substrates falling below a certain length (6-8 kDa) are not detected by the

turbidimetric method but do affect the results of the colorimetric assay. To test this hypothesis, the effect of pH on the composition of the oligosaccharide mixtures produced by digestion of hyaluronate with hyaluronidase under various conditions was investigated in detail in further studies (cf. chapter 6).

5.3.3.1.2 Determination of the turbidity reducing unit (TRU)

To allow for a comparison with the *S. agalactiae* hyaluronate lyase the TRU of Neopermease® was determined at optimal pH (pH 5.8) according to the procedure described for the bacterial enzyme (cf. section 5.3.2.2) using identical incubation conditions.

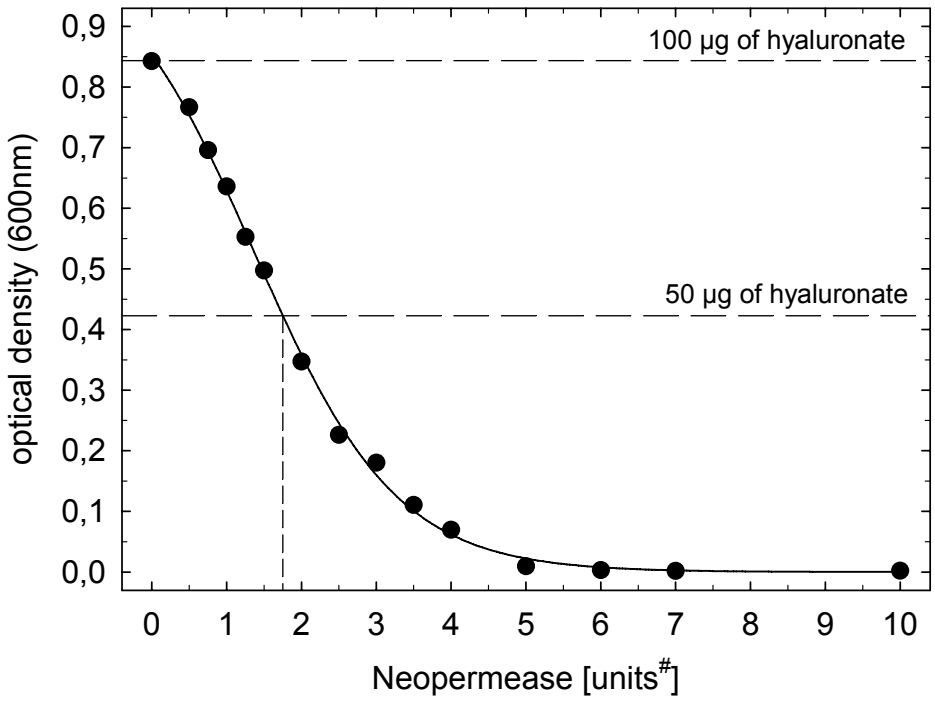

Fig. 5-12: Turbidity as a function of the amount of Neopermease® in the incubation mixture. 50 µl of enzyme solution (appropriately diluted to achieve the required concentrations in the incubation mixture) were incubated with incubation mixture 5-IV (400 µl; pH 5.8) for 30 min at 37 °C. The dashed lines indicate the turbidity induced by incubation mixtures containing 100 µg and 50 µg of hyaluronate, respectively, in the absence of enzyme.

The resulting plot of the measured turbidities as a function of the amount of enzyme in the incubation mixture revealed that the TRU of Neopermease® corresponds to 1.75 units according to the declaration of the supplier (Fig. 5-12). As done in case of the bacterial enzyme, the TRU of Neopermease® was related to the unit (U) as defined according to the International union of Biochemistry with reference to the results of the colorimetric assay (cf.

[#] as declared by the supplier

chapter 3). Thus, 1 TRU of Neopermease® amounts to 0.0875 mU, i.e. it will catalyse the formation of $0.0875 \cdot 10^{-3}$ µmol of N-acetyl-D-glucosamine (NAG) at the reducing ends of sugars per minute under the conditions used in our experiments (citrate-phosphate buffer, pH 3.6). In this context it has to be noted that the quantification of enzyme activity in terms of TRU and in units according to the definition of the International union of Biochemistry has been performed at optimum pH for the enzyme in the respective assay, i.e. at pH 3.6 in the colorimetric assay and at pH 5.8 in the turbidimetric assay, respectively. The relation between the different terms used for the declaration of the activity of Neopermease® is shown in table 5-2.

Table 5-2: Correlation between the different units used for the declaration of the activity of Neopermease®

Turbidity reducing units (TRU)	Units (U) according to the definition of the International union of Biochemistry	Units according to the declaration of the supplier
1 TRU	0.0875 mU	1.75

The liberation of NAG per one designated unit (as declared by the respective supplier) of the *S. agalactiae* enzyme and Neopermease® was found to differ by a factor of 60 (cf. chapter 3). It is interesting to notice that in the turbidimetric assay the equiactive concentration of the bacterial enzyme was only by a factor of 3.86 lower when compared with Neopermease® (tables 5-1 and 5-2). This result reflects the different catalytic mechanisms of bacterial and bovine hyaluronidases (cf. chapter 1). In case of BTH, which acts as an endolytic enzyme (Vercruysse et al., 1994), every cleavage is accompanied by both the formation of NAG and the formation of two shorter substrate chains, i.e. precipitation of the substrate with CTAB is continuously reduced. By contrast, in case of the bacterial enzyme, after the first endolytic cleavage, the following exolytic and processive release of unsaturated disaccharide (Baker and Pritchard, 2000; Li and Jedrzejas, 2001) does not effectively decrease the length of the substrate chain and thus its capability to precipitation with CTAB.

In view of the fact that in the turbidimetric assay the activity of hyaluronidase is quantified according to its ability to reduce the MW of the substrate chains, it can be assumed that equiactive concentrations of bacterial and bovine enzyme as determined by the turbidimetric method are more likely to be pharmacologically equieffective when used as a spreading factor

compared with concentrations being equiactive in the colorimetric assay. Table 5-3 summarises the activities of Neopermease® and the *S. agalactiae* hyaluronate lyase, determined in the colorimetric and the turbidimetric assay, respectively.

Table 5-3: Comparison of the enzyme activities of Neopermease® and *S. agalactiae* hyaluronate lyase

Enzyme activities were determined in the colorimetric assay (liberation of NAG, cf. chapter 3) and in the turbidimetric assay (TRU), respectively, at optimal pH for each enzyme, using identical incubation conditions, which are described in the materials and methods section (incubation mixture 5-IV).

Hyaluronidase preparation	Designated units per mg	Activities per designated unit		Specific activities	
		Liberation of NAG per designated unit (μmol min^{-1})	TRU per designated unit	Liberation of NAG per mg (μmol min^{-1} mg^{-1})	TRU per mg
Neopermease®	50 000	$5 \cdot 10^{-5}$	0.57	2.5[a]	28 500[a]
***S. agalactiae* hyaluronate lyase**	350 000	$2.9 \cdot 10^{-3}$	2.2	1015[b]	770 000[b]

[a] for calculation the protein content, determined by the method of Bradford, was used
[b] for calculation the amount of enzyme protein per vial, as indicated by the supplier, was used

5.3.3.2 Hylase® "Dessau"

5.3.3.2.1 Effect of pH on enzyme activity

Comparison of the two pharmaceutical preparations Neopermease® and Hylase® "Dessau" with respect to the effect of pH on enzyme activity in the colorimetric assay revealed that they are characterised by almost identical complex pH profiles (cf. chapter 3).

As shown in Fig. 5-13, by turbidimetric determination of hyaluronidase activity similar pH activity profiles were obtained for the two BTH preparations, i.e., as observed in the case of Neopermease®, the pH activity profile of Hylase® "Dessau" was also found to depend on the type of assay. However, while the turbidimetrically determined curves of the different BTH preparations were consistent with respect to the relatively broad maximum at pH 5.8, the second maximum at pH 3.5 was with 80 % of maximal activity more pronounced in case of

Hylase® "Dessau". This difference in the relative activities of the two enzyme preparations at acidic pH may reflect the fact that they are complex mixtures of proteins, which do not contain identical enzymatically active proteins, as has been shown by SDS PAGE (cf. chapter 3).

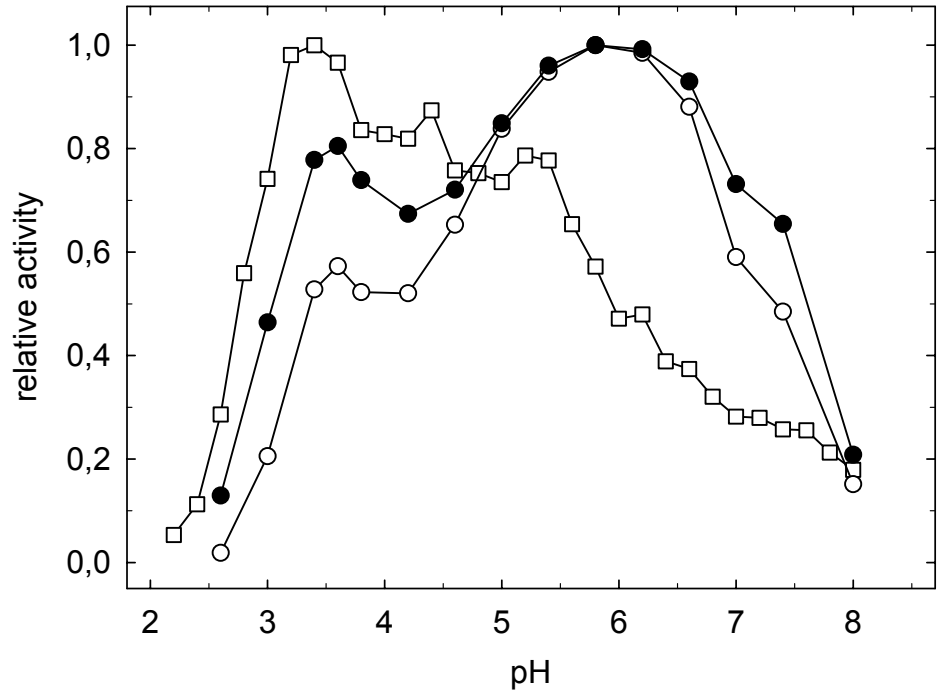

Fig. 5-13: Effect of pH on the activities of the basic materials of Hylase® "Dessau" (—●—) and Neopermease® (—○—), determined by the turbidimetric method. 50 µl of enzyme solution (Hylase® "Dessau": 1: 143 dilution of the basic material; Neopermease® (basic material): 1.75 units[#]) were incubated with incubation mixture 5-IV (400 µl) for 1h at 37 °C. pH activity profile of the basic material of Hylase® "Dessau", obtained by the colorimetric method (—□—; cf. Oettl (2000)).

[#] as declared by the supplier

5.3.4 Viscosimetric estimation of hyaluronidase activity

According to theoretical considerations concerning polymer degradation, there is a linear relationship between the reciprocal value of the number average molecular mass (M_n) and the reaction time at the initial stage of the reaction, provided that a polymer sample is degraded in an at random way (Tanford, 1961). The slope k_n of this relationship provides the reaction rate, which can be expressed as the number of bonds broken per unit time.

Vercruysse et al. (1994) investigated the degradation of HA by BTH using size exclusion chromatography. Plotting the reciprocal value of the experimentally determined M_n as a function of the reaction time yielded a linear relationship with the slope k_n indicating an at random degradation of HA by BTH in the intial stage of the reaction. In addition, the authors have shown a linear relationship for plots of the reciprocal value of the viscosity average molecular mass (M_v) against reaction time. The slope k_v of the plot of $1/M_v$ as a function of the reaction time was shown to be related to k_n (slope of the plot of $1/M_n$ versus reaction time) according to eq. 5-4.

$$k_n = k_v\left[\Gamma(2+\alpha)\right]^{1/\alpha} \qquad \text{(eq. 5-4)},$$

where α is the exponent of the Mark-Houwink equation (eq. 5-6, see below).

Eq. 5-4 is in accordance with the relationship theoretically predicted for an at random degradation of a polymer (Peebles, 1971). Therefore, by applying eq. 5-4 the reaction rate expressed as the number of bonds broken per unit time (k_n) can be calculated from the slope k_v of the plot of $1/M_v$ versus reaction time.

Based on this relationship Vercruysse et al. (1995) conceived a viscosimetric approach to determine the activity of BTH, expressed as the number of bonds cleaved per unit time: using an Ubbelohde capillary viscosimeter the authors determined the decrease in the relative viscosity (η_r) of the substrate (HA) solution during the enzymatic reaction by measuring the the outflow times (t) after increasing incubation times (T). From η_r the intrinsic viscosity [η] was calculated using the Martin equation (eq. 5-5), which has been shown to be appropriate to describe the relationship between η_r and [η] in their test system.

$$\log\left(\frac{\eta_r - 1}{C}\right) = \log[\eta] + k_M[\eta]\cdot C \qquad \text{(eq. 5-5)},$$

with C being the concentration of HA and k_M being the viscosimetric constant (0.138), which has been determined under the test conditions.

From the calculated values of [η] the authors obtained the decrease in the viscosity average molecular mass M_v during the enzymatic reaction using the Mark-Houwink equation (eq. 5-6) and the Mark-Houwink constants of HA, determined under the test conditions at 37 °C ($\alpha = 0.72$; $K = 0.036$).

$$[\eta] = K \cdot M_V^{\alpha} \qquad \text{(eq. 5-6)}$$

The reciprocal values of M_v were then plotted as a function of the reaction time yielding a linear relationship with a slope k_v. The reaction rate expressed as the number of bonds broken per unit time (k_n) was calculated from k_v according to eq. 5-4.

The reactions rates, expressed as the number of cleaved bonds per unit time (in the following termed absolute reaction rate), obtained by the viscosimetric approach were found to be in good agreement with the reaction rates obtained by size exclusion chromatography, i.e. the reaction rates obtained from the slopes of the plots of $1/M_n$ as a function of the reaction time.

In continuation of the determination of the absolute reaction rate Vercruysse et al. (1995) applied an empirical method for the estimation of enzyme activity from the decrease of η_r of the substrate (HA) solution during the enzymatic reaction. In brief, $(\ln \eta_r)^{-1}$ was plotted as a function of the reaction time (T+t/2) and an empirical reaction rate was determined from the slope of the plot which showed good linearity at the initial stage of the reaction.

By comparison of the empirical reaction rates with the absolute reaction rates, expressed as the number of cleaved bonds per unit time, it was demonstrated that the slope of the plot of $(\ln \eta_r)^{-1}$ as a function of the reaction time (T+t/2) is related to the number of bonds broken per unit time, and thus provides a good estimate of enzyme activity.

5.3.4.1 Comparison of the activities of Neopermease® and S. agalactiae hyaluronate lyase

Comparison of the enzyme activities of Neopermease® and *S. agalactiae* hyaluronate lyase by the colorimetric and the turbidimetric method revealed that the higher activity of the bacterial enzyme compared to the bovine enzyme was less pronounced in the turbidimetric assay than in the colorimetric assay, i.e. the equiactive concentrations of the two hyaluronidase preparations were found to depend on the type of assay (cf. table 5-3).

Considering the desired pharmacological effect when hyaluronidase is applied as a spreading factor, the reduction in viscosity of hyaluronic acid by an enzyme preparation is the most direct physicochemical correlate of the therapeutic action (Humphrey and Jaques, 1953). Therefore, Neopermease® and hyaluronate lyase from *S. agalactiae* were also investigated with respect to their ability to reduce the viscosity of hyaluronate solutions. Using an Ubbelohde viscometer the reduction of the viscosity of substrate solution induced by the action of the hyaluronidase preparations was measured as a function of the reaction time during incubation under similar conditions as used in case of the colorimetric and turbidimetric method, respectively. Taking into account the results of Daubenmerkl et al. (1957) who found that the activities of hyaluronidase preparations, measured in the turbidimetric assay, are in good correlation with the activities of the respective preparations in a viscosimetric assay, it was expected that turbidimetrically equiactive concentrations of Neopermease® and *S. agalactiae* hyaluronate lyase would reduce the viscosity of the incubation mixture to an equal extent.

Indeed, as it is shown in Fig. 5-14, after a reaction time of 30 min, viscosity was reduced to the same degree. However, interestingly, the viscosity reduction curves intersect at this point. While in the beginning of the reaction the bovine enzyme caused a faster reduction in viscosity of the incubation mixture, in the course of the reaction the viscosity reduction rate of the bovine enzyme decreased below that of the bacterial enzyme.

An explanation for this phenomenon might be found when looking at the different catalytic mechanisms of the investigated enzymes. As an endolytic enzyme bovine hyaluronidase effectively reduces the molecular weight of the high molecular HA chains with every cleavage step. In case of the bacterial hyaluronate lyase, after the first endolytic attack the molecular weight of the HA chains is reduced rather ineffectively by the processive release of disaccharides until one chain is fully degraded and the next endolytic attack occurs.

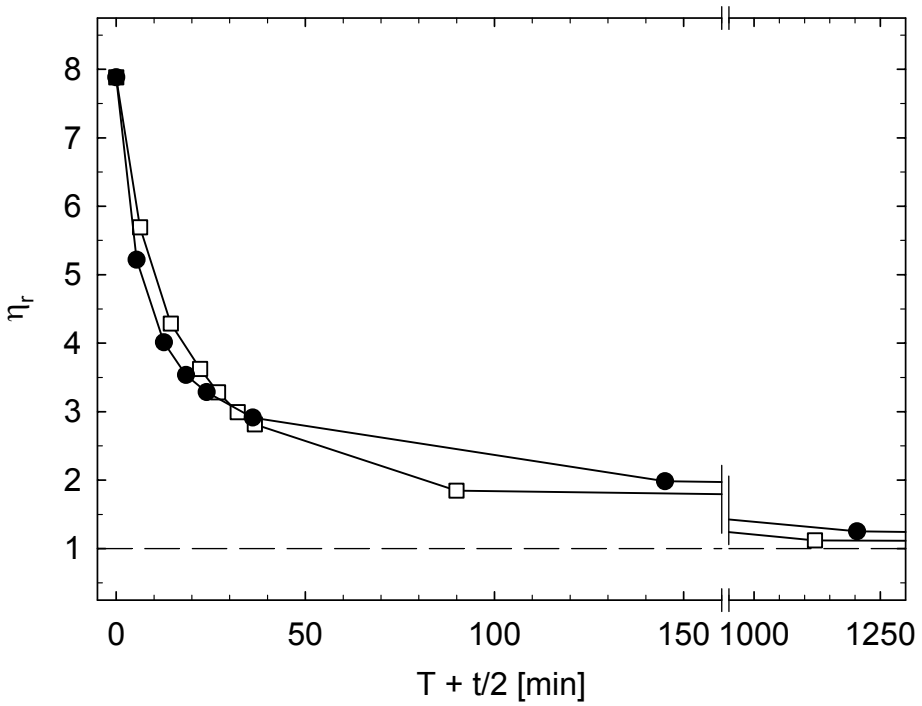

Fig. 5-14: Reduction of the viscosity of substrate (HA) solution induced by the action of Neopermease® (—●—) and *S. agalactiae* hyaluronate lyase (—□—). 100 µl of enzyme solution (Neopermease®: 9 units#; hyaluronate lyase: 2.34 units#) were incubated with incubation mixture 5-V (pH 5.0, 18 ml) at 37 °C and the relative viscosity (η_r) of the incubation mixture was determined as a function of the reaction time (cf. 5.2.2.2). The dashed line indicates the η_r of the reference mixture in the absence of substrate.

The fact that the molecular weight of the high molecular HA chains is more effectively reduced by the endolytic action of BTH explains the higher rate of viscosity reduction observed for the bovine enzyme compared to the bacterial enzyme in the initial stage of the reaction. However, in the further course of the reaction the higher rate of HA cleavage by the bacterial enzyme compared to the bovine enzyme (cf. chapter 3) may become the decisive factor for the rate of viscosity reduction. The latter explains the observation that the viscosity reduction rate of the bacterial enzyme exceeded that of the bovine enzyme.

Interestingly, when plotting the viscosity reduction curves in Fig. 5-14 according to the method applied by Vercruysse et al. (1995) good linearity is obtained for prolonged reaction time only for the bacterial enzyme, whereas the curve of the bovine enzyme tends to bend downward already at the initial stage of the reaction (Fig. 5-15).

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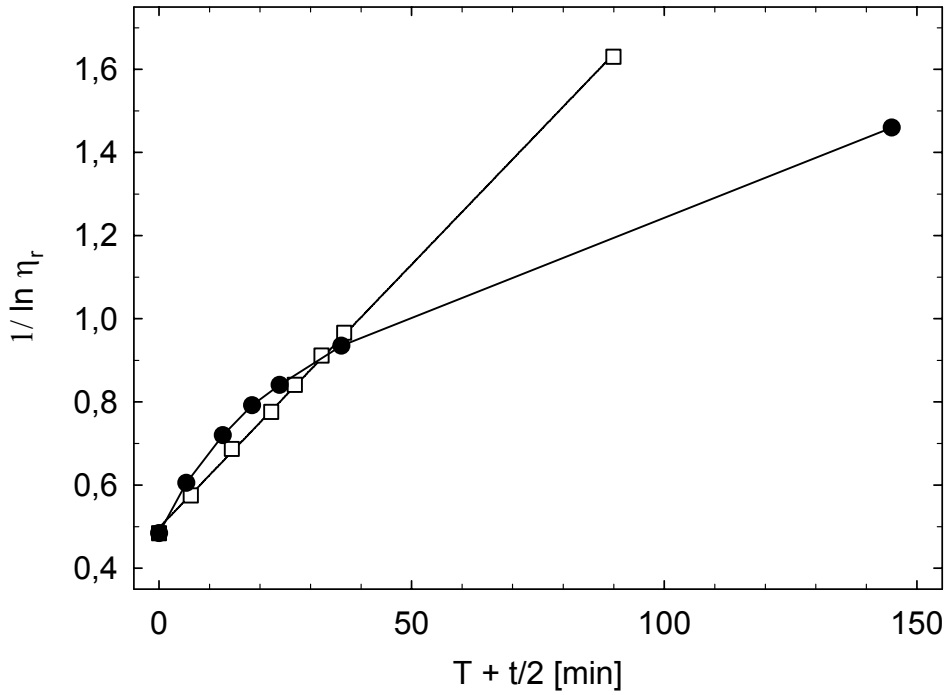

Fig. 5-15: Activity of Neopermease® (—●—) and *S. agalactiae* hyaluronate lyase (—□—) against HA monitored by viscosimetry. For the experimental conditions cf. Fig. 5-14. The reciprocal value of ln η_r is plotted as a function of the reaction time.

This observation is rather surprising, since according to the results of Vercruysse et al. (1995) linearity would be expected for the bovine enzyme preparation but not necessarily for the bacterial enzyme considering the processive, i.e. non random, degradation mechanism of the hyaluronate lyase. The deviance from linearity in case of the investigated BTH preparation may be explained by the fact that Neopermease® contains three enzymatically active proteins differing in molecular mass, specific activity and with respect to their pH activity profiles (cf. chapter 3 and section 5.3.3.1.1).

5.3.4.2 Effect of pH on the activity of Neopermease®

In our previous investigations concerning the effect of pH on the activity of Neopermease® almost inverted pH activity profiles were obtained by the colorimetric (maximum at pH 3.6) and the turbidimetric (maximum at pH 6.0) hyaluronidase assay (cf. Fig. 5-10). The determination of hyaluronidase activity as a function of pH by an additional method was therefore of particular interest.

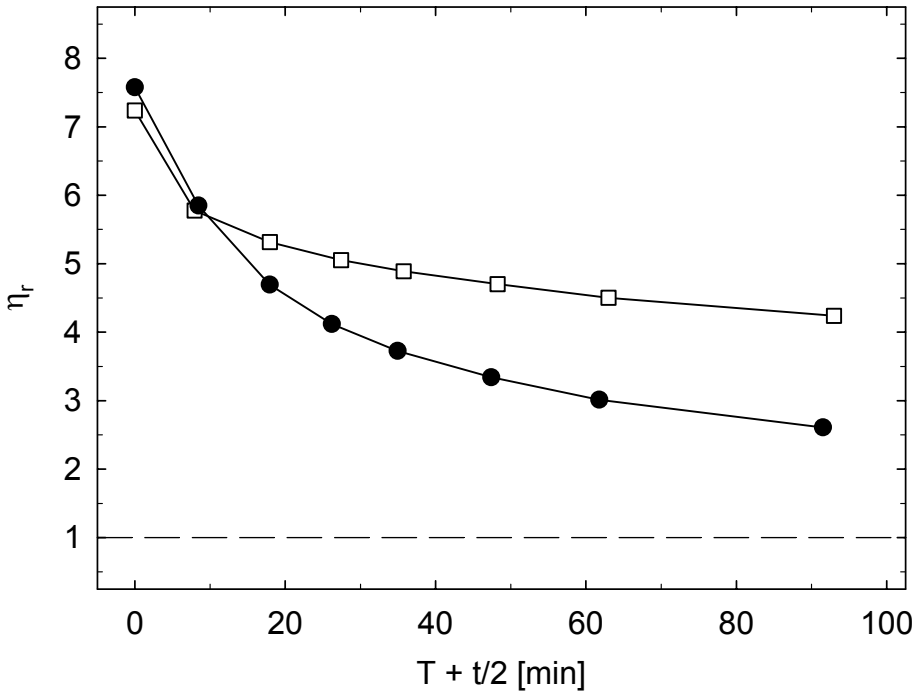

Fig. 5-16: Reduction of the viscosity of substrate (HA) solution induced by the action of Neopermease® during incubation at pH 3.8 (—□—) and pH 6.0 (—●—), respectively. 90 µl of enzyme solution (3.6 units#) were added to incubation mixture 5-V (16 ml) at 37 °C and the relative viscosity (η_r) of the incubation mixture was determined as a function of the reaction time (cf. 5.2.2.2). The dashed line indicates the η_r of the reference mixture in the absence of substrate.

Fig. 5-16 exemplarily shows the viscosity reduction curves obtained by the incubation of Neopermease® with hyaluronan at pH 3.6 and pH 6.0 using similar incubation conditions as used in the colorimetric and the turbidimetric assay. While the curves run nearly in parallel within the first 10 min, in the further course of the reaction the viscosity was reduced to a considerably higher extent at pH 6.0. Thus, when analysing the data by analogy to the colorimetric and turbidimetric determination of pH activity profiles, i.e. with respect to the extent of viscosity reduction after an incubation time of 60 min, a pH activity profile was obtained, which is in quite good agreement with the results of the turbidimetric assay, whereas after an incubation time of 8 min the highest viscosity reduction was found at pH 5 (Fig. 5-17).

The phenomenon that the decrease in the initial reaction rate is more rapid at lower pH values in the viscosimetric assay was also observed by Salegui et al. (1967) who report that BTH shows an initial optimum activity for short time periods (2 min) at pH 3.5, which shifts with time to a broad plateau between pH 4.5 and 6.

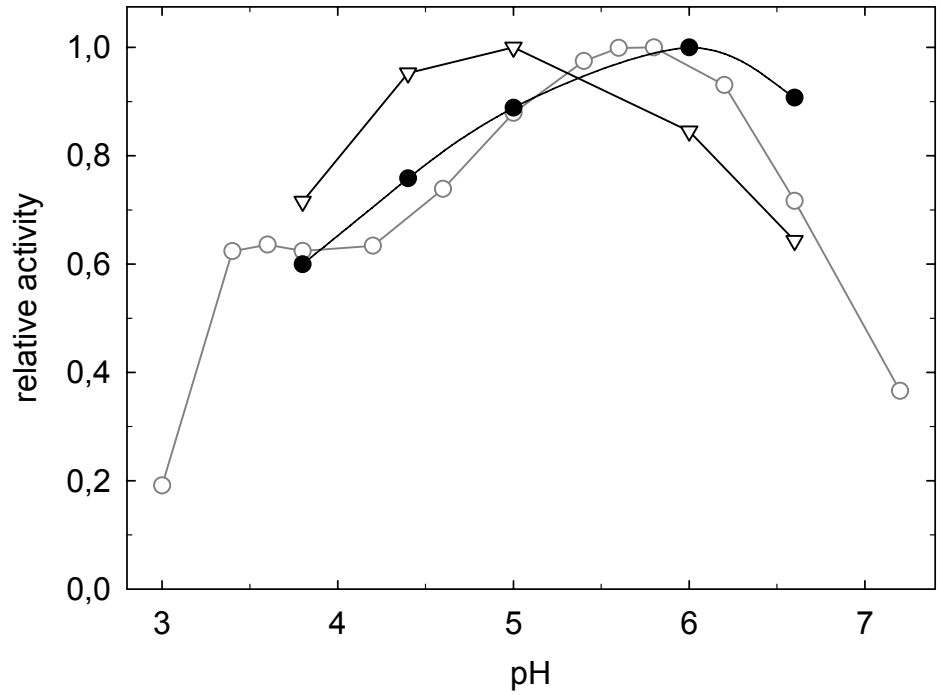

Fig. 5-17: Effect of pH on the activity of Neopermease® determined by viscosimetry after a reaction time of 8 min (—▽—) and 60 min (—●—), respectively. For experimental conditions cf. Fig. 5-16. pH activity profile as obtained by the turbidimetric method (—○—; cf. Fig. 5-10)

From the discrepancy between the viscosimetrically and the colorimetrically determined pH activity profiles of Neopermease® it can be concluded that the ability of the enzyme to reduce the viscosity of the substrate solution does not correlate with the liberation of NAG at the reducing ends of sugars. The pH activity profile obtained by the colorimetric method showed that the amount of reducing NAG end groups produced by the action of BTH is higher at pH 3.8 compared to pH 6.0. However, the enzymatically active fraction being responsible for the high product formation at acidic pH might preferably cleave HA chains of low molecular weight and thus contribute less to viscosity reduction. Another factor contributing to the discrepancy between the viscosimetrically and colorimetrically determined pH activity profiles may be the transglycosylase activity of BTH. The transglycosylation, which is favoured at neutral and weakly acidic pH (Saitoh et al., 1995), interferes with the colorimetric assay but may have only a marginal if any effect on the viscosity of the incubation mixture as it occurs in the low molecular weight region of HA fragments.

The fact that the mechanism of HA degradation by Neopermease® is not identical at pH 3.8 and pH 6.0, can also be seen from the different shapes of the respective viscosity reduction curves (Fig. 5-16). Interestingly, when plotting the reciprocal value of ln η_r as a function of

the reaction time to estimate an empirical reaction rate from the slope of the plot at the initial stage of the reaction according to Vercruysse et al. (1995), a rather abrupt bend was observed after a reaction time of 10 min in case of the curve obtained at pH 3.8, whereas the slope of the curve obtained at pH 6.0 was found to decrease only slightly within 90 min (Fig. 5-18). Furthermore, a comparison of the plots of the viscosity reduction data obtained at pH 5.0 and pH 6.6 revealed intersecting curves and thus shows that at the initial stage of the reaction the empirical reaction rate was higher at pH 5.0 (Fig. 5-18), although the extent of viscosity reduction after a reaction time of 60 min was found to be slightly higher at pH 6.6 (Fig. 5-17).

Another noteworthy observation is the fact that with increasing pH of the reaction mixture better linearity becomes obvious in the plots of the reciprocal value of ln η_r as a function of the reaction time, independent from the slope of the particular curve. In contrast to the curves obtained at pH 5.0 and below, the prolonged linearity of the curves resulting from measurements at weak acidic pH (pH 6.0 and pH 6.6) is in good agreement with the results reported by Vercruysse et al. (1995), who investigated the degradation of HA by BTH at a pH of 6.4.

As outlined above, Vercruysse et al. suggest an at random mechanism for the degradation of HA by BTH at pH 6.4. One could thus speculate that the deviance from linearity of the curves obtained at more acidic pH might result from non at random degradation. As mentioned in the context with the discrepancy of the colorimetrically and viscosimetrically determined pH activity profiles it is conceivable that the enzyme fraction being active at pH 3.8 preferably cleaves HA chains of low molecular weight. However, the correlation between the kinetics of viscosity reduction and the underlying degradation mechanism of HA by hyaluronidase appears to be quite complex, considering the fact that a linear relationship between the reaction time and the reciprocal value of ln η_r was obtained in case of two different degradation mechanisms, i.e. by incubation with both the endolytically acting Neopermease® (at pH 6.6) and the *S. agalactiae* hyaluronate lyase (cf. Fig. 5-15) which is known to act on HA in a non random way.

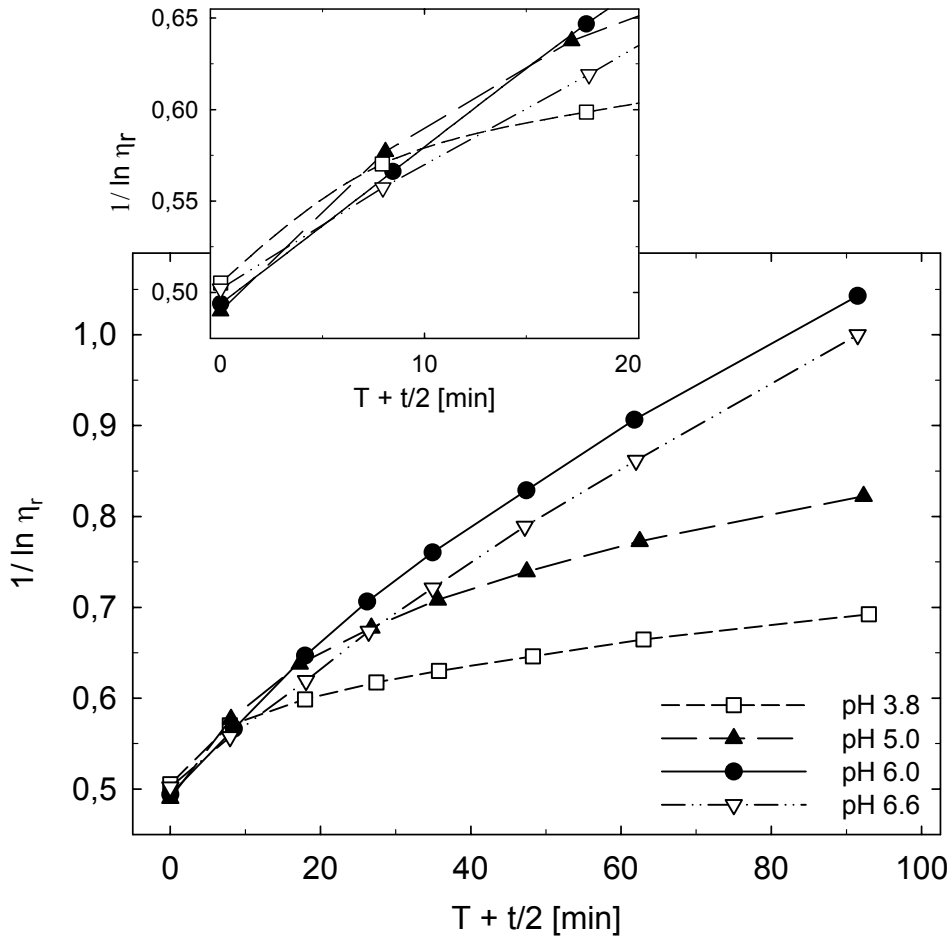

Fig. 5-18: Activity of Neopermease® in the viscosimetric assay at various pH values. For the experimental conditions cf. Fig. 5-16. The reciprocal value of ln η_r is plotted as a function of the reaction time. *Inset:* magnified view of the initial part of figure 5-18.

In the context with the viscosimetric investigation of the effect of pH on the activity of hyaluronidase another aspect to be considered is the fact that aqueous solutions of HA vary in their rheological properties depending on the pH and on the ionic strength of the solution. Previous rheological studies (Balazs, 1966; Gibbs et al., 1968) revealed that by a decrease in pH to a value of 2.5 the dynamic viscoelastic properties of HA solution reached a maximal value, whereas the limiting viscosity number (intrinsic viscosity) and the sediment volume reached a minimal value. Investigations on the optical properties of HA in aqueous solution (Chakrabarti and Balazs, 1973) revealed that with increasing hydrogen ion concentration the circular dichroic band at 187.5 nm changes abruptly to the positive side below pH 4 and reaches a maximum value at pH 2.5. The observed changes in both the rheological and the optical properties of solutions of HA at low pH thus indicate the occurrence of conformational

changes which can be explained by the formation of ordered crosslink regions due to the decrease in the ionization of the carboxyl groups of HA (Chakrabarti and Balazs, 1973).

It can be speculated that the observed differences in the kinetics of viscosity reduction by the action of Neopermease® depending on the pH of the incubation mixture might rather be ascribed to the different rheological properties of the incubation mixture than to the pH dependent differences in degradation mechanism. However, it seems unlikely that this assumption is true, given the fact that in case of HA degradation with bee venom hyaluronidase the reduction of viscosity was found to obey kinetics being consistent within the investigated pH region (pH 3.8 – pH 6.0, cf. section 5.3.5).

5.3.4.3 Effect of a H_2O/D_2O exchange in the incubation mixture

In view of NMR studies on hyaluronan degradation by the different hyaluronidases the activities of Neopermease® and *S. agalactiae* hyaluronate lyase were measured by the viscosimetric method under incubation conditions, applicable for monitoring the enzyme reactions by NMR spectroscopy (cf. chapters 6 and 7).

Incubation was carried out at 21 °C in phosphate buffer, pH 6.0, containing NaCl and BSA. To study the effect of H_2O/D_2O replacement on the activity of the bovine and bacterial enzyme viscosimetric measurements were carried out in both solvents. As shown in Fig. 5-19 the activity of the *S. agalactiae* hyaluronate lyase was not affected by the replacement of H_2O with D_2O. However, in case of Neopermease® reduced activity was measured under D_2O conditions: by comparison of the slopes of the plots of the reciprocal value of ln η_r versus reaction time the empirical reaction rates differed by a factor of 1.5.

The observation that in contrast to the bacterial enzyme the activity of the bovine enzyme preparation is affected by replacing H_2O with D_2O reflects the different mechanisms of HA degradation by the two hyaluronidases. In case of Neopermease®, which acts as a hydrolase, water molecules are directly involved in the degradation process insofar as they are transferred to the substrate molecule during the hydrolytic cleavage via a nucleophilic attack (cf. chapter 1). In case of the bacterial hyaluronate lyase water molecules are participating the degradation process, too, however, not as nucleophiles but as proton donors and acceptors, respectively, in the course of the regeneration of the catalytic residues involved in the ß-elimination reaction (cf. chapter 1).

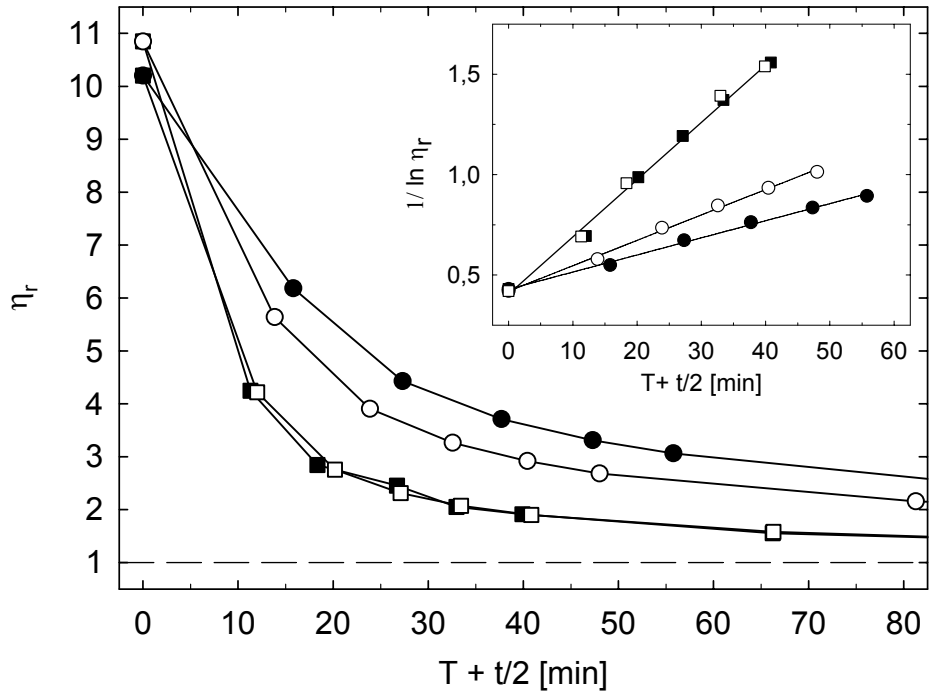

Fig. 5-19: Reduction of the viscosity of the substrate (HA) solution induced by the action of Neopermease® (○/●) and *S. agalactiae* hyaluronate lyase (□/■) as a function of the solvent (H_2O (○/□); D_2O (●/■)) in the incubation mixture. 100 µl of enzyme solution (Neopermease®: 46 units#; hyaluronate lyase: 12 units#) were incubated with the incubation mixtures 5-VII (18 ml) and 5-VIII (18 ml) at 21 °C. The relative viscosities (η_r) of the incubation mixtures and the reciprocal value of ln η_r (inset) were plotted as a function of the reaction time (cf. 5.2.2.2). The dashed line indicates the η_r of the reference mixtures (in H_2O or D_2O, respectively) in the absence of substrate.

5.3.4.4 Effect of NaCl on the activities of Neopermease® and S. agalactiae hyaluronate lyase

The viscosimetric determination of the activities of Neopermease® and *S. agalactiae* hyaluronate lyase in the absence and in the presence of NaCl (0.067 M) revealed that the activities of the two hyaluronidase preparations were inversely affected by addition of NaCl to the incubation mixture. Comparison of the slopes of the plots of the reciprocal value of ln η_r as a function of the reaction time showed that the empirical reaction rate of the bovine enzyme was by a factor of 3.3 higher in the presence of NaCl, whereas in case of the bacterial enzyme activity was decreased by 33 % (cf. Fig. 5-20).

as declared by the supplier

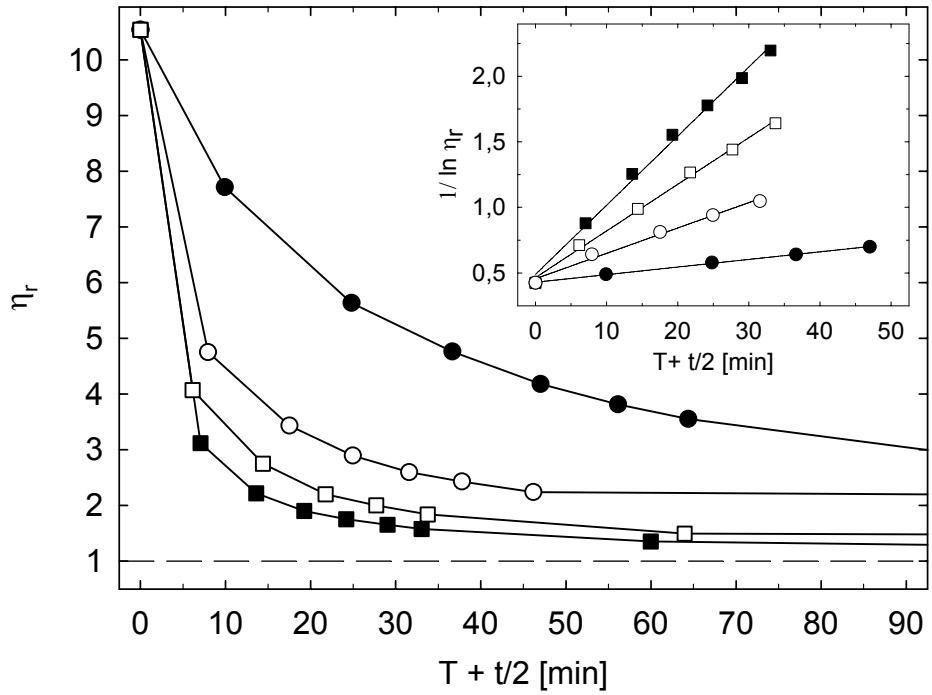

Fig. 5-20: Reduction of the viscosity of substrate (HA) solution induced by the action of Neopermease® (○/●) and *S. agalactiae* hyaluronate lyase (□/■) in the presence (○/□) and in the absence (●/■) of NaCl (0.67 M) in the incubation mixture. 100 µl of enzyme solution (Neopermease®: 46 units#; hyaluronate lyase: 12 units#) were incubated with the incubation mixtures 5-VI (18 ml) and 5-VII (18 ml) at 21 °C. The relative viscosities (η_r) of the incubation mixtures and the reciprocal value of ln η_r (inset) were plotted as a function of the reaction time (cf. 5.2.2.2). The dashed line indicates the η_r of the reference mixtures (with or without NaCl, respectively) in the absence of substrate.

For the bacterial enzyme this result is qualitatively in agreement with measurements of product formation at 232 nm (cf. chapter 3) under identical conditions revealing a 45 % decrease in activity when NaCl (0.067 M) was added to the incubation mixture (results not shown). With Neopermease® no additional assay was performed under identical conditions in this work. However, the results obtained by viscosimetry are supported by the data of Oettl (2000), who determined an optimal NaCl concentration in the region from 0.045 M to 0.100 M for the activity of Neopermease® in the colorimetric assay.

as declared by the supplier

5.3.5 Characterisation of a hyaluronidase from bee venom (BVH)

5.3.5.1 Activity of BVH

In addition to the studies on hyaluronidase preparations from *S. agalactiae* and bovine testes a recombinant bee venom hyaluronidase (BVH), expressed in baculovirus-infected insect cells, was investigated with respect to its activity in the different hyaluronidase assays. By analogy with the bovine and bacterial enzymes (cf. chapter 3) the activity of BVH was determined by measuring the liberated NAG at the reducing ends of sugars after various incubation periods by the colorimetric method of Reissig et al. (1955), using standards with known NAG concentration. Catalysing the formation of 56.4 µmol of reducing NAG moieties per min and mg, the bee venom enzyme showed a specific activity that was 22 times higher compared to Neopermease® and 18 times lower compared to the *S. agalactiae* hyaluronate lyase, respectively.

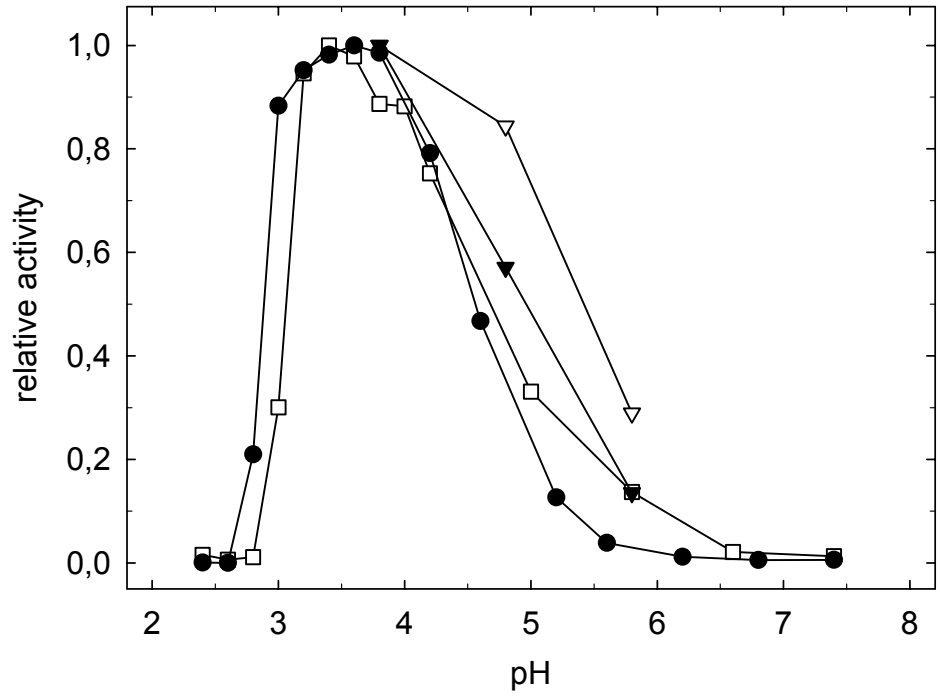

Fig. 5-21: Effect of pH on the activity of recombinant hyaluronidase from bee venom determined by the colorimetric (—□—), turbidimetric (—●—) and viscosimetric (▼/▽) method. 50 µl of enzyme solution (colorimetric assay: 34. 29 ng; turbidimetric assay: 5.14 ng) were incubated with incubation mixture 5-IV (400 µl) for 1h at 37 °C. For the experimental conditions of the viscosimetric assay cf. Fig. 5-22. The relative activities in the viscosimetric assay were calculated by comparing both the reduction of viscosity after an incubation time of 60 min (—▽—; cf. 5.2.2.2) and the slopes of the plots of $1/\ln \eta_r$ as a function of the reaction time (0 – 30 min, —▼—).

The determination of the activity of BVH as a function of pH yielded nearly identical pH activity profiles in the colorimetric and the turbidimetric assay showing maximum activity at pH 3.5 (Fig. 5-21).

The bee venom enzyme and bovine testicular hyaluronidase, which has been shown to be a fragment of the membrane bound PH-20 enzyme (Meyer et al., 1997), belong to the same family of glycosyl hydrolases (EC 3.2.1.35, glycosyl hydrolase family 56). In addition BVH has been found to be homologous to the mammalian PH-20 enzyme showing 37 % sequence similarity and conservation of the active site residues, suggesting a similar catalytic mechanism for the two enzymes (Gmachl and Kreil, 1993; Markovic-Housley et al., 2000). Thus it is surprising that the hyaluronidase from bee venom did not show the inverted pH activity profiles which were obtained for Neopermease® in the colorimetric and the turbidimetric assay, respectively (cf. Fig. 5-10). While Neopermease® showed enzyme activity over a broad range (pH 3 – 8) and was characterised by complex pH activity profiles corresponding to the sum of the pH profiles of different enzymatically active fractions (cf. chapter 3 and section 5.3.3.1.1), in case of BVH no activity was detected at pH > 6 and the pH profiles were almost symmetrically, indicating that the preparation contains only a single enzymatically active protein.

An attempt to explain the shift of the pH optimum of BTH from pH 3.5 in the colorimetric assay to pH 6 in the turbidimetric assay was the transglycosylase activity of BTH, which, in contrast to hydrolysis, does not result in the formation of new reducing NAG end groups and thus affects the results of the colorimetric assay, whereas in the turbidimetric assay changes of the substrate length in the low MW region are not detected. Presuming that the transglycosylase activity is higher at weak acidic and neutral pH compared to pH 3.5 (Saitoh et al., 1995), this provides a plausible explanation for the difference in the ratios of activity at pH 3.5 and pH 6 as measured in the different assays in case of BTH. By contrast, in case of the bee venom enzyme the low level of detection of reducing NAG in the colorimetric assay at weak acidic pH cannot be ascribed to transglycosylase activity since also in the turbidimetric assay, which is not affected by the transglycosylation reaction, only very low activity was obtained around pH 6.

Taken together, the results obtained by the viscosimetric determination of the activity of BVH at varying pH were in agreement with the pH profiles of the colorimetric and the turbidimetric assay (cf. Fig. 5-21). The viscosimteric data were analysed by two methods which yielded

qualitatively the same results: the relative activities were calculated both by comparing the extent of viscosity reduction after an incubation time of 60 min (cf. 5.2.2.2) and by comparing the slopes of the plots of 1/ln η_r as a function of the reaction time (0-30 min), i.e. the empirical reaction rates according to Vercruysse et al. (1995). The latter method of analysis was possible since, in contrast to the investigations with Neopermease®, linear curves were obtained at acidic pH, too, when plotting 1/ln η_r as a function of the reaction time (cf. Fig. 5-22).

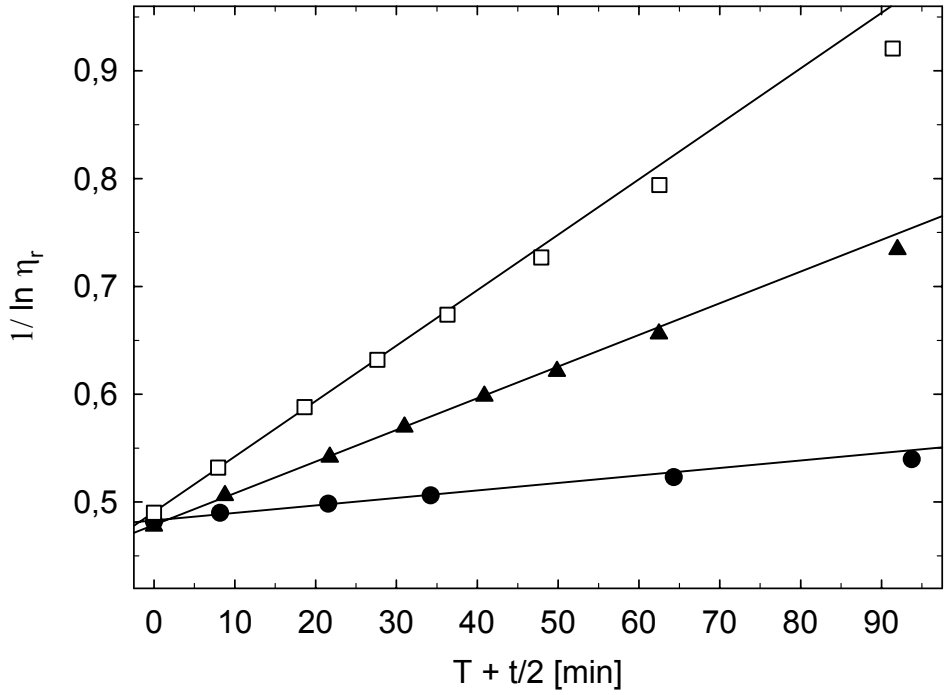

Fig. 5-22: Activity of recombinant hyaluronidase from bee venom in the viscosimetric assay at pH 3.8 (□), pH 4.8 (▲) and pH 5.8 (●), respectively. 90 µl of enzyme solution (21.6 ng) were incubated with incubation mixture 5-V (16 ml) at 37 °C and the relative viscosity (η_r) of the incubation mixture was determined as a function of the reaction time (cf. 5.2.2.2). The reciprocal value of ln η_r is plotted as a function of the reaction time.

Two remarkable differences in comparison to the bovine enzyme preparation were observed in the viscosimetric assay of BVH: the kinetics of the reduction of viscosity was independent on the pH of the incubation mixture and the extent of the reduction in viscosity at different pH was in good correlation with the liberation of reducing NAG end groups.

In summary our investigations demonstrate that no discrepancies in the apparent activity of recombinant BVH were observed when comparing the pH activity profiles obtained by the different methods under identical incubation conditions. However, Allalouf et al. (1975) who

determined the activity of bee venom hyaluronidase, isolated from venom sacs, by the colorimetric method in the absence of NaCl and BSA, reported a pH activity profile, which shows maximum activity at pH 4.5 and remarkable activity at neutral pH but no activity at pH 3.5. The discrepancy to our results indicates that, as it is known for BTH (Gacesa et al., 1981; Oettl, 2000), also the activity of bee venom hyaluronidase is affected by the composition of the incubation buffer.

5.3.5.2 SDS-PAGE

To obtain information about the purity of the investigated recombinant bee venom hyaluronidase and the molecular mass of the contained proteins the enzyme preparation was separated by SDS-PAGE.

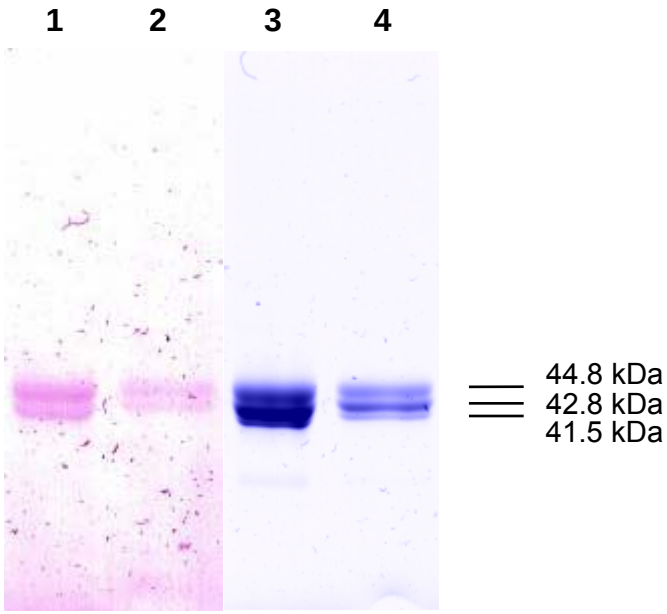

Fig. 5-23: SDS-PAGE of recombinant bee venom hyaluronidase (BVH). BVH (2.5 µg (lanes 1; 3) and 1.25 µg (lanes 2; 4)) was separated on an 8 % gel. The gel was stained according to the periodic acid-Schiff (PAS) technique (lanes 1; 2) prior to staining with Coomassie Brilliant Blue G-250 (lanes 3; 4). Molecular masses were assigned by the use of marker proteins.

As shown in Fig. 5-23 (lanes 3; 4) Coomassie staining revealed the existence of three proteins slightly differing in molecular mass. Glycosylation was demonstrated for the two proteins with higher molecular mass by staining according to the periodic acid-Schiff (PAS) technique (lanes 1; 2). The main constituent of the recombinant BVH was a 43 kDa glycoprotein which is in agreement with the molecular mass of the hyaluronidase isolated from honey bee venom

(Kemeny et al., 1984), whereas the weak band at 41.5 kDa can be ascribed to the nonglycosylated form of bee venom hyaluronidase (Soldatova et al., 1998). The finding that the recombinant BVH, which was expressed in baculovirus-infected insect cells, did not appear as single band on SDS-PAGE is consistent with the results of Soldatova et al. (1998), who report on the appearance of "two to three bands at 43 to 44 kDa in SDS-PAGE" corresponding to proteins which were shown to differ at the N-terminus due to the fact that in addition to the cleavage of the signal sequence at the proper cleavage site additional cleavage occurred after the fourth and the tenth position from the first cleavage.

5.4 Conclusion

Characterisation of the BTH preparation Neopermease® and a preparation of hyaluronate lyase from *S. agalactiae* with respect to their activities in the turbidimetric assay revealed that 1 TRU corresponds to 1.75 designated units of Neopermease® and to 0.45 designated units of the bacterial hyaluronate lyase preparation, respectively, i.e. in the turbidimetric assay the equiactive concentration of the bacterial enzyme preparation was by a factor of 3.86 lower compared to Neopermease®. Concentrations of the two enzyme preparations being equiactive in the turbidimetric assay were found to reduce the viscosity of the substrate solution to an equal extent in the viscosimetric assay. Comparison of these results with the activities determined in the colorimetric assay (cf. chapter 3) revealed that in the turbidimetric and the viscosimetric assay the higher activity of the bacterial enzyme compared to the bovine enzyme was less pronounced than in the colorimetric assay, where the equiactive concentrations differed by a factor of 60. Thus, it was shown that equiactive concentrations of the two enzyme preparations depend on the type of assay.

In the turbidimetric and the viscosimetric assay hyaluronidase activity is quantified according to the ability of the enzyme to reduce the molecular mass of substrate chains. The aforementioned results thus demonstrate that, when one compares preparations of bovine and bacterial hyaluronidase, equiactive concentrations with respect to product formation, as determined in the colorimetric assay, are not equieffective in reducing the molecular mass of the substrate chains and, thus, in reducing the viscosity of the hyaluronate-enriched extracellular matrix.

The fact that in the turbidimetric and the viscosimetric assay the activity of the bacterial hyaluronate lyase was relatively low compared to its high activity in the colorimetric assay

partly explains the observation that, in constrast to Neopermease®, the hyaluronate lyase from *S. agalactiae* did not show the spreading effect when applied to enhance the intratumoral accumulation of melphalan (cf. chapter 4). However, this explanation is compromised by the fact that the activity administered in the in vivo experiments with the bacterial hyaluronate lyase was, compared to the activity applied in the corresponding experiments with Neopermease®, by a factor of 19.3 higher when extrapolated from the results of the turbidimetric and viscosimetric assay.

The turbidimetrically determined pH activity profile of hyaluronate lyase from *S. agalactiae* with a maximum at pH 5.0 is in good agreement with the results of the colorimetric assay. By contrast, in case of the two BTH preparations Neopermease® and Hylase® "Dessau" the different methods yielded almost inverted pH profiles showing maximum activity at pH 3.6 in the colorimetric assay and at pH 6.0 in the turbidimetric assay, respectively. Comparison of the isolated enzymatically active proteins contained in Neopermease® with respect to their pH profiles by both methods revealed that in both assays the complex effect of pH on the activity of Neopermease® can be explained as the sum of the pH profiles of the individual proteins differing in specific activity. While the pH profiles of the 33 kDa and the 77 kDa fraction (maximum activity at pH 6) were not affected by the type of assay, the main 58 kDa fraction gave the inverse profiles typical for the whole preparation.

The determination of pH-dependency of the activity of Neopermease® by viscosimetry confirmed the pH profile of the turbidimetric assay (activity optimum at pH 6.0).

The higher activity of BTH at pH 6.0 compared to pH 3.6 in the turbidimetric and the viscosimetric assay indicates that the molecular mass of the substrate chains is more effectively reduced at pH 6.0 than at pH 3.6, regardless of the higher rate of liberation of NAG end groups detected in the colorimetric assay at pH 3.6. It can thus be speculated that at pH 3.6 oligosaccharides are preferentially generated in the presence of high molecular mass HA, resulting in the low activity in the turbidmetric and the viscosimetric assay, whereas at pH 6.0 all types of molecules of the polydisperse substrate are processed to the same extent. Another possible explanation for the different pH optima of BTH, depending on the type of assay, may be the transglycosylation activity of BTH which is favoured at neutral and weak acidic pH (Saitoh et al., 1995) and interferes with the colorimetric but not with the turbidimetric and the viscosimetric assay.

Determination of the activity of a recombinant bee venom hyaluronidase (BVH) as a function of pH yielded nearly identical pH activity profiles in the colorimetric and turbidimetric assay showing maximum activity at pH 3.5. Considering that BVH and BTH belong to the same family of glycosyl hydrolases (EC 3.2.1.35) and show 37 % sequence similarity, it is surprising that BVH does not show the inverted pH profiles which were obtained with BTH under identical conditions.

The colorimetrically and turbidimetrically obtained pH profile of BVH was confirmed by viscosimetry (maximum activity at pH 3.8). In the viscosimetric assay of BVH the kinetics of viscosity reduction was independent of the pH of the incubation mixture (linear curves were obtained when plotting $1/\ln \eta_r$ versus reaction time within the range of pH 3.8 to pH 5.8), thus indicating that the pH-related differences in the kinetics of viscosity reduction, which were observed in the viscosimetric assay of Neopermease®, are most probably not due to different rheological properties of the incubation mixture at various pH but reflect pH-dependent differences in the mechanism of HA degradation by BTH.

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6 pH-dependent differences in the mechanism of hyaluronate degradation by bovine testicular hyaluronidase

6.1 Introduction

Since several different methods are used for the determination of enzymatic activity of bovine testicular hyaluronidase (BTH), the data reported in the literature are inconsistent in part or even contradictory: optimum activity of BTH was found at acidic pH (pH 3.5 - 4.0) (Bonner and Cantey, 1966; Muckenschnabel et al., 1998a; Seaton et al., 2000) as well as at weakly acidic pH (pH 5.0 - 6.0) (Dorfman and Ott, 1948; Allalouf et al., 1975; Highsmith et al., 1975) and at neutral pH (Tung et al., 1994; Csoka et al., 1998).

Comparative analysis of the two BTH preparations Neopermease® and Hylase® "Dessau" with respect to their activity in two frequently used hyaluronidase assays - the colorimetric assay and the turbidimetric assay - revealed that the two BTH preparations are characterised by nearly identical complex pH activity profiles. However, the pH profiles obtained by the different methods were almost inverted showing maximum activity at pH 3.6 (colorimetric assay) and at pH 6.0 (turbidimetric assay), respectively (cf. chapter 5, figures 5-10 and 5-13).

For Neopermease® the turbidimetrically obtained pH profile was confirmed by the viscosimetric determination of hyaluronidase activity. In addition, comparison of the viscosity reduction curves (plots of viscosity reduction induced by hyaluronidase versus reaction time) at varying pH showed that the kinetics of viscosity reduction by Neopermease® depended on the pH of the incubation medium, thus indicating pH-related differences in the mechanism of HA degradation by BTH (cf. chapter 5, figures 5-16, 5-17 and 5-18).

In addition to hydrolysis, BTH catalyses the reverse reaction, i.e. the transglycosylation (cf. chapter 1, Fig. 4-1). It has been reported that the optimal pH for the transglycosylation reaction is about pH 7.0, whereas hydrolysis is favoured at pH < 5.0. Furthermore, highest transglycosylase activity was found in the absence of NaCl, whereas transglycosylation was nearly completely inhibited at concentrations of NaCl higher than 0.5 M (Saitoh et al., 1995).

It can be assumed that the discrepancy between the pH activity profiles obtained for BTH in the different assays results from the fundamentally different principles of the colorimetric

assay on the one hand and the turbidimetric and the viscosimetric assay on the other hand. The turbidimetric and the viscosimetric assay are physicochemical methods in which the activity of hyaluronidase is quantified according the reduction of the size of high molecular mass HA molecules by measuring the changes in the physicochemical properties of HA solutions (reduction of turbidity (precipitation) with quartery ammonium salts or reduction of viscosity, respectively). While hydrolysis of high molecular mass HA is sensitively detected by the physicochemical methods, hydrolysis and transglycoslation reactions on HA fragments of low molecular mass are not measured in these assays. In the colorimetric assay the activity of hyaluronidase is quantified by the determination of N-acetyl-D-glucosamine (NAG) at the reducing ends of HA fragments generated by HA hydrolysis and transglycoslation. In contrast to the physicochemical methods, the colorimetric method detects hydrolysis of high and low molecular mass HA molecules in equal measure. However, the results of the activity measurements are affected by the transglycosylase activity of BTH, which, in contrast to hydrolysis, does not lead to the formation of new reducing NAG end groups.

Taking the different principles of the assays into account, the discrepancy in the pH activity profiles obtained for BTH by the different methods might on one hand be explained by the pH related transglycosylase activity of BTH, which is detected in colorimetric assay but not by the physicochemical methods. On the other hand the discrepancy might result from different affinities of the enzyme to high and low molecular mass HA, depending on the pH.

To find out, if the phenomenon that the pH profile of BTH depends on the type of assay indeed results from pH-related differences in the mechanism of HA degradation by BTH, in the present study the composition of the oligosaccharide mixtures produced by the action of Neopermease® at varying pH was analysed by CE and anion exchange HPLC.

6.2 Materials and methods

6.2.1 Chemicals

Neopermease® and the pure stabiliser (cf. chapter 3) were gifts from Sanabo (Vienna, Austria) whereas bee venom hyaluronidase (BVH, cf. chapter 5) was kindly provided by Dr. Zora Markovic-Housley, University of Basel (Switzerland). Hyaluronic acid (HA) from *Streptococcus zooepidemicus* was purchased from Aqua Biochem GmbH (Dessau, Germany). HA from rooster comb, HA from human umbilical cord and bovine serum albumin (BSA)

were purchased from Serva (Heidelberg, Germany). HA from bovine trachea was from Sigma (Deisenhofen, Germany). Cetyltrimethylammonium bromide (CTAB) was from Roth (Karlsruhe, Germany). 2,5-dihydroxybenzoic acid (DHB) and dextrin 15 were kindly provided by GSG Mess- und Analysengeräte GmbH (Bruchsal, Germany). Deuteriumoxide (D_2O, isotopic enrichment 99.98 %) was purchased from Deutero GmbH (Kastellaun, Germany). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Eschborn, Germany).

6.2.2 Measurement of hyaluronidase activity

6.2.2.1 Colorimetric method

The colorimetric determination of the activity of Neopermease® was based on the method of Reissig et al. (1955) and performed as described in the material and methods section of chapter 3. The composition of the buffer solutions and the various incubation mixtures used is given in the following:

citrate-phosphate buffer 1: solution A: 0.2 M Na_2HPO_4/0.1M NaCl, solution B: 0.1 M citric acid/0.1 M NaCl; solutions A and B were mixed in appropriate portions to adjust the required pH;

citrate-phosphate buffer 2: solution A: 0.2 M Na_2HPO_4/0.05 M NaCl, solution B: 0.1 M citric acid/0.05 M NaCl; solutions A and B were mixed in appropriate portions to adjust the required pH;

citrate-phosphate buffer 3: 1.003 g of NaCl were added to 100 ml of citrate-phosphate buffer 2 of pH 3.6;

incubation mixture 6-I: 100 µl of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 200 µl of citrate-phosphate buffer 1, 50 µl of BSA (0.2 mg BSA per ml of water: solution 1), 50 µl of water;

incubation mixture 6-II: 100 µl of substrate (1 mg HA from *S. zooepidemicus* per ml of water), 200 µl of citrate-phosphate buffer 1, 50 µl of BSA (solution 1), 50 µl of water;

incubation mixture 6-III: 50 µl of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 200 µl of citrate-phosphate buffer 2, 50 µl of BSA (solution 1), 100 µl of water;

incubation mixture 6-IV: 50 µl of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 200 µl of citrate-phosphate buffer 3, 50 µl of BSA (solution 1), 100 µl of water;

incubation mixture 6-V: 50 µl of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 200 µl of citrate-phosphate buffer 1, 50 µl of BSA (solution 1), 100 µl of water.

6.2.2.2 Turbidimetric method

The turbidimetric determination of the activity of Neopermease® was based on the method of Di Ferrante (1956) and performed as described in the material and methods section of chapter 5. The following incubation mixtures were used:

incubation mixture 6-VI: 100 µl of substrate (1 mg HA from *S. zooepidemicus* per ml of water), 200 µl of citrate-phosphate buffer 2, 50 µl of BSA (solution 1), 50 µl of water;

incubation mixture 6-VII: 100 µl of substrate (1 mg HA from *S. zooepidemicus* per ml of water), 200 µl of citrate-phosphate buffer 3, 50 µl of BSA (solution 1), 50 µl of water.

6.2.3 Incubation mixtures of constant ionic strength (I)

For the determination of the effect of pH on the activity of Neopermease® under conditions of constant ionic strength the difference in the ionic strength of incubation mixture 6-III (cf. 6.2.2.1, colorimetric method) at different pH (pH 3.6 and 6.0) was calculated, and compensated by the addition of NaCl.

The ionic strengths of the incubation mixtures were calculated taking into account the ionic species present in citrate-phosphate buffer 2 (cf. 6.2.2.1) of the respective pH (citric acid: pK_{a1} = 3.06; pK_{a2} = 4.74; pK_{a3} = 5.4; phosphoric acid: pK_{a1} = 2.12; pK_{a2} = 7.21; pK_{a3} = 12.32) and the substrate hyaluronate containing negatively charged carboxylic groups (pK_a = 3.18) with associated sodium ions. The concentration of the polymeric hyaluronate was expressed as the concentration of the disaccharide units (1.38 mM). The contribution of BSA was neglected due to the very low concentration (0.67 µM). Ionic strength was calculated with the Maple software based on the law of mass action and using the electroneutrality condition and the mass balance condition.

The calculation revealed ionic strengths of 0.080 M (pH 3.6) and 0.156 M (pH 6.0), respectively. Compensation of the calculated difference was achieved by the use of citrate-phosphate buffer 3, containing 0.22 M NaCl (cf. 6.2.2.1), instead of citrate-phosphate buffer

2, containing 0.05 M NaCl, for preparation of the incubation mixture at pH 3.6 (= incubation mixture 6-IV, cf. 6.2.2.1).

Since hyaluronate was found to make only a minor contribution to the ionic strength of the incubation mixture 6-III (1.5 % at pH 3.6; 0.89 % at pH 6), also in case of incubation mixture 6-VI (cf. 6.2.2.2, turbidimetric method), which differs from incubation mixture 6-III only with respect to a 2.5 times lower hyaluronate concetration (0.552 mM), the ionic strength of pH 3.6 was made up to that of pH 6.0 by the use of citrate-phosphate buffer 3 (= incubation mixture 6-VII).

In the aforementioned calculations the single ion activity coefficients were not taken into account, since according to the present state of the theory in case of complex electrolyte systems an explicit calculation of the single ion activity coefficients is impossible (Wittmann, H.-J.; personal communication).

6.2.4 Analysis of oligosaccharide mixtures produced by the digestion of hyaluronate with hyaluronidase

6.2.4.1 Separation of hyaluronate oligosaccharides by anion exchange HPLC

6.2.4.1.1 Digestion of hyaluronate

The incubation mixture (incubation mixture 6-VIII) consisted of 100 µl of citrate-phosphate buffer 1 (pH 3.5, 5.0, 5.8 and 6.0; cf. 6.2.2.1), 150 µl of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 50 µl of BSA (solution 1, cf. 6.2.2.1) and 100 µl of water. 400 µl of incubation mixture 6-VIII were incubated with 50 µl of enzyme (Neopermease®: 100 units[#], bee venom hyaluronidase: 171.45 ng, each dissolved in BSA (solution 1)) at 37 °C. Incubation was carried out for 1 h, 16.5 h and 62 h, respectively. With Neopermease® additionally a 7-d incubation was performed. In this case enzyme was added in two portions (each 25 µl, containing 100 units[#]) at the beginning and after 4 d of incubation and incubation was carried out at 30 °C. The enzyme reactions were stopped by heating at 100 °C for 15 min. For the subsequent analysis by HPLC protein was removed from the mixtures by ultrafiltration using the Nanosep® centrifugal device with a molecular weight cutoff of 10 kDa (Nanosep® 10 K, Pall Life Sciences, New York, USA). In addition, reference mixtures

[#] as declared by the supplier

consisting of incubation mixture 6-VIII + 50 µl of BSA (solution 1) instead of enzyme were prepared at various pH and treated in the same way as described for the digestion mixtures.

