THE PHENOLOXIDASES* OF THE ASCOMYCETE *PODOSPORA ANSERINA*. COMMUNICATION VI. GENETIC REGULATION OF THE FORMATION OF LACCASE†

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IN communication III (ESSER 1966) of this series of papers, eleven loci belonging to six different linkage groups are described. These loci not only influence morphological characteristics, but also the formation of melanin pigments in Podospora. A preliminary biochemical analysis of these mutants has shown that at least two of these loci, the unlinked genes zonata and flexuosa, control the synthesis of the phenoloxidases which are responsible for the pigment formation. Mutations in these genes result in a different spectrum of the laccases. Both mutants form only small amounts of high molecular weight laccases, which account for most of the phenoloxidases in the wild strain (ESSER, DICK and GIELEN 1964). In addition the mutants produce laccases hitherto undemonstrable in the wild-type strain (ESSER 1966). On the basis of these first results it was not possible to explain the defects in these strains. The prerequisites for such a model, purification and characterization of the laccases of both mutants, are reported in the present paper. The data presented support the hypothesis that both loci control the aggregation of enzyme monomers to the high molecular weight laccase.

MATERIALS AND METHODS

Material: Wild strain: s_1- of *Podospora anserina*; for details concerning the origin, ontogenesis and genetics see ESSER (1956a, 1959). Both mutants contain single gene mutations which exhibit pleiotropic effects. By 7 subsequent backcrosses with the wild strain, they have been made highly isogenic. All strains used for the production of mycelia possