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Purification and Some Physical Properties of a Chymotrypsin-Like Protease of the Larva of the Hornet, *Vespa orientalis*

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A chymotrypsin-like endopeptidase has been purified by ion-exchange chromatography, affinity chromatography, and gel filtration. The enzyme preparation is homogeneous in the ultracentrifuge and disc electrophoresis. The enzyme is proved to be free from any other proteolytic activities. The molecular weight of the proteinase as determined with several techniques (ultracentrifugation, gel filtration and electrophoresis without dodecylsulfate) is in the range between 13000—14000; however, by dodecylsulfate gel electrophoresis a molecular weight of 23000 was obtained. The obtained physical constants of hornet chymotrypsin are: sedimentation coefficient $s^{0}_{20,w} = 1.96 \times 10^{-13}$ s, diffusion coefficient $D^{0}_{20,w} = 131$ [im^2 s^{-1}, partial specific volume v ^ 0.737 ml g^{-1}, frictional ratio $f/f_{min} = 104$, and degree of hydration = 0.1 g per g protein.

During our investigation of the evolution of endopeptidases from invertebrates, Sonneborn *et al.* [1] have characterized a proteinase from the midgut of the larva of the hornet, *Vespa orientalis.* This protease is homologous with mammalian chymotrypsin by such criteria as the complete inhibition by Phenylmethylsulphonyl fluoride and iV*-tosyl-L-phenylalanine chloromethane and the cleavage specificity. However, by gel filtration on Sephadex the Molecular weight was found to be 12800. This molecular weight is much lower than that of previously known chymotrypsins from vertebrates and other invertebrates. Therefore the question arises, whether it is a true molecular weight or an artefact which could be result from interactions between Sephadex gel and the protein.

The present paper describes a modified purification method of the hornet chymotrypsin and reports a study of its size and shape.

MATERIALS AND METHODS

Ion-exchange resins, Sephadex G-75 and Sepharose 4B were obtained from Deutsche Pharmacia and Bio-Gel P 100 from Bio-Rad Laboratories.

Enzymes. **Bovine chymotrypsin (EC 3.4.4.5); trypsin (EC 3.4.4.4); carboxypeptidase A (EC 3.4.2.1); lactate dehydrogenase (EC 1.1.1.27); malate dehydrogenase (EC 1.1.1.37); lysozyme (EC 3.2.2.17).**

Cyanogen bromide and phenylbutylamine were products of Fluka. Material for electrophoresis and the proteins used as molecular standards were purchased from Serva, Heidelberg. Substrates and all the other substances were chemicals from E. Merck, Darmstadt.

The lyophilized midguts of the larva were a gift from Dr J. Ishay.

Measurement of Enzymatic Activities

Proteolytic activity was estimated by the casein-digestion method at pH 8.2 and 25 °C as described by Sonneborn *et al.* [1] and expressed in Kunitz units. Chymotrypsin activity was determined photometrically at 405 nm with gluratyl-L-phenylalanine-p-nitroanilide as substrate [2]. The reaction mixture contained 400 fi.1 4 mM substrate in 0.2 M Tris-HCl pH 8.4 and 25 [xl enzyme solution. The reaction was carried out at 25 °C and the liberation of p-nitroanilide was monitored. The millimolar absorption coefficient of p-nitroanilide was assumed to be 9.62 mM^{-1} cm^{-1}.

Trypsin activity was assayed in the same manner using benzoyl-DL-arginine-p-nitroanilide as substrate [3]. Carboxypeptidase A activity was measured with hippuryl-L-phenylalanine as substrate according to the method of Folk and Schirmer [4]. 0.1 ml of the enzyme solution was added to 2.9 ml of the substrate (1 mM hippurylphenylalanine dissolved in 50 mM

Tris-HCl • buffer pH 7.5 containing 0.2 M NaCl) and the rate of hydrolysis was determined at 254 nm in a Zeiss PM Q II.

Lactate dehydrogenase and malate dehydrogenase activities were measured as described by Bergmeyer [5].

The unit of enzyme activity (U) was defined throughout as the amount of enzmye which hydrolyses 1 fimol substrate per min.

Preparation of Phenylbutylamine-Sepharose 4B

The procedure used for CNBr activation of Sepharose and the covalent coupling of phenylbutylamine was carried out as described by Cuatrecasas [6] and Stevens and Landman [7]. 40 ml of settled Sepharose 4B were suspended in 40 ml 0.1 M XaHC0$_3$ buffer, pH 9.0 and the Sepharose was activated with 4 g CNBr at pH 10.5 during 12 min. Phenylbutylamine (4 g) dissolved in 40 ml dimethylformamide was added to the activated Sepharose. Coupling proceeded for 20 h at 6 °C and pH 9.5. The phenylbutylamine-Sepharose was washed extensively with 0.1 M NaHC0$_3$ buffer pH 9.0 containing 30% (v/v) dimethylformamide to dissolve the uncoupled phenylbutylamine. Before use the gel was pretreated with 0.1 M acetic acid pH 3.0 and then with 0.02 M sodium phosphate buffer pH 7.8.