6.2.4.1.2 Instrumentation

The HPLC analysis was performed with a TSP (tsp Thermo Separation Products GmbH, Egelsbach, Germany) liquid chromatograph consisting of a SN 4000 controller, a P 4000 pump, an SCM 400 degasser, an AS 3000 autosampler and a Spectra Focus UV-VIS detector. The chromatograms were recorded with the TSP software on a microcomputer running on OS2/Warp. The separation of the oligosaccharides was carried out on two different stationary phases: (1) Luna 3µ NH_2 (150 mm x 3.0 mm i.d.) column equipped with a security guard cartridge (NH_2, 4 mm x 2.0 mm i.d.) and (2) Sphereclone 5µ NH_2 (250 mm x 4.6 mm i.d.) column equipped with a security guard cartridge (NH_2, 4 mm x 3.0 mm i.d.). In both cases the column and the security guard cartridge were from Phenomenex (Aschaffenburg, Germany).

6.2.4.1.3 HPLC conditions

The separation of the hyaluronate oligosaccharides was based on the method described by Tawada et al. (2002), i.e. oligosaccharides were eluted by application of a linear phosphate gradient (16 – 800 mM NaH_2PO_4) under constant flow at 40 °C. The flow rate was 0.5 ml/min for stationary phase 1 (Luna-NH_2) and 1.0 ml/min in case of stationary phase 2 (Spereclone-NH_2). Solvent A was Milli-Q water, solvent B consisted of 800 mM NaH_2PO_4, dissolved in Milli-Q water, and was filtrated (GH Polypro membrane disc filter, 0.45 µm, Pall Life Sciences, Dreieich, Germany) prior to use. A linear gradient was applied which consisted of 2 to 100 % of solvent B over 70 min for stationary phase 1. In case of stationary phase 2 the percentage of solvent B was linearly increased from 2 to 33 % within the first 40 min and then up to 100 % within the following 15 min. In between the different runs the columns were re-equilibrated with 16 mM NaH_2PO_4 (2 % of solvent B) for 60 min. Detection was carried out at 205 nm. Peaks were collected manually, pooled according to their retention times and lyophilised.

6.2.4.2 Desalting of the oligosaccharide fractions by size exclusion chromatography

Size exclusion chromatography was performed on a Superdex Peptide column (10 mm x 300 mm, Amersham Biosciences, Freiburg, Germany) with a Pharmacia LKB Pump P-500, a Waters 2410 refractive index detector (Waters GmbH, Milford, USA), a Servogor 210 recorder (Metrawatt GmbH, Nürnberg, Germany) and a Pharmacia Biotech valve V-7,

equipped with a 200 µl loop. For desalting the lyophilised oligosaccharide fractions were redissolved in Milli-Q water and filtrated. 200 µl of each sample were applied onto the column, equilibrated with mobile phase for at least 2 h. The mobile phase consisted of 0.1 M NH_4OAc, dissolved in Milli-Q water, and was filtrated (GH Polypro membrane disc filter, 0.45 µm, Pall Life Sciences, Dreieich, Germany) and degassed by ultrasound prior to use. Chromatography was carried out at room temperature at a flow rate of 30 ml per h, and the eluent was monitored by refractive index detection. Fractions containing the oligosaccharide were manually collected, lyophylised and stored at -20 °C until analysis by mass spectroscopy.

6.2.4.3 ESI-MS

HA oligosaccharides were analysed by ESI-MS on a ThermoQuest Finnigan TSQ 7000 spectrometer (Thermo Electron GmbH, Dreieich, Germany). The desalted and lyophilised oligosaccharide samples were dissolved in a 1:10 mixture of water and MeOH containing 10 mM NH_4OAc and manually injected into the ion source. Analysis was carried out in the negative ion mode with a spray voltage of 3 kV. The temperature of the heated capillary was 200 °C.

6.2.4.4 MALDI-TOF MS

6.2.4.4.1 Characterisation of the oligosaccharide fractions from anion exchange HPLC

MALDI-TOF MS was carried out on a GSG/future linear MALDI-TOF mass spectrometer (GSG Mess- und Analysengeräte GmbH, Bruchsal, Germany). For sample preparation the GSG/SPA sample preparation system was used. The desalted and lyophilised oligosaccharide samples were dissolved in methanol/water (50:50). 2 µl of oligosaccharide solution were mixed with 6 µl of matrix solution (10 mg of 2,5-dihydroxybenzoic acid (DHB) dissolved in 1 ml of methanol/ water (50:50)). 1 µl of the mixture was applied onto one of the mesas on the sample target and allowed to dry. Spectra were recorded in the positive ion mode over the m/z range from 0 to 3000 Da. For each mesa a number of mass spectra were acquired and summed (50-100, depending on the signal intensity of the single spectra). The laser energy required to give a detectable signal varied from 5 to 7 µJ and was modified during the measurement to attain optimal signal intensity. The calibration of the spectrometer was performed over the m/z range from 0 to 3000 Da with a dextrin 15 standard (1 mg dissolved in methanol/water (50:50)) and the DHB matrix solution described above using a ratio

(standard:matrix) of 1:3 (v/v).

6.2.4.4.2 Effect of boiling on the stability of HA hexasaccharide

6.2.4.4.2.1 Preparation of HA hexasaccharide

HA hexasaccharide was kindly provided by Edith Hofinger. Briefly, 3.32 ml of Neopermease® (6640 units#, dissolved in BSA (solution 1, cf. 6.2.2.1)) were added to an incubation mixture consisting of 10 ml of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 6.66 ml of acetate buffer (0.1 M NH_4OAc/HOAc, pH 4.0), 3.32 ml of BSA (solution 1) and 6.66 ml of water. After an incubation period of 86 h at 37 °C the enzyme reaction was stopped by boiling for 10 min. Protein was removed from the incubation mixture by centrifugation (12000 g; 20 min) and the supernatant containing a mixture of HA oligosaccharides was lyophilised. The lyophilised oligosaccharides were redissolved in H_2O (10 mg/ml) and separated by size exclusion chromatography on a Superdex Peptide column (10 mm x 300 mm, Amersham Biosciences, Freiburg, Germany) at a flow rate of 0.5 ml/min using 0.1 M NH_4OAc as mobile phase. The sample volume was 200 µl. Elution was monitored by UV detection at 210 nm, and fractions containing HA hexasaccharide were manually collected and then lyophilised.

6.2.4.4.2.2 MALDI-TOF MS

Lyophilised HA hexasaccharide was dissolved in millipore water at a concentration of 11.78 mg/ml (hexasaccharide solution A). 100 µl of solution A were boiled for 15 min (hexasaccharide solution A^*). For MALDI-TOF MS analysis solutions A and A^*, respectively, were diluted by a factor of 110 with methanol/water (50:50). MALDI-TOF MS was carried out as described in section 6.2.4.4.1.

Additonally, the effect of boiling on HA hexasaccharide was investigated under the conditions prevalent in the incubation mixtures analysed by anion exchange HPLC. 100 µl of hexasaccharide solution A were mixed with 100 µl of BSA (solution 1, cf. 6.2.2.1), 200 µl of water and 100 µl of citrate-phosphate buffer 1 (pH 3.6 or pH 6.0, cf. 6.2.2.1) yielding the hexasaccharide solutions B (pH 3.6) and C (pH 6.0). 200 µl of solutions B and C were boiled for 15 min yielding hexasaccharide solutions B^* and C^*, respectively. Protein was removed from solutions B, C, B^* and C^* by ultrafiltration using the Nanosep® 10 K centrifugal device

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(cf. 6.2.4.1.1). For MALDI-TOF MS analysis the ultrafiltrated samples were diluted by a factor of 4 with methanol/water (50:50). MALDI-TOF MS was carried out as described in section 6.2.4.4.1.

6.2.4.5 Separation of hyaluronate oligosaccharides by capillary electrophoresis (CE)

6.2.4.5.1 Digestion of hyaluronate

150 µl of Neopermease® (300 units#, dissolved in BSA (solution 1, cf. 6.2.2.1)) were incubated with 1200 µl of incubation mixture 6-IX, consisting of 600 µl of acetate buffer (pH 3.65 and pH 5.8; solution A: 0.1 M acetic acid, solution B: 0.1 M NaOAc, solutions A and B were mixed in appropriate portions to adjust the required pH), 450 µl of substrate (5 mg HA from *S. zooepidemicus* per ml of water) and 150 µl of BSA (solution 1) at 37 °C. Three samples (samples a, b and c) were prepared at each pH. After an incubation time of 28 h in case of samples a and b the enzyme reactions were stopped by boiling the mixtures for 15 min (a) or 2 h (b), respectively, before protein was removed from the mixtures by ultrafiltration using the Nanosep® 10 K centrifugal device (cf. 6.2.4.1.1), whereas in case of samples c the boiling procedure was omitted. In addition, reference mixtures consisting of incubation mixture 6-IX + 150 µl of BSA (solution 1) instead of Neopermease® were prepared. Two samples (samples b and c) were prepared at pH 3.65 and pH 5.8, respectively, and treated in the same way as described for the digestion mixtures. A 1 ml volume of each ultrafiltrated sample was then lyophilised and redissolved in 1 ml of CE sample buffer (cf. 6.2.4.5.2) for subsequent CE analysis.

6.2.4.5.2 CE conditions

CE analysis was carried out in the normal mode on a Biofocus 3000 capillary electrophoresis system (Biorad, München, Germany) equipped with an uncoated fused silica capillary (75 cm x 50 µm; Chrompack). Operating buffer: 50 mM Na_2HPO_4, 20 mM $Na_2B_4O_7$, pH 9; sample buffer: operating buffer 1:10 diluted with Milli-Q water. The sample was injected by pressure (20 psi · s) and analysis was performed at a capillary temperature of 15 °C applying a run voltage of 15 kV. The detector wavelength was 195 nm. Between the single analyses the capillary was washed with 0.1 M NaOH (200 s), Milli-Q water (200 s) and finally with the operating buffer (300 s).

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6.2.4.6 Analysis of hyaluronate oligosaccharides by NMR spectroscopy

6.2.4.6.1 Digestion of hyaluronate

6.2.4.6.1.1 Exhaustive digestion

Hyaluronate was digested with Neopermease® at pH 3.8 and pH 6.0 for subsequent characterisation of the produced oligosaccharide mixtures by NMR spectroscopy. The incubation mixture (incubation mixture 6-X) consisted of 6 ml of citrate-phosphate buffer 1 (pH 3.8 and 6.0; cf. 6.2.2.1), 4.5 ml of substrate (6 mg HA from *S. zooepidemicus* per ml of water), 1.5 ml of BSA (0.4 mg BSA per ml of water) and 150 µl of water. 12.15 ml of incubation mixture 6-X were incubated with l.35 ml of Neopermease® (135 000 units[#]; 1 vial of Neopermease® was dissolved in 2 ml of water) at 37 °C. In addition reference mixtures were prepared by incubation with 1.35 ml of the pure stabiliser of Neopermease® (1 vial of the stabiliser was dissolved in 2 ml of water). After an incubation time of 30 h both the digestion and the reference mixtures were frozen with liquid N_2 and lyophilised. The lyophilised digestion mixtures were redissolved in 1 ml of D_2O and 15 µl of internal standard solution (50 mg of TSP (3-(trimethylsilyl)-propionic acid-2,2,3,3-d4 sodium salt) per ml of D_2O) were added. Since considerable turbidity was observed at pH 3.8, the mixture was 1:1 diluted with D_2O. To obtain solutions free of turbidity, both digestion mixtures were centrifuged and the supernatants used for NMR analysis. The lyophilised reference mixtures were dissolved in 4 ml of D_2O. For NMR analysis 250 µl of the solution were diluted with 750 µl of D_2O and 15 µl of internal standard solution were added.

6.2.4.6.1.2 Incubation conditions for monitoring the digestion by NMR spectroscopy

The degradation of hyaluronate by the action of Neopermease® was investigated at pH 3.8 and pH 6.0 by monitoring the changes in the digestion mixture by ^{1}H NMR spectroscopy. For the digestion at pH 6.0 incubation conditions, which had been elaborated in preliminary studies by viscosimetry (cf. chapter 5), were applied. The incubation mixture (mixture 6-XI) was prepared as follows: 10 mg of substrate (HA from *S. zooepidemicus*) were dissolved in 6 ml of 0.133 M phosphate buffer (pH 6) containing 0.1 M NaCl and 0.044 mg/ml BSA (140.93 mg NaH_2PO_4, 22.73 mg Na_2HPO_4, 0.44 mg BSA and 5.84 mg NaCl per 10 ml of D_2O). To 1 ml of incubation mixture 6-X 10 µl of internal standard solution (TSP, cf. 6.2.4.6.1.1) were added and a reference spectrum of the undigested incubation mixture was recorded. At

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t = 0 min 50 µl of Neopermease® (2.7 units#, dissolved in BSA (0.2 mg of BSA per ml of D_2O)) were added to the mixture directly into the NMR tube, and after thorough mixing spectra were recorded at increasing reaction times (t = 0-16 h, at intervals of 10 min within the first two hours, and 60 min, respectively). Since under the described conditions (2.55 units#/ml, t = 16 h) no changes were detected in the digestion mixture, additional experiments were performed using higher concentrations of enzyme and longer reaction times (13.3 units#/ml, t = 187 h; 26.67 units#/ml, t = 60 h).

The incubation mixture for digestion at pH 3.8 (incubation mixture 6-XII) was prepared as follows: 16.7 mg of substrate (HA from *S. zooepidemicus*) were dissolved in 10 ml of 0.1 M formate buffer (pH 3.8) containing 0.1 M NaCl and 0.044 mg/ml BSA (68.01 mg of sodium formate and 58.4 mg of NaCl were dissolved in 9.9 ml of D_2O. To this solution 100 µl of BSA (4.44 mg per ml of D_2O) were added. The pH was adjusted by addition of 28 µl of formic acid.) 1.5 ml of incubation mixture 6-XII was mixed with 10 µl of internal standard solution (TSP, cf. 6.2.4.6.1.1). The digestion was started at t = 0 min by the addition of 100 µl of Neopermease® (20 units#, dissolved in BSA (0.2 mg of BSA per ml of D_2O)). After thorough mixing 1 ml of the digestion mixture was transferred into a NMR tube and spectra were recorded at increasing reaction times (t = 17 min, 4 h, 7 h). Since no changes were detected in the digestion mixture additional enzyme (20 units#) was added into the NMR tube at t = 7.5 h and another spectrum recorded at t = 23 h. In a next step digestion was promoted by adding even more enzyme (40 units# at t = 23.5 h; 80 units# at t = 96 h; 100 units# at t = 120 h) and increasing the incubation temperature from 21 °C to 30 °C (at t = 23.5 h) and to 37 °C (at t = 96 h). A final spectrum was recorded at t = 145 h.

6.2.4.6.2 NMR spectroscopy

In case of the exhaustively digested incubation mixtures (cf. 6.2.4.6.1.1) measurements were performed on an Avance 600 spectrometer operating at 600.13 MHz for ^1H (Bruker Biospin GmbH, Rheinstetten, Germany). All spectra were recorded at 300 K and the intense water signal was suppressed by the application of presaturation on the water resonance frequency. Chemical shifts were referenced to internal TSP. The peak assignment of the ^1H spectra was made via the plots obtained from 2D experiments, i.e. double quantum filter correlation spectroscopy (DQF COSY), rotating frame nuclear Overhauser effect spectroscopy (ROESY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC).

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The kinetic experiments, i.e. the monitoring of hyaluronate digestion by Neopermease® (cf. 6.2.4.6.1.2) by ^{1}H NMR spectroscopy, were carried out on a Bruker Avance 400 spectrometer, operating at 400.13 MHz for ^{1}H, at 294 K.

6.3 Results and discussion

6.3.1 Effect of substrate concentration on the pH activity profile of Neopermease®

In the course of the comparative studies on hyaluronidases from different sources (bovine testes, bee venom, *S. agalactiae*) with respect to their apparent activity in different hyaluronidase assays (cf. chapter 5), it turned out that in case of the investigated bovine enzyme preparations (Neopermease®, Hylase® "Dessau") the effect of pH on the apparent activity depended on the type of assay. Almost inverted pH activity profiles were obtained by the colorimetric and the turbidimetric method, respectively (cf. chapter 5, Fig. 5-10). From previous investigations it was known that changes in the composition of the incubation mixture with respect to the type of buffer, NaCl concentration and BSA concentration, cause a shift of the pH optimum of BTH (Gorham et al., 1975; Gacesa et al., 1981; Oettl, 2000). Therefore, in the preceding chapter the turbidimetric determination of the pH activity profile was performed under identical incubation conditions as used for the colorimetric assay, except for the substrate (hyaluronic acid, HA) concentration, which was chosen by a factor of 2.5 lower in the turbidimetric assay to guarantee a linear relationship between HA concentration and the induced turbidity (cf. chapter 5, Fig. 6).

Hyaluronic acid has, depending on the pH of the medium, a polyanionic character due to the carboxylic groups on the glucuronic acid residues, the pK_a of which is 3-4, again depending on the type and concentration of the ions in the medium (Hascall and Laurent, 1997). To rule out the possibility that, due to the effect of the ionic strength of the incubation mixture, the different HA concentrations account for the observed inversion of the pH activity profile of BTH, additional pH profiles of BTH (Neopermease®) were determined by the colorimetric method using a) the substrate concentration of the turbidimetric assay (0.22 mg/ml) and b) a substrate concentration of 1.11 mg/ml, which was twice as high compared to the standard conditions of the colorimetric assay.

The results, which are depicted in Fig. 6-1, clearly show that varying substrate concentrations did not affect the pH profile of Neopermease® in the colorimetric assay. Therefore, it can be ruled out that the discrepancy between the turbidimetrically and colorimetrically determined pH activity curves originates from different HA concentrations in the incubation mixtures.

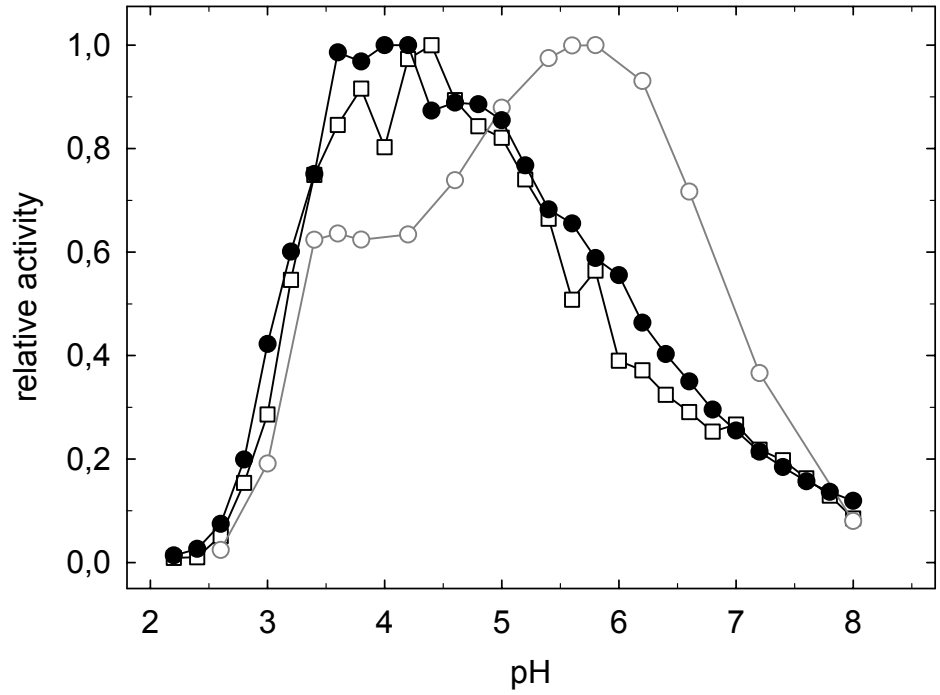

Fig. 6-1: Effect of pH on the activity of Neopermease® determined by the colorimetric method at different substrate concentrations. 50 µl of enzyme solution (25 units[#]) were incubated with incubation mixture 6-I (—●—; 0.22 mg/ml of HA) for 3 h and with incubation mixture 6-II (—□—; 1.11 mg/ml of HA) for 30 min, respectively, at 37 °C. pH activity profile of Neopermease® as obtained by the turbidimetric method (—○—; cf. chapter 5, Fig. 5-10).

[#] as declared by the supplier

6.3.2 Activity of Neopermease® in incubation mixtures of constant ionic strength

The determination of the pH activity profiles in the preceding experiments was performed using Mc Ilvaine's citric acid-phosphate buffer (McIlvaine, 1921). Since the buffer system covers a broad range from pH 2.2 to 8.0 it allowed for the determination of the pH activity profiles without changing the buffer system. However, the ionic strength of McIlvaine's buffer varies depending on pH resulting in a difference in the ionic strength of the incubation mixtures at pH 3.6 and pH 6.0 by a factor of approximately 1.8.

The ionic strength of the standard incubation mixture of the colorimetric assay (mixture 6-V, cf. 6.2.2.1) was calculated by two methods: a) taking into account the activity coefficients estimated according to the extended Debye-Hückel equation (pH 3.6: I = 0.105 M; pH 6.0: I = 0.195 M) and b) without consideration of the activity coefficients (pH 3.6: I = 0.102 M; pH 6.0: I = 0.178 M). Comparison of the results showed that at pH 3.6 almost identical values of I were obtained by both methods, whereas at pH 6.0 the calculated value of I was about 10 % higher when taking the activity coefficients into account compared to the value obtained by the calculation disregarding the activity coefficients.

To ensure that the activity maxima obtained for BTH in the different assays really reflect the effect of pH and are not caused by differences in the ionic strength of the incubation mixtures, enzyme activity was determined at pH 3.6 and pH 6.0 in mixtures of constant ionic strength, which were prepared by raising the ionic strength of the pH 3.6-mixture to that of the pH 6.0-mixture by the addition of NaCl. Since the standard incubation mixture (mixture 6-V) already contains a relatively high concentration of NaCl (44 mM), it was decided to use an incubation mixture with a lower concentration of NaCl (22 mM; mixture 6-III) for the correction of I. For this mixture (incubation mixture 6-III) the values of I, calculated without consideration of the activity coefficients, are 0.080 M (pH 3.6) and 0.156 M (pH 6.0), respectively (cf. 6.2.3).

Indeed, the activity as measured by the colorimetric method after the incubation of Neopermease® (10 and 20 units[#], respectively) in the mixture which was corrected for ionic strength was 25 % (20 units[#]) - 30 % (10 units[#]) lower compared to the value obtained with the uncorrected mixture at pH 3.6 under otherwise identical conditions (Fig. 6-2). However, although the difference between the activity at pH 3.6 and pH 6.0 was found to be less

[#] as declared by the supplier

pronounced than observed without correction for ionic strength, Neopermease® still showed higher activity at pH 3.6 compared to pH 6.0.

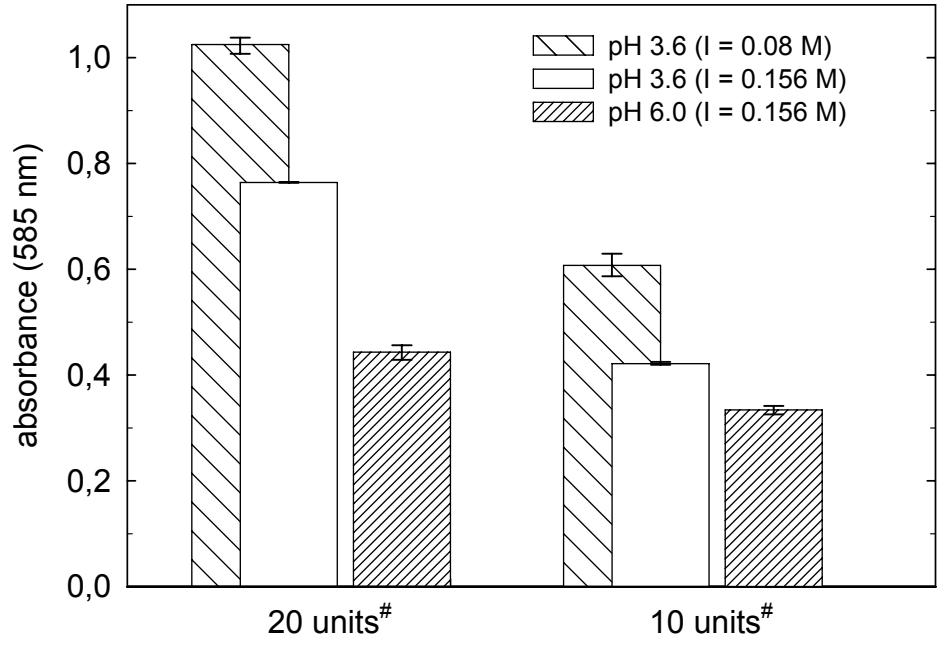

Fig. 6-2: Effect of pH on the activity of Neopermease® in the colorimetric assay using incubation mixtures of constant ionic strength. 50 µl of enzyme solution (10 and 20 units#, respectively) were incubated with incubation mixture 6-III (pH 6.0, I = 0.156 M) and incubation mixture 6-IV (pH 3.6, I = 0.156 M) for 1 h at 37 °C. For comparison the activity obtained at pH 3.6 without correction for ionic strength is shown (I = 0.08 M, incubation mixture 6-III, pH 3.6).

Interestingly, the difference in the activities at pH 3.6 and pH 6.0 was less pronounced in case of incubation with 10 units# than in case of incubation with 20 units# of Neopermease®. This finding may be explained by the transglycosylase activity of Neopermease®, which is favoured at pH 6.0 (Saitoh et al., 1995) and may occur to a higher extent in the incubation mixture containing 20 units# of enzyme due to the fact that in this mixture a higher amount of low MW substrate being susceptible to the transglycosylation reaction is produced within the given incubation time (1h).

The effect of pH on the activity of Neopermease® in incubation mixtures of constant ionic strength measured by the turbidimetric method is presented in figure 6-3. Like in the colorimetric assay a significant decrease (34 %) in activity was observed in the incubation mixture which was corrected for ionic strength compared to the uncorrected mixture at pH 3.6. The difference between the activity at pH 3.6 and the activity maximum of the

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turbidimetric assay at pH 6.0 was thus even more pronounced when using incubation mixtures of constant ionic strength.

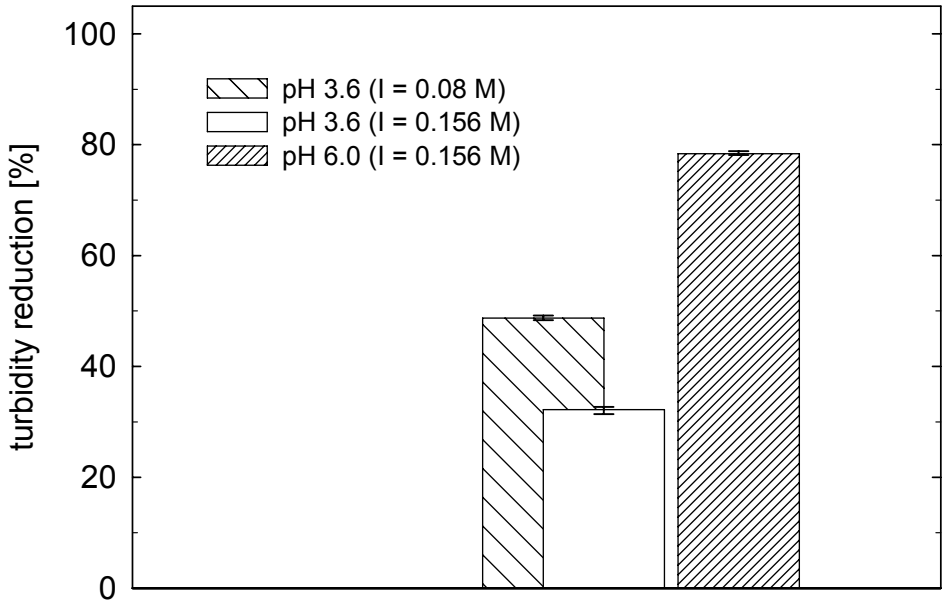

Fig. 6-3: Effect of pH on the activity of Neopermease® in the turbidimetric assay using incubation mixtures of constant ionic strength. 50 µl of enzyme solution (1.5 units[#]) were incubated with incubation mixture 6-VI (pH 6.0, I = 0.156 M) and incubation mixture 6-VII (pH 3.6, I = 0.156 M) for 1 h at 37 °C. For comparison the activity obtained at pH 3.6 without correction for ionic strength is shown (I = 0.08 M, incubation mixture 6-VI, pH 3.6).

Taken together, the results of the colorimetric and the turbidimetric assay demonstrate that the correction for the ionic strength of the incubation mixtures at pH 3.6 and 6.0 did not alter the activity optima of Neopermease® in the respective assay but resulted in a quantitative change, insofar, as the activity optimum of the colorimetric assay at pH 3.6 was found to be less pronounced, while the activity optimum of the turbidimetric assay at pH 6.0 was found to be even more pronounced compared to the measurements in McIlvaine's buffer without correction for ionic strength.

This means that the activity maxima obtained for BTH in our preceding investigations qualitatively reflect the effect of pH on enzyme activity, although the effect of pH was superimposed by an effect caused by the different ionic strength in the incubation mixtures.

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6.3.3 Anion exchange HPLC of hyaluronate depolymerisation mixtures

6.3.3.1 Digestion of hyaluronate with Neopermease®

From preceding experiments it was assumed that the discrepancies in the apparent activity of BTH, which were observed, when assaying the enzyme with the colorimetric and the turbidimetric method, might be ascribed to the pH related transglycosylase activity of BTH. To test this hypothesis, the composition of the oligosaccharide mixtures produced by the action of Neopermease® at various pH (using incubation mixtures analogous to those of the activity assays) was analysed by anion exchange HPLC according to the procedure described by Tawada et al. (2002). Using a linear phosphate gradient the even numbered negatively charged HA oligosaccharides were eluted from the column in the order of increasing chain length as shown by MALDI-MS (cf. 6.3.4). Figures 6-4 and 6-5 show the separation of the mixtures obtained after incubation at pH 3.6 (Fig. 6-4) and pH 6.0 (Fig. 6-5) for 1 h (B) and 16.5 h (C), respectively.

Comparison of the 1 h digests clearly shows that HA degradation at pH 3.6 yielded a considerably higher amount of low molecular HA oligosaccharides (peaks **2**, **3**, **4**) than incubation at pH 6.0. This observation is in agreement with the results of the colorimetric assay, in which a higher amount of reducing NAG end groups was detected after incubation at pH 3.6 compared to pH 6.0. On the other hand the peaks which were detected in the pH 6.0-1 h digestion mixture at retention times up to 30 min, i.e. corresponding to HA fragments with a molecular mass up to 6 kDa (15 disaccharide units), indicate that that the high molecular HA was degraded to a mixture of HA fragments being too short to precipitate with CTAB. This provides an explanation for the high apparent activity of Neopermease® at pH 6.0 in the turbidimetric assay.

Analysis of the 16.5 h digestion mixtures revealed an increase in the low molecular weight oligosaccharides associated with a decrease in the oligosaccharides of higher molecular weight compared to the 1 h digests, both at pH 3.6 and pH 6.0. The digest at pH 3.6 (Fig. 6-4C) contained a mixture of only four different oligosaccharides with the tetra **2** - and the hexasaccharide **3** as the main digestion products accompanied by a small amount of the octasaccharide **4** and the disaccharide **1**.

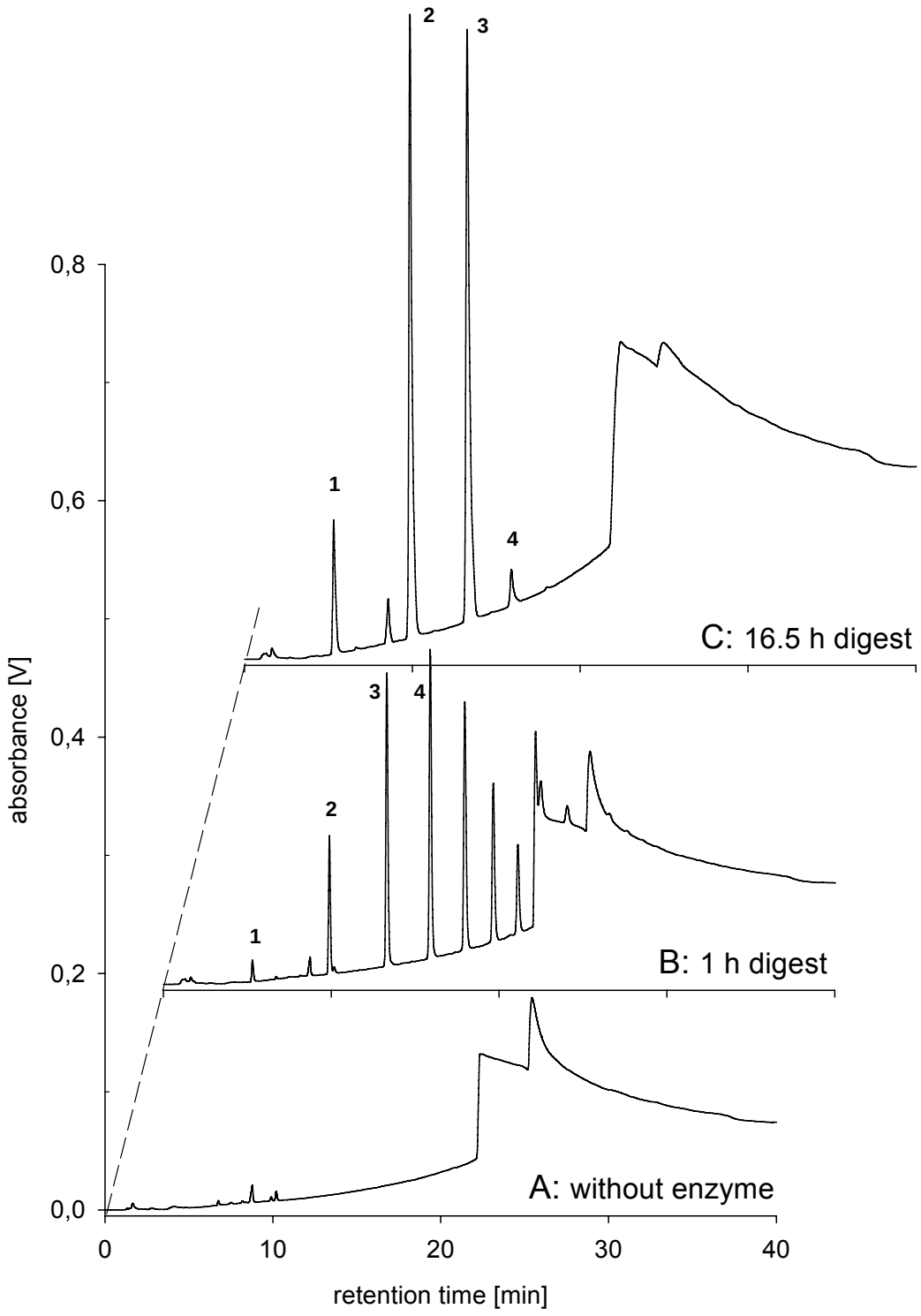


Fig. 6-4: Anion exchange HPLC chromatograms of hyaluronate (HA) oligosaccharides produced by the digestion of HA with Neopermease® at pH 3.6 for 1 h (B) and 16.5 h (C), respectively. A: without enzyme. Separation was performed on stationary phase 1 (Luna-NH_2) as described in the materials and methods section (cf. 6.2.4.1). The injection volume was 10 µl. **1** = HA disaccharide: βGlcUA1-3βGlcNAc; **2** = HA tetrasaccharide: $(\beta GlcUA1\text{-}3\beta GlcNAc1\text{-}4)_2$; **3** = HA hexasaccharide: $(\beta GlcUA1\text{-}3\beta GlcNAc1\text{-}4)_3$; **4** = HA octasaccharide: $(\beta GlcUA1\text{-}3\beta GlcNAc1\text{-}4)_4$. Peaks **2**-**4** were collected and assigned to the respective HA oligosaccharides by mass spectrometry (cf. 6.3.4). Peak **1** was identified by comparison with the chromatogram of unsaturated HA disaccharide (△4,5GlcUA1-3βGlcNAc) obtained after exhaustive digestion of HA by bacterial hyaluronate lyase (cf. chapter 7).

