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The Phenoloxidases of the Ascomycete *Podospora anserina*

VII. Quantitative Changes in the Spectrum of Phenoloxidases during Growth in Submerged Culture *

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Summary. In order to learn the internal conditions for the production of the various phenoloxidases produced by the Ascomycete *Podospora anserina* the wild strain has been grown under controlled conditions in a fermenter for a period of 34 days. Samples were withdrawn at regular intervals and assayed for mycelial yield and intra- and extracellular phenoloxidase production.

Maximal yield was obtained at the following age of the culture: Mycelial production 9 d, tyrosinase 4 d, the high molecular weight laccase I between 9 and 19 d. The low molecular weight laccases II and III, initially present in medium concentrations, dropped to an early minimum after 4 days, followed by an increase with a maximum in the late autolytic phase.

The changes in the phenoloxidase spectrum and the antiparallel production curve for the high molecular weight against the low molecular weight laccases are discussed in relation to the earlier observed genetical and physiological control of phenoloxidase synthesis and in relation to the possibility of laccase I being composed of active subunits of low molecular weight laccases.

As shown in the previous papers of this series[1] the wild strain of *Podospora anserina* produces besides tyrosinase (E.C. 1.10.3.1) different species of laccase (E.C. 1.10.3.2). When the rate of mycelial production is at a maximum, a high molecular weight laccase I is formed in high amounts, while the low molecular weight laccases II and III and the tyrosinase are produced in very low amounts only. This *spectrum of phenoloxidases* can be *affected* in a quantitative manner by alterations of the *genetic information* and of the *environmental conditions*.

The pleiotropic morphological mutant *zonata (z)*, which originated from a point mutation (Lysek and Esser, 1970) exhibits under comparable growth conditions a phenoloxidase spectrum inverse to that of the wild strain: small amounts

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1 References for communications I—VI of this series are given at the beginning of the reference list; they are referred to in the text as "part I, part II" etc.

of laccase I and high amounts of the other phenoloxidases. Comparable alterations in the production of phenoloxidases are also achieved by other morphological mutants not linked to the *z*-locus (part VI, Esser, unpubl.).

By variation of external conditions, e.g. low pH and modification of the carbon source of the nutritive medium, the morphological effect of the zonata mutation is phenocopied in the wild strain (Esser, 1969a; Lysek and Esser, 1971) and the phenoloxidase spectrum (part V) is influenced as well.

The production of phenoloxidases depends in addition to these parameters also on *internal conditions*, e.g. on the age of the mycelium as shown by preliminary experiments. Therefore, it seemed desirable to analyse in a systematic manner the spectrum of phenoloxidases formed in the wild strain during its vegetative growth under defined genetic and environmental conditions. These experiments were necessary not only to establish the conditions required for optimal yield of each of these enzymes but also in connection with other biochemical and genetical problems being under study, e.g. correlation of phenoloxidases with morphogenesis (Esser, 1968), subunit composition of the high molecular weight laccase I (part V). The results reported in this paper show that the *relative proportions of the various phenoloxidases depend strongly on the age of the mycelium.*

Material and Methods

Wild Strain s_{1-}. For details concerning origin, ontogenesis and genetics see Esser (1956b, 1959).

Cultivation of Mycelia. The mycelia were grown in a 300 l fermenter (Chemap AG., Männedorf/Switzerland) containing 200 l of corn meal extract prepared according to Esser (1969b). The pH of the medium was adjusted to 6 after sterilization. The fermenter was inoculated with 18 l of a culture (5 d) containing about 22 g of mycelium (dry weight) grown in 3 carboys in aerated liquid medium (part I). Agitation of the medium by sterilized air (12.3 l air/l medium/h) and temperature (26° C) were kept constant.