Analytical Gel Filtration

Molecular weight and Stokes' radius were determined by the gel filtration method using Sephadex G-75 or Bio-Gel P 100 [8,9]. The columns (1.5 x 100cm) were equilibrated with 50 mM Tris-HCl buffer pH 7.5 containing 0.2 M NaCl. Elution was performed at a flow rate of 6 ml/h and 0.8 ml fractions were collected. For calibration the following proteins were used: human serum albumin (M_r 69000), bovine serum albumin (67000), ovalbumin (44000), pepsin (35500), chymotrypsin A (24500), trypsin (23400), myoglobin (17 800), ribonuclease (13400), and cytochrome *c* (12400). The distribution coefficient $\#_{a,v}$ was calculated from the formula

$$\#_{a,v} = \frac{v_e - v_o}{v_t - v_o}$$

The volume, F_0, was estimated using dextran blue 2000, and the total volume, V_t, was calculated as described by Erlanson and Borgstrom [10]. The Stokes radii of the standard proteins were taken from [9].

Sucrose Gradient Centrifugation

Marker proteins (cytochrome c, lysozyme, myoglobin, malate dehydrogenase, lactate dehydrogenase) and hornet chymotrypsin were dissolved in 0.02 M sodium phosphate buffer pH 7.2. 0.1 ml (100 (xg) of each sample was layered on the top of a 12-ml linear sucrose gradient (5—20% sucrose in the same buffer). The gradients were centrifuged for 20 h at 40000 rev./min in a WKF-Hitachi centrifuge. 0.3-ml fractions were collected using an Isco gradient fractioner and assayed for cytochrome *c* and myoglobin by measurement of the absorption at 405 nm. The other proteins were identified by their enzymatic activity. The molecular weight was estimated according to the method of Martin and Ames [11].

Ultracentrifugation

Ultracentrifugation analyses were performed with a Spinco model E centrifuge equipped with schlieren optics. An aluminium double-sector cell in an AnD rotor at 68000 rev./min was used for sedimentation velocity measurements. Diffusion studies were carried out in a synthetic boundary cell at 4400 rev./min. All runs were performed at 20 °C. The protein was dissolved in 0.02 M sodium phosphate buffer pH 7.2. The apparent values were converted to $s_{20,w}$ or $D_{20,w}$ respectively in the usual manner [12].

Partial Specific Volume

The partial specific volume, v, was calculated from the density of a 1.1 % solution of hornet chymotrypsin in 0.02 M sodium phosphate buffer pH 7.2. Density measurements were carried out at 20 °C with the digital precision densitometer DMA-02 CE, made by A. Paar KG (Graz, Austria) [13].

Electrophoresis

Dodecylsulfate gel electrophoresis was carried out as described by Laemmli [14]. The samples (1 mg/ml) were denatured at 60 °C for 3 h in a solution containing 3% sodium dodecylsulfate and 5% mercaptoethanol. For electrophoresis 20 μl of this solution was mixed with 5 (il 0.1 % bromphenol blue and the proteins were separated during 4—5 h at a current of 3 mA per tube. The gels were stained with coomassie blue for 30 min and destained in 7.5% acetic acid. Calibration of the gels for molecular weight determination was carried out according to Weber and Osborn [15].

Electrophoresis without dodecylsulfate was carried out at pH 4.3 with the buffer system of Reisfeld *et al* [16].

Amino-acid Analysis

Amino-acid analysis was performed by the method of Spackman *et al.* [17] on the Bio Cal amino-acid analyzer model BC 200. For amino-acid composition determination approx. 1 mg of the protein was hydrolyzed in 6 M HC1 for 20, 40, 80, and 160 h in evacuated sealed tubes at 108 °C. Cysteine was determined as cysteic acid after performic acid oxidation [18]. Tryptophan was estimated after p-toluenesulfonic acid hydrolysis as described by Liu and Chang [19]. Serine and threonine contents were calculated by extraxpolation to zero time.

Absorption Coefficient

Absorption spectra were obtained in a Beckman DM recording spectrometer with samples dissolved in 0.02 M sodium phosphate buffer pH 7.2.

Protein Concentration

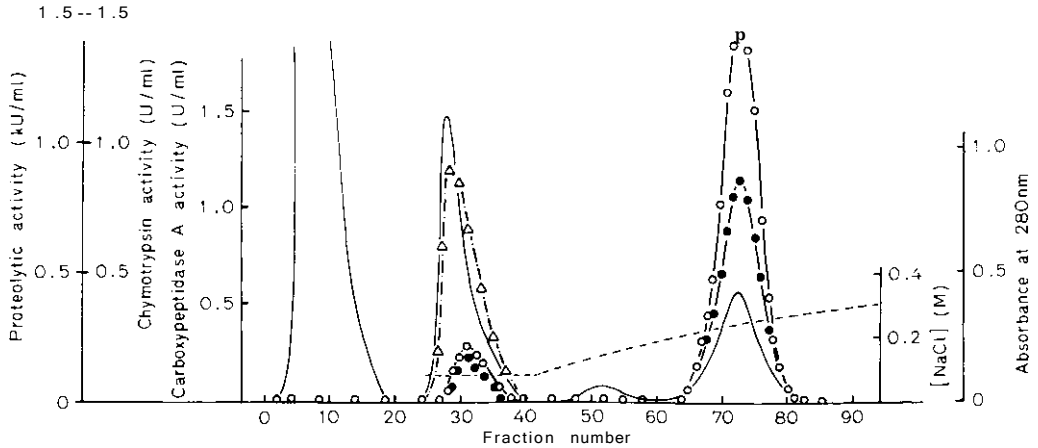
Generally the protein dissolved in buffer was determined by a microbiuret procedure (Hagens, personal communication) or by the Lowry method [20]. The determination of the partial specific volume and the ultracentrifuge experiments, however, were performed with lyophilized protein which were then dried in a drying pistol (60 °C, P_2O_5 under vacuum) to remove all bound water.