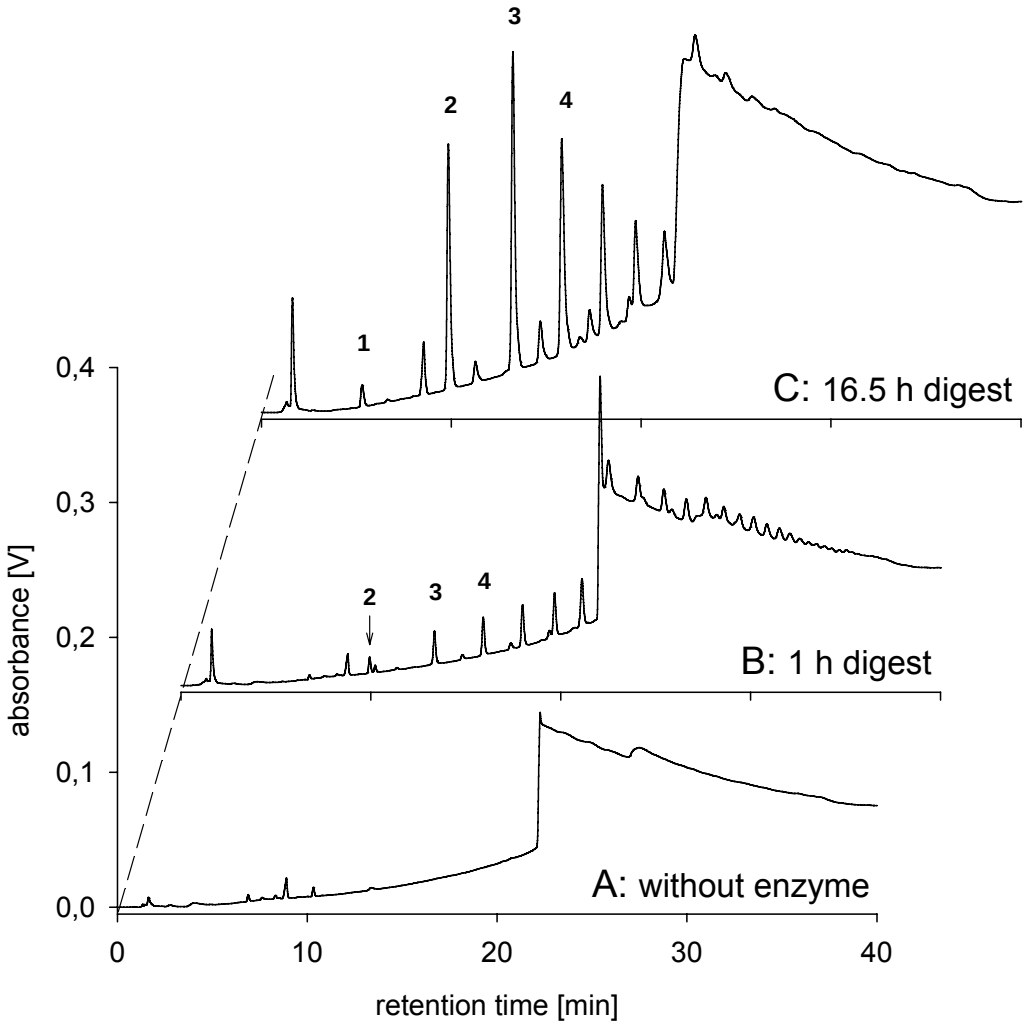


Fig. 6-5: Anion exchange HPLC chromatograms of hyaluronate (HA) oligosaccharides produced by the digestion of HA with Neopermease® at pH 6.0 for 1h (B) and 16.5 h (C), respectively. A: without enzyme. For HPLC conditions and peak assignment confer to figure 6-4 (**1** = HA disaccharide; **2** = HA tetrasaccharide; **3** = HA hexasaccharide; **4** = HA octasaccharide).

In contrast to the tetra-, hexa- and octasaccharide, which were collected and characterised by MALDI-MS, peak **1** was identified by comparison with the chromatogram of an exhaustive digest of HA with hyaluronate lyase from *Streptococcus agalactiae*, which is known to yield unsaturated HA disaccharides as the final product (cf. chapter 7). Compared to the digest at pH 3.6 the oligosaccharide mixture obtained after an incubation period of 16.5 h at pH 6.0 was much more heterogeneous (Fig. 6-5C). In addition to the peaks corresponding to oligosaccharides of higher molecular mass additional small peaks were detected at retention times between those of the even numbered oligosaccharides, and were not present in the chromatograms of digestion mixtures at pH 3.6.

So far it has not been reported in the literature that BTH catalyses the formation of odd numbered oligosaccharides during the hydrolysis of HA. However, the question arose, if the observed intermediate peaks resulted from odd numbered HA oligosaccharides, since the elution profiles of the digestion mixtures at pH 6.0 resembled those reported by Nebinger et al. (1983), who investigated the separation of even- and odd-numbered HA oligosaccharides by anion exchange HPLC. Mahoney et al. (2001) separated a product mixture obtained after digestion of HA with hyaluronidase from ovine testes and found a small amount of odd-numbered oligosaccharides, the formation of which they ascribed to the presence of a contaminating hydrolase in the ovine testicular hyluronidase preparation used. Unfortunately due to a limited column life span it was not possible to collect enough material for characterisation of the intermediate peaks eluted from the Luna-NH_2 stationary phase, which was used for the separations depicted in figures 6-4 and 6-5. However, the identification of the intermediate peaks as odd-numbered HA oligosaccharides having glucuronic acid at the reducing end was successful after separation of pH 6.0-digestion mixtures on a different stationary phase (Sphereclone-NH_2), which showed a slightly altered selectivity (cf. Fig. 6-9). The peak eluted in the void volume might thus be ascribed to the elution of free uncharged NAG. Investigations concerning the formation of the odd-numbered oligosaccharides, which might be formed during the 15 min boiling procedure performed to stop the enzymatic reaction, are subject of another section in this chapter (cf. 6.3.5).

The chromatograms in figure 6-6 show the separation of the oligosaccharide mixtures obtained after exhaustive digestion (7 d) of HA with Neopermease® at varying pH (pH 3.6; 5.0; 6.0). At all pH values the tetrasaccharide **2** was the main product. However, while at pH 3.6 the substrate was degraded to the tetrasaccharide, hexasaccharide and a considerable amount of disaccharide, at pH 6.0 the digestion mixture still contained the octasaccharide and trace amounts of oligosaccharides of higher molecular mass. In addition, odd-numbered oligosaccharides and the presumptive free NAG (eluted in the void volume) were detected in the digest at pH 6.0 and to a lower extent also in the digest at pH 5.0. Analysis of the changes in the product spectrum from pH 3.6 over pH 5.0 to pH 6.0 revealed decreasing amounts of disaccharide associated with increasing amounts of hexasaccharide with increasing pH, whereas a maximum concentration of tetrasaccharide was found at pH 5.0.

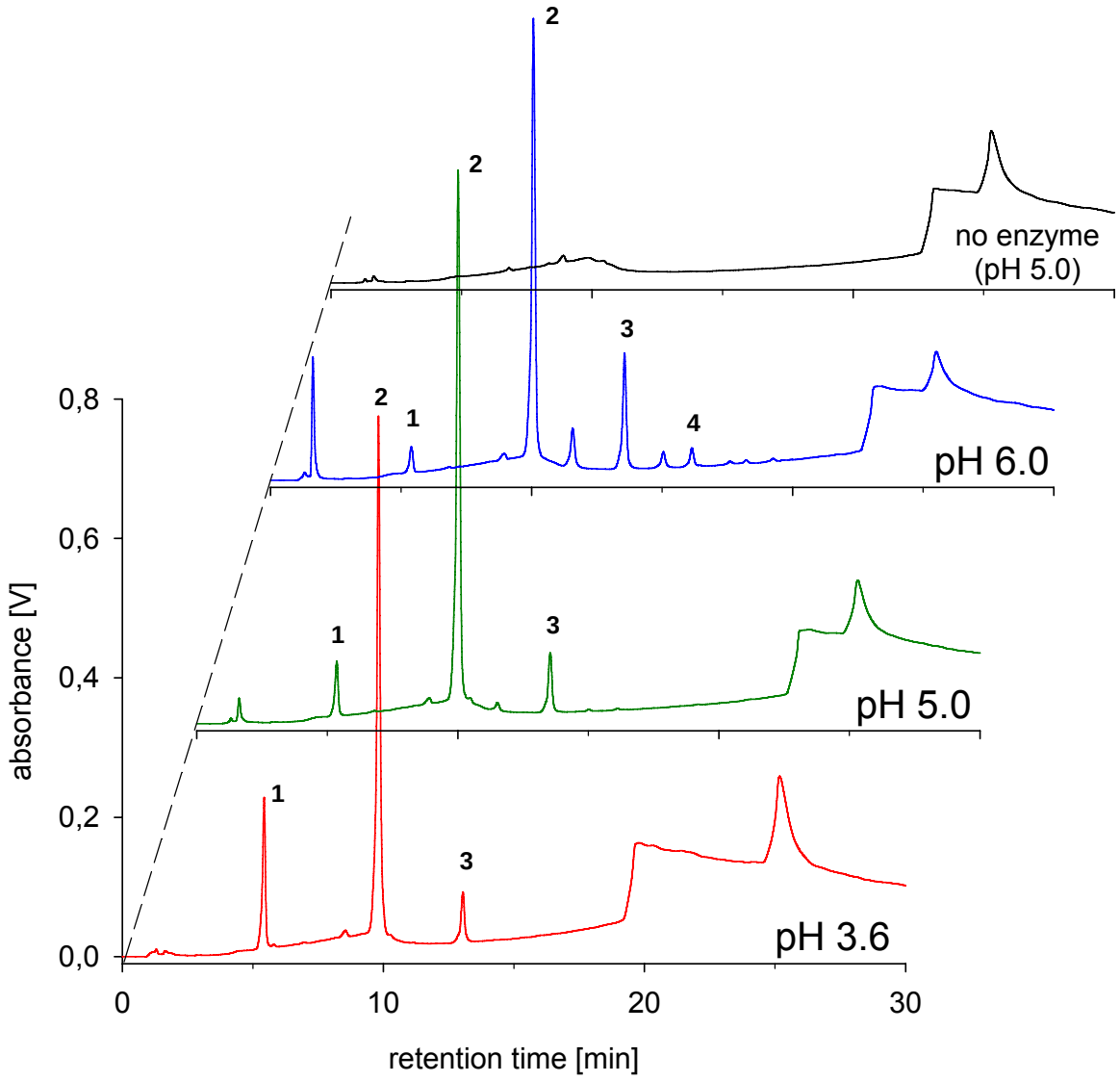


Fig. 6-6: Effect of pH on the exhaustive digestion of hyaluronate (HA) by Neopermease®. Anion exchange HPLC chromatograms of the produced HA oligosaccharides. For HPLC conditions and peak assignment see figure 6-4 (**1** = HA disaccharide; **2** = HA tetrasaccharide; **3** = HA hexasaccharide; **4** = HA octasaccharide).

These results most probably reflect the pH related transglycosylase activity of Neopermease® taking place under the weak acidic conditions at pH 6.0 but not at lower pH values and are thus in agreement with the results reported by Saitoh et al. (1995). This consistency could not be anticipated because of the different composition of the incubation buffer used in our experiments compared with that used by Saitoh and coworkers, concerning ionic strength and the presence of BSA.

In summary, the analysis of the product mixtures obtained after time dependent HA digestion with Neopermease® at varying pH values demonstrates that the pH of the incubation medium

does not only affect the rate of hydrolysis but also the incidence of the transglycosylation reaction and, thus, the composition of the resulting mixture of oligosaccharides. The observation that the amount of low molecular mass oligosaccharides was rather low at pH 6.0 after 1 h of incubation suggests that the transglycosylation reaction, involving low molecular mass oligosaccharides, makes only a minor contribution to the product spectrum at this stage of the reaction.

The observed discrepancies in the apparent pH optima of BTH in the colorimetric assay (pH 3.6) on the one hand and in the turbidimetric (pH 6.0) and the viscosimetric assay (pH 6.0) on the other hand are thus presumably not caused by the transglycosylation reaction. However, the different pH optima may be explained as follows: although HA chains with a molecular mass > 10 kDa were not detected by anion exchange HPLC as they were removed from the digestion mixtures by ultrafiltration prior to analysis, it is most probable that at pH 3.6 oligosaccharides are preferentially generated in the presence of high molecular mass HA, resulting in the low activity detected in the turbidimetric and the viscosimetric assay at pH 3.6. Provided that, in contrast to the hydrolysis at pH 3.6, at pH 6.0, the enzyme does not prefer small HA fragments, i.e. all types of molecules of the polydisperse substrate are processed to the same extent, the higher activity of BTH in the turbidimetric and the viscosimetric assay can be explained, regardless of the higher rate of NAG exposure at the reducing end (the activity criterion in the colorimetric assay) at pH 3.6.

6.3.3.2 Digestion of hyaluronate with bee venom hyaluronidase (BVH)

By analogy with the oligosaccharide mixtures obtained by the degradation of HA with Neopermease®, the digestion products resulting from incubation of HA with bee venom hyaluronidase (BVH) at varying pH were also separated by anion exchange HPLC to reveal possible differences in the degradation of HA by the two hyaluronidases, including the production of odd-numbered oligosaccharides.

Figure 6-7A shows the chromatogram of a 16.5 h digest at pH 3.6 containing a mixture of di-, tetra-, hexa- and octasaccharides, which was in good agreement with the product mixture obtained with the bovine enzyme under the same conditions (Fig. 6-4C). By contrast at pH 6.0 after an incubation time of 16.5 h the substrate was degraded to a considerably lesser extent by the bee venom hyaluronidase (Fig. 6-7B) than by the bovine enzyme (Fig. 6-5C), when using the same enzyme concentrations as at pH 3.6. This result was expected because of the low activity of BVH in the colorimetric, turbidimetric and viscosimetric assay. However, after

incubation for 62 h at pH 5.8 the product mixture formed by the action of BVH (Fig. 6-7C) was similar to that obtained by the exhaustive digestion of HA with Neopermease® at pH 6.0 (Fig. 6-6). The odd-numbered oligosaccharides and the peak eluted in the void volume, the presumptive NAG, were also detected, indicating that the formation of these odd-numbered products was not specific for the action of BTH.

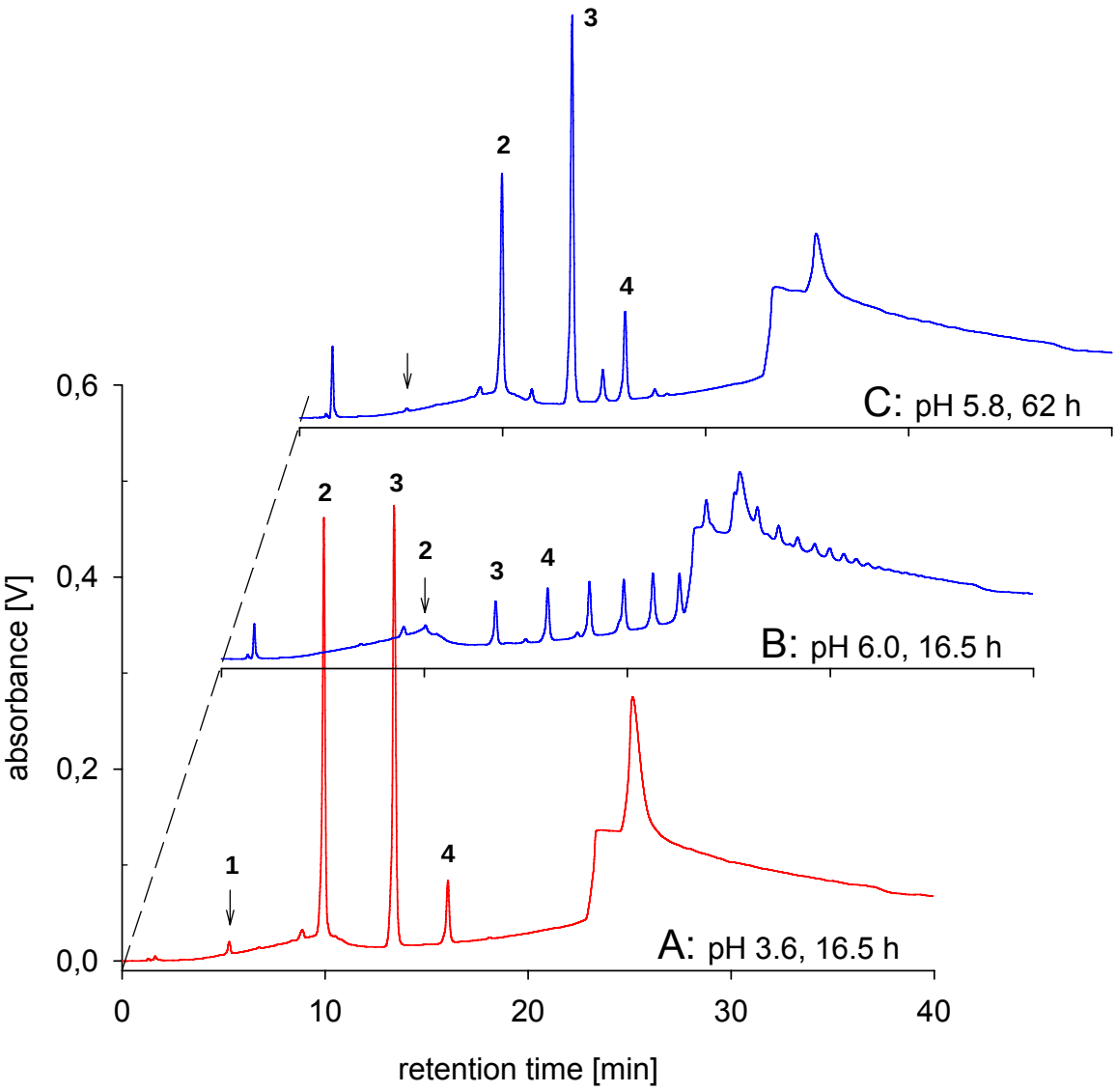


Fig. 6-7: Anion exchange HPLC chromatograms of hyaluronate (HA) oligosaccharides produced by the digestion of HA with bee venom hyluronidase under various conditions. A: pH 3.6, 16.5 h; B: pH 6.0, 16.5 h; C: pH 5.8; 62 h. For HPLC conditions and peak assignment see figure 6-4 (**1** = HA disaccharide; **2** = HA tetrasaccharide; **3** = HA hexasaccharide; **4** = HA octasaccharide).

Except for the presence of the intermediate peaks the chromatogram of the 62 h digest at pH 5.8 was very similar to that of the 16.5 h digest at pH 3.6 (Fig. 6-7A and C). However, as shown for Neopermease®, it can be assumed that the octasaccharide is further degraded by BVH at pH 3.6, too, when the incubation time is increased. Unfortunately we were not able to

further investigate the obtained product mixtures by exhaustive digestion of HA with BVH at varying pH, since the stationary phase of the Luna-NH_2 column turned out to be unstable when exposed to the aqueous mobile phase of high ionic strength necessary for the elution of the negatively charged analytes. Therefore it is still unclear if BVH also catalyses pH related transglycosylation as demonstrated for the bovine enzyme.

6.3.3.3 Digestion of hyaluronate from different sources

Although the hyaluronate present in various mammalian tissues and bacteria has a uniform chemical structure (cf. chapter 1), the preparations of hyaluronate isolated from various sources may differ with respect to their molecular mass and may be contaminated by other glycosaminoglycans, for example chondroitin sulphate. The degradation of HA by BTH is affected by both the molecular mass of the substrate molecules, which may differ in their susceptibility to degradation by BTH, and the presence of chondroitin sulphate, which is also substrate for BTH (Meyer, 1971) and may act as a competitive inhibitor.

Therefore it was investigated, if the product mixtures obtained by the BTH digestion of HA from different sources showed differences in oligosaccharide composition. Considering the fact that no reports concerning the formation of odd-numbered oligosaccharides by the digestion of HA with BTH and BVH exist in the literature so far it was of particular interest to find out, if odd-numbered oligosaccharides, which were found in the incubation mixtures after digestion of HA from *S. zooepidemicus* at pH 6.0, might be produced exclusively with the bacterial HA as substrate. Figures 6-8 and 6-9 show the chromatograms of the oligosaccharide mixtures obtained by the digestion of four different HA preparations with Neopermease® at pH 3.6 and pH 6.0, respectively, using the same incubation buffer as in the preceding experiments. The slight difference of the elution pattern compared to the former chromatograms results from the use of a new, slightly more hydrophilic stationary phase (Sphereclone-NH_2), which was, according to the supplier, supposed to be more stable against the required mobile phase.

Comparison of the product spectra obtained by the digestion of the different HA preparations at pH 3.6 and pH 6.0 revealed no differences for HA from *S. zooepidemicus*, rooster comb and human umbilical cord, whereas in the mixtures with HA from bovine trachea within the limits of detection no oligosaccharides were identified. The reason for the low activity of Neopermease® towards HA from bovine trachea was not further investigated. However, solutions of HA from bovine trachea showed much lower viscosity compared to solutions of

the other HA preparations of the same concentration, indicating a lower molecular mass of the HA from bovine trachea. The low activity may thus be explained by a low affinity of Neopermease® to the low molecular mass substrate.

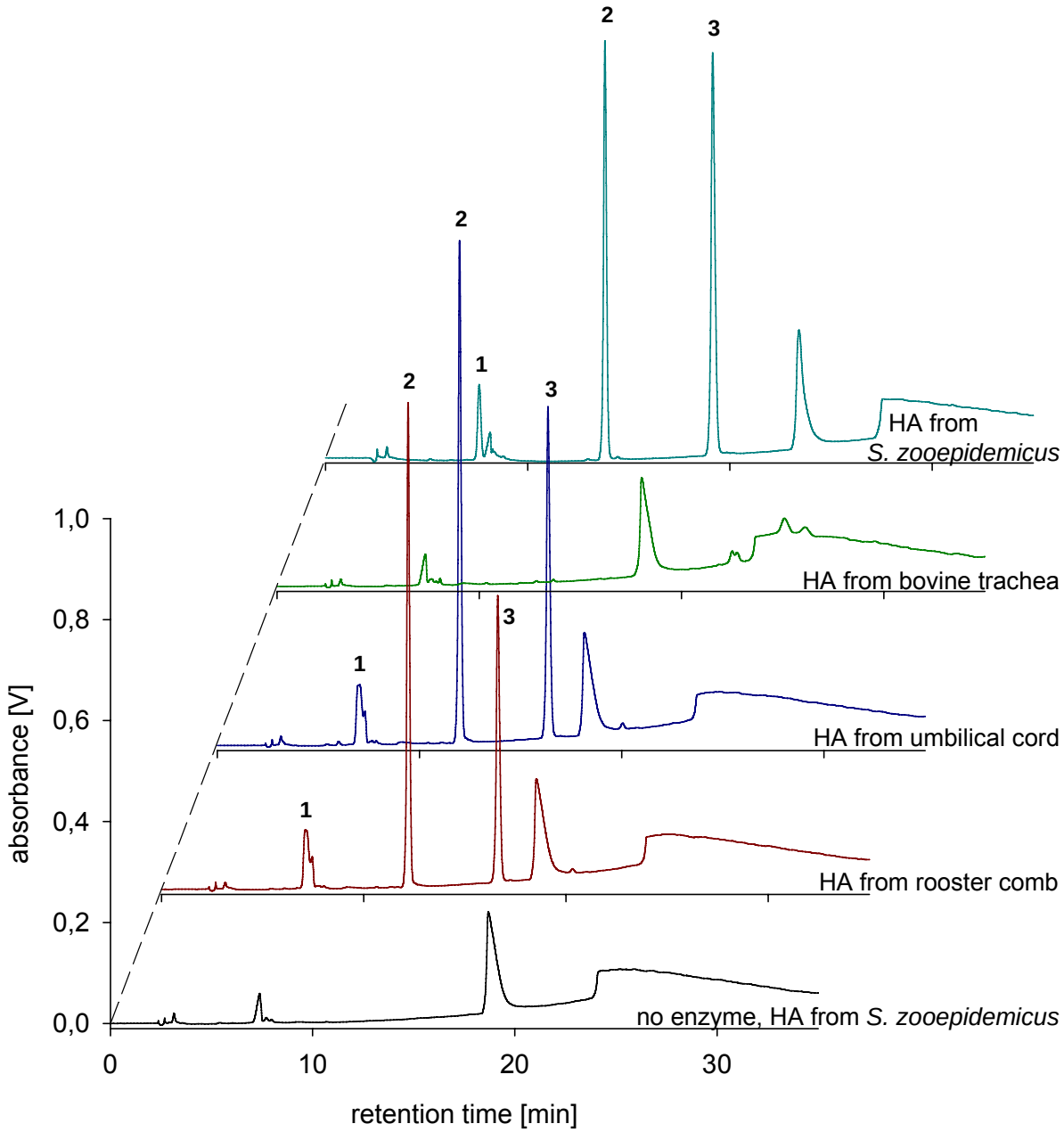


Fig. 6-8: Anion exchange HPLC chromatograms of hyaluronate (HA) oligosaccharides produced by the digestion of HA from various sources with Neopermease® at pH 3.6 for 16.5 h. Separation was performed on stationary phase 2 (Sphereclone-NH_2) as described in the materials and methods section (cf. 6.2.4.1). The injection volume was 30 µl. **1** = HA disaccharide; **2** = HA tetrasaccharide; **3** = HA hexasaccharide; **4** = HA octasaccharide. Peaks **2-4** were collected and assigned to the respective HA oligosaccharides by mass spectrometry (cf. 6.3.4), whereas peak **1** was identified by comparison with the chromatogram of unsaturated HA disaccharide obtained after exhaustive digestion of HA by bacterial hyaluronate lyase (cf. chapter 7).

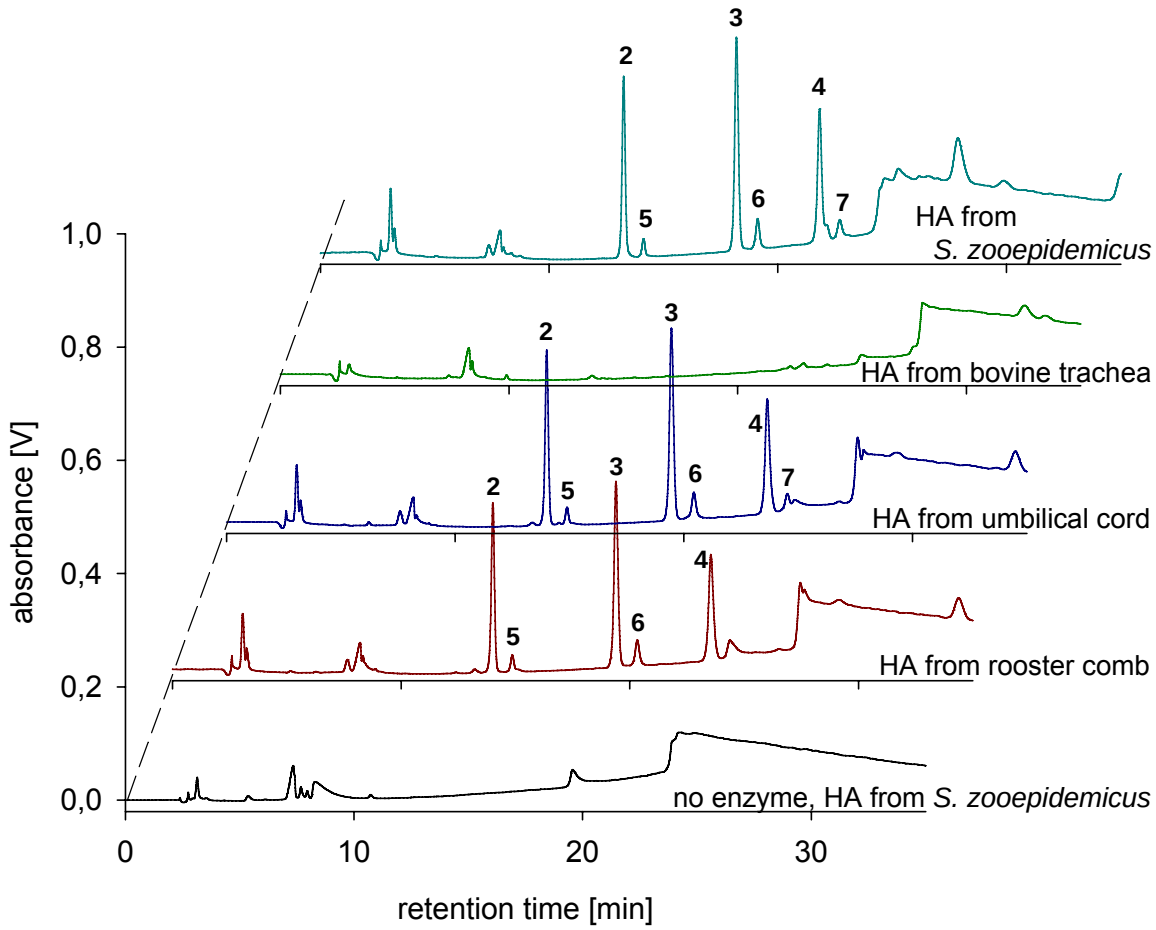


Fig. 6-9: Anion exchange HPLC chromatograms of hyaluronate (HA) oligosaccharides produced by the digestion of HA from various sources with Neopermease® at pH 6.0 for 16.5 h. For HPLC conditions and peak assignment see figure 6-8. **1** = HA disaccharide; **2** = HA tetrasaccharide; **3** = HA hexasaccharide; **4** = HA octasaccharide; **5** = HA trisaccharide: βGlcUA1-3βGlcNAc1-4GlcUA; **6** = HA pentasaccharide: βGlcUA1-3(βGlcNAc1-4βGlcUA1-3)$_2$; **7** = HA heptasaccharide: βGlcUA1-3(βGlcNAc1-4βGlcUA1-3)$_3$.

As mentioned in section 6.3.3.1 the peaks (**2**-**7**) eluted from the Sphereclone-NH$_2$ column were collected from several runs, pooled and characterised by mass spectroscopy (cf. 6.3.4). The small intermediate peaks were identified as odd-numbered oligosaccharides of HA, with glucuronic acid (GlcUA) at the reducing end. Due to their higher charge to mass ratios the odd-numbered oligosaccharides eluted shortly after the respective even-numbered oligosaccharide containing an additional N-acetylglucosamine (GlcNAc) at the reducing end, i.e. the trisaccharide **5** eluted shortly after the tetrasaccharide **2**. The observed retention behaviour is in accordance with the results reported in the literature for the separation of even- and odd-numbered HA oligosaccharides by chromatography on different anion exchange columns (Nebinger et al., 1983; Mahoney et al., 2001).

6.3.4 Characterisation of the anion exchange HPLC fractions by mass spectrometry

The assignment of the fractions collected from anion exchange HPLC to the respective HA oligosaccharides was accomplished by mass spectrometry. Prior to the analysis by ESI-MS and MALDI-TOF MS, phosphate was removed from the chromatography fractions by size exclusion chromatography on a Superdex Peptide column (cf. 6.2.4.2). The calculated monoisopotic masses of the HA oligosaccharides are given in table 6-1.

Table 6-1: Calculated monoisotopic masses of hyaluronate oligosaccharides:

	Molecular formula	**Monoisotopic mass [Da]**
HA trisaccharide βGlcUA1-3βGlcNAc1-4GlcUA	$C_{20}H_{31}NO_{18}$	573.15
HA tetrasaccharide $(\beta GlcUA1\text{-}3\beta GlcNAc1\text{-}4)_2$	$C_{28}H_{44}N_2O_{23}$	776.23
HA pentasaccharide $\beta GlcUA1\text{-}3(\beta GlcNAc1\text{-}4\beta GlcUA1\text{-}3)_2$	$C_{34}H_{52}N_2O_{29}$	952.27
HA hexasaccharide $(\beta GlcUA1\text{-}3\beta GlcNAc1\text{-}4)_3$	$C_{42}H_{65}N_3O_{34}$	1155.34
HA heptasccharide $\beta GlcUA1\text{-}3(\beta GlcNAc1\text{-}4\beta GlcUA1\text{-}3)_3$	$C_{48}H_{73}N_3O_{40}$	1331.38
HA octasaccharide $(\beta GlcUA1\text{-}3\beta GlcNAc1\text{-}4)_4$	$C_{56}H_{86}N_4O_{45}$	1534.46

Fig. 6-10 presents the negative-ion ESI-MS spectra of the peaks **2** and **5** from anion exchange HPLC, which were identified as the HA tetrasaccharide and the trisaccharide having glucuronic acid (GlcUA) at the nonreducing and the reducing end, respectively. The spectrum of the tetrasaccharide fraction (peak **2** from anion exchange HPLC), which proved to be quite pure, showed the mono- and the dianion as the main peaks and an additional minor peak, corresponding to the dianion plus Na^+. Also in the spectrum of the trisaccharide fraction (peak **5** from anion exchange HPLC) the trisaccharide was unambiguously identified by the presence of the peaks corresponding to the mono- and dianion and the corresponding Na^+-adduct. However, a number of additional peaks was present, two of which were assigned to

the dianion and the Na^+-adduct of a 655 Da molecule, which was not further characterised. The impurities observed in the ESI-MS spectrum of the trisaccharide fraction might be ascribed to stationary phase material, which was dissolved in the mobile phase buffer and coeluted with the respective peak during anion exchange HPLC.

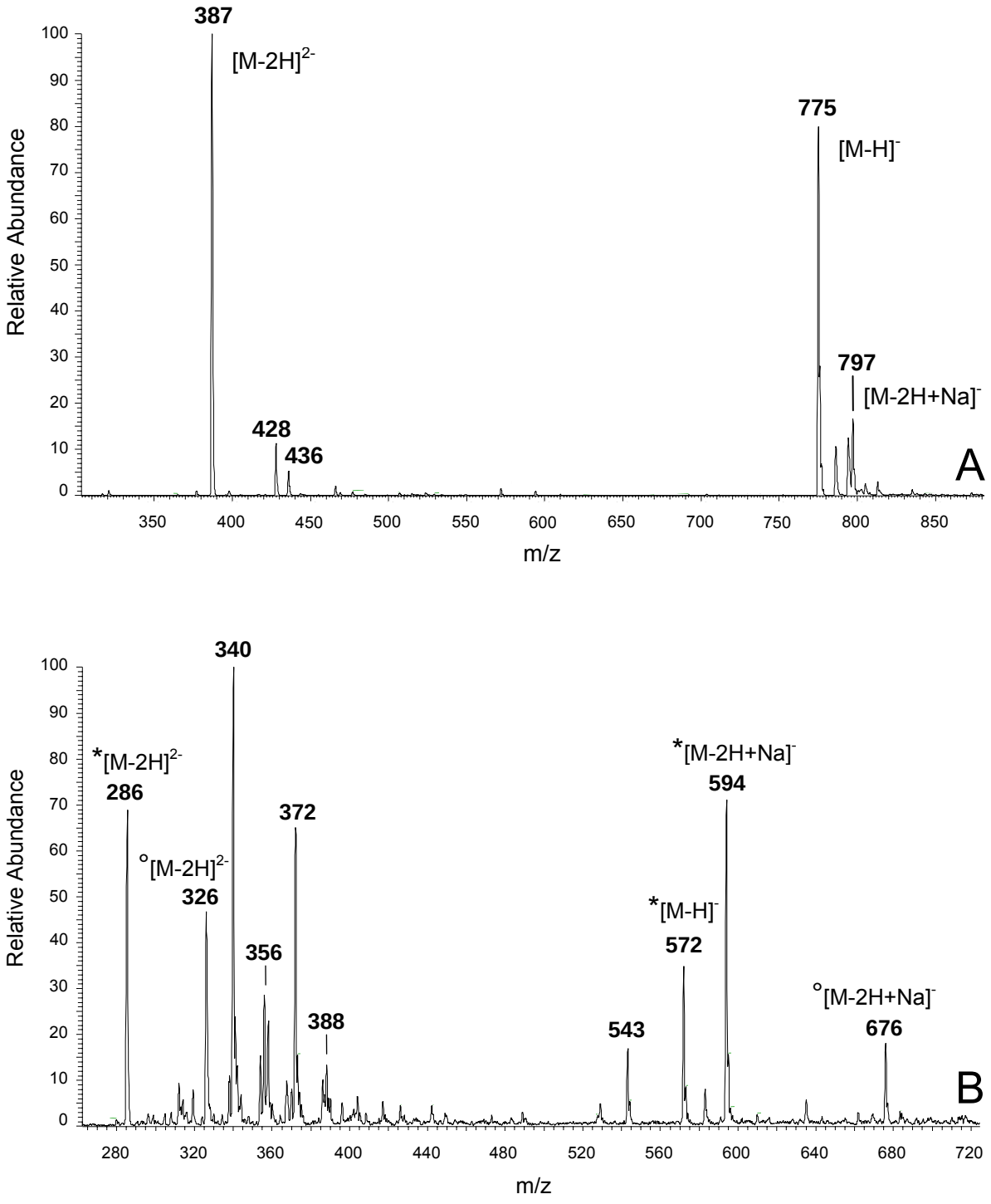


Fig. 6-10: Negative ion ESI-MS spectra of (A) HA tetrasaccharide: $C_{28}H_{44}N_2O_{23}$, calculated monoisotopic mass (M) = 776.23 Da (peak **2** from anion exchange HPLC) and (B) *HA trisaccharide: $C_{20}H_{31}NO_{18}$, M = 573.15 Da (peak **5** from anion exchange HPLC).