Material for Biochemical Assays. At the indicated intervals (see results) samples were taken from the fermenter under sterile conditions. The mycelium was separated from the culture medium by filtration through a double layer of cheese cloth and further treated as described in parts I, II and V. After treatment with protamine-sulfate the supernatant was concentrated by ultrafiltration. The culture filtrate was cleared by centrifugation (20 min, 2,200 × g). Both mycelial extract and culture filtrate were prepared and kept at 4° C.

Analytical Methods. These have been described mostly in the previous parts of this series: Colorimetric determination of enzyme activity and heat inactivation (part I), analytical disc electrophoresis (part III), agar gel electrophoresis and immunological methods (part III, Esser, 1963). Isoelectric focusing in polyacrylamid gel was performed according to Wrigley (1968) and the spectrophotometric determination of protein according to Warburg and Christian (1941). Dry weight was determined by drying mycelial samples in triplicate in a vacuum-oven at 80° C to constant weight.

Substrates. Compounds used for determination of enzyme activity were: DL-3,4-dihydroxyphenylalanine (DOPA) which reacts with both laccase and

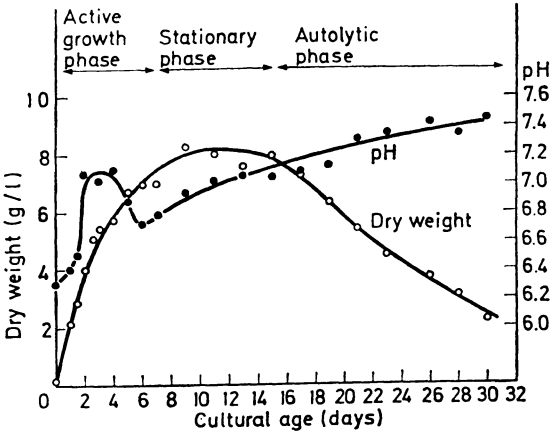

Fig. 1. Mycelial production and pH of the culture medium during growth in the fermenter

tyrosinase; potassium ferrocyanide (PFC) which is specific for laccase; p-cresol was used for qualitative tests; it produces with tyrosinase a red pigment and with laccase a white precipitate consisting of dicresols.

Definition of Enzyme Units. One enzyme unit corresponds to a ΔE of 0.2/min at 436 nm in a light path of 1 cm. Specific activity (SPA) is equivalent to the number of enzyme units per milligram of protein.

Results

In order to establish whether or not there is a correlation between mycelial age and the production of the various phenoloxidases in submerged growth, samples were taken from the fermenter in intervals of one to three days.

I. Mycelial Growth and Total Phenoloxidase Activity

As prerequisite for the examination of the spectrum of phenoloxidases we have first assayed the samples for mycelial yield, pH and total extra- and intra-cellular phenoloxidase activity. These data presented in Figs. 1 and 2 allow the following statements:

1. The *growth curve* can be divided into a phase of *active growth* (day 1—7), a *stationary phase* (day 8—15) and an *autolytic phase* (beginning with day 15).

Mycelial growth was determined by dry weight measurements. A comparison of these values with wet weight determinations (as used for other purposes in part I to VI) showed that the dry matter content of the fungal cells decreases from 26% in the early active growth phase to an almost constant value of about 17% in the