RESULTS

Purification Procedure

All operations were carried out at 8 °C. Lyophilized midguts (10 g) were suspended in 50 ml 50 mM Tris-HCl buffer pH 7.8 and stirred mechanically for 12 h. The suspension was centrifuged at 15000 rev./min for 60 min, and the supernatant passed through a column of DEAE-Sephadex A-50 (2 x 30 cm) which had been equilibrated with 50 mM Tris-HCl buffer pH 7.8. Hornet chymotrypsin and carboxypeptidase A were not absorbed to the gel and were therefore eluted without retardation. A considerable of the dark-colored impurities were removed at this step. The fractions (185 ml) containing chymotrypsin and carboxypeptidase A activities were dialysed against McIlvaine buffer [21] pH 6.5 (1:3 diluted) for 14 h changing the buffer several times. The dialysed solution was applied to a CM-Sephadex C-25 column (1.5x50 cm) equilibrated with the same buffer. The column was washed free of unabsorbed protein using the diluted McIlvaine buffer. Subsequently the same buffer but containing 0.075 M NaCl was applied which eluted the whole carboxypeptidase A. The hornet chymotrypsin, however, was eluted using a linear NaCl gradient (0.075—0.3 M NaCl in the same buffer). The enzyme emerged from the column as one peak (Fig. 1). The chymotrypsin solution obtained was applied to a phenylbutylamine-Sepharose 4B column (2 x 15 cm) which had been equilibrated with 0.02 M sodium phosphate buffer pH 7.2. The column was then washed with 300 ml of the same buffer containing 0.8 M NaCl to elute any protein not specifically absorbed. Application of 0.1 M acetic acid pH 3.0 resulted in the elution of the chymotrypsin (Fig. 2). Since the hornet chymotrypsin undergoes denaturation in acid solution the effluent fraction were collected in tubes containing 2 ml of 0.5 M Tris-HCl buffer pH 9.0. The active fractions were brought up to pH 7.8 and solid ammonium sulphate was added under stirring up to 80% saturation. After 6 h the precipitate formed was collected by centrifugation. The pellet was dissolved in 2 ml of 0.1 M bicarbonate buffer pH 7.8 and chromatographed on a column of Sephadex G-75 equilibrated with 0.1 M bicarbonate buffer pH 7.8 (2x95 cm). A chromatographic pattern is shown in Fig. 3. The first inactive protein peak contained the acid-denaturated part of the chymotrypsin. The active chymotrypsin emerged between fraction 27—37 with a constant specific activity of 2.2 U mg^{-1}. The purification procedure is summarized in Table 1. Specific activities increased 20.9-fold toward glutaryl-L-phenylalanme-p-nitroanilide and 17.8-fold toward casein as substrate.

More interesting is the comparison of the specific activities of bovine chymotrypsin and hornet



Pig. 1. ***CM-Sephadex C-25 chromatography of hornet chymotrypsin.*** **Flow rate was 42 ml/h and fractions of 10 ml were collected. (•———•) Proteolytic activity (casein as substrate) (O———O) chymotrypsin activity (glutaryl-L-phenylalanine-p-nitroanilide as substrate); (A———A) carboxypeptidase A activity (hippuryl-i/-phenylalanine as substrate); (———) absorption at 280 nm; (———) NaCl gradient**

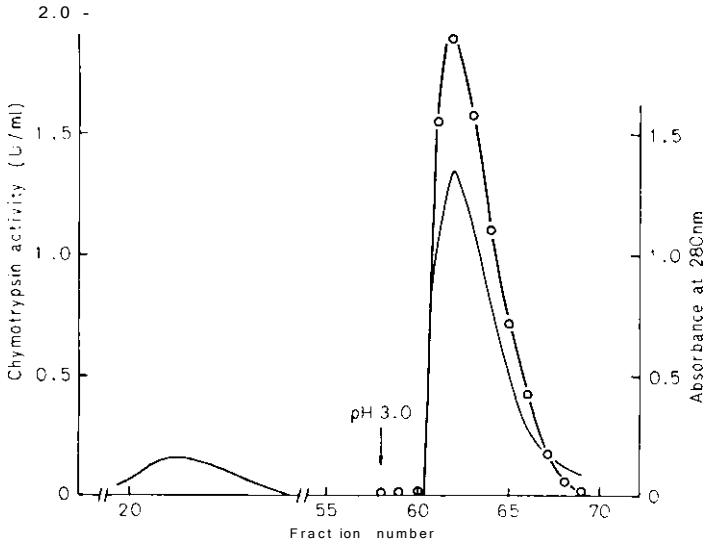


Fig. 2. *Affinity chromatography of hornet chymotrypsin on PBA-Sepharose 4 B.* Flow rate was 35 ml/h and fractions of 7.8 ml were collected. Curves are designated as described in Fig. 1