In a recent publication Prebyl and coworkers (Prebyl et al., 2003) investigated the fragmentation pattern of HA oligosaccharides during ESI-MS. By comparison of ESI-MS spectra with those obtained by MALDI-MS of the respective oligosaccharide samples they observed, depending on the cone voltage applied, the generation of odd-numbered oligosaccharides during ESI-MS of samples containing exclusively even-numbered oligosaccharides. To ascertain that our ESI-MS spectra reflect the masses of the oligosaccharides originally present in the respective fractions and not the masses of fragmentation products that might form during the electrospray process, the anion exchange HPLC fractions were additionally investigated by MALDI-MS, which has been proven to be a suitable and reliable method for the characterisation of HA oligosaccharides in the work of Prebyl et al. (2003).

The MALDI-MS spectra, which were recorded in the positive ion mode, for peaks **2**-**7** from anion exchange HPLC are depicted in Fig. 6-11 (even-numbered oligosaccharides) and Fig. 6-12 (odd-numbered oligosaccharides), respectively. Analysis of the spectra revealed the derivative $[M+Na]^+$ of the respective oligosaccharides as the predominant species accompanied by a number of Na^+- and K^+-adducts as indicated in detail for the tetrasaccharide (Fig. 6-11A) and trisaccharide (Fig. 6-12A) fraction. All of the oligosaccharide fractions collected from anion exchange HPLC were thus unambiguously identified. The results proved the presence of odd-numbered oligosaccharides, having reducing and nonreducing terminal GlcUA residues in the pH 6.0 digests of HA with Neopermease®. Since the formation of odd-numbered oligosaccharides is not in agreement with the mechanism of action reported in the literature for BTH, it was assumed that the odd-numbered degradation products might be generated by a process independent of hyaluronidase. One possibility would be the presence of contaminating hydrolytic enzymes in the hyaluronidase preparation, as suggested by Mahoney et al., who observed odd-numbered oligosaccharides after digestion of HA with a preparation of hyaluronidase from ovine testes (Mahoney et al., 2001). However, we found odd-numbered oligosaccharides in mixtures digested with various hyaluronidase preparations (BTH, BVH), and a contamination of the different preparations with the same kind of hydrolase seems rather unlikely. We therefore investigated the possibility that the formation of odd-numbered products might occur during the 15 min boiling procedure performed with the digestion mixtures to stop the enzyme reaction at the end of the incubation period.

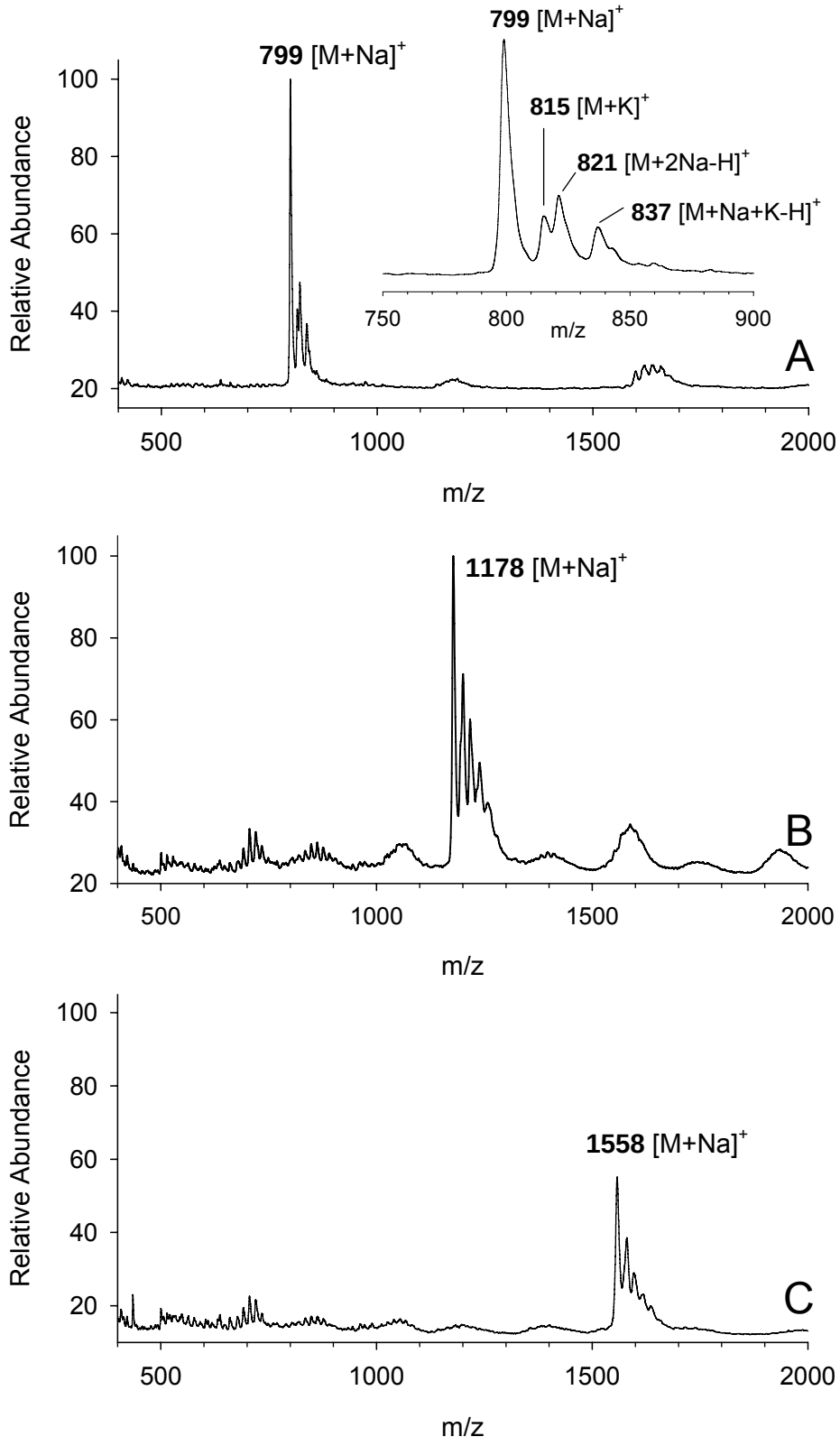


Fig. 6-11: Positive ion MALDI-TOF mass spectra of (A) HA tetrasaccharide: $C_{28}H_{44}N_2O_{23}$, calculated monoisotopic mass (M) = 776.23 Da (peak **2** from anion exchange HPLC), *Inset:* magnified view of the m/z-range 750-900, (B) HA hexasaccharide: $C_{42}H_{65}N_3O_{34}$, M = 1155.34 Da (peak **3** from anion exchange HPLC) and (C) HA octasaccharide: $C_{56}H_{86}N_4O_{45}$, M = 1534.46 Da (peak **4** from anion exchange HPLC).

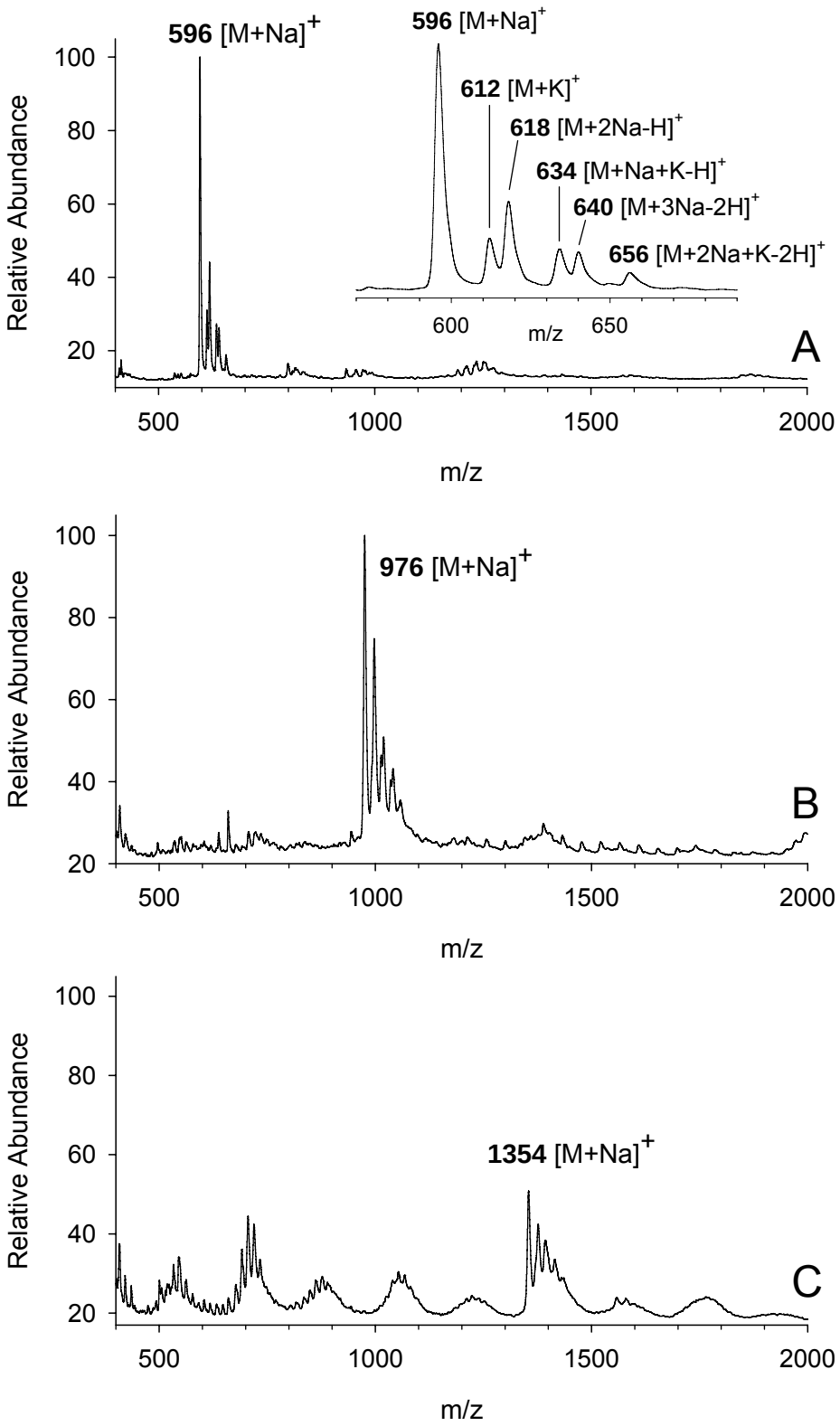


Fig. 6-12: Positive ion MALDI-TOF mass spectra of (A) HA trisaccharide: $C_{20}H_{31}NO_{18}$, calculated monoisotopic mass (M) = 573.15 Da (peak **5** from anion exchange HPLC), *Inset:* magnified view of the m/z-range 570-690, (B) HA pentasaccharide: $C_{34}H_{52}N_2O_{29}$, M = 952.27 Da (peak **6** from anion exchange HPLC) and (C) HA heptasaccharide: $C_{48}H_{73}N_3O_{40}$, M = 1331.38 Da (peak **7** from anion exchange HPLC).

6.3.5 Investigations on the effect of boiling on hyaluronate oligosaccharides

6.3.5.1 Capillary electrophoresis (CE) of hyaluronate depolymerisation mixtures

Anion exchange HPLC of the oligosaccharide mixtures produced by the enzymatic digestion of hyaluronate and subsequent characterisation of the individual oligosaccharide fractions by mass spectrometry revealed the presence of odd-numbered oligosaccharides having reducing and nonreducing terminal GlcUA residues in the pH 6.0 digests of HA with Neopermease®. Since the formation of odd-numbered oligosaccharides is not in agreement with the mechanism of HA degradation reported in the literature for BTH, in the following experiments it was investigated if the formation of the odd-numbered products might result from the 15 min boiling procedure performed at the end of the incubation period to stop the enzyme reaction. For this purpose digestion mixtures obtained by incubation of HA with Neopoermease® and subsequent removal of protein by ultrafiltration were compared with digestion mixtures obtained by incubation of HA with Neopoermease® under identical conditions and subsequent boiling for 15 min or 2 h before ultrafiltration. As the application of anion exchange HPLC was impractical due to the instability of the stationary phase (cf. 6.3.3), the comparative analysis of the oligosaccharide mixtures was carried out by capillary electrophoresis. Based on the protocol of Grimshaw et al. (1994) the CE analysis was performed in the normal polarity mode using an alkaline running buffer (phosphate-borate buffer, pH 9). Under these conditions the negatively charged HA oligosaccharides are separated showing increasing migration times with increasing chain length (Carney and Osborne, 1991; Grimshaw et al., 1994; Grimshaw, 1997).

Figure 6-13 shows the electropherograms of the oligosaccharide mixtures obtained by 28 h digestion of HA with Neopoermease® at pH 3.65. The digest was boiled for 2 h at the end of the incubation period (Fig. 6-13B), whereas in case of the digest shown in figure 6-13A the boiling procedure was omitted. Comparison of the electropherograms depicted in Fig. 6-13 A and B reveals no difference in the oligosaccharide composition of the digestion mixtures, thus indicating that no odd-numbered oligosaccharides were produced during the 2 h boiling procedure at pH 3.65. This result was expected, since also by anion exchange HPLC analysis the formation of odd-numbered degradation products was not observed in digestion mixtures at pH 3.6, which had been boiled for 15 min to stop the enzyme reaction.

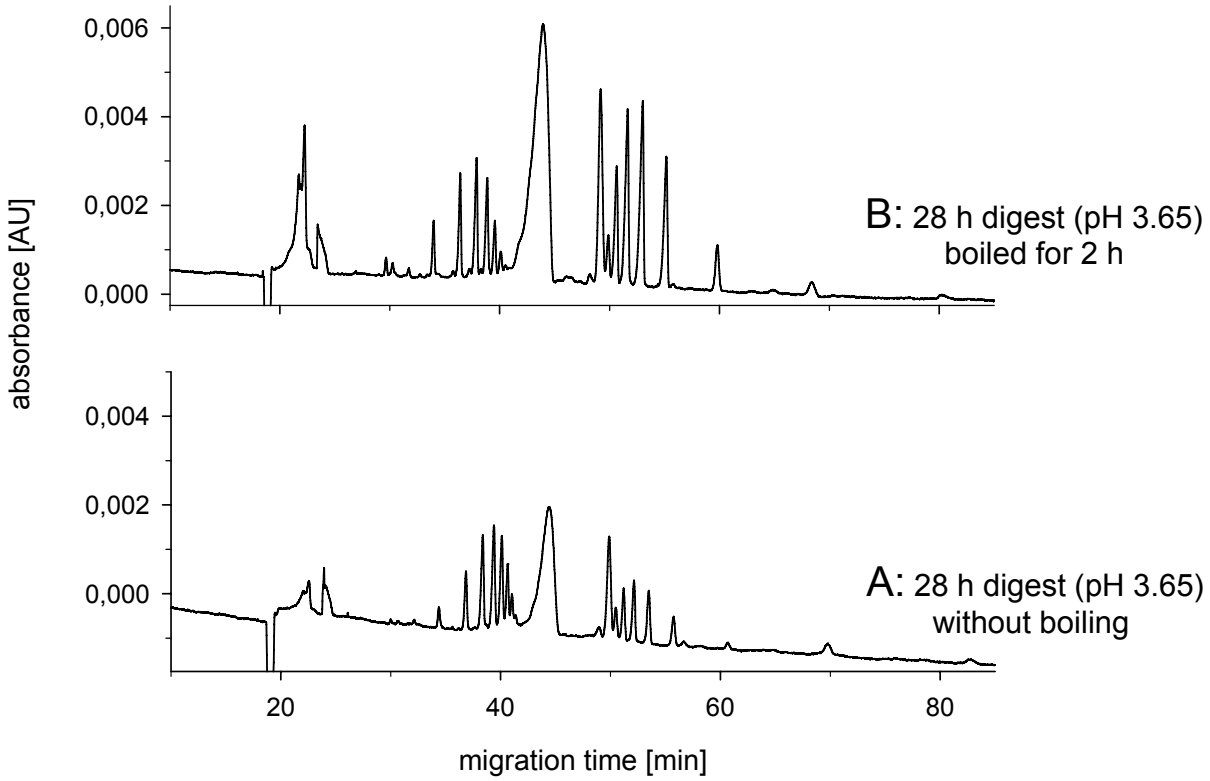


Fig. 6-13: Electropherograms of hyaluronate (HA) oligosaccharides produced by the digestion of HA with Neopermease® at pH 3.65 for 28 h. A: the digest was ultrafiltrated (MWCO 10 kDa) immediately after the incubation period; B: the digest was boiled for 2 h prior to ultrafiltration. CE analysis was performed as described in the materials and methods section (cf. 6.2.4.5). The detector wavelength was 195 nm.

The electropherograms of the oligosaccharide mixtures obtained by 28 h digestion of HA with Neopermease® at pH 5.8 are presented in figure 6-14. As in case of the digestion mixtures at pH 3.65, also at pH 5.8 no additional peaks were detected in the digest which was boiled for 2 h at the end of the incubation period (Fig. 6-14B), compared to the digest which was not boiled prior to analysis (Fig. 6-14A). This indicates that the boiling procedure does not result in the formation of additional oligosaccharides. This in turn suggests that the presence of the odd-numbered oligosaccharides, which have been found in the digestion mixtures at pH 6.0 by anion exchange HPLC (cf. Fig. 6-9), is not caused by the 15 min boiling procedure.

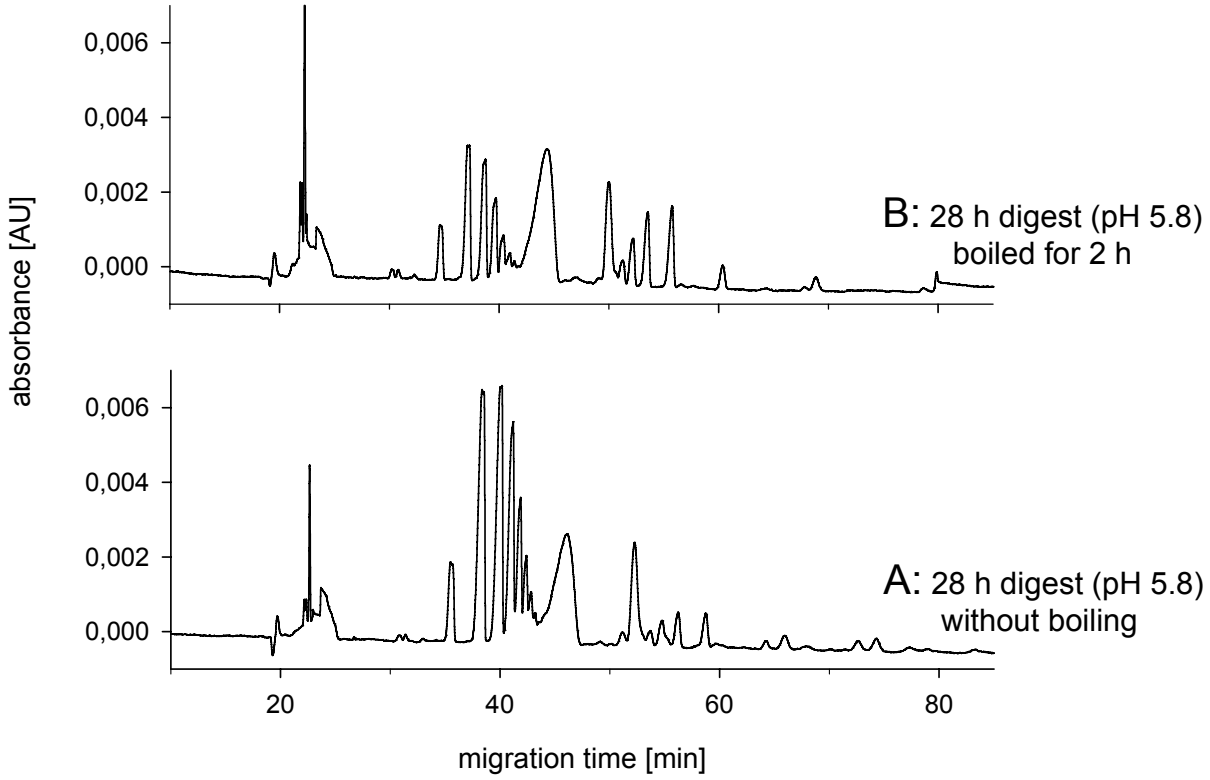


Fig. 6-14: Electropherograms of hyaluronate (HA) oligosaccharides produced by the digestion of HA with Neopermease® at pH 5.8 for 28 h. A: the digest was ultrafiltrated (MWCO 10 kDa) immediately after the incubation period; B: the digest was boiled for 2 h prior to ultrafiltration. CE analysis was performed as described in the materials and methods section (cf. 6.2.4.5). The detector wavelength was 195 nm.

Anion exchange HPLC analysis of the product mixtures obtained by digestion of HA with Neopoermease® revealed at pH 6.0 the presence of a peak eluted in the void volume that was not observed at pH 3.6 and was assumed to result from free NAG.

CE analysis of the digestion mixtures revealed the presence of a peak eluted near the electroosmotic flow (migration time t approx. 23.8 min) that might be ascribed to free NAG and is more pronounced at pH 5.8 (Fig. 6-14 A, B) than at pH 3.65 (Fig. 6-13 A, B). Furthermore, comparison of figures 6-14A and 6-14B shows, that the peak is remarkably higher in the digest after the 2 h boiling procedure.

To investigate if this peak indeed results from free NAG the digestion mixtures were spiked with NAG and analysed by CE. The resulting electropherograms are shown in figure 6-15 (pH 3.65) and figure 6-16 (pH 5.8), respectively.

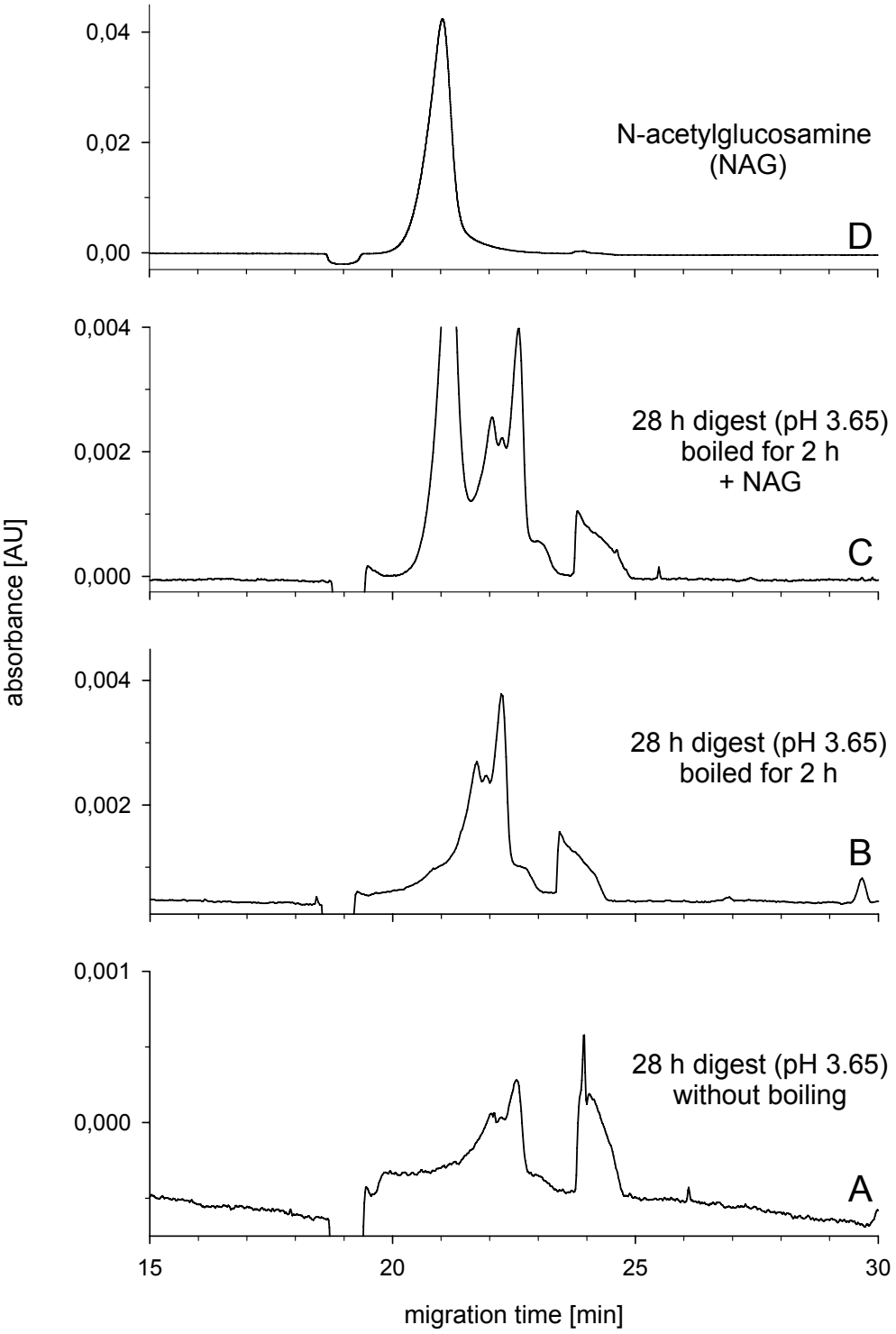


Fig. 6-15: A, B: Magnified view of the electropherograms A and B of figure 6-13; C: electropherogram of the same sample as in B, spiked with N-acetylglucosamine (NAG) (10 µl of NAG (2 mg/ml, dissolved in operating buffer) were added to 90 µl of the sample); D: electropherogram of NAG (2 mg/ml, dissolved in running buffer). CE analysis was performed as described in the materials and methods section (cf. 6.2.4.5). The detector wavelength was 195 nm.

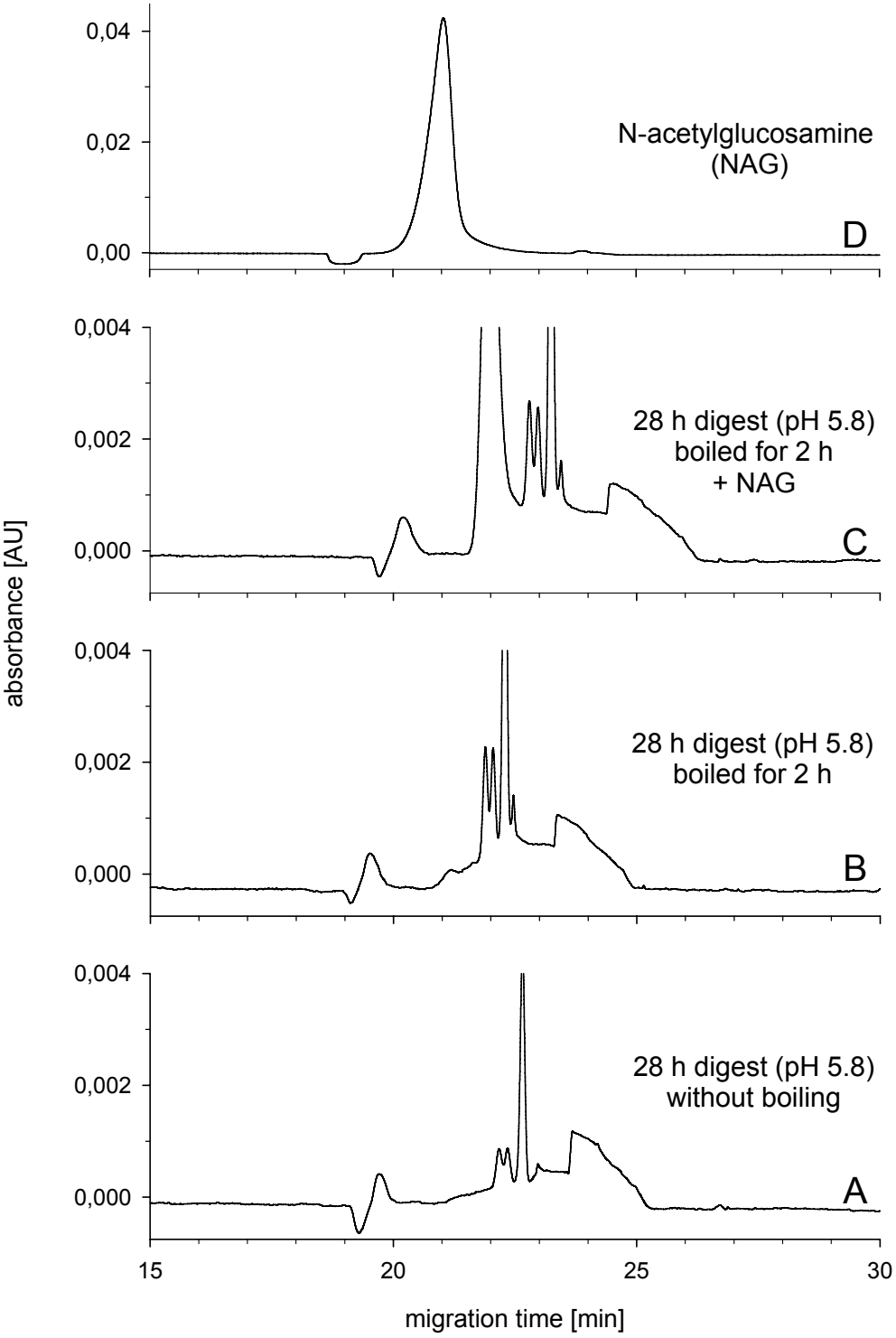


Fig. 6-16: A, B: Magnified view of the electropherograms A and B of figure 6-14; C: electropherogram of the same sample as in B, spiked with N-acetylglucosamine (NAG) (10 µl of NAG (2 mg/ml, dissolved in operating buffer) were added to 90 µl of the sample); D: electropherogram of NAG (2 mg/ml, dissolved in running buffer). CE analysis was performed as described in the materials and methods section (cf. 6.2.4.5). The detector wavelength was 195 nm.

The electropherograms clearly show that NAG did not coelute with any substance originally present in the digestion mixtures, indicating that the main peak of the digestion mixture at pH 5.8 after 2 h boiling does not result from NAG.

When heated under alkaline conditions (pH 9; 100 °C) NAG residues at the reducing ends of HA fragments are removed from the HA chains according to the Morgan-Elson reaction yielding chromogens I and II (anhydro-NAG) (cf. Fig. 6-17).

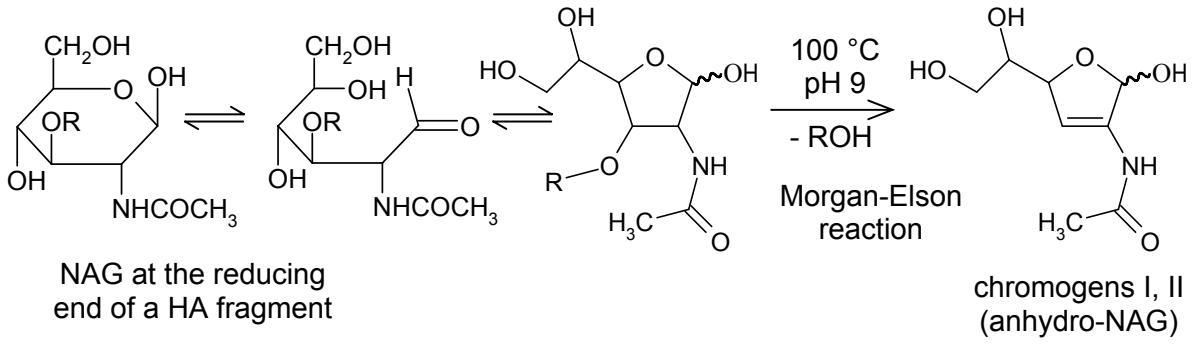


Fig. 6-17: Mechanism of the Morgan-Elson reaction, adapted from Muckenschnabel et al. (1998b).

It can be speculated that also under the weak acidic conditions in the digestion mixture at pH 5.8 even-numbered HA oligosaccharides having NAG at the reducing end are cleaved during the boiling procedure yielding odd-numbered HA oligosaccharides having GlcUA at the reducing end and anhydro-NAG. The main peak of the digestion mixture at pH 5.8 after 2 h boiling may thus be assigned to anhydro-NAG. However, further investigations are necessary to test this hypothesis.

6.3.5.2 MALDI-TOF mass spectrometry of HA hexasaccharide

HA hexasaccharide was obtained by digestion of HA with Neopermease® and subsequent separation of the produced mixture of oligosaccharides by size exclusion chromatography. Samples of the pure HA hexasaccharide, dissolved in Milli-Q water, were boiled for 15 min and then analysed by MALDI-TOF mass spectrometry with respect to the presence of potential degradation products which may have formed during the boiling procedure.

Figure 6-18 shows the MALDI-TOF mass spectrum of HA hexasaccharide after the 15 min boiling procedure (Fig. 6-18B) in comparison with the spectrum of an untreated hexasaccharide sample (Fig. 6-18A) and the spectrum of the matrix (Fig. 6-18C).

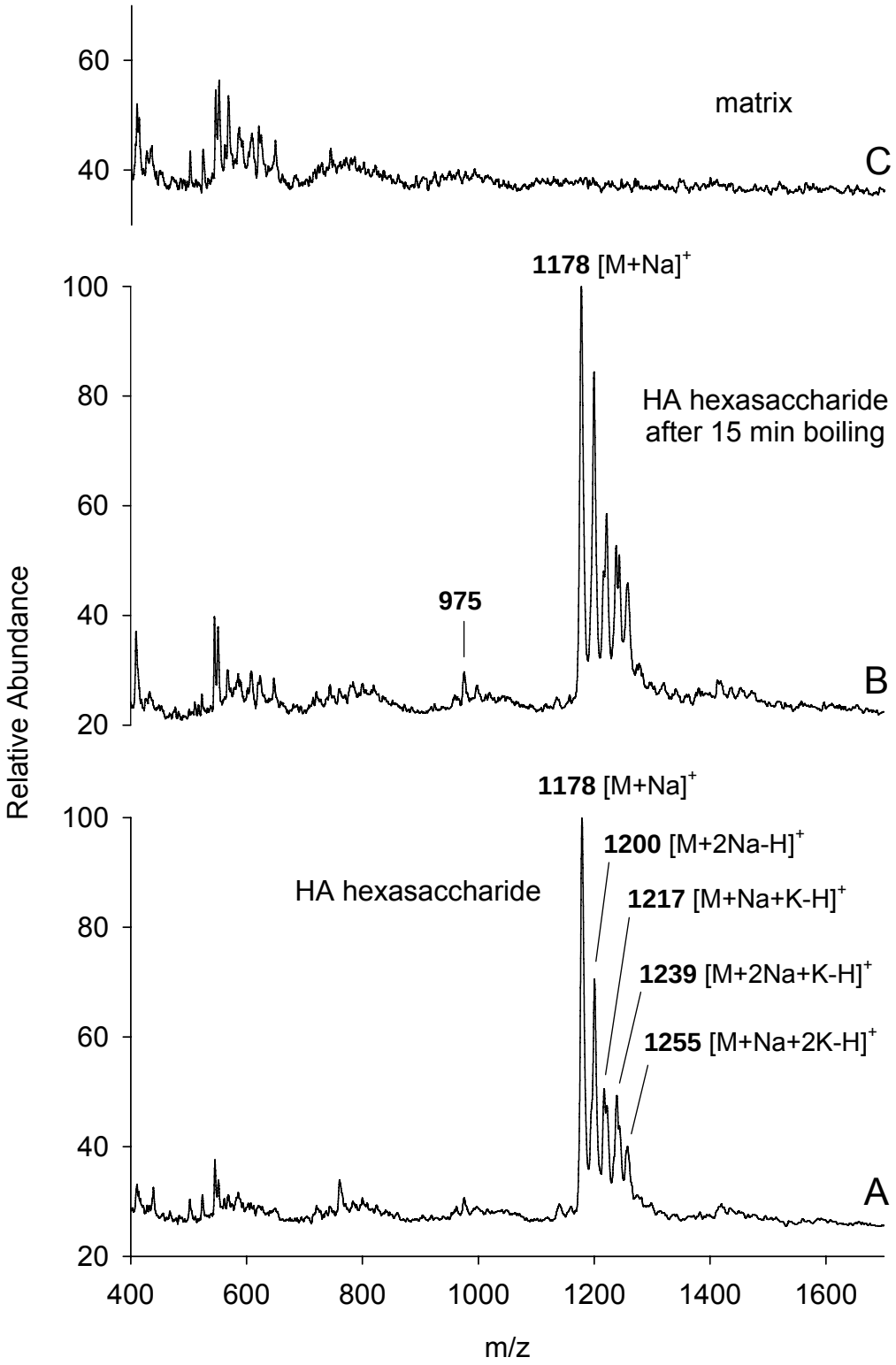


Fig. 6-18: Positive ion MALDI-TOF mass spectra of (A) HA hexasaccharide: $C_{42}H_{65}N_3O_{34}$, calculated monoisotopic mass (M) = 1155.34 Da (solution A, cf. 6.2.4.4.2.2), (B) HA hexasaccharide after boiling for 15 min (solution A*, cf. 6.2.4.4.2.2). C: Positive ion MALDI-TOF mass spectrum of the matrix (DHB, M = 154.03).