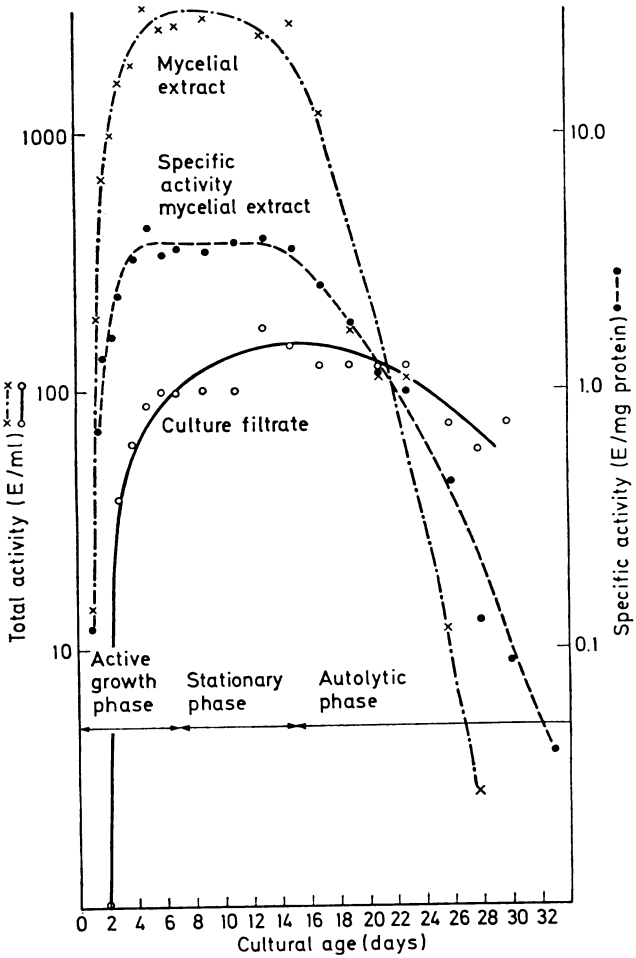

Fig. 2. Activity of phenoloxidases during growth in the fermenter. The values for activity are calculated for the mycelial content of 1 l culture medium and for its culture filtrate

stationary phase, indicating the physiological changes which result in vacuolization of the mycelium.

2. The *pH of the culture medium* exhibits a peak during active growth phase and after a minimum around day 6 increases continuously towards the late autolytic phase.

3. The peak of the *total intracellular phenoloxidase activity*, comprising the tyrosinase and laccase content of the mycelial extract, occurs in the

stationary phase. One may see from the curve for specific activity that during this phase also an optimal enzymatic activity yield is obtained.

Pigmentation of the mycelium as result of the activity of the intracellular phenoloxidases increases with mycelial production and reaches its maximum in the stationary phase.

4. The *total extracellular phenoloxidase activity* present in the culture filtrate is extremely low and reaches only about 5% of the mycelial activity.

This confirms our earlier observation that *Podospora anserina*, in contrast to many other laccase producing fungi (Lyr, 1958; Fåhraeus and Reinhammar, 1967), does not secrete appreciable amounts of phenoloxidases into the medium.

These comparatively stable extracellular laccases appeared later and in much smaller concentrations.

II. Changes of the Phenoloxidase Spectrum during Mycelial Growth

The observed changes of activity of the phenoloxidases can be due to changes in the absolute amount of these enzymes and/or changes in their relative proportion since they exhibit different specific activities (s. Table). It was therefore desirable to follow the production of these enzymes individually. The identification of the various phenoloxidases does not require a fractionation of the mycelial extract. As established in parts I, IV, V and VI, there are sufficient parameters to characterize tyrosinase and the three laccases in one preparation (Table).

However, in using these methods for quantitative purposes one obtains figures with different dimensions, e.g. heat inactivation data may be expressed as enzyme units, whereas the various enzyme bands appearing after disc and agar gel electrophoresis, electrofocusing or after the immunological reaction can only be evaluated according to the intensities of staining. Since the specific activity of the different phenoloxidases is characteristic (Table), staining intensity resulting from the enzymatic activity may also be used to indicate the concentration of the respective enzymes in addition to the information coming from protein staining with amido black after separation of the enzymes by electrophoresis.

In order to condense this paper we have pooled the data of the different assays and expressed the results for enzyme activity in 5 arbitrary units ranging from 0 (no activity) to 4 (maximum activity) for the respective enzyme.

In Fig. 3 we have summarized the data calculated from the different assays. For reason of comparison we have included the curve for specific activity of intracellular phenoloxidases from Fig. 1.