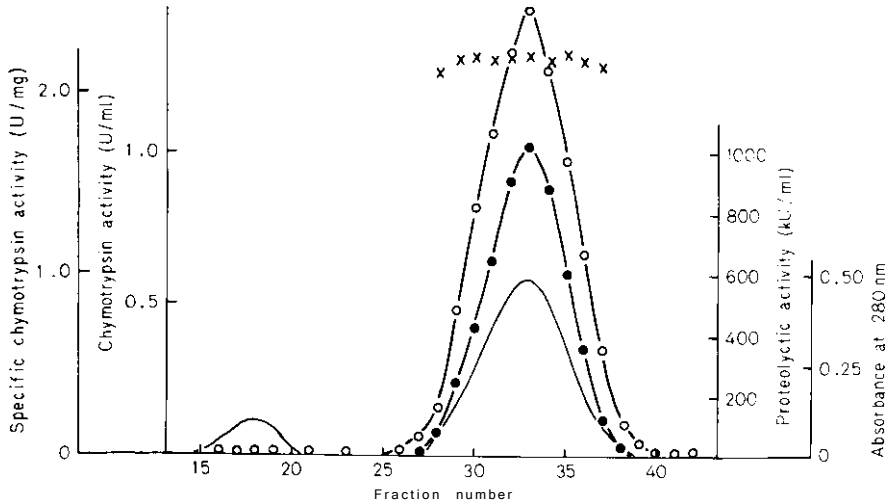


Fig. 3. *Chromatography of hornet chymotrypsi?i on Sephadex G-75.* Flow rate was 35 ml/h and fractions of 9.5 ml were collected. Curves are designated as in Fig. 1. (x X X x) Specific chymotrypsin activity (U. mg-¹)

Table 1. *Purification of chymotrypsin*
Activity was measured using glutaryl-L-phenylalanine-p-nitroanilide as substrate

Step	Total activity	Total protein	Specific activity	Yield	Purification
	U	mg	U mg^{-1}	/o	-fold
Crude extract	109.73	1035	0.106	100	1.0
DEAE-Sephadex A-50	98.86	261	0.380	90	3.6
Dialysis	98.00	257	0.381	90	3.6
CM-Sephadex C-25	85.43	65	1.314	78	12.4
PBA-Sepharose 4 B	60.13	35	1.668	55	15.7
Sephadex G-75	57.12	26	2.219	51	20.9

chymotrypsin. Hornet chymotrypsin hydrolyzes 2.2 (JLMOI glutaryl-L-phenylalanine per mg protein, while bovine chymotrypsin hydrolyzes only 0.074 (xmol mg protein^{-1}. Bovine chymotrypsin hydrolyzes proteins more quickly than hornet chymotrypsin. The specific activities toward casein are 4400 kU for bovine chymotrypsin and 1600 kU for hornet chymotrypsin. However, the cleavage specificity of the B-chain of bovine insulin is almost the same [1],

Homogeneity of Purified Chymotrypsin

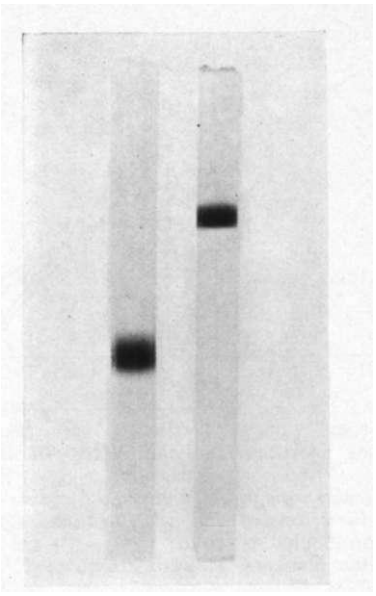
It can be seen in Fig. 3 that the enzyme eluted as a single symmetric peak with constant specific activity suggesting a homogeneous preparation. Finally, the preparation lacks tryptic, carboxypeptidase A or B activities even when large amounts of enzyme were added to the test mixtures for a long incubation time.

Physical evidence for homogeneity was further provided by disc electrophoresis and sedimentation velocity ultracentrifugation. The electrophoretic pattern of the enzyme is shown in Fig. 4. The sample moved as one band in 7.5% and 15% gels at pH 4.3. A single symmetrical peak was obtained by sedimentation velocity ultracentrifugation which indicates a monodisperse system (Fig. 5).

Determination of Molecular Weight

Gel Filtration. Gel filtration was carried out on Sephadex G-75 and Bio Gel P-100. The result is Presented in Fig. 6 and the molecular weight was estimated to be 13000. Using a buffer containing 0-5 M NaCl instead of 0.2 M for the elution resulted in the same molecular weight. This molecular weight is much smaller than that of bovine chymotrypsin (M_r 24500). However, the elution position of a protein during gel filtration is better related to the Stokes radius than to the molecular weight [22,23] and therefore the Stokes radius of hornet chymotrypsin was determined. Plotting $(-\log i f_{s,w})^{1/1}$ ***vs*** Stokes radii of the standard proteins gives a Stokes radius of 1-65 nm. The Stokes' radius was further calculated by the method of Laurent and Killander [9] and a value of 1.61 nm was obtained. In addition, bovine chymotrypsin gives a Stokes radius of 2.12 nm, a value which agrees well with the reported value of 2.09 nm [9].