Comparison of the spectra shows that no additional peaks were detected in the sample subjected to the 15 min boiling procedure compared to the untreated sample, indicating that the HA hexasaccharide was not degraded during the boiling procedure. The small peak at m/z = 975 might result from the Na^+-adduct $[M+Na]^+$ of HA pentasaccharide having GlcUA at the reducing end (M = 952). However, this peak is also present in the spectrum of the untreated hexasaccharide sample suggesting that the corresponding molecule did not result from the boiling process.

Anion exchange HPLC analysis revealed the presence of odd-numbered oligosaccharides in incubation mixtures at pH 6.0 but not at pH 3.6 (cf. 6.3.3). The effect of boiling on HA hexasaccharide was therefore additionally investigated at various pH under the conditions prevalent in the incubation mixtures, which had been analysed by anion exchange HPLC. Hexasaccharide was dissolved in citrate-phosphate buffer (pH 3.6 or pH 6.0), containing NaCl and BSA, and MALDI-TOF mass spectra were recorded of samples after a 15 min boiling process and of samples left at room temperature. Like in the aforementioned experiment, where hexasaccharide was dissolved in Milli-Q water (cf. Fig. 6-18), no difference was observed between the spectra of the boiled and the untreated samples of HA hexasaccharide, dissolved in citrate-phosphate buffer at pH 3.6 or pH 6.0 (results not shown).

Taken together, the results of the investigations performed to study the effect of boiling on oligosaccharides of HA (CE analysis of mixtures of HA oligosaccharides and MALDI-TOF MS analysis of HA hexasaccharide) do not confirm the hypothesis of Prebyl et al. (2003), who proposed that the presence of odd-numbered oligosaccharides in product mixtures obtained by BTH digestion of HA can be ascribed to the boiling procedure performed to stop the enzyme reaction.

6.3.6 NMR spectroscopy

An additional approach to investigate the differences in the product mixtures derived from the digestion of hyaluronate (HA) with Neopermease® at different pH was made by means of NMR spectroscopy. Figure 6-20 shows the ^{1}H NMR spectra obtained for hyaluronate exhaustively digested with Neopermease® at pH 3.8 and pH 6.0 using incubation mixtures analogous to those of the activity measurements, i.e. citrate-phosphate buffer containing NaCl and BSA. For NMR spectroscopy, after exhaustive digestion, the mixtures were lyophilised and redissolved in D_2O.

The peak assignment of the ^{1}H spectra via the plots of 2D experiments (DQF COSY, ROESY, HMQC and HMBC) revealed that in both cases, i.e. at pH 3.8 and pH 6.0, the main product was the HA tetrasaccharide (Fig. 6-19) occurring at a ratio of the anomers of $\alpha : \beta \approx 1.6 : 1$. The spectra of the digests depicted in figure 6-20 were found to be in very good agreement with the NMR data (chemical shift, coupling constants) reported for hyaluronate tetrasaccharide dissolved in D_2O by Toffanin et al. (1993). The results are thus in agreement with those obtained by anion exchange HPLC with respect to the main degradation product (cf. Fig. 6-6). However, the additional presence of minor amounts of di- and hexasaccharides at a different ratio depending on pH, which was observed in anion exchange HPLC, was not detected by the NMR measurements. This may be ascribed to the relatively low sensitivity of NMR spectroscopy and to the fact that buffer components (citrate, phosphate) may have interfered with the recording of highly resolved NMR spectra.

In addition to the characterisation of the exhaustively digested incubation mixtures described above, efforts were made to elucidate pH related kinetic differences in the degradation of HA with Neopermease® by monitoring the product formation at pH 3.8 and pH 6.0 by NMR spectroscopy, i.e. after the addition of enzyme to the incubation mixture ^{1}H NMR spectra were recorded at increasing reaction times.

For the experiment at pH 6.0 the incubation conditions which had been elaborated in preliminary studies by viscosimetry (cf. chapter 5), were applied. However, although under these conditions the relative viscosity of the substrate solution had been reduced by about 80 % after 60 min and 90 % after 150 min (cf. chapter 5, Fig. 5-19), product formation was not observed in the ^{1}H NMR spectra even after an incubation time of 16 h. When raising the

enzyme concentration by a factor of 5 (13.3 units[#]/ml), product formation was detected by the appearance of the peaks corresponding to free anomeric protons (δ = 5.16 ppm; 4.73 ppm), however only to a small extent, which was not further increased even after prolonged incubation (91h, 187 h). Analysis of the obtained spectra with respect to the length of the produced oligosaccharides was not possible due to the overlaying peaks resulting from high molecular mass HA (results not shown). By further elevation of the enzyme concentration (26.27 units[#]/ml) spectra similar to those of the exhaustive digests were obtained after 60 h. However, during the digestion process the overlaying peaks of high molecular mass HA interfered with the identification of the digestion products.

Similar observations were made in the course of the investigation of HA degradation at pH 3.8, which is described in detail in the materials and methods section (6.2.4.6.1.2). The use of high concentrations of enzyme and raising the incubation temperature from 21 °C to 37 °C yielded spectra similar to those of the exhaustive digests after prolonged incubation times (results not shown). Since, like in case of the experiments at pH 6.0, described above, during the digestion process the peak assignment of the degradation products was impossible due to interfering peaks of high molecular mass HA, NMR spectroscopy was found to be an unsuitable method to monitor the action of hyaluronidase on HA.

[#] as declared by the supplier

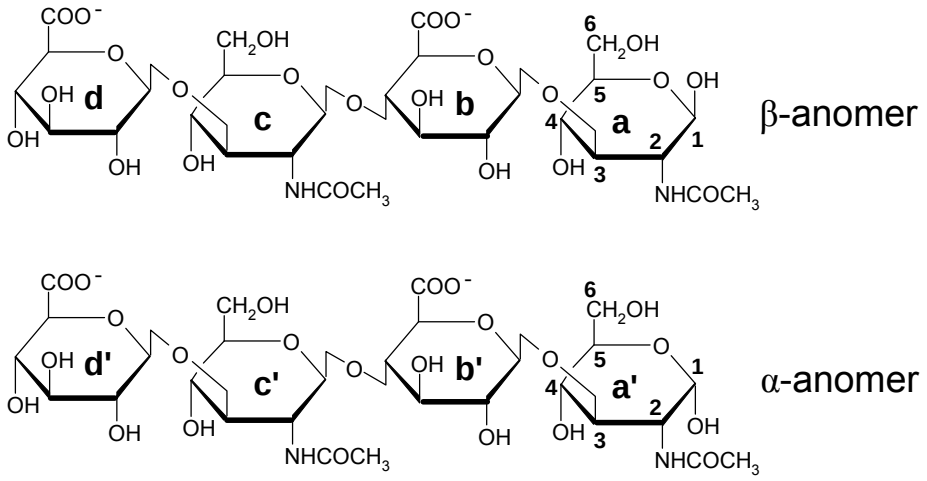


Fig. 6-19: Structure of hyaluronate tetrasaccharide

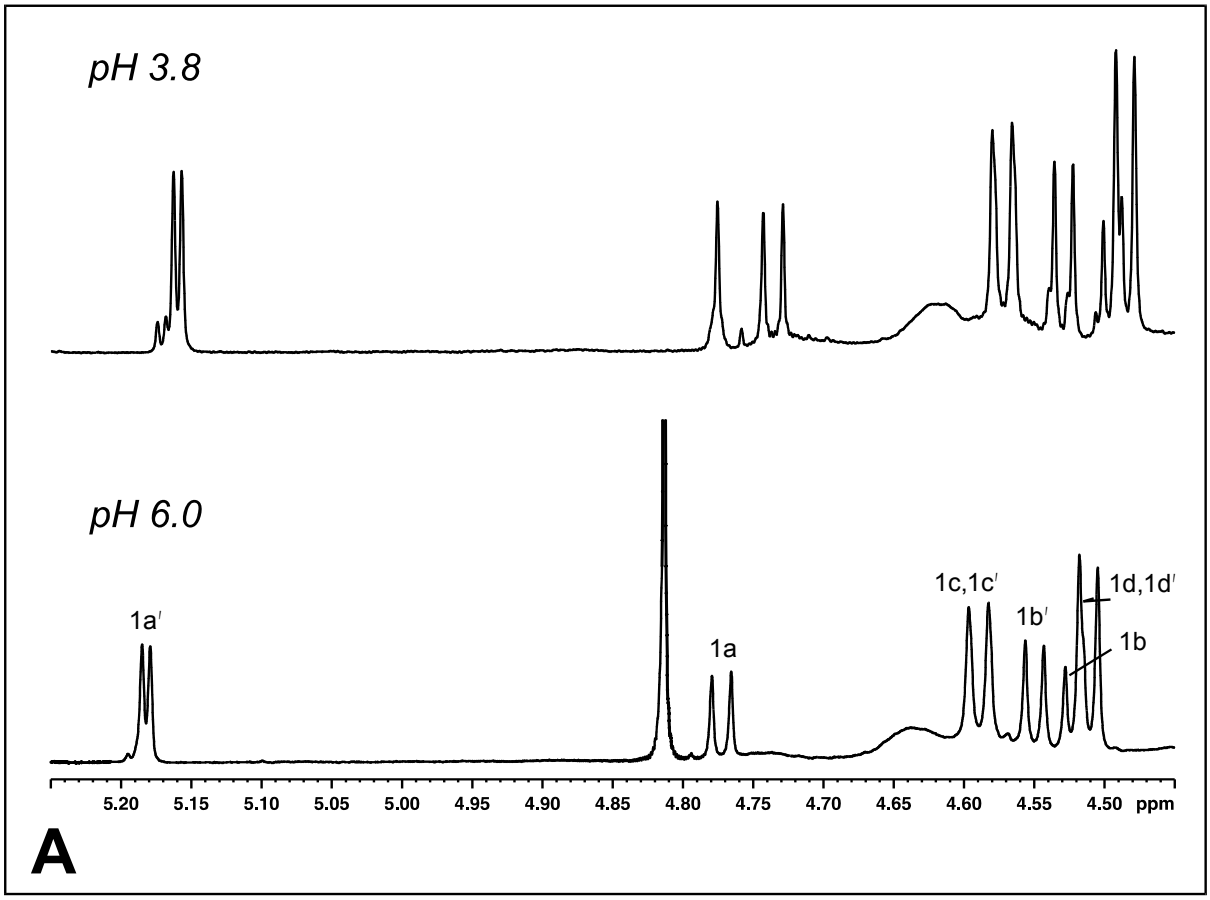
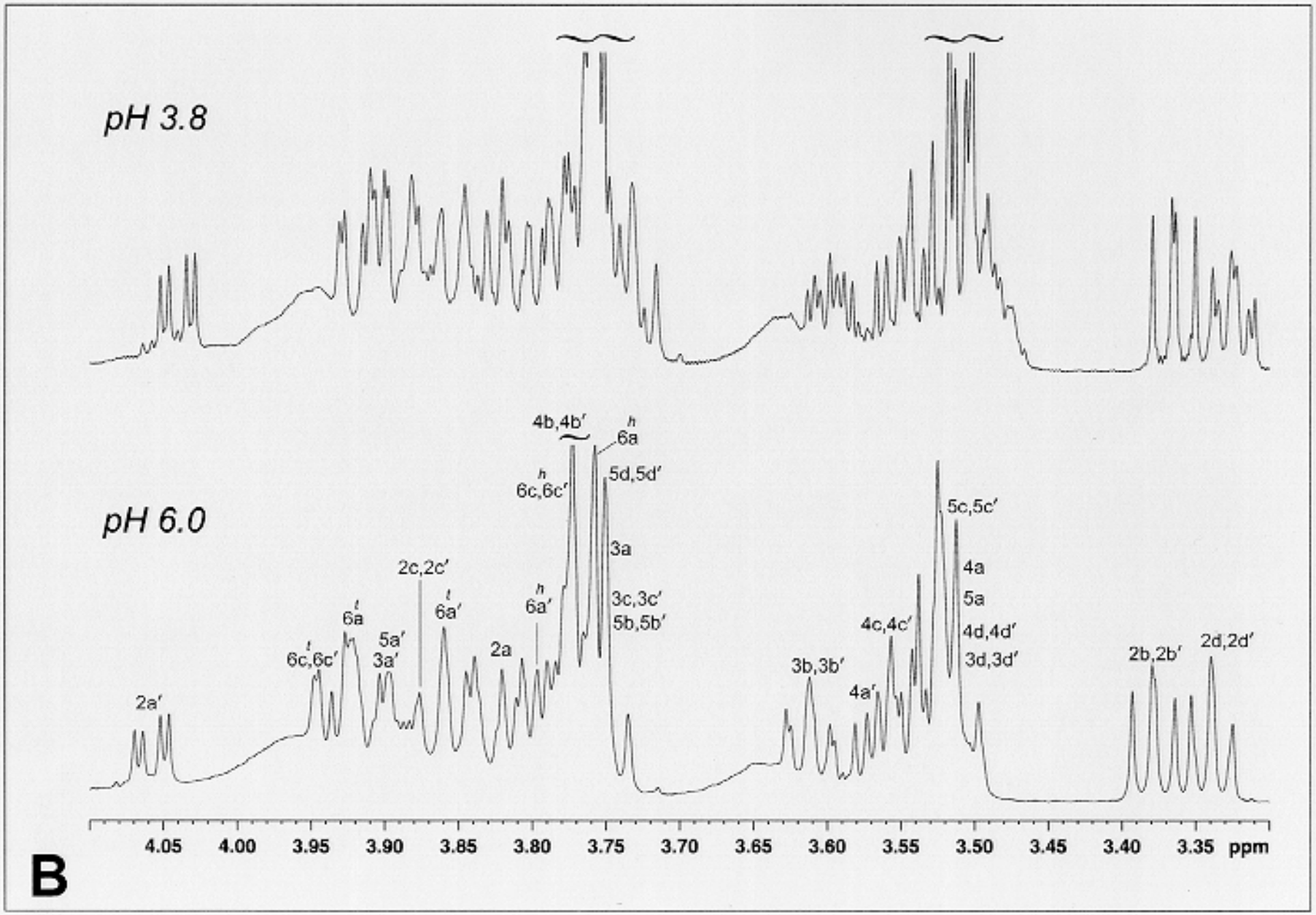


Fig. 6-20: 600-MHz ^{1}H NMR spectra of the product mixtures obtained by the digestion of hyaluronate (HA) with Neopermease® at pH 3.8 and pH 6.0 as described in the materials and methods section (see 6.2.4.6.1.1). The main component was HA tetrasaccharide (ratio of the anomers α : β ≈ 1.6 : 1). The peaks were assigned according to figure 6-19. A: H-1 signals (anomer region); B (see following page): signals of H-2,3,4,5,6.


pH 3.8
pH 6.0
4b,4b′
h
6a
5d,5d′
h
6c,6c′
3a
3c,3c′
5b,5b′
2c,2c′
t
6a
t
6a′
5a′
3a′
t
6c,6c′
2a
h
6a′
2a′
5c,5c′
4a
5a
4d,4d′
3d,3d′
4c,4c′
3b,3b′
4a′
2b,2b′
2d,2d′
4.05
4.00
3.95
3.90
3.85
3.80
3.75
3.70
3.65
3.60
3.55
3.50
3.45
3.40
3.35
ppm
B

6.4 Conclusion

The colorimetric determination of the activity of Neopermease® as a function of pH yielded identical pH activity profiles (maximum at pH 3.6) at various substrate (HA) concentrations, including the substrate concentration of the turbidimetric assay. Therefore, it can be ruled out that the discrepancy between the colorimetrically and the turbidimetrically (maximum at pH 6.0) determined pH activity curves of Neopermease® reported in the preceding chapter (cf. chapter 5, Fig. 5-10) originates from different HA concentrations in the incubation mixture.

In addition, it was demonstrated that the activity maxima obtained for BTH in the different assays indeed reflect the effect of pH and are not caused by differences in the ionic strength of the incubation mixtures at varying pH. Adjusting the ionic strength at pH 3.6 to that at pH 6.0 by NaCl supplementation of the buffers resulted in a decrease in the activity of Neopermease® at pH 3.6 in both assays. However, also at constant ionic strength activity was still higher at pH 3.6 compared to pH 6.0 in the colorimetric assay, whereas in the turbidimetric assay highest activity was measured at pH 6.0.

To investigate, if the different pH activity profiles of BTH obtained by the colorimetric and the turbidimetric assay can be ascribed to the pH related transglycosylase activity of BTH, the composition of the oligosaccharide mixtures produced by the action of Neopermease® at varying pH was analysed by anion exchange HPLC. Indeed, the product mixtures obtained after exhaustive digestion of HA at varying pH indicated transglycosylase activity of Neopermease® at pH 6.0 but not at pH 3.6. However, analysis of the product mixtures obtained after HA digestion for 1 h revealed that the amount of low molecular mass oligosaccharides was rather low at pH 6.0. This suggests that the transglycosylation reaction involving low molecular mass oligosaccharides makes only a minor contribution to the product spectrum at this stage of the reaction and does, thus, presumably not account for the different pH optima of BTH in the colorimetric and the turbidimetric assay. However, comparison of the 1 h digests at various pH suggests that the pH optima of BTH in the various assays may be explained as follows:

HA degradation at pH 3.6 yielded a considerably higher amount of short oligosaccharides than incubation at pH 6.0. This explains the pH profile obtained in the colorimetric assay, where a higher amount of reducing NAG end groups is detected after incubation at pH 3.6 compared to pH 6.0. Although at pH 6.0 after 1 h of incubation the formation of short

oligosaccharides was relatively low, the anion exchange HPLC analysis indicates that HA is degraded to HA fragments which are not precipitated in the turbidimetric assay. Assuming that at pH 3.6 oligosaccharides are preferentially generated in the presence of high molecular mass HA, whereas at pH 6.0 all types of molecules of the polydisperse substrate are processed to the same extent, the higher activity detected in the turbidimetric assay and also in the viscosimetric assay (cf. chapter 5) at pH 6.0 can be explained regardless of the higher rate of NAG formation detected in the colorimetric assay at pH 3.6.

At pH 6.0 odd-numbered oligosaccharides having reducing and nonreducing terminal GlcUA residues were identified in the product mixtures obtained by the degradation of HA with Neopermease®. Since the formation of odd-numbered oligosaccharides is not in agreement with the mechanism of HA degradation reported in the literature for BTH it was investigated if the odd-numbered products might be formed during the 15 min boiling procedure performed at the end of the incubation period to stop the enzyme reaction, as proposed by Prebyl et al. (2003). However, this assumption could not be confirmed.

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7 Investigations on hyaluronate degradation by hyaluronate lyase from *Streptococcus agalactiae*

7.1 Introduction

Streptococcus agalactiae is a human pathogenic bacterium, which is one of the major causes of meningitis and septicemia, often leading to death in neonates (Dillon et al., 1987; Baker and Edwards, 1988). A major virulence factor of *S. agalactiae* is hyaluronate lyase, which breaks down the biophysical barrier of the host connective tissue by degrading the hyaluronic acid of the extracellular matrix thereby facilitating the invasion and the spreading of the bacteria and the associated toxins (Li and Jedrzejas, 2001). In recent years the search for potent inhibitors of hyaluronate lyase, which could be useful tools in studying the role of hyaluronan and hyaluronidases in bacterial infections and may be promising agents with respect to the investigation of a novel auxiliary antibacterial therapy, was started (Salmen, 2003; Botzki, 2004; Botzki et al., 2004).

In the course of the characterisation of inhibitors of hyaluronate lyase from *S. agalactiae* with respect to their activity at different pH values it turned out that in many cases lower IC_{50} values were obtained at physiological pH (pH 7.4) compared to pH 5.0, where the enzyme shows maximum activity (Salmen, 2003). Motivated by this observation the present study was carried out to find out, if the pH dependency of the inhibitory activity can be ascribed to a different affinity of the enzyme to the substrate depending on pH. The degradation of hyaluronan by *S. agalactiae* hyaluronate lyase was therefore investigated with respect to the effect of pH on the kinetic parameters V_{max} and K_m.

As described in detail in chapter 1, hyaluronate lyase from *S. agalactiae* is supposed to degrade the polymeric substrate via a processive mode of action. In brief, the enzyme randomly binds to a hyaluronate molecule and cleaves it into two pieces by β-elimination. After this initial endolytic cleavage one of the two generated hyaluronan fragments is released from the enzyme and may serve as substrate for another enzymatic attack, whereas the other fragment remains a the binding site of the enzyme to be processively degraded to unsaturated disaccharides (Baker and Pritchard, 2000; Li and Jedrzejas, 2001).

In ordinary enzyme catalysed reactions the enzyme E binds to the substrate S, forming an enzyme substrate complex ES, and the substrate is converted in the following step into a product P, which is finally released from the enzyme, as shown by equation 7-1. In this case the kinetic parameters V_{max} and K_m can be derived from the experimentally determined initial rates of product formation v_0 at various concentrations of substrate according to the Michaelis Menten equation (eq. 7-2).

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P \qquad \text{(eq. 7-1)}$$

$$v_0 = \frac{V_{max} \cdot [S]}{K_m + [S]} \qquad \text{(eq. 7-2)}$$

Hyaluronate degradation by *S. agalactiae* hyaluronate lyase, involving an initial endolytic cleavage followed by rapid and processive release of unsaturated disaccharide, differs from the common mechanism underlying the Michaelis Menten equation, since the substrate remains at the enzyme during the processive release of unsaturated disaccharide products. To check the validity for the estimation of the kinetic parameters V_{max} and K_m of the Michaelis Menten formalism from a set of initial rates of product formation measured at various substrate concentrations it was demonstrated by theoretical considerations that hyaluronate degradation by *S. agalactiae* hyaluronate lyase obeys Michaelis Menten kinetics, regardless of the processive character of the exolytic cleavage of the substrate.

In preliminary studies the product mixtures resulting from partial and exhaustive digestion of HA by hyaluronate lyase from *S. agalactiae* were analysed by anion exchange HPLC and NMR spectroscopy to confirm the aforementioned processive mode of action.

7.2 Materials and methods

7.2.1 Chemicals

Stabilised hyaluronate lyase, i.e. 200 000 units[#] of lyophilised hyaluronidase (0.572 mg) from *Streptococcus agalactiae*, strain 4755, plus 2.2 mg BSA and 37 mg Tris-HCl per vial, was kindly provided by id-pharma GmbH (Jena, Germany). Hyaluronic acid (HA) from *Streptococcus zooepidemicus* was purchased from Aqua Biochem GmbH (Dessau, Germany). Bovine serum albumin (BSA) was from Serva (Heidelberg, Germany). Deuteriumoxide (D_2O, isotopic enrichment 99.98 %) was purchased from Deutero GmbH (Kastellaun, Germany). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Eschborn, Germany).

7.2.2 Separation of hyaluronate oligosaccharides by anion exchange HPLC

7.2.2.1 Digestion of hyaluronate

For the subsequent analysis of the degradation products by anion exchange HPLC digestion of hyaluronate with *S. agalactiae* hyaluronate lyase was carried out under the same incubation conditions as used in case of the analogous experiments with hyaluronidases from bovine testes and bee venom, respectively, which are described in chapter 6. The incubation mixture (incubation mixture 7-I) consisted of 100 µl of citrate-phosphate buffer, pH 5.0 (solution A: 0.2 M Na_2HPO_4/0.1M NaCl, solution B: 0.1 M citric acid/0.1 M NaCl; solutions A and B were mixed in appropriate portions to adjust the required pH), 150 µl of substrate (5 mg HA from *S. zooepidemicus* per ml of water), 50 µl of BSA (0.2 mg BSA per ml of water: solution 1) and 100 µl of water. 400 µl of incubation mixture 7-I were incubated with 50 µl of enzyme (*S. agalactiae* hyaluronate lyase: 5 units[#], dissolved in BSA (solution 1)) at 37 °C for 1 h and 16.5 h, respectively. The enzyme reaction was stopped by heating at 100 °C for 15 min. Protein was removed from the mixtures by ultrafiltration (Nanosep® 10 K centrifugal device, Pall Life Sciences, New York, USA). In addition, a reference mixture, consisting of incubation mixture 7-I plus 50 µl of BSA (solution 1) instead of enzyme, was prepared and treated in the same way as described for the digestion mixture.

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7.2.2.2 Instrumentation

HPLC analysis was performed with the equipment specified in the materials and methods section of chapter 6 (see 6.2.4.1.2). As stationary phase the Luna 3µ NH_2 (150 x 3.0 mm i.d.) column equipped with a security guard cartridge (NH_2, 4 x 2.0 mm i.d.) was used (Phenomenex, Aschaffenburg, Germany).

7.2.2.3 HPLC conditions

The separation of the hyaluronate oligosaccharides was performed by the application of a linear phosphate gradient (16 - 800 mM NaH_2PO_4) at 40 °C according to the method described for the Luna-NH_2 stationary phase in the materials and method section of chapter 6 (see 6.2.4.1.3). Detection was carried out at 205 nm and 232 nm, respectively.

7.2.3 Analysis of hyaluronate degradation products by NMR spectroscopy

7.2.3.1 Digestion of hyaluronate

7.2.3.1.1 Exhaustive digestion

For the characterisation of the final degradation products by means of NMR spectroscopy HA was exhaustively digested with *S. agalactiae* hyaluronate lyase. The incubation mixture (incubation mixture 7-II) consisted of ammonium acetate buffer (0.2 M; pH 5.6), containing 1.67 mg/ml of HA from *S. zooepidemicus* and 0.04 mg/ml of BSA. 30 ml of incubation mixture 7-II were incubated with 20 units[#] of hyaluronate lyase (dissolved in solution 1). After incubation for 5 days at room temperature the mixture was lyophilised. For NMR analysis 26.7 mg of the lyophilised sample were dissolved in 0.6 ml of D_2O.

7.2.3.1.2 Incubation conditions for monitoring the digestion by NMR spectroscopy

To investigate product formation during HA degradation with *S. agalactiae* hyaluronate lyase the changes in the digestion mixture were monitored by NMR spectroscopy. The digestion was performed under the conditions, which had been investigated in preliminary studies by viscosimetry (see chapter 5). The incubation mixture (incubation mixture 7-III) was prepared as follows: 10 mg of substrate (HA from *S. zooepidemicus*) were dissolved in 6 ml of 0.133 M phosphate buffer (pH 6) containing 0.044 mg/ml BSA (140.93 mg NaH_2PO_4, 22.73 mg Na_2HPO_4 and 0.44 mg BSA per 10 ml of D_2O). To 700 µl of incubation mixture 7-III 10 µl of

[#] according to the declaration of the supplier

internal standard solution (50 mg of TSP (3-(trimethylsilyl)-propionic acid-2,2,3,3-d4 sodium salt) per ml of D_2O) were added, and a reference spectrum of the undigested incubation mixture was recorded. At t = 0 the enzyme (50 µl, 0.5 units[#], dissolved in solution 1) was added to the mixture directly into the NMR tube and after thorough mixing spectra were recorded at increasing reaction times (t = 6 min - 7 h, at intervals of 10 min within the first 3 hours and 60 min, respectively). Additional spectra were acquired at t = 24 h and t = 4 d.

7.2.3.2 NMR spectroscopy

Measurements were performed on a Bruker Avance 400 spectrometer, operating at 400.13 MHz for ^1H (Bruker Biospin GmbH, Rheinstetten, Germany). All spectra were recorded at 294 K and the intense water signal was suppressed by the application of presaturation on the water resonance frequency. Chemical shifts were referenced to internal TSP. The peak assignment of the ^1H spectrum of the exhaustive digest was made via the plots obtained from 2D experiments, i.e. double quantum filter correlation spectroscopy (DQF COSY) and rotating frame nuclear overhouser effect spectroscopy (ROESY).

7.2.4 Determination of hyaluronidase activity by UV spectroscopy

The activity of *S. agalactiae* hyaluronate lyase was determined by quantifying the unsaturated degradation product 2-acetamido-2-deoxy-3-O-(ß-D-gluco-4-enepyranosyluronic acid)-D-glucose (△DiHA) photometrically at 232 nm according to Greiling (1957). The incubation mixture (incubation mixture 7-IV) consisted of 400 µl of citrate-phosphate buffer (pH 5 and pH 7.4, respectively, see 7.2.2.1), 100 µl of BSA (solution 1, see 7.2.2.1) and 300 µl of substrate (varying concentrations of HA from *S. zooepidemicus* dissolved in H_2O). HA solutions of varying concentrations in the range from 0.1 to 6 mg/ml were prepared by dilution of a stock solution (6 mg/ml). The measurements were performed as described in the materials and methods section of chapter 4 (see 4.2.2.2) with a Cary 100 spectrophotometer (Varian, Darmstadt, Germany). Instead of tandem cuvettes a set of matched cuvettes (Semi-Micro Cells, Hellma®, No. 104-QS, Müllheim, Germany) with pathlength a l = 10 mm were used. The reference cuvette contained incubation mixture 7-IV and 100 µl of BSA (solution 1). The amount of enzyme used is given in the results section in the context with the respective experiments.

[#] according to the declaration of the supplier

From the increase in absorbance (△A) at 232 nm per time (△t) the velocity of the enzymatic reaction was calculated according to the following equation:

$$v\ [\mu mol \cdot l^{-1} \cdot min^{-1}] = \triangle A / (\triangle t \cdot \varepsilon \cdot l) \qquad \text{(eq. 7-3)}$$

where $\varepsilon = 4550\ l \cdot mol^{-1} \cdot cm^{-1}$ (see 4.2.2.2). For the calculation of the specific activity of *S. agalactiae* hyaluronate lyase the amount of enzyme per vial, as indicated by the supplier, was used.

7.3 Results and discussion

7.3.1 Analysis of the products of hyaluronate digestion

7.3.1.1 Anion exchange HPLC

HA degradation by *S. agalactiae* hyaluronate lyase is reported in the literature to occur via a mechanism which involves an initial endolytic cleavage of a HA chain followed by rapid exolytic and processive release of unsaturated disaccharide (△DiHA) being the sole product (Pritchard et al., 1994; Baker and Pritchard, 2000; Li and Jedrzejas, 2001; Mello et al., 2002).

To confirm that HA degradation by the *S. agalactiae* hyaluronate lyase preparation investigated in our laboratory is in agreement with the commonly assumed mechanism product mixtures resulting from partial and exhaustive digestion of HA were analysed by anion exchange HPLC by analogy to the analysis of the product mixtures obtained by HA digestion with hyaluronidases from bovine testes and bee venom, respectively (cf. to chapter 6). Separation of the exhaustively digested incubation mixture showed that HA was degraded to the disaccharide **1** as the only product (Fig. 7-1A). The disaccharide **1** was found to be the predominant product also after partial digestion. However, additional peaks corresponding to the tetra **2** - hexa **3** - and octasaccharide **4** were detected (Fig. 7-1B).

Detection of the peaks at 232 nm (Fig. 7-2A) indicated that they correspond to the oligosaccharides having 4,5 - unsaturated glucuronic acid residues at their nonreducing ends resulting from the β-elimination catalysed by the bacterial hyaluronidases. Presuming a similar retention time for the saturated and unsaturated HA oligosaccharides the peaks in figures 7-1 and 7-2 were assigned according to the peaks in the chromatograms of the saturated oligosaccharides which have been identified by mass spectroscopy (cf. to chapter 6).

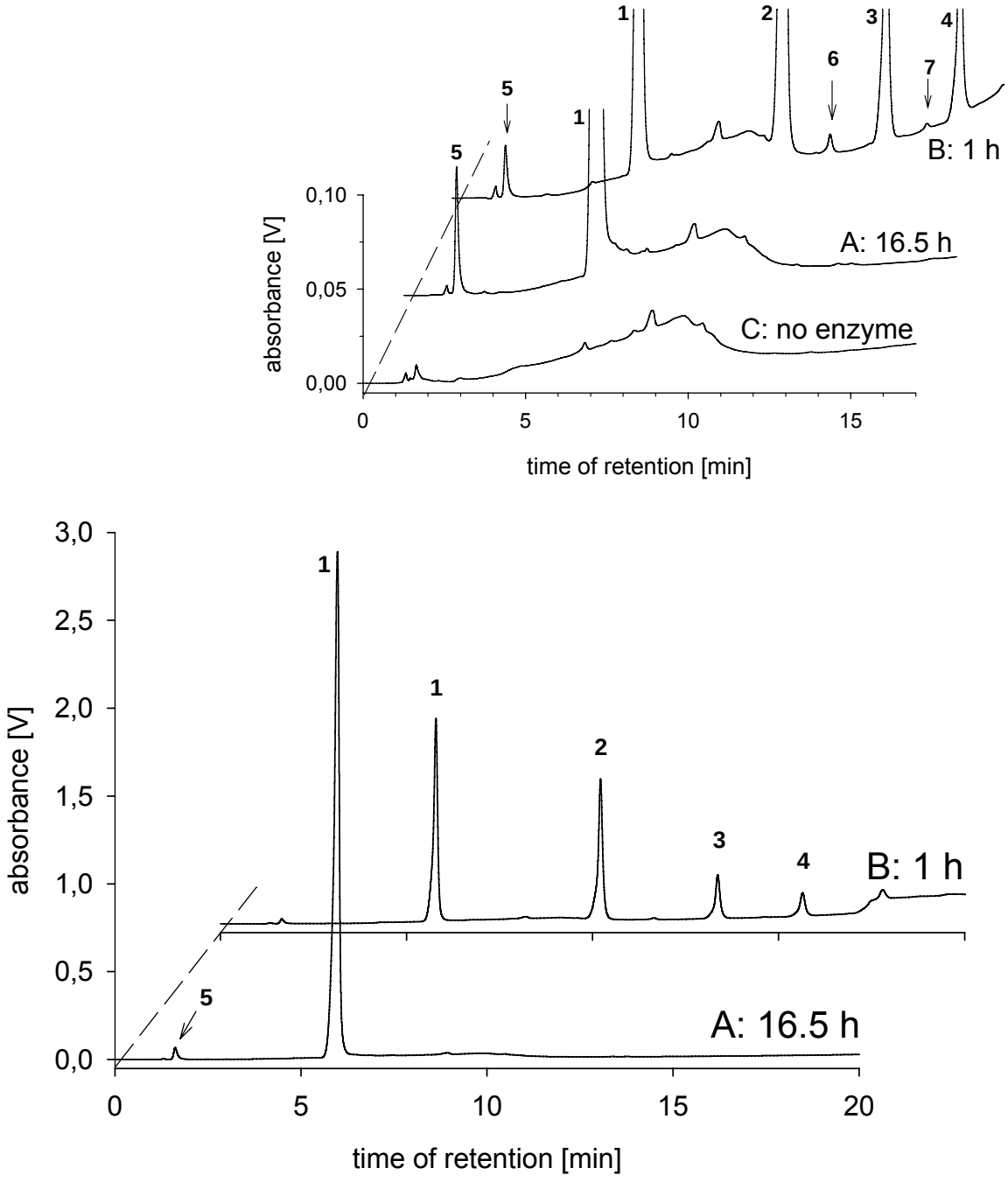

Fig. 7-1: Anion exchange HPLC chromatograms of hyaluronate (HA) oligosaccharides produced by the digestion of HA with *S. agalactiae* hyaluronate lyase at pH 5.0 for 16.5 h (A) and 1h (B), respectively. Separation was performed as described in the materials and method section. The injection volume was 10 µl and the detection wavelength was 205 nm. *Inset*: magnified view of lanes A and B; C: chromatogram of the reference mixture (see 7.2.2.1).