The results shown in Fig. 3 permit the following statements:

1. Heatstable Phenoloxidases. The easiest method to differentiate the high molecular weight laccase I from the low molecular weight laccases II and III and from tyrosinase is to determine the heat stability of their

Table. *Characteristics which are used for identification of the phenoloxidases*[a] *in the mycelial extract*

Parameter	Tyrosinase	Laccase I	Laccase II	Laccase III	References
Molecular weight	42,000–100,000[b]	390,000	70,000	80,000	part IV, V, VI
Specific activity	270	150–180	40	30	part IV, V, VI
Activation by heat (60° C)	yes	no	no	no	part I, IV, V, VI
Half life time of activity at 60° C (min)	60–100	5	60	110	part I, IV, VI
Reaction with DOPA	red pigment	red pigment	red pigment	red pigment	
Reaction with PFC	–	yellow pigment	yellow pigment	yellow pigment	
Reaction with p-cresol	red pigment	white precipitate	white precipitate	white precipitate[c]	
Disc electrophoresis (anodic) R_f-values	several bands between 0.02–0.30[d]	0.10[e]	–	0.33[e]	part III, IV, V, VI
Disc electrophoresis (cathodic) R_f-values, pH 4.3	–	–	0.10–0.20[f]	–	part III, VI
Isoelectric point (Electrofocusing)	–	4.9	7–10[f]	4.0	part V, VI
Agar gel electrophoresis direction of migration	anodic	anodic	cathodic	anodic	part V, VI
Immuno electrophoresis reaction with AE–I[g]	–	+	–	(+)[h]	
AE–II	–	–	+	–	
AE–III	–	(+)[h]	–	+	

[a] Purified from mycelium harvested at the time of maximal mycelial yield; laccase I from wild strain mycelium, tyrosinase, laccase II and laccase III from mutant *z*.

[b] Depending on degree of aggregation.

[c] Only at higher enzyme concentration.

[d] Depending on degree of purification; electrophoresis at pH 7.

[e] Electrophoresis at pH 8.9.

[f] Due to microheterogeneity.

[g] AE = antienzyme.

[h] Weak reaction.