Dodecylsulfate Gel Electrophoresis. Polyacrylamide gel electrophoresis on 12.5% gels after denaturation of the proteinase with dodecylsulfate results in one sharp band (Fig. 7). Hornet chymotrypsin migrates between bovine chymotrypsinogen and myoglobin in a position corresponding to a molecular weight of 23000. Dodecylsulfate electrophoresis in 4 M urea results in the same molecular weight. This high value could be due to a smaller dodecylsulfate binding capacity in comparison to the standard



A B

Fig.4. *Polyacrylamide-disc electrophoresis of purified hornet chymotrypsin.* (A) **7.5% gel; (B) 15% gel. 80 (jig protein was applied to each column, running pH 4.3. Direction of migration is from anode (top) to cathode (bottom). Pattern were** obtained **by staining in coomassie blue**

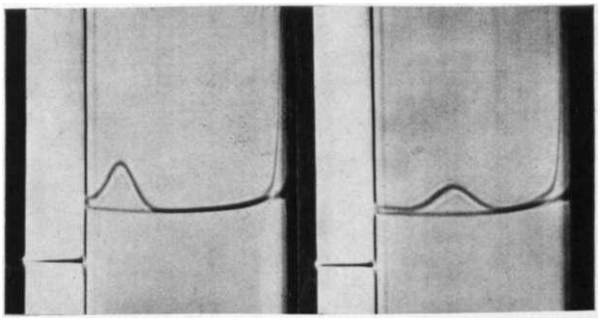


Fig.5. *Sedimentation velocity patterns of hornet chymotrypsin (lOmgjml) in 0.02 M sodium phosphate buffer pH 7.2.* **Pictures were taken after 40 and 72 min maintaining maximum speed of 68000 rev./min. Sedimentation from left to right**

proteins. In this case increasing the acrylamide percentage in the gel would cause the apparent molecular weight to shift to lower values [24]. As shown in Fig. 8, the molecular weight does not decrease with increasing gel concentration. The molecular weight of the protease remains constant.

Electrophoresis at pH 4.3. The molecular weight was also determined electrophoretically according to the method of Hedrick and Smith [25]. The electrophoresis was carried out on gels of increasing acrylamide concentration at pH 4.3. The rate of the change of the protein mobility relative to the tracking dye (R_m) was measured. Since log R_m varies as

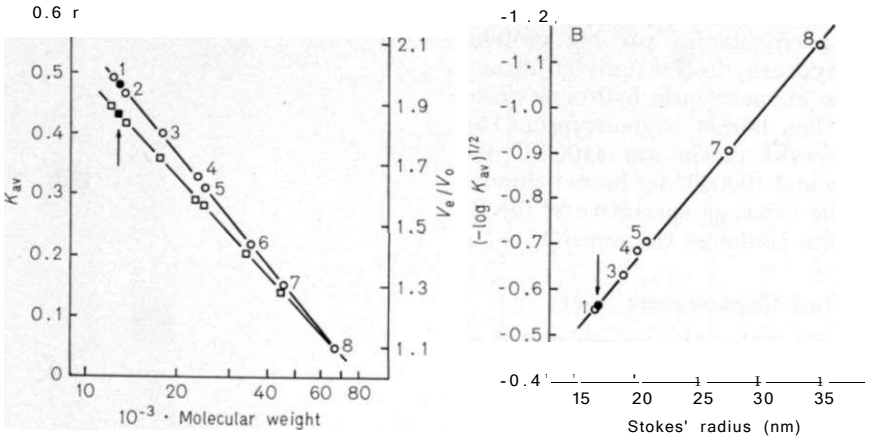


Fig.6. ***Molecular weight (A) and Stokes' radius (B) of hornet chymotrypsin estimated by gel filtration.*** **(O—-—O) Gel filtration on Sephadex G-75; (•———•) gel filtration on Biogel P-100. Markers: 1, cytochrome c; 2, ribonuclease; 3, myoglobin; 4, trypsin; 5, bovine chymotrypsin; 6, pepsin; 7, ovalbumin; 8, bovine serum albumin. The molecul** weight and the Stokes' radius of hornet chymotrypsin arc indicated by the arrows**