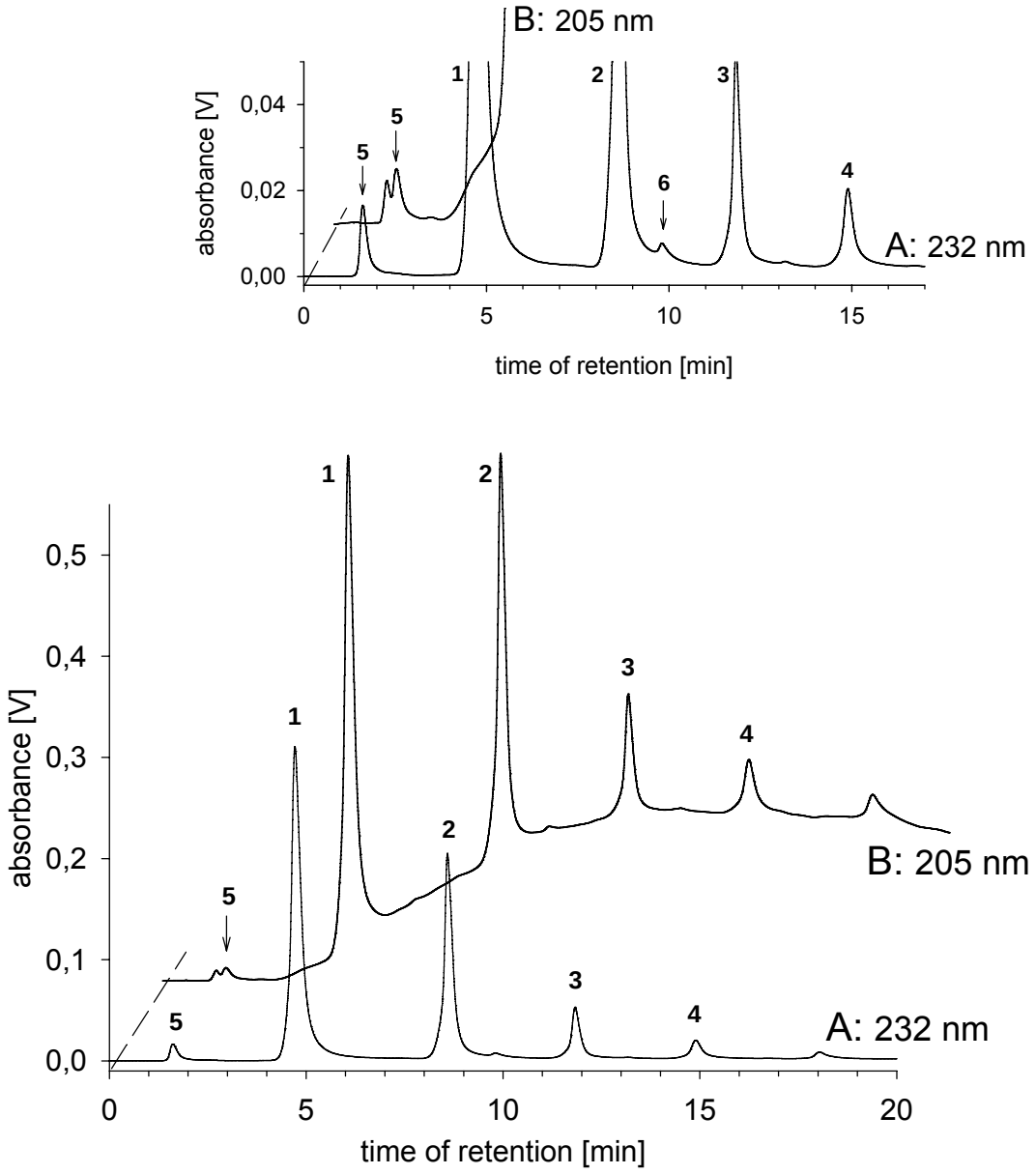

Fig. 7-2: Anion exchange HPLC of hyaluronate (HA) oligosaccharides produced by the digestion of HA with *S. agalactiae* hyaluronate lyase for 1h at pH 5.0. Separation was performed as described in the materials and method section. The injection volume was 10 µl. UV detection was carried out at 232 nm (A) and 205 nm (B), respectively. *Inset*: magnified view of lanes A and B.

By comparison of the peak areas detected at 232 nm (Fig. 7-2A) the relative amounts of the unsaturated oligosaccharides compared to △DiHA in the incomplete digest were calculated as 56 % (tetrasaccharide), 13.7 % (hexasaccharide) and 6 % (octasaccharide), respectively.

At first sight the presence of unsaturated oligosaccharides in addition to the disaccharide in the partial digest seems to disagree with a processive action pattern of *S. agalactiae*

hyaluronate lyase. According to this mechanism the digestion mixture is expected to contain only the processively released unsaturated disaccharides and HA chains of high molecular mass. However, the transient accumulation of unsaturated tetra- and hexasaccharides is consistent with the results of Baker and Pritchard (2000), who proposed the following mechanism for the degradation of hyaluronan by *S. agalactiae* hyaluronate lyase: after an initial random endolytic attack the enzyme remains at the substrate chain and processively releases unsaturated disaccharides until the chain is degraded to small oligosaccharides, which are released and finally cleaved endolytically and more slowly.

Compared to the results of Baker and Pritchard (2000), who reported a maximum accumulation of unsaturated oligosaccharides of 2.5 % (relative to △DiHA) in partially digested incubation mixtures, the amount of unsaturated oligosaccharides detected in the 1 h digest of our experiment was quite high (76 % relative to △DiHA). This discrepancy may be explained by differences in the molecular mass and the polydispersity of the HA used as substrate and by a different ratio of enzyme to substrate in the incubation mixture. Considering that according to the described mechanism each initial endolytic cleavage leads to the release of a small unsaturated oligosaccharide, before this is finally cleaved into disaccharides, a higher ratio of enzyme to substrate, which is associated with an increased number of initial chain cleavages, would result in a higher transient accumulation of unsaturated oligosaccharides. However, this explanation remains speculative, since the digestion conditions used by Baker and Pritchard are not described in detail in their publication. In addition, unfortunately we were not able to further investigate the effect of varying enzyme concentrations on the accumulation of unsaturated oligosaccharides by anion exchange HPLC, since the stationary phase turned out to be unstable against the aqueous mobile phase of high ionic strength. The continuous decrease in column efficiency becomes obvious when comparing the chromatograms of Fig. 7-1B and Fig. 7-2B, which show the separation of the identical sample, applied onto a new column (Fig. 7-1) and reanalysed after about 100 working hours of the column (Fig. 7-2).

The inset in Fig. 7-1 shows that in the 1 h digest in addition to the peaks of the even-numbered oligosaccharides (**1** – **4**) small peaks (**6**; **7**) were detected, which are not present in the reference mixture and the exhaustive digest. Taking the results of chapter 6 into account it is likely that the small peaks result from odd-numbered oligosaccharides, as shown for the intermediate peaks in the chromatograms of the oligosaccharide mixtures resulting from HA digestion with bovine testicular hyaluronidase (BTH). The presence of odd-numbered

oligosaccharides in oligosaccharide mixtures derived from HA by hyaluronate lyase treatment has been reported by Price et al. (1997), too, who identified the odd-numbered products as oligosaccharides having 4,5-unsaturated glucuronic acid at the nonreducing and glucuronic acid at the reducing end. This structure is in agreement with the odd-numbered oligosaccharides found in the BTH digests (saturated glucuronic acid at the nonreducing and the reducing end). However, due to the limited column life we were not able to collect enough material to characterise the intermediate peaks of the hyaluronate lyase digest.

The absence of peaks **6** and **7** in the mixture which had been exhaustively digested to △DiHA indicates that the odd-numbered products might have formed from an even-numbered oligosaccharide by the loss of one sugar unit presumably during the 15 min boiling procedure performed to stop the enzymatic reaction. Provided that the odd-numbered oligosaccharides contain glucuronic acid at the reducing end, as reported by Price et al. (1997), the peak eluted with the void volume of the column (**5**) could be assigned to free NAG. However, a compound is co-eluted, which is also detected at 232 nm (Fig. 7-2A), indicating that it contains a chromophore, like the 4,5-unsaturated glucuronic acid, the putative cleavage product of △DiHA. Considering that glucuronic acid contains a negatively charged carboxyl group whereas NAG is uncharged under the conditions of the described anion exchange HPLC analyses (pH 4.6) it is unlikely that the two compounds are eluted at the same time. Thus, the identification of peak **5** and the correct assignment of peaks **6** and **7** require further investigation.

7.3.1.2 NMR spectroscopy

An additional approach to investigate product formation during HA degradation with *S. agalactiae* hyaluronate lyase was made by NMR spectroscopy. Figure 7-4 shows an ^{1}H NMR spectrum of the product mixture obtained by HA digestion which has been allowed to proceed to completion. Assignment of the peaks via the plots of 2D spectra (DQF COSY, ROESY) revealed the 4,5-unsaturated hyaluronate disaccharide (△DiHA, see Fig. 7-3), occurring at a ratio of the anomers of $\alpha : \beta \approx 2 : 1$, as the sole product of exhaustive digestion (see table 7-1) and thus confirmed the results of anion exchange HPLC (see Fig. 7-1A). Similar ^{1}H NMR spectra are also reported for HA after digestion with a hyaluronate lyase from *S. intermedius* (Homer et al., 1994).

To further elucidate the mechanism of HA degradation by hyaluronate lyase from *S. agalactiae* with respect to the transient accumulation of unsaturated oligosaccharides in the

digestion mixture, as reported by Baker and Pritchard (2000), and as observed in the course of our investigations by anion exchange chromatography (see Fig. 7-1B), an attempt was made to monitor product formation by NMR spectroscopy. So after the addition of enzyme to the incubation mixture ^{1}H NMR spectra were recorded at increasing reaction times.

For this purpose incubation conditions which had been investigated in preliminary viscosimetric studies (cf. to chapter 5) were applied. After a reaction time of 16 min product formation was detected by the appearance of the signals characteristic for the 4-position olefinic proton of 4,5-unsaturated hyaluronate derived oligosaccharides (δ = 5.87 ppm) and the signals corresponding to the free anomeric protons (δ = 5.17 ppm; 4.77 ppm; results not shown). The observation that the signals corresponding to the free anomeric protons of the α-anomer occurred earlier than the respective signals of the β-anomer was surprising, since the anomeric configuration of the NAG residue is known to be retained during the cleavage of the β-1,4 glycosidic bond by the β-elimination process, which is catalysed by *S. agalactiae* hyaluronate lyase (Jedrzejas, 2000). However, it may be explained by a fast achievement of the mutarotation equilibrium ($\alpha : \beta \approx 2 : 1$).

With increasing reaction time additional signals corresponding to ^{1}H resonances of $\triangle$DiHA emerged due to a decrease of the signals resulting from high molecular mass HA. The final spectrum, which was recorded after a reaction time of 4 days, resembled the spectrum of the exhaustive digest which has been shown to contain $\triangle$DiHA as the sole product (Fig. 7-4). However, a transient accumulation of hyaluronate degradation products other than the $\triangle$DiHA, as it had been found by anion exchange HPLC, was not observed in the ^{1}H NMR spectra in the course of the reaction. The absence of signals corresponding to unsaturated HA oligosaccharides may be explained by the relatively low sensitivity of NMR spectroscopy. In view of the fact that the ratio of enzyme to substrate was relatively low in the NMR incubation mixture (0.4 units[#] per mg of HA) compared to the mixture which had been analysed by anion exchange chromatography (6.65 units[#] per mg of HA), it is conceivable that also the amount of transiently released unsaturated oligosaccharides was lower than that determined by anion exchange HPLC. According to the degradation mechanism discussed in the preceding section (7.3.1.1) a lower enzyme to substrate ratio is accompanied by a decreased number of initial chain cleavages and thus a decreased transient release of unsaturated oligosaccharides. Further investigations using varying enzyme concentrations would be necessary to support this hypothesis. However, due to the relatively low sensitivity

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of the method and the fact that during the course of the digestion overlaying signals of high molecular HA would interfere with peak assignment of the produced HA oligosaccharides, no further studies were carried out by NMR spectroscopy.

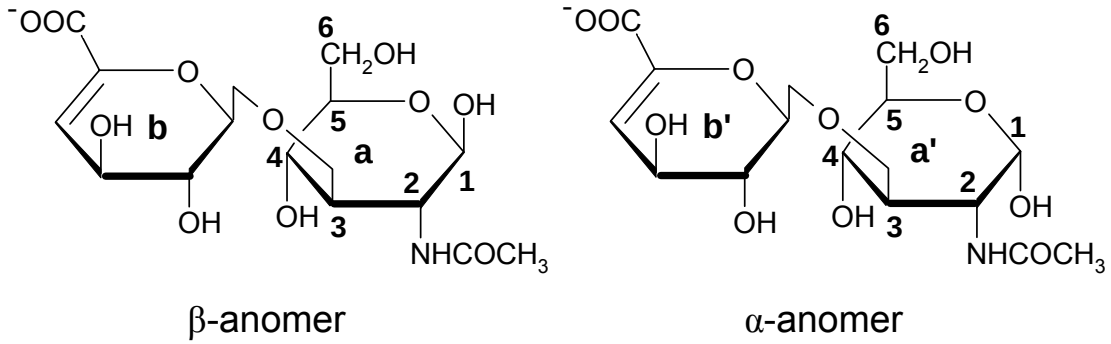

Fig. 7-3: Structure of the 4,5-unsaturated hyaluronate disaccharide (△DiHA).

Table 7-1: Chemical shifts (δ in ppm) of the ^{1}H resonances of △DiHA in D_2O.

^{1}H position[a]	δ in ppm		^{1}H position[a]	δ in ppm	
a/a'	**β - anomer**	**α - anomer**	**b/b'**	**β - anomer**	**α - anomer**
1	4.77	5.17	**1**	5.20	5.18
2	3.80 – 3.84	4.02	**2**	3.77	
3	3.80 – 3.84	4.00	**3**	4.16	
4	3.52	3.55	**4**	5.87	
5	3.77	3.90			
6	ca. 3.7 – 3.9	ca. 3.8 – 3.9			
NHCO**CH$_3$**	2.07				

[a] designated according to Fig. 7-3

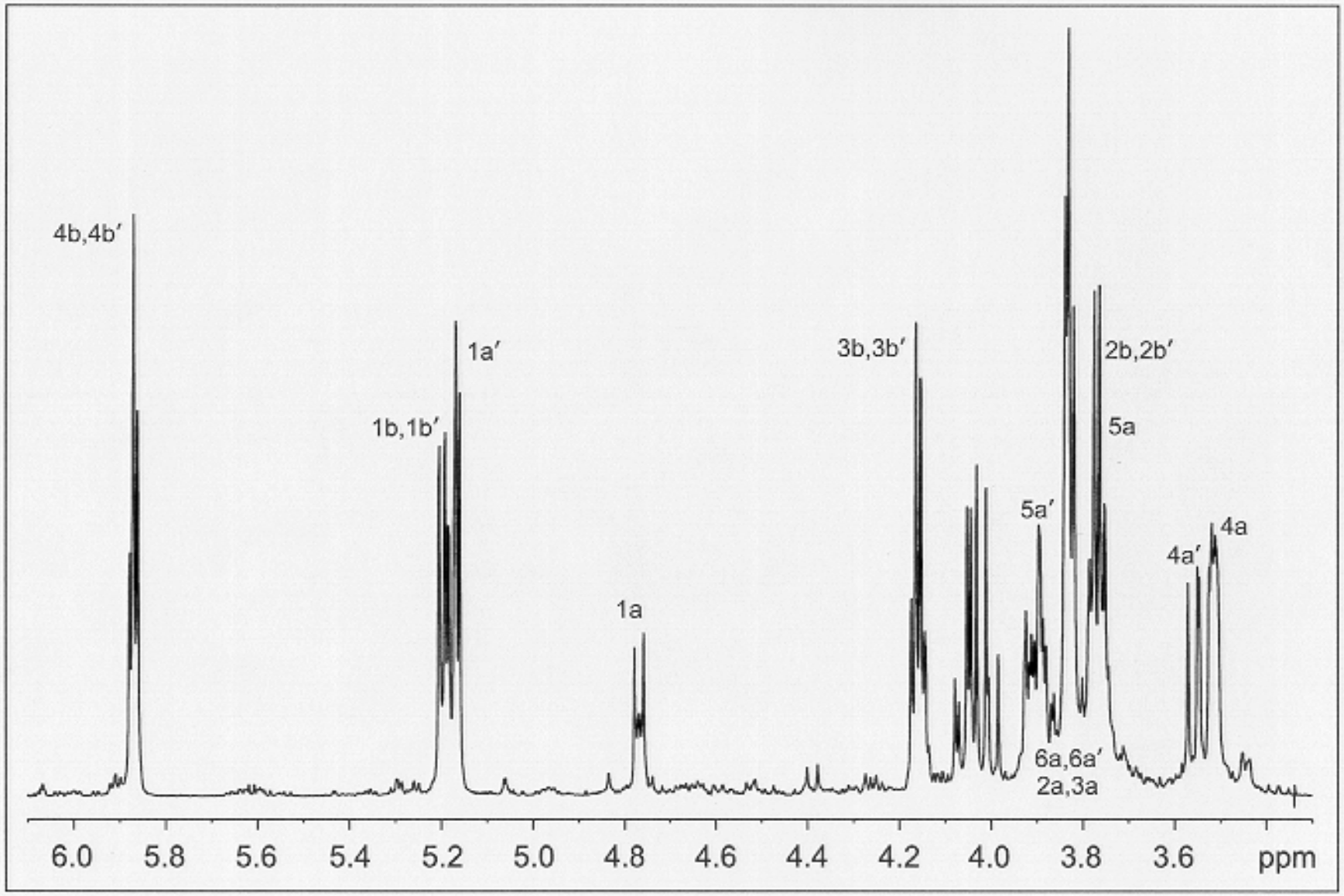

Fig. 7-4: 400-MHz ^{1}H NMR spectrum of the unsaturated disaccharide (ΔDiHA) obtained by exhaustive digestion of hyaluronate (HA) with *S. agalactiae* hyaluronate lyase as described in the materials and methods section (see 7.2.3). The peaks were assigned according to figure 7-3. Ratio of the anomers: $\alpha : \beta \approx 2 : 1$.

7.3.2 Determination of the kinetic parameters V_{max} and K_m

7.3.2.1 Theoretical considerations

For the determination of the kinetic parameters V_{max} and K_m the initial rates of product formation were determined at different substrate concentrations by measuring the increase in absorbance at 232 nm. To check if the experimental data can be interpreted according to the Michaelis Menten equation the mechanism of hyaluronate degradation by *S. agalactiae* hyaluronate lyase was expressed by the following set of equations:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP^0 + S' \qquad \text{(eq. 7-4)}$$

$$EP^0 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP^{0*} \xrightarrow{k_4} EP^1 + D \qquad \text{(eq. 7-5)}$$

$$EP^1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP^{1*} \xrightarrow{k_4} EP^2 + D \qquad \text{(eq. 7-6)}$$

$$\vdots$$

$$EP^{n-1} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP^{n-1*} \xrightarrow{k_4} P^n + E + D \qquad \text{(eq. 7-7)}$$

Equation 7-4 describes the binding of the enzyme E to high molecular mass hyaluronate S followed by the cleavage of the hyaluronate chain into a fragment S', which is released, and a fragment P^0 which consists of n disaccharide units and remains at the binding site of the enzyme to be processively degraded to unsaturated disaccharides D. Equation 7-5 describes the formation of disaccharide, starting with a translocation step, in which the truncated hyaluronate chain in the enzyme substrate complex EP^0 is moved by one disaccharide unit to yield the enzyme substrate complex EP^{0*}, in which the substrate is placed in the right position, so that the following cleavage step results in the formation of an unsaturated disaccharide, which is released, and a hyaluronate fragment P^1, which remains at the enzyme for the next round of catalysis (equation 7-6). Processive degradation of the enzyme bound substrate chain analogous to equations 7-5 and 7-6 occurs n-times until the whole chain is degraded. The final degradation product P^n is released from the enzyme, as indicated by equation 7-7. The enzyme is now available for the binding of new substrate (S or S', respectively).

According to the mechanism described by equations 7-4 to 7-7 the changes in the concentrations of substrate (S; S'), product (D) and the different enzyme forms with time were derived:

$$\frac{dS}{dt} = -k_1 [E][S] + k_{-1} [ES] \quad \text{(eq. 7-8)}$$

$$\frac{dS'}{dt} = k_2 [ES] \quad \text{(eq. 7-9)}$$

$$\frac{dD}{dt} = k_4 \sum_{i=1}^{n-1} [EP^{i*}] + k_4 [EP^{0*}] \quad \text{(eq. 7-10)}$$

$$\frac{dE}{dt} = -k_1 [E][S] + k_{-1} [ES] + k_4 [EP^{n-1*}] \quad \text{(eq. 7-11)}$$

$$\frac{dES}{dt} = k_1 [E][S] - k_{-1} [ES] - k_2 [ES] \quad \text{(eq. 7-12)}$$

$$\frac{dEP^0}{dt} = k_2 [ES] - k_3 [EP^0] + k_{-3} [EP^{0*}] \quad \text{(eq. 7-13)}$$

$$\frac{dEP^i}{dt} = k_4 [EP^{i-1*}] - k_3 [EP^i] + k_{-3} [EP^{i*}] \quad \text{(eq. 7-14)}$$

$$\frac{dEP^{0*}}{dt} = k_3 [EP^0] - k_{-3} [EP^{0*}] - k_4 [EP^{0*}] \quad \text{(eq. 7-15)}$$

$$\frac{dEP^{i*}}{dt} = k_3 [EP^i] - k_{-3} [EP^{i*}] - k_4 [EP^{i*}] \quad \text{(eq. 7-16)}$$

Under steady state conditions the expressions for the rates of changing concentrations of the different enzyme forms become zero, yielding equations 7-17 to 7-20.

$$[EP^0] = \frac{k_2\,[ES] + k_{-3}\,[EP^{0*}]}{k_3} \qquad \left(\text{for } \frac{dEP^0}{dt} = 0\right) \qquad \text{(eq. 7-17)}$$

$$[EP^{0*}] = \frac{k_3 \cdot [EP^0]}{k_{-3} + k_4} \qquad \left(\text{for } \frac{dEP^{0*}}{dt} = 0\right) \qquad \text{(eq. 7-18)}$$

$$[EP^i] = \frac{k_4\,[EP^{i-1*}] + k_{-3}\,[EP^{i*}]}{k_3} \qquad \left(\text{for } \frac{dEP^i}{dt} = 0\right) \qquad \text{(eq. 7-19)}$$

$$[EP^{i*}] = \frac{k_3 \cdot [EP^i]}{k_{-3} + k_4} \qquad \left(\text{for } \frac{dEP^{i*}}{dt} = 0\right) \qquad \text{(eq. 7-20)}$$

Expression of the different enzyme forms as a function of [ES] yields equations 7-21 to 7- 28.

$$[EP^0] = \frac{k_2(k_{-3} + k_4)\,[ES]}{k_3 \cdot k_4} \qquad \text{(eq. 7-21)}$$

(from eq. 7-17: substitution of $[EP^{0*}]$ according to eq. 7-18)

$$[EP^{0*}] = \frac{k_2 \cdot [ES]}{k_4} \qquad \text{(eq. 7-22)}$$

(from eq. 7-18: substitution of $[EP^0]$ according to eq. 7-21)

i = 1: $$[EP^1] = \frac{k_2(k_{-3} + k_4)\,[ES]}{k_3 \cdot k_4} \qquad \text{(eq. 7-23)}$$

(from eq. 7-19: substitution of $[EP^{i*}]$ according to eq. 7-20;
substitution of $[EP^{i-1*}]$ (= $[EP^{0*}]$) according to eq. 7-22)

$$[EP^{1*}] = \frac{k_2 \cdot [ES]}{k_4} \qquad \text{(eq. 7-24)}$$

(from eq. 7-20: substitution of $[EP^i]$ according to eq. 7-23)

$$i = 2: \quad [EP^2] = \frac{k_2 (k_{-3} + k_4)\,[ES]}{k_3 \cdot k_4} \qquad \text{(eq. 7-25)}$$

(from eq. 7-19: substitution of $[EP^{i*}]$ according to eq. 7-20;
substitution of $[EP^{i-1*}]$ (= $[EP^{1*}]$) according to eq. 7-24)

$$[EP^{2*}] = \frac{k_2 \cdot [ES]}{k_4} \qquad \text{(eq. 7-26)}$$

(from eq. 7-20: substitution of $[EP^i]$ according to eq. 7-25)

By analogy, the expressions for $[EP^i]$ and $[EP^{i*}]$ as a function of [ES] can be derived for i = 3 to i = n-1, yielding the general expressions (eqs. 7-27 and 7-28).

$$[EP^i] = \frac{k_2 (k_{-3} + k_4)\,[ES]}{k_3 \cdot k_4} \qquad \text{(eq. 7-27)}$$

$$[EP^{i*}] = \frac{k_2 \cdot [ES]}{k_4} \qquad \text{(eq. 7-28)}$$

With $[EP^{i*}] = [EP^{0*}]$ (cf. eqs. 7-22 and 7-28) the rate equation for product formation (eq. 7-10) can be rewritten as follows:

$$\frac{dD}{dt} = k_4 \sum_{i=1}^{n-1} [EP^{i*}] + k_4 [EP^{0*}] = n \cdot k_4 \cdot \frac{k_2 \cdot [ES]}{k_4} = n \cdot k_2 [ES] \qquad \text{(eq. 7-29)}$$

The concentration of free enzyme E can be expressed as follows:

$$[E] = [E_T] - [ES] - \sum_{i=0}^{n-1} [EP^i] + \sum_{i=0}^{n-1} [EP^{i*}] \qquad \text{(with } [E_T]\text{: total enzyme concentration)}$$

$$= [E_T] - [ES] - n \cdot \frac{k_2}{k_4} \left(1 + \frac{k_{-3} + k_4}{k_3}\right) \cdot [ES] \qquad \text{(eq. 7-30)}$$

(according to eqs. 7-27 and 7-28)

$$[E] = \frac{k_{-1} + k_2}{k_1} \cdot \frac{[ES]}{[S]} \qquad (\text{for } \frac{dES}{dt} = 0, \text{ cf. eq. 7-12}) \qquad \text{(eq. 7-31)}$$

By combining eqs. 7-30 and 7-31, the concentration of the enzyme substrate complex can be expressed as a function of $[E_T]$ and [S]:

$$[ES] = \frac{[S] \cdot [E_T]}{\frac{k_{-1} + k_2}{k_1} + [S]\left(1 + n \cdot \frac{k_2}{k_4}(1 + \frac{k_{-3} + k_4}{k_3})\right)} \qquad \text{(eq. 7-32)}$$

In the following, the terms $\frac{k_{-1} + k_2}{k_1}$ and $\frac{k_{-3} + k_4}{k_3}$ are defined as K_S and K_P, respectively.

Multiplication of eq. 7-32 with $(n \cdot k_2)$ yields an expression for the rate of product formation as a function of $[E_T]$ and [S]:

$$\frac{dD}{dt} = v_0 = n \cdot k_2[ES] = \frac{\frac{n \cdot k_2 \cdot k_4}{k_4 + n \cdot k_2(1 + K_P)} \cdot [E_T] \cdot [S]}{\frac{k_4 \cdot K_S}{k_4 + n \cdot k_2(1 + K_P)} + [S]} \qquad \text{(eq.7-33)}$$

Equation 7-33 is in agreement with the Michaelis Menten equation of the general form:

$$v_0 = \frac{V_{max} \cdot [S]}{K_m + [S]} \qquad \text{(eq. 7-2)}$$

According to eq. 7-33, the kinetic parameters V_{max} and K_m of hyaluronate degradation by *S. agalactiae* hyaluronate lyase are given by the following expressions:

$$V_{max} = \frac{n \cdot k_2 \cdot k_4}{k_4 + n \cdot k_2 (1 + K_P)} \cdot [E_T] \qquad \text{(eq. 7-34)}$$

$$K_m = \frac{k_4 \cdot K_S}{k_4 + n \cdot k_2 (1 + K_P)} \qquad \text{(eq. 7-35)}$$

The term K_m is used according to the simple operational definition of the Michaelis constant K_m as the substrate concentration at which the reaction velocity is halfmaximal.

The catalytic constant (turnover number) k_{cat} is given by the following expression:

$$k_{cat} = \frac{V_{max}}{E_T} = \frac{n \cdot k_2 \cdot k_4}{k_4 + n \cdot k_2 (1 + K_P)} \qquad \text{(eq. 7-36)}$$

At substrate concentrations [S] <<< K_m equation 7-2 reduces to a second order rate equation with the second order rate constant k_{cat}/K_m (specificity constant):

$$v_0 = \frac{V_{max} \cdot [S]}{K_m} = \frac{k_{cat}}{K_m} \cdot [E_T] \cdot [S] \qquad \text{(eq. 7-37)}$$

According to eqs. 7-35 and 7-36, for hyaluronate degradation by *S. agalactiae* hyaluronate lyase k_{cat}/K_m is given by the following expression:

$$\frac{k_{cat}}{K_m} = \frac{n \cdot k_2}{K_S} = \frac{n \cdot k_1 k_2}{k_{-1} + k_2} \qquad \text{(eq. 7-38)}$$

The theoretical considerations concerning the mechanism of hyaluronate degradation by *S. agalactiae* hyaluronate lyase revealed that according to the Michaelis Menten theory the kinetic parameters V_{max} and K_m can be derived from the experimentally determined rates of product formation under steady state conditions ($dD/dt = v_0$) at various concentrations of substrate. Since equation 7-33 was derived under the assumption of steady state conditions, its validity is warranted only in the steady state phase of the reaction. Concerning the duration of the steady state the investigated enzyme reaction shows an extraordinary property. In usual enzyme catalysed reactions, where a particular substrate is converted into a particular product, the reaction rate decreases as the substrate is consumed. The maintenance of the steady state is thus limited by the depletion of substrate. On the contrary, in case of the investigated degradation of hyaluronate the concentration of substrate remains constant, since the released cleavage product of the first step of the enzyme reaction (S', cf. equation 7-4) again serves as substrate for another enzymatic attack.

$$[S_{total}] = [S] + [S'] \qquad \text{(eq. 7-39)}$$

$$\frac{dS_{total}}{dt} = \frac{dS}{dt} + \frac{dS'}{dt} = -k_1\,[E][S] + k_{-1}\,[ES] + k_2\,[ES] \qquad \text{(eq. 7-40)}$$

$$\text{With } [ES] = \frac{k_1}{k_{-1} + k_2}\,[E]\,[S] \quad \left(\text{for } \frac{dES}{dt} = 0, \text{ cf. eq. 7-12}\right)$$

$$\frac{dS_{total}}{dt} = -k_1\,[E][S] + (k_{-1} + k_2) \cdot \frac{k_1}{k_{-1} + k_2}\,[E]\,[S] = 0 \qquad \text{(eq. 7-41)}$$

This constancy of the substrate concentration accounts for a prolonged maintenance of the steady state and thus of the linear part of the product versus time curve, even when low substrate concentrations are applied. As will be seen in the following chapter the prolonged linearity of the progress curve allows for the reliable determination of the steady state reaction rates ($dD/dt = v_0$), which are used for the calculation of the kinetic parameters V_{max} and K_m.

7.3.2.2 Determination of v_0

The determination of the steady state rate (v_0) of hyaluronate (HA) degradation by *S. agalactiae* hyaluronate lyase was carried out by monitoring the increase of the unsaturated degradation product (△DiHA) photometrically at 232 nm as described in the materials and methods section.

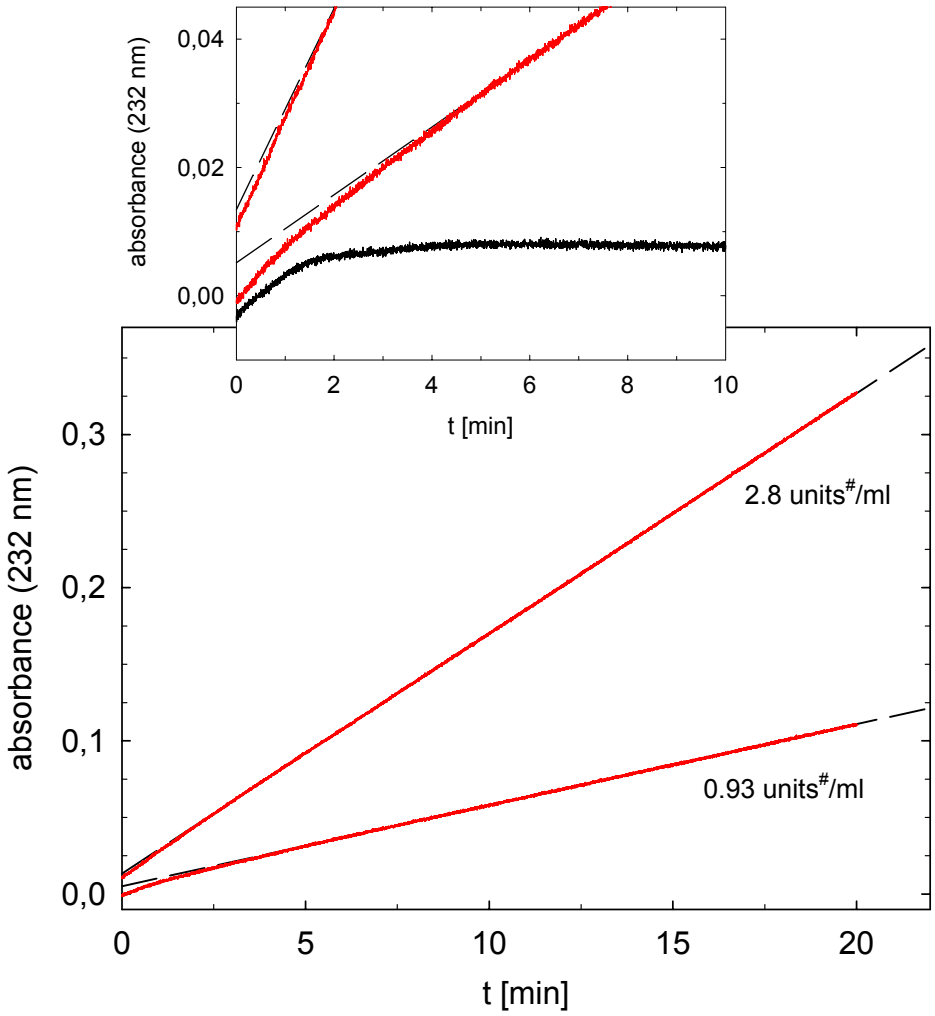

Fig. 7-5: Degradation of HA by *S. agalactiae* hyaluronate lyase, monitored at 232 nm (—). 100 µl of enzyme solution (2.5 units[#] and 0.83 units[#], dissolved in BSA (solution 1)) were incubated with 800 µl of incubation mixture 7-IV (pH 5.0; c (HA): 0.67 mg/ml). The dashed lines indicate the slope of the linear part of the respective absorbance curve. *Inset:* magnified view of the initial part (t = 0-10 min) of figure 7-5; black curve: 100 µl of BSA (solution 1) were incubated with incubation mixture 7-IV.

Figure 7-5 shows the kinetics of product formation obtained with two different concentrations of enzyme at a fixed concentration of substrate (0.67 mg/ml) under identical incubation conditions. For both enzyme concentrations a prolonged linear increase in absorbance is

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observed which indicates a long-lasting maintenance of the steady state of the reaction. This is in agreement with the theoretical considerations outlined above. As expected, the rate of product formation (slope of the linear part of the curve) was found to be proportional to the enzyme concentration. The unexpected observation that the prolonged linear phase does not coincide with the initial phase of the respective progress curve (cf. inset of Fig. 7-5) was found to arise from the fact that at the initial stage of the reaction the increase in absorbance at 232 nm due to the formation of △DiHA is overlaid by an effect induced by BSA, contained in the enzyme solution as a stabiliser, causing a slight turbidity due to precipitation with HA (inset of Fig. 7-5, black line).

Aiming at the determination of the kinetic parameters V_{max} and K_m a set of activity curves was recorded using various concentrations of substrate and the standard incubation mixture (see 7.2.4) at optimum pH for *S. agalactiae* hyaluronate lyase (pH 5). With 2.8 units[#]/ml an enzyme concentration was chosen, which ensured both access of the substrate and a measurable increase in absorbance at 232 nm also at low concentrations of the substrate. Assuming a molecular mass of $1.5 \cdot 10^6$ Da for hyaluronate (according to the declaration of the supplier) the lowest molar concentration of hyaluronate (0.133 mg/ml; $8.87 \cdot 10^{-8}$ M), which was taken into account for the calculation of V_{max} and K_m, was by a factor of 1000 higher compared to the enzyme ($9.35 \cdot 10^{-11}$ M).