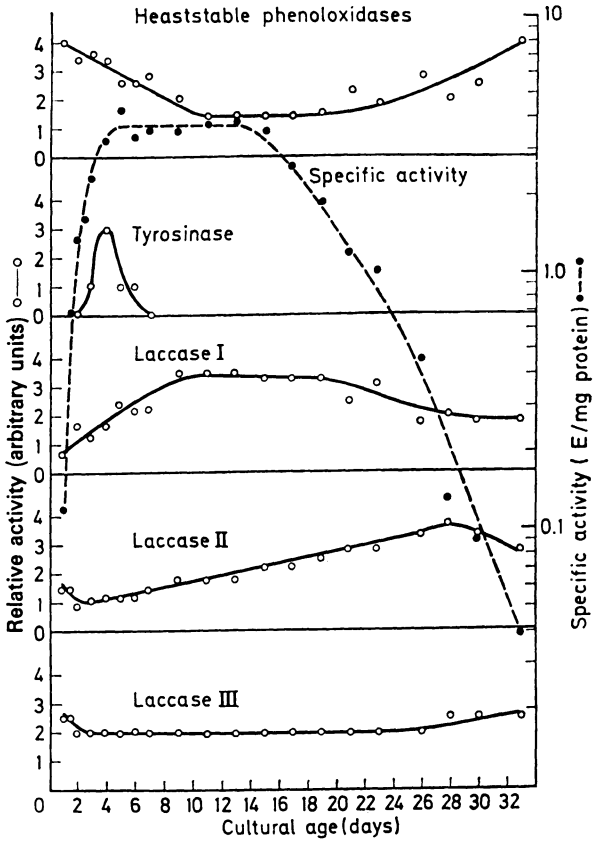

Fig. 3. Changes in the relative activity of the phenoloxidases with age. The curve for specific activity of total phenoloxidases, based on absolute enzyme units, comes from Fig. 1. For definition of arbitrary units see text. The activity curves for the individual phenoloxidases were determined using the following methods: *Heatstable Phenoloxidases:* Heatinactivation curves at 60° C with DOPA and PFC as substrates. *Tyrosinase:* Cresol test before and after incubation at 60° C for 12 min (for presence and activation of tyrosinase). The activity with PFC (specific for laccases) and DOPA (reaction with tyrosinase and laccases) was determined before and after heating at 60° C for 12 and 30 min. (The difference provides an estimate of both tyrosinase and laccase activities.) *Laccase I:* Heatinactivation curve at 60° C, determination of amount of enzyme with half live time of 5 min at 60° C. Disc electrophoresis at pH 8.9 and electrofocusing between pH 3 and 10, staining with DOPA and p-cresol. *Laccase II:* Disc electrophoresis at pH 4.3 (p-cresol) and electrofocusing between pH 3 and 10 (p-cresol); agargel electrophoresis (DOPA, p-cresol), immuno electrophoresis against antienzyme II, protein staining with amido black. *Laccase III:* Disc electrophoresis at pH 8.9 (DOPA) and electrofocusing between pH 3 and 10 (DOPA); immuno electrophoresis against antienzyme III, protein staining with amido black

enzymatic activity. From the Table follows that only tyrosinase is first activated by heat and then inactivated with a comparatively long half life time similar to that of the heatstable laccases II and III. In contrast to that, laccase I is rather heatlabile.

The curve for heatstable phenoloxidases in Fig. 3 shows a decrease of heat stability from the early active growth phase to a minimum in the stationary phase, followed by an increase in the autolytic phase. The low overall heat stability of the phenoloxidases during the stationary phase suggests a high proportion of laccase I. Only a specific determination of the phenoloxidases can show to which of the heatstable enzymes the high percentage of heatstable activity can be attributed.

2. Tyrosinase. This enzyme is produced in detectable amounts only in the active growth phase with a maximum on day 4. Its presence therefore is mainly responsible for the observed high proportion of heatstable phenoloxidases in the early growth phase. The increase in heatstable activity in the autolytic phase must be due then to laccase II and/or laccase III.

3. Laccase I. The activity curve for laccase I starts with low amounts in the early growth phase and shows a continuous increase leading to an activity plateau from day 9 to day 19, followed by a slow decrease. The beginning of maximal laccase I activity coincides with maximal mycelial yield, maximal total intracellular phenoloxidase activity, maximal content of heat labile phenoloxidases and with the second half of the plateau of specific activity.

4. Laccase II. Starting with moderate activity, laccase II decreases to a minimum around day 3, followed by a slow but continuous increase until a maximum in the late autolytic phase. This was supported by determination of laccase II protein concentration from immuno electrophoresis against antienzyme II. The activity curve of laccase II thus shows a pattern contrary in some respects to that of laccase I.

5. Laccase III. After a small initial decrease in activity laccase III stays level from day 3 to day 26 and then also starts increasing. Results from immuno electrophoresis against antienzyme III support this finding.

The activity curves for laccases II and III correspond (as one should expect) with the occurrence of heatstable phenoloxidases.

Summing up the results of the experiments on the changes in the phenoloxidase spectrum one can say that *tyrosinase shows a single sharp maximum during active growth, laccase I a broad maximum during stationary growth, whereas laccase II and laccase III both decrease slightly in the beginning of active growth and show an increase again in the autolytic phase.*

The high amounts of laccases II and III and the low amounts of laccase I, found during early growth in the fermenter, cannot be due to laccases introduced

with the mycelium of the inoculum, since this showed at the time used (maximal mycelial yield) an inverse quantitative relationship for these phenoloxidases (see part V).

A repetition of the growth experiment in the fermenter exhibited the same growth curve and phenoloxidase activities with only a slight (2 day) shift in time.

Discussion

The fact that enzyme production depends on the genetic information and on environmental conditions is generally known and already described by many authors for laccase.