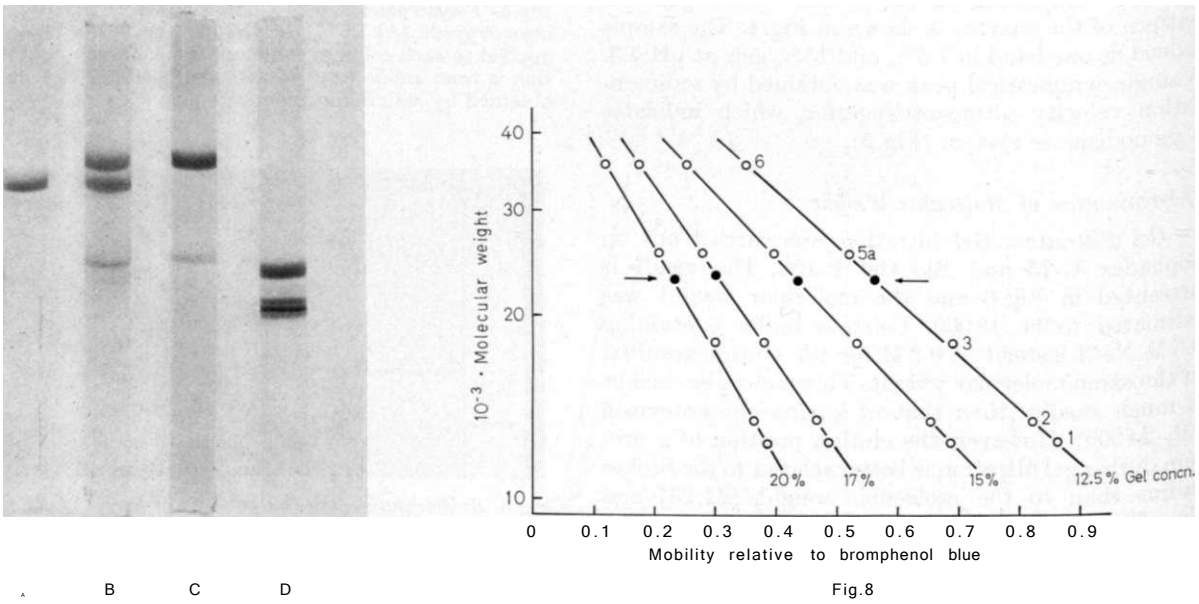


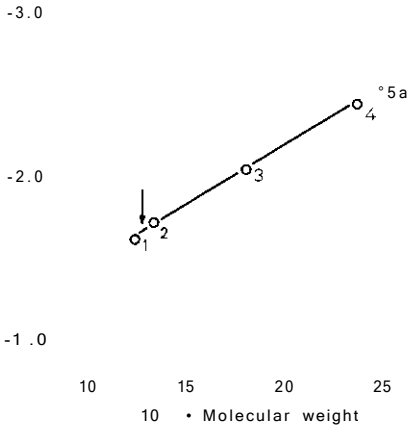
Fig. 7 Fig.8

Fig 7 ***Dodecylsulfate gel electrophoresis of hornet chymotrypsin (A, 25 ug), ^electrophoresis of hornet chymotrypsin & bovine chymotrypsinogen (B, 50 fig), bovine chymotrypsinogen (C, 25 p,g), and bovine chymotrypsin (D, 50 ug)***

Fie 8 ***Molecular weight determination of hornet chymotrypsin dodecylsulfate disc electrophoresis with different acrylamide centaqes.*** **Markers: 1, cytochrome c; 2, ribonuclease; 3, myoglobin; 5a, bovine chymotrypsinogen; 6, pepsin. The positio hornet chymotrypsin is indicated by the arrow. Bromophenol blue served as marker**

a function of acrylamide concentration, a plot of log R_m ***vs*** gel concentration gives a series of points from which the regression line can be calculated. Also a linear relationship exists between the slopes of this line and the molecular weight, which one allows to estimate the molecular weight. The plot of the slopes from the regression lines of proteins of known molecular weights is shown in Fig.9. The molecule weight of hornet chymotrypsin obtained in tin* manner was found to be 12800.

Sucrose-Density Centrifugation. The molecular weight of hornet chymotrypsin was determine* 1 i" be 14200 by sucrose density ultracentrifugation (Table 2).



Molecular weight determination by disc electrophoresis without dodecylsulfate. **Markers are designated as in Fig.6, bovine chymotrypsinogen. The molecular weight of hornet chymotrypsin is indicated by the arrow-**

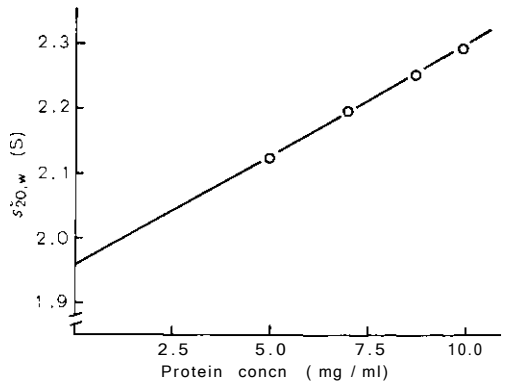


Fig. 10. ***Concentration dependence of s°20,w values of hornet chymotrypsin.*** **For experimental conditions see Methods. *s*°20w = 1.96 S**

Table 2. ***Molecular weight as determined by sucrose density centrifugation***

Reference protein	calculated	
	s20.w	*Mr*
	S	
Cytochrome *c*	2.14	13500
Lysozyme	1.92	14300
Myoglobin	1.63	12000
palate dehydrogenase	1.83	14800
^actate dehydrogenase	1.88	16600
Average	1.88	14200

Sedimentation Coefficient

Sedimentation velocity experiments were performed at concentrations ranging from 0.5—1.0°/₀ solution of hornet chymotrypsin in 0.02 M sodium Phosphate buffer pH 7.2. The sedimentation coefficient increases linearly with increasing protein concentration and extrapolation to zero concentration ieads to a value of $s°_{20w}$ = 1.96 X 10"¹³ s (Fig. 10).