In figure 7-6 a selection of the recorded activity curves is presented. With decreasing concentrations of substrate an increase in the discrepancy between the slope of the initial part of the curve and the slope of the prolonged linear part is observed, since the rate of increase in absorbance at 232 nm due to product formation becomes relatively low compared to the unspecific effect of BSA.

At first sight the observed bending of the activity curves seems to make the curves unusable for the determination of the kinetic constants of the enzyme reaction, since due to the interfering effect of BSA, it is impossible to determine the rate of product formation at the initial stage of the reaction at t = 0. However, figure 7-6 also shows that even at low hyaluronate concentrations the steady state (linear increase in product formation) is maintained during the recorded reaction time (10 and 15 min, respectively) - due to the extraordinary situation that in the course of the investigated enzyme reaction the concentration of substrate remains constant. The prolonged linearity allows for the reliable

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determination of the steady state rate of product formation (v_0), which is the required rate for deriving the kinetic parameters V_{max} and K_m according to the Michaelis Menten equation, by taking into account the slope of a later segment of the product formation curve (t > 5 min) and omitting the initial part, which is affected by the influence of BSA.

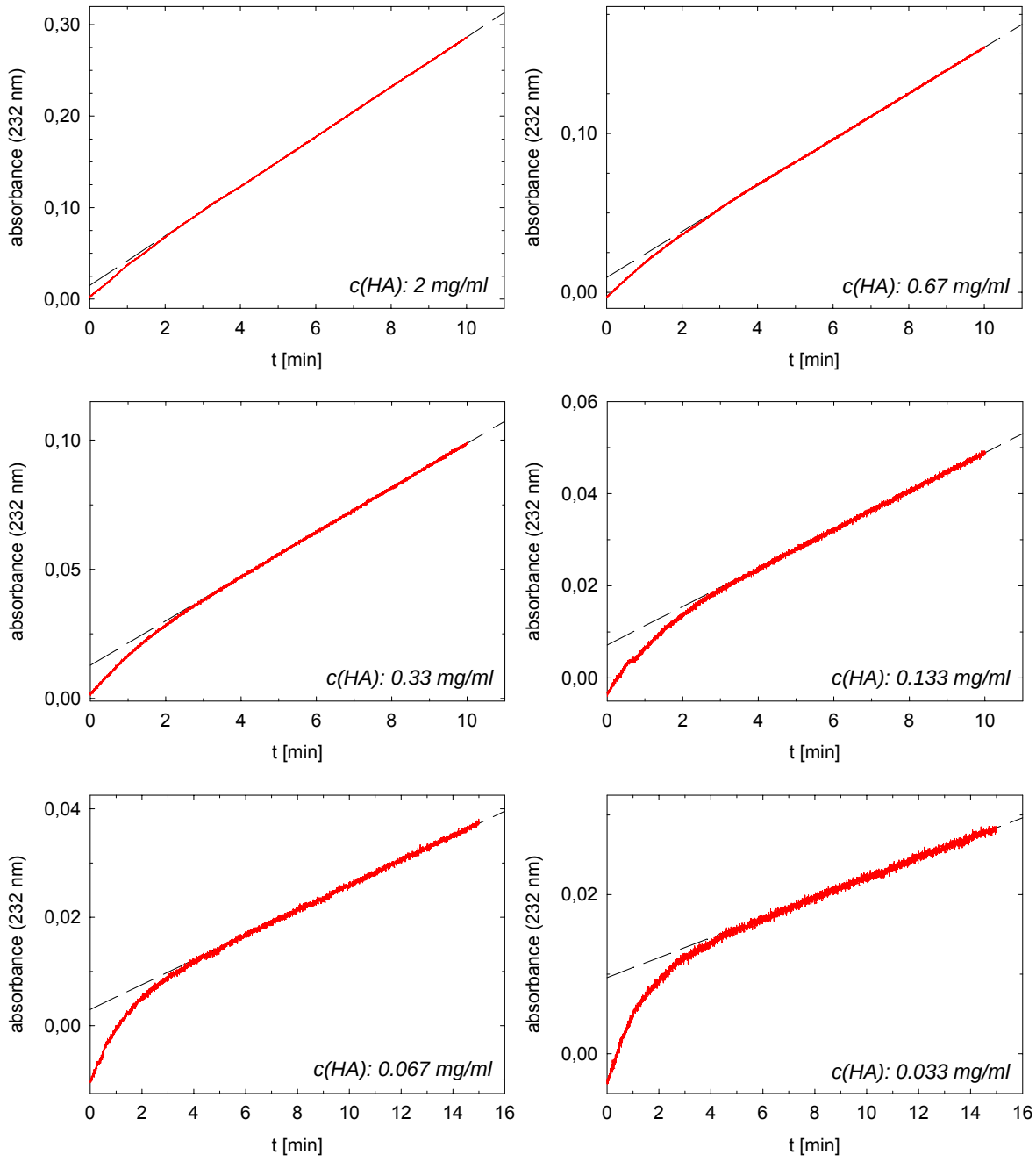

Fig. 7-6: Degradation of HA by *S. agalactiae* hyaluronate lyase, monitored at 232 nm (—). 100 µl of enzyme solution (2.5 units[#], dissolved in BSA (solution 1)) were incubated with 800 µl of incubation mixture 7-IV (pH 5.0), containing various concentrations of HA. The dashed lines indicate the slope of the linear part of the respective absorbance curve.

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7.3.2.3 Estimation of V_{max} and K_m

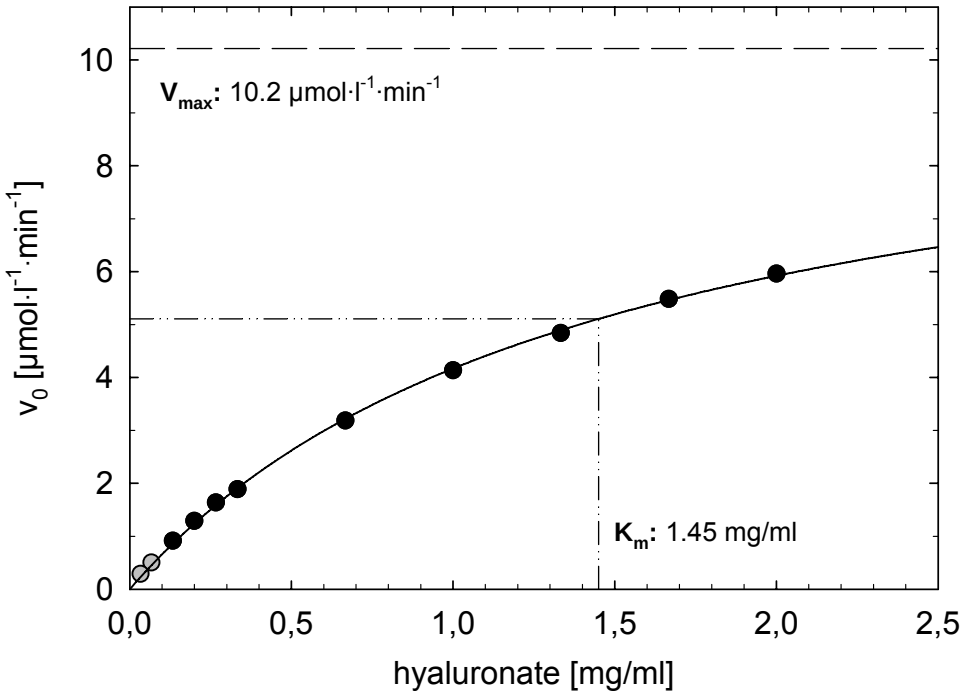

Fig. 7-7: Activity of *S. agalactiae* hyaluronate lyase as a function of the concentration of HA (at pH 5.0). The rate of product formation was determined photometrically at 232 nm as indicated in figure 7-6. The data (●) were fitted to the Michaelis Menten equation by nonlinear regression ($R^2 = 0.9993$). The data for c(HA) < 0.1 mg/ml (●) were omitted from the fit due to a molar ratio of substrate to enzyme < 1000.

To characterise the degradation of HA by *S. agalactiae* hyaluronate lyase with respect to V_{max} and K_m the kinetic parameters were estimated according to the Michaelis Menten equation (eq. 7-2) from a series of steady state reaction rates (v_0) measured at various substrate concentrations as described in the preceding section.

$$v_0 = \frac{V_{max} \cdot [S]}{K_m + [S]} \qquad \text{(eq. 7-2)}$$

In figure 7-7 v_0 is plotted as a function of the substrate concentration. Fitting the data according to the Michaelis Menten equation by a nonlinear regression program (SigmaPlot) yielded values of 10.2 µmol · l^{-1} · min^{-1} for V_{max} and 1.45 mg/ml for K_m, respectively. The turnover number k_{cat} was calculated according to eq. 7-36 yielding a value of $1.1 \cdot 10^5$ min^{-1}. To ensure that the concentration of enzyme bound substrate [ES] can be disregarded compared to the concentration of free substrate [S], the data obtained with HA concentrations

< 0.1 mg/ml (molar ratio of substrate to enzyme < 1000) were excluded from the fitting process.

The double reciprocal plot of the data according to equation 7-42 (obtained from eq. 7-2 by taking reciprocals of both sides) is presented in figure 7-8.

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} \qquad \text{(eq. 7-42)}$$

As expected for a reaction that obeys Michaelis Menten kinetics the datapoints lie on a straight line with intercepts $1/V_{max}$ on the $1/v_0$ axis and $-1/K_m$ on the 1/[S] axis.

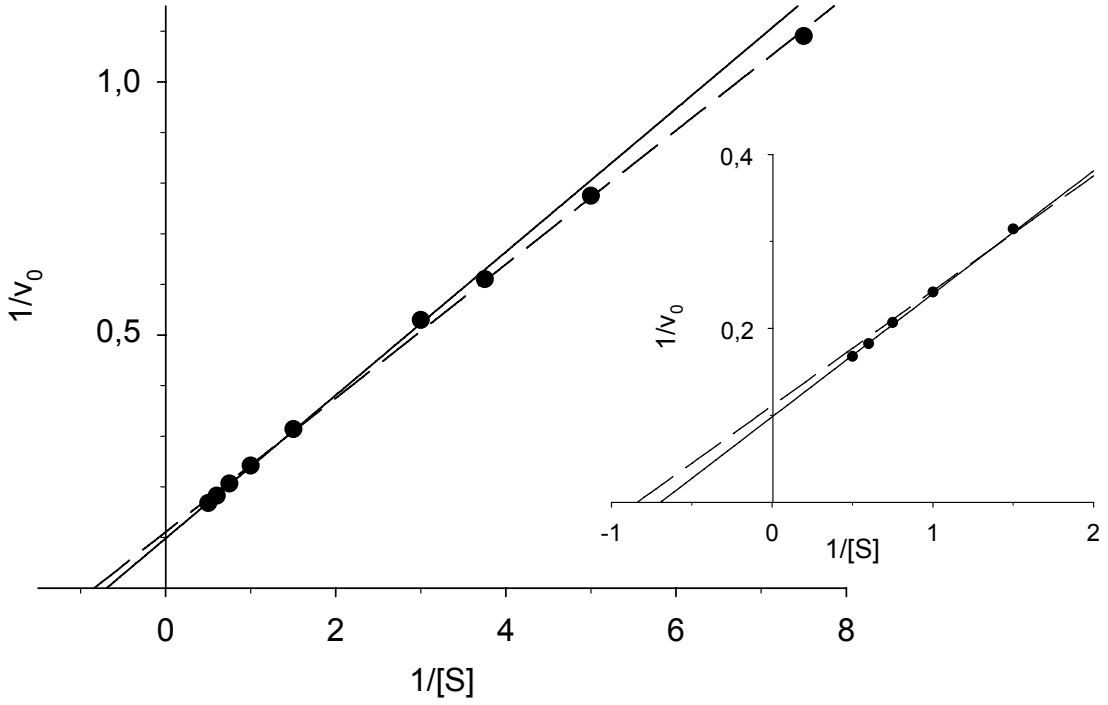

Fig. 7-8: Double reciprocal (Lineweaver-Burk) plot of the data presented in figure 7-7 (●). The data were fitted to the reciprocal form of the Michaelis Menten equation (eq. 7-42) by unweighted linear regression (dashed line) and by weighted linear regression (solid line) using the weighting factor $w(1/v_0) = v_0^4$ as recommended by Cornish-Bowden (1995b, chapter 2). *Inset:* magnified view of the region of high substrate concentrations in figure 7-8. [S]: hyaluronate in mg/ml; v_0 in $\mu mol \cdot l^{-1} \cdot min^{-1}$;

Fitting the data to eq. 7-42 by weighted linear regression using the weighting factor $w(1/v_0) = v_0^4$ as recommended by Cornish-Bowden (1995b, chapter 2) yielded values of 10.2 $\mu mol \cdot l^{-1} \cdot min^{-1}$ for V_{max} and 1.44 mg/ml for K_m, respectively, which are in very good agreement with the results estimated from the hyperbolic curve in figure 7-7. The line obtained by the weighted linear regression (Fig. 7-8, solid line) appears to the eye to fit rather

badly compared to the line obtained by unweighted linear regression (Fig. 7-8, dashed line). However, as discussed in detail by Cornish-Bowden (1995a; 1995b), the use of suitable weights provides results, which are more reliable compared to those obtained by unweighted linear regression, as it takes into account that for small values of v_0 small errors in v_0 lead to enormous errors in $1/v_0$, whereas for large values of v_0 the same small errors in v_0 lead to barely noticeable errors in $1/v_0$. Indeed, the values for V_{max} (9.0 µmol · l^{-1} · min^{-1}) and K_m (1.19 mg/ml), which were estimated from the unweighted regression line, were found to deviate from the results of the hyperbolic plot (Fig. 7-7).

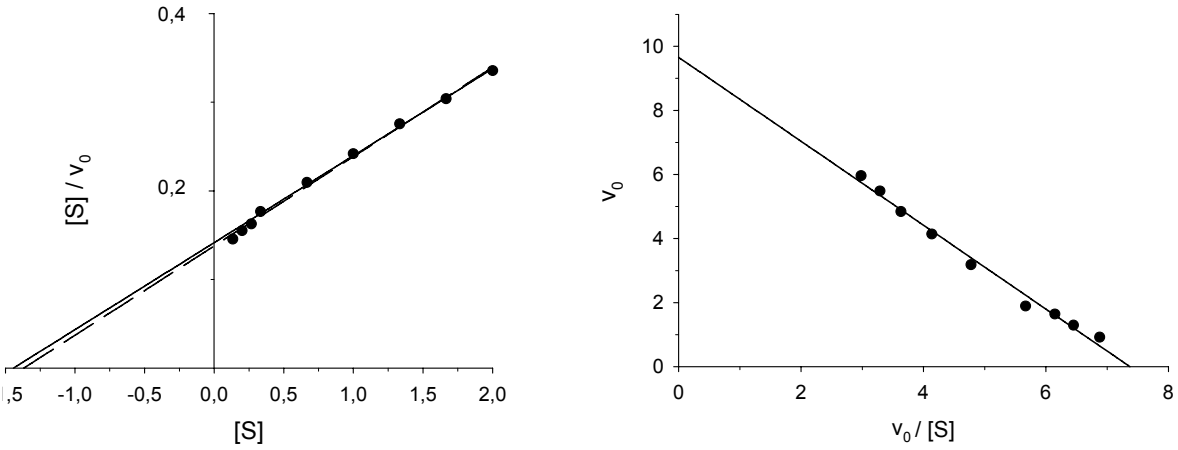

Fig. 7-9: *Left:* plot of the data presented in figure 7-7 (●) according to eq. 7-43 (Hanes plot). The data were fitted to eq. 7-43 by unweighted linear regression (dashed line) and by weighted linear regression (solid line) using the weighting factor $w([S]/v_0) = v_0^4/[S]^2$ as recommended by Cornish-Bowden (1995b, chapter 2). *Right:* plot of the data presented in figure 7-7 (●) according to eq. 7-44 (Eadie-Hofstee plot), fitted by linear regression (unweighted).

Figure 7-9 shows two additional straight line plots of enzyme kinetic data according to the Michaelis Menten equation. In the Hanes plot $[S]/v_0$ is plotted versus [S] according to eq. 7-43 (obtained from eq. 7-42 by multiplying both sides by [S]) yielding a straight line with slope $1/V_{max}$ and intercept $-K_m$ on the [S] axis.

$$\frac{[S]}{v_0} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \cdot [S] \qquad \text{(eq. 7-43)}$$

As done in case of the Lineweaver Burk plot the datapoints of the Hanes plot were fitted to the underlying equation (eq. 7-43) according to the recommendations of Cornish-Bowden, i.e. by weighted linear regression using the weighting factor $w([S]/v_0) = v_0^4/[S]^2$ (Cornish-Bowden, 1995b, chapter 2). The obtained values for V_{max} (10.2 µmol · min^{-1} · mg^{-1}) and K_m (1.44 mg/ml) were identical to those estimated from the double reciprocal plot. The

observation that in case of the Hanes plot there is only a minor deviation between the weighted (solid line) and the unweighted regression line (dashed line) shows that the errors in the quotient $[S]/v_0$ provide a faithful reflection of those in v_0 (Cornish-Bowden, 1995a). In this respect the Hanes plot provides an advantage over the double reciprocal plot, which, as aforementioned, gives a misleading impression of the experimental error. The kinetic parameters estimated from the unweighted regression line were $V_{max} = 9.9\ \mu mol \cdot l^{-1} \cdot min^{-1}$ and $K_m = 1.36$ mg/ml, respectively.

In the third straight line plot, the Eadie-Hofstee plot, v_0 is plotted versus $v_0/[S]$ according to eq. 7-44 (obtained from eq. 7-42 by multiplying both sides by v_0V_{max} and rearranging) yielding a straight line with slope $-K_m$ and intercept V_{max} on the v_0 axis.

$$v_0 = V_{max} - K_m \cdot \frac{v_0}{[S]} \qquad \text{(eq. 7-44)}$$

Compared to the other straight line plots (in particular the double reciprocal plot) in the Eadie-Hofstee plot deviations of the datapoints from the straight line become more obvious, especially if there are systematic deviations. It is therefore a useful tool for the detection of potential deviations from Michaelis Menten behaviour (Cornish-Bowden, 1995a).The finding that in the Eadie-Hofstee plot of our experimental data (Fig. 7-9, right plot) no remarkable deviation from the straight line is observed supports the validity of the theoretically derived assumption that the investigated hyaluronate degradation by *S. agalactiae* hyaluronate lyase obeys Michaelis Menten kinetics.The Eadie-Hofstee plot also clearly shows that the highest v_0 determined in our kinetic experiments is much lower than V_{max}, which indicates that additional experiments covering a higher range of substrate concentrations should be performed. However, in our case the increase in the substrate concentration was limited to 2 mg/ml by the high viscosity of hyaluronate solutions.

Our results are not in agreement with those reported by Rodig et al. (2000), who also investigated the degradation of HA by a purified *S. agalactiae* hyaluronate lyase and determined a K_m value of $8 \cdot 10^{-4}$ mg/ml, i.e. a value by a factor of about 1800 lower compared to the value estimated by us. In addition, Rodig and coworkers observed decreasing initial velocities with increasing concentrations of substrate (substrate inhibition) at concentrations of HA exceeding 0.1 mg/ml, whereas in the course of our investigations no deviation from Michaelis Menten kinetics was observed up to HA concentration of 2 mg/ml. These discrepancies are surprising, particularly, since in both cases HA from *S.*

zooepidemicus, obtained from Aqua Biochem GmbH, was used as substrate. However, the enzyme concentration used by Rodig et al. was by a factor of 167 higher compared to the concentration applied in our experiments, as far as can be seen from the experimental part of their publication. Therefore, it is questionable if their experimental data allow for a reliable estimation of K_m, since the precondition that substrate is present in much higher concentration compared to the enzyme is not warranted. Other factors that might account for the discrepancy in the determined K_m values are the differences in the incubation conditions used by Rodig et al. (0.1 M NaOAc, pH 6) and in our experiments (citrate-phosphate buffer, pH 5, containing NaCl and BSA). However, it seems unlikely that the 1800 times lower K_m determined by Rodig et al. can be ascribed to the effect of pH, considering that in the course of our experiments an increase in K_m was observed, when increasing the pH from pH 5 to pH 7.4 under otherwise identical conditions (see below).

In the course of the characterisation of inhibitors of *S. agalactiae* hyaluronate lyase with respect to their inhibitory potency at different pH it turned out that in many cases lower IC_{50} values were obtained at physiological pH (pH 7.4) compared to pH 5, where the enzyme showed maximum activity (Salmen, 2003). To investigate if the observed pH dependency of the inhibitory potency can be ascribed to the effect pH on the affinity of the substrate to the enzyme, the attempt was made to determine K_m also at pH 7.4.

By analogy with the method applied for the determination of V_{max} and K_m at pH 5 the rate of product formation v_0 was measured photometrically at 232 nm at various concentrations of substrate. However, due to the lower activity of *S. agalactiae* hyaluronate lyase at pH 7.4 the enzyme concentration in the incubation mixture was increased to obtain a readily measurable increase in absorbance at 232 nm within a reaction time of 10 min. The results of this first approach to determine the kinetic parameters at physiological pH are presented in figure 7-10. As described in the context with the results of pH 5 (Fig. 7-7) the data obtained with HA concentrations providing a molar ratio of substrate to enzyme > 1000 were fitted to the Michaelis Menten equation (eq. 7-2) by a nonlinear regression program (SigmaPlot).

The obtained values for V_{max} and K_m were 18.7 $\mu mol \cdot l^{-1} \cdot min^{-1}$ and 10.5 mg/ml, respectively. However, figure 7-10 clearly demonstrates that the existing dataset, which contains only four datapoints within the relevant range of substrate concentrations, does not allow for a reliable estimation of V_{max} and K_m. Due to the increased concentration of enzyme the lower limit of

the substrate concentrations yielding a molar ratio of substrate to enzyme > 1000 was 0.2 mg/ml, resulting in a limited range of substrate concentrations differing only by a factor of 10 (0.2 – 2mg/ml). Thus it seems questionable if the simple repetition of the experiment using additional substrate concentrations within the relevant range would allow for a reliable determination of the kinetic parameters. The results are expected to be unsatisfactory, particularly, since the data presented in Fig. 7-10 already indicate that the applicable substrate concentrations cover only the steep increasing initial part of the hyperbolic curve.

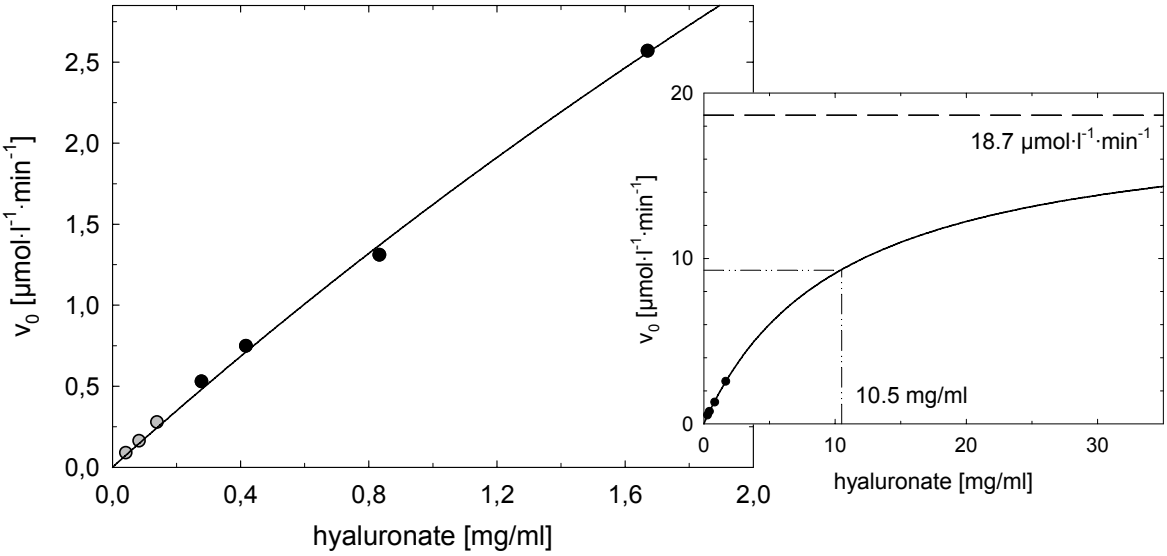

Fig. 7-10: Activity of *S. agalactiae* hyaluronate lyase as a function of the concentration of HA (at pH 7.4). The rate of product formation was determined photometrically at 232 nm as indicated in figure 7-6, using 5 units[#] of enzyme and incubation mixture 7-IV (pH 7.4). The data (●) were fitted to the Michaelis Menten equation by nonlinear regression ($R^2 = 0.9970$). The data for c(HA) < 0.2 mg/ml (●) were omitted from the fit due to a molar ratio of substrate to enzyme < 1000. *Inset:* extended view of the fitted hyperbola of figure 7-10.

The results of the first approach to determine K_m at pH 7.4 thus showed that further efforts must be made to obtain data, which allow for a reliable estimation. The problem with such experiments is the fact that the range of substrate concentrations is limited due to the high viscosity of hyaluronate solutions at concentrations exceeding 2 mg/ml. However, although an exact determination of K_m at pH 7.4 was impossible, the present data indicate that the K_m is higher at pH 7.4 compared to the value obtained at pH 5.0 (1.44 mg/ml), suggesting that the higher activity of inhibitors at pH 7.4 may be explained by the lower affinity of the enzyme to the substrate at physiological pH.

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7.4 Conclusion

The mixtures of products resulting from partial and exhaustive digestion of HA by hyaluronate lyase from *S. agalactiae* were analysed by anion exchange HPLC and NMR spectroscopy. Showing the unsaturated disaccharide as the only product after exhaustive digestion and as the main product after partial digestion, the mixtures proved to be in agreement with the mechanism reported in the literature for the degradation of HA by hyaluronate lyase from *S. agalactiae*. Theoretical analysis of this mechanism, involving an initial endolytic cleavage followed by rapid and processive release of unsaturated disaccharide, revealed that the kinetics of HA degradation by *S. agalactiae* hyaluronate lyase can be described according to the Michaelis Menten equation.

At the optimum pH for *S. agalactiae* hyaluronate lyase (pH 5.0) estimation of the kinetic parameters according to the Michaelis Menten formalism from a series of steady state reaction rates, measured at various substrate concentrations, yielded a K_m value of 1.45 mg/ml and a turnover number (k_{cat}) of $1.1 \cdot 10^5$ min^{-1}. In addition, no deviation from Michaelis Menten behaviour was observed when plotting the experimental data in a Lineweaver-Burk, Hanes and Eadie-Hofstee diagram.

The determination of K_m at physiological pH turned out to be problematic, since it would have required a further elevation of the substrate concentration, which was impractical due to the high viscosity of high molecular mass HA at concentrations exceeding 2 mg/ml. However, the data obtained at substrate concentrations ≤ 2 mg/ml already indicated a lower affinity of the enzyme to the substrate at pH 7.4 compared to pH 5.0, which might explain the higher activity of inhibitors of the enzyme at pH 7.4 compared to pH 5.0, as it was observed by Salmen (2003).

For the investigations presented in this chapter high molecular mass HA from *S. zooepidemicus* was used as substrate. In addition to the high viscosity, this substrate implies the problem of being polydisperse, which makes an exact determination of K_m impossible. At present, efforts are made to prepare low molecular mass HA of defined chain length (hyaluronate hexasaccharide), which may be used as substrate for the exact determination of K_m in continuative studies.

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8 Summary

Pharmaceutical preparations of bovine testicular hyaluronidase (BTH) have been therapeutically applied in the fields of orthopaedia, ophthalmology and internal medicine for many years (Baumgartner and Moritz, 1988; Menzel and Farr, 1998). In the context with the risk of BSE, companies stopped the production of BTH preparations resulting in a shortage of this drug, which is the only highly effective antidote in the treatment of extravasations of vinca alkaloids. In this case hyaluronidase prevents local tissue necrosis by improving the absorption of the paravasate (Bertelli et al., 1994; Albanell and Baselga, 2000).

To find a substitute for the poorly characterised BTH preparations, in the first part of this thesis two preparations of bovine testicular hyaluronidase (Neopermease®; Hylase® "Dessau") and a hyaluronate lyase from *Streptococcus agalactiae* were compared with respect to purity (composition) and enzyme activity. As revealed by SDS-PAGE and size exclusion chromatography the BTH preparations were complex mixtures of proteins with enzymatic activity residing in different fractions. Although identical enzymatically active proteins were not detected, Neopermease® and Hylase® "Dessau" proved to be hyaluronidase preparations with nearly the same enzymatic properties. In the Morgan-Elson assay both preparations were characterised by almost identical though complex pH profiles showing activity over a broad range (pH 3 - 8) with maximum activity at pH 3.6. For Neopermease® it was shown that the complex effect of pH on the activity of the whole preparation can be explained as the sum of the pH profiles of several proteins, differing in specific activity. The bacterial enzyme preparation was separated into two main proteins [100 kDa (pI = 8.9) and 85 kDa (pI = 9.2)], which were enzymatically active in SDS substrate-PAGE. By measuring product formation both in the Morgan-Elson assay and by UV difference spectroscopy enzyme activity of hyaluronate lyase from *S. agalactiae* was detected in the range from pH 4.5 to pH 8, showing a maximum at pH 5.0. When comparing the preparations with respect to their specific activities, the bacterial hyaluronidase preparation was 410-times more active than Neopermease® and 5100-times more active than Hylase® "Dessau", respectively. These results suggested that hyaluronate lyase from *S. agalactiae*, as it was found to meet the requirements for a pharmaceutical preparation with respect to purity and specific activity, might be an alternative for BTH.

Therefore it was investigated if the bacterial enzyme may serve as an appropriate substitute for BTH as a spreading factor and activity enhancer in cancer chemotherapy in preclinical in vitro and in vivo studies. In contrast to the combination therapy with BTH (Spruß et al., 1995; Muckenschnabel et al., 1996; Gürtler, 2001), the co-administration of hyaluronate lyase from *S. agalactiae* did neither enhance the efficacy of regional chemotherapy of human malignant melanoma, implanted in nude mice, nor improve the intratumoral accumulation of peritumorally (s.c.) injected chemotherapeutics. The bacterial enzyme was administered at concentrations yielding in the Morgan-Elson assay 150 to 300 times higher activiy compared to the activity applied in the corresponding experiments with BTH (Neopermease®). It was assumed that the apparent discrepancy between the activities determined in the colorimetric assay, which detects product formation (release of reducing N-acetylglucosamine (NAG)), and the observed spreading effect, which depends on viscosity reduction of the extracellular matrix, may be explained by the different mechanisms of HA degradation by the bacterial and the bovine enzyme: while in case of the endolytic action of BTH every cleavage step (release of reducing NAG) may be conducive to viscosity reduction, the processive release of disaccharides catalysed by the bacterial enzyme is supposed to reduce the viscosity rather ineffectively, regardless of the higher rate of product formation.

To test this hypothesis, in subsequent studies Neopermease® and hyaluronate lyase from *S. agalactiae* were compared with respect to their activity in a turbidimetric and viscosimetric assay. According to Humphrey and Jaques (1953) the results obtained in these assays, which are physicochemical methods quantifying the activity of hyaluronidase according to the reduction of the size of high molecular mass HA, are supposed to be well correlated with the pharmacological activity of the enzymes when applied as a spreading factor. Indeed, it turned out that in the turbidimetric and the viscosimetric assay the activity of the bacterial enzyme was relatively low compared to its high activity in the colorimetric assay, which may partly explain the observation that the hyaluronate lyase did not show the desired spreading effect when applied to enhance the intratumoral accumulation of melphalan. However, this explanation is compromised by the fact that the activity administered in the in vivo experiments with the bacterial hyaluronate lyase was, compared to the activity applied in the corresponding experiments with Neopermease®, still by a factor of 19.3 higher, when extrapolated from the results of the turbidimetric and viscosimetric assay.

The turbidimetrically determined pH activity profile of hyaluronate lyase from *S. agalactiae* showed a maximum at pH 5.0 and was in good agreement with the results of the colorimetric

assay. By contrast, in case of BTH the pH profile, obtained in the turbidimetric assay showing maximum activity at pH 6.0, was almost inverted compared to the pH profile obtained by the colorimetric method (activity optimum at pH 3.6). Comparison of the isolated enzymatically active proteins contained in Neopermease® with respect to their activity in both assays revealed that the main 58 kDa fraction showed the highest specific activity in both assays and gave the inverse profiles typical for the whole preparation. In addition, the turbidimetrically obtained pH profile of Neopermease® was confirmed by viscosimetry.

Anion exchange HPLC analysis of the oligosaccharide mixtures produced by HA digestion with Neopermease® at various pH values suggests that the pH optima of BTH in the different assays may be explained as follows: after an incubation time of 1 h (incubation time used for the determination of the pH activity profiles) HA degradation at pH 3.6 yielded a considerably higher amount of short oligosaccharides than incubation at pH 6.0, which explains the pH profile of the colorimetric assay. Although at pH 6.0 the formation of short oligosaccharides was relatively low after 1 h of incubation, anion exchange HPLC analysis indicates that HA is degraded to HA fragments, which are not precipitated in the turbidimetric assay. Assuming that at pH 3.6 oligosaccharides are preferentially generated in the presence of high molecular mass HA, whereas at pH 6.0 all types of molecules of the polydisperse substrate are processed to the same extent, the higher activity detected in the turbidimetric and the viscosimetric assay at pH 6.0 can be explained regardless of the higher rate of NAG formation detected in the colorimetric assay at pH 3.6.

Since in the present work HA chains with molecular masses > 10 kDa were not detected by anion exchange HPLC as they were removed from the digestion mixtures by ultrafiltration prior to analysis it was not proven that at pH 3.6 oligosaccharides were formed in the presence of high molecular mass HA. To confirm the hypothesis that BTH shows different affinities to high and low molecular mass HA, depending on pH, additional investigations are required. Size exclusion chromatography analysis of the digestion mixtures, obtained at various pH as a function of the incubation time, may provide valuable information on the time-dependent changes of the composition of the product mixtures. By this method HA fragments of high molecular mass and undegraded substrate are also taken into account. In addition, it seems worthwhile to investigate in continuative studies if in the colorimetric and the turbidimetric assay the pH profile of BTH varies with incubation time. Viscosimetric measurements revealed that the activity optimum of BTH shifted from pH 5.0 after an incubation time of 8 min to pH 6.0 after an incubation time of 60 min.

The investigations on hyaluronan degradation by hyaluronate lyase from *S. agalactiae* with respect to the effect of pH on the kinetic parameters V_{max} and K_m suggest that the enzyme shows lower affinity to the substrate at pH 7.4 than at pH 5.0. This may explain the higher activity observed for a number of inhibitors of the enzyme at pH 7.4 compared to pH 5.0 (Salmen, 2003). However, an exact determination of K_m as a function of pH has to be done in future experiments using HA of defined chain length as substrate.

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9 Appendix

9.1 List of abbreviations

2D	two-dimensional
△DiHA	2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose
[η]	intrinsic viscosity
η_r	relative viscosity
BSA	bovine serum albumin
BTH	bovine testicular hyaluronidase
BVH	bee venom hyaluronidase
CD	circular dichroism
CE	capillary electrophoresis
CTAB	cetyltrimethylammonium bromide
DHB	2,5-dihydroxybenzoic acid
DQF COSY	double quantum filter correlation spectroscopy
ECM	extracellular matrix
ESI-MS	electrospray ionization mass spectrometry
GAG	glycosaminoglycan
GBS	group B streptococcal
GlcNAc	N-acetyl-D-glucosamine
GlcUA	glucuronic acid
HA	hyaluronic acid
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
i.d.	inner diameter
i.p.	intraperitoneal
I	ionic strength
IC_{50}	concentration of an inhibitor required to give 50 % inhibition of enzyme activity
IEF	isoelectric focussing

IgG	immunoglobulin G
I.S.	internal standard
k_{cat}	catalytic constant; turnover number
K_m	Michaelis constant; concentration of a substrate at which reaction rate is halfmaximal
L-PAM	melphalan, L-Phenylalanine mustard
MALDI-TOF MS	matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
MW	molecular weight
MWCO	molecular weight cutoff
NAG	N-acetyl-D-glucosamine
NMR	nuclear magnetic resonance
pI	isoelectric point
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
ROESY	rotating frame nuclear Overhauser effect spectroscopy
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
TSP	3-(trimethylsilyl)-propionic acid-2,2,3,3-d4 sodium salt
Tris	tris(hydroxymethyl)aminomethane
TRU	turbidity reducing unit
USP	United States Pharmacopeia
UV	ultra violet
v_0	steady state reaction rate
V_{max}	maximum velocity

9.2 List of publications and abstracts

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