Genetic control of laccase production is evident from part III and VI and from the work of Fox *et al.* (1963). A series of papers shows the influence of environmental conditions as aeration and agitation (Bocks, 1967), medium composition (Esser, 1956a; Malmström *et al.*, 1958; Cheung and Marshall, 1969), addition of inducers or inhibitors (for references see Grabbe *et al.*, 1968).

Our results also confirm earlier observations that phenoloxidase production is strongly dependent on mycelial age (Lyr, 1964; Černá and Musílek, 1967; Grabbe *et al.*, 1968; Burnett *et al.*, 1969; Cheung and Marshall, 1969; Stafford, 1969). However, the data coming from most of these papers consider only the influence of these factors on the overall content of laccase or even only on the phenoloxidases as a whole. In contrast to that, we were able to show that the changes do not only concern the total content of phenoloxidases but also the particular enzyme species, independently from each other (compare also Burnett, 1969, for tyrosinase and Cheung and Marshall, 1969, for laccase).

Since *Podospora anserina* is able to produce at least three different laccases whose quantitative control is dependent on genetic and environmental factors (part III, V, IV; Fox *et al.*, 1963), the present paper adds information on the influence of mycelial age on the phenoloxidase production. The experiments have revealed that the production of the high molecular weight laccase I increases during early growth phase, reaches its maximum in the stationary phase and drops again during autolytic phase. In contrast to this, the concentration maxima for the low molecular weight laccases II and III are found in the beginning of growth and in the autolytic phase, when laccase I concentration is low.

These results might indicate that the production of these enzymes is under independent control. However, there may be an alternative explanation: Preliminary experiments (part V) suggest that laccase I is a polymer associated from subunits resembling laccases II and III and that this polymer may dissociate again into the same components. The concentration curves of the three laccases during growth of *P. anserina* would then appear as the result of the association and dissociation of the laccase I polymer.

The fact that the curves for laccase II and laccase III show only the same pattern but are not parallel could be explained by the following considerations: Synthesis of laccases II and III may be independent from the aggregation process. A minimum concentration of either enzyme may be the limiting factor for aggregation. Furthermore it is not yet possible to differentiate the low molecular weight laccases in the cells before aggregation from those arising through dissociation. The relative proportion of the subunits of laccase I is still unknown.

Cheung and Marshall (1969) working with three extracellular laccases (a high molecular weight species and two low molecular weight species) of the fungus *Trametes* interprete the results of the laccase production during growth and of the enzyme characterization also with a monomer-polymer relationship between these laccases.

Another point to be mentioned in this connection is the fact that in the mycelium of the wild strain and the mutant *zonata* there exists an inverse quantitative relationship between the high and low molecular weight laccases for the point of maximal mycelial yield. Apart from the known influence of genetical and other factors like pH of the culture (part V), the present data show that at a certain mycelial age (early active growth of the wild strain) the phenoloxidase spectra of the two strains are similar. Conducting a long range growth experiment with mutant *zonata* like that reported here for the wild strain could answer the question whether the genetic constitution of the mutated *zonata* gen principally prevents the formation of larger amounts of the high molecular weight laccase or if this enzyme is synthesized only at a certain stage of mycelial development.

In addition to the establishment of the connection between mycelial age and phenoloxidase production our data served also practical purposes:

1. Cultivation in the fermenter proved to be superior to culture in carboys as used before. Mycelial yield and total intracellular phenoloxidase production were both increased by a factor of about 8. Almost no differences were found in specific activity and pigmentation.

2. The harvesting time for maximal yield of mycelium and total intracellular phenoloxidase activity was established and found to be about 6 days later than in the carboy culture.

3. Since laccase II and III are present only in small amounts in the wild strain mycelium harvested at maximum mycelial yield from carboy culture, they could not be investigated in detail (part VI). In order to find out whether laccase I is a polymer of laccase II and III it is, however, necessary to purify and characterize these low molecular weight laccases and to prove their identity with the dissociation products of laccase I. The superior yield of phenoloxidases in the fermenter and the establishment of the production maxima of laccases II and III now renders these experiments promising.

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