Diffusion Coefficient

All the schlieren patterns obtained in diffusion experiments were symmetrical and the diffusion constants were calculated by the height-area method. As shown in Fig. 11 the apparent diffusion coefficients are slightly concentration-dependent. By extrapolation to infinite dilution a value of $D\%_{ow}$ = 131 (Am² • s⁻¹ was obtained. The diffusion coefficient was also calculated from the Stokes' radius using the Stokes-Einstein equation [26],

$$D = \frac{kT}{6\pi\eta a}$$

where k is the Boltzmann constant, T the absolute temperature, and a the Stokes' radius. Based on a

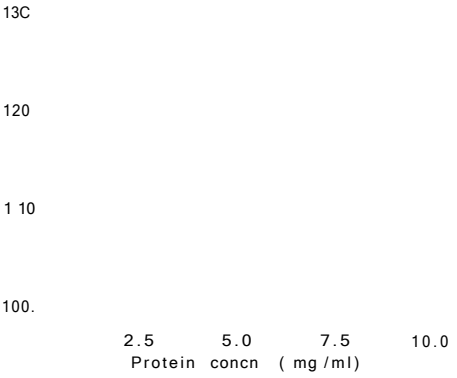


Fig. 11. ***Plot of diffusion constants ($D_{20,w}$) versus protein concentration.*** **For experimental conditions see Methods. 131 (Am² • s⁻¹**

Stokes' radius of 1.62 nm the diffusion coefficient was calculated to be 133 [xm² • s⁻¹. This value agrees well with that from the ultracentrifugation studies.

The partial specific volume of the protease was determined to be 0.737 ml g⁻¹.

The molecular weight calculated from the Svedberg equation using s°₀w = 1.96 S, $D°_{0f}$ = 131 fj.m² s⁻¹, and v = 0.737 ml g"¹ gives a value of 13800.

Shape and Size

The values for $s°_{20,w}$ and $D°_{20,w}$ obtained by ultracentrifugation may be used together with the partial specific volume to calculate the hydrodynamic shape and size of hornet chymotryptin. The frictional ratio, f/f_{min}, [27] ***i.e.*** the ratio of the observed frictional coefficient, f, to the minimal frictional coefficient of the hypothetical unhydrated particle was calculated for the protease to be 1.04, a value typical for globular proteins. However, the ratio of f/f_{min} depends on two factors; the shape and the

Table 3. *Physical properties of hornet chymotrypsin*

Parameter	Value
Sedimentation coefficient $s^{\circ}_{20,w}$	1.96 x 10^{-13} s
Diffusion coefficient $Z)^{\circ}_{a(w}$ *	131 ^xm^{2} s^{-1}
Partial specific volume *v*	0.737 ml g^{-1}
Frictional ratio ///mm	1.04
Axial ratio *(a/b)*	2.1
Stokes radius	1.62 nm
Molecular weight:	
Gel filtration on Sephadex G-75 or Bio-Gel P-100	13000
Electrophoresis without dodecylsulfate	12 800
Ultracentrifugation using *4o.*>* ^So.w and *v* data	13800
Sucrose density	14200
Dodecylsulfate electrophoresis	23000
Absorption maximum	278 nm
Ratio of absorption (280/260 nm)	1.82

degree of hydration of the protein [27]. Assuming the protease is unhydrated and only the axial ratio ***(aft)*** of an ellipsoid (asymmetry of shape) generates this value of ///$_{a}$j$_{c}$> we can calculate a value of ***a/b*** = 2.1 for both prolate and oblate ellipsoids. On the other side, if the shape is spherical and ///min indicates only the degree of hydration, the maximal hydration is found to be 0.1 g per g hornet chymotrypsin. This value is something lower than for other globular proteins.

The physical properties of the hornet chymotrypsin are summarized in Table 3.

Amino-acid Analyses

The results of the amino-acid analyses of hornet chymotrypsin are presented in Table 4. The values reported for valine, leucine, and isoleucine are given by the 80-h and 160-h hydrolysis times, and serine and threonine have been extrapolated to zero hydrolysis time. No methionine was found after hydrolysis with p-toluenesulfonic acid or after performic acid oxidation. Also no glucosamine and galactosamine was detected on the amino-acid analyzer.

The minimal molecular weight calculated from these data is 13984 which agrees with the value obtained by sedimentation analysis. Also listed are the amino-acid composition of some other chymotrypsins.

Absorption Spectrum

Hornet chymotrypsin has a typical protein absorption with a maximum at 278 nm and a minimum at 250 nm. The ratio of the absorbances at 280/260 nm is 1.82. The millimolar absorption coefficient at 280 nm of the hornet chymotrypsin was found to be 12.4 mM^{-1} · cm^{-1} by taking a molecular weight of 13800.

DISCUSSION

The chymotrypsin-like protease from the larva of the hornet, ***Vespa orientalis,*** was isolated by a modified purification method now involving cation-exchange chromatography and affinity chromatography. In the previous method [1] the enzyme preparation was contaminated with carboxypeptidase A. Carboxypeptidase A was now clearly separated from the chymotrypsin-like enzyme by cation-exchange chromatography. The resulting chymotrypsin preparation is proved to be free from any other proteolytic activities. Subsequently affinity chromatography and gel filtration resulted in homogeneous hornet chymotrypsin.

The amino-acid composition shows that hornet chymotrypsin contains no methionine and only four half-cystine residues. This suggests that this chymotrypsin has only two disulfide bridges. In contrast bovine chymotrypsin A contains 10 half-cystine residues, all of which are present as intrachain disulfide bonds [28].

By gel filtration on Sephadex on molecular weight of 13000 was estimated which agrees well with the reported value of Sonneborn ***et al.*** [1]. This molecular weight is surprising, since no chymotrypsin of that size has ever been found. Generally molecular weights of 17000—26000 were reported for invertebrates and vertebrates [28]. This low molecular weight, however, could be only an artefact in a sense of interactions between Sephadex gel and the protein as reported for basic proteins [33], glycoproteins [34] or amylases [35]. Therefore the molecular weight determination was carried out by several independent methods. The values obtained by gel electrophoresis without dodecylsulfate (i// 12800) by ultracentrifugation ***(M,*** 13800, 14200), and by gel filtration on Bio Gel P-100 ***(M,*** 13000) all are in fair agreement within the range of experimental error.

In addition to the observed Stokes' radius obtained by gel filtration on Sephadex G-75 the redetermination of the Stokes radius on Bio Gel P-100 leads to a value of 1.64 nm. The calculation from diffusion coefficient using the Stokes-Einstein equation gives a value of 1.63 nm. All these values are in agreement an and indicate the correctness of the molecular weight determination on Sephadex gel. The increasing of the sedimentation constants with increasing protein concentration suggests an aggregation of hornet chymotrypsin. Other chymotrypsins are know *o associate to polymeric forms as the protein concentration increases [30,36]. The low degree of association observed for hornet chymotrypsin, however, is probably due to the much lower ionic strength used for this experiment. An interesting feature is the anomalous behaviour of hornet chymotrypsin on electrophoresis in dodecylsulfate. This molecular weight determination, a method widely used for polypeptide chains, gives a molecular weight of

Table 4. *Amino-acid composition of hornet chymotrypsin*

Amino acid	Amount*	minm M_r	Residues (nearest integer)	Nearest integer Xminm Mr	Integral Noxifr of residue	Residues molecule chymotrypsin or chymotrypsinogen					
						Bovine A [25]	Porcine C [4]	Human [29]	Fin Whale [31]	Dogfish [30]	*Streptomyces[32]*
	gAOOg		g/13800g								
Aspartic acid	10.89	1047	13.07 (13)	13611	1495	23	22	23	12	20	13.70
Threonine	4.60	2176	6.29 (6)	13056	606	23	14	19	8	14	18.00
Serine	5.80	1516	9.20 (9)	13644	783	28	20	21	11	21	19.00
Glutamic acid	9.37	1365	10.02 (10)	13650	1290	15	21	18	7	13	7.33
Proline	4.18	2298	5.94 (6)	13788	582	9	12	15	8	13	3.59
Glycine	5.36	1073	13.00 (13)	13949	741	23	25	25	12	21	26.48
Alanine	3.05	2351	5.93 (6)	14106	426	22	12	24	11	22	18.50
Half-cystine	2.81	3669	3.81 (4)		408	10	7	8	6	8	2.66
Valine	8.61	1163	12.00 (12)	13956	1188	23	19	22	12	22	11.10
Methionine	0		0 (0)			2	2	2	2	4	0.50
Isoleucine	8.19	1392	10.00 (10)	13920	1130	10	12	11	7	10	7.02
Leucine	8.20	1392	10.05 (10)	13920	1130	19	18	18	10	10	8.76
Tyrosine	4.83	3403	4.08 (4)	13612	652	4	6	3	2	6	6.25
Phenylalanine	3.15	4711	2.85 (3)	14136	441	6	4	7	4	3	4.48
Histidine	3.62	3816	3.65 (4)	15264	548	2	5	4	1	4	2.86
Lysine	7.55	1711	8.15 (8)	13688	1024	14	7	16	6	10	0
Axginine	5.54	2842	4.80 (5)	14210	780	4	7	8	2	7	5.82
Tryptophan	2.49	7388	1.87 (2)	14776	368	8	8	5	5	12	1.70 8
Total	99.05		125	13984	13610*						
Mr						25700	23800	25800	;17000	24500	15700

[a] Average of three determinations.
[b] Molecular weight corrected for 1 molecule of water.

23000 which is twice as high the other values obtained. The estimation of molecular weights on dodecylsulfate gels based on the hypothesis that all proteins bind fairly equal amounts of the detergent and that the complexes formed between protein and detergent adopt the same shapes ***(i.e.*** conformations), such that the electrophoretic mobility is a function of the molecular weight and the pore size of the gel. If a protein does not show such a behavior, the determination of the molecular weight becomes erroneous. Known examples of such proteins include polypeptides with unusual charge [37,38], conformation [39] or with unreduced disulfide bridges [40] and glycoproteins [41]. In the case that the protein binds less detergent, the apparent molecular weight decreases with increasing gel concentration, since the charge of the complex becomes less important to the sieving effect of the gels [24]. This is obviously not the case with hornet chymotrypsin, since the molecular weight remains constant at 23000 in the range of 12.5—20% acrylamide. The ambiguity of this method in the case of hornet chymotrypsin could be that this protein deviates from the general behavior of proteins in dodecylsulfate solution.

During preincubation of the proteins for dodecylsulfate gel electrophoresis carried out as described in Materials and Methods the proteins dissociate into their polypeptide chains and disulfide bridges are reduced. Bovine chymotrypsin consists of three polypeptide chains which are held together by disulfide bridges and therefore on dodecylsulfate gel electrophoresis two protein bands are obtained (Fig. 7). In comparison hornet chymotrypsin gives only a single band. This result suggests that hornet chymotrypsin is a single polypeptide chain. However, further investigations are in progress to substantiate this conclusion and to explain the anomalous behavior of hornet chymotrypsin on dodecylsulfate gel electrophoresis.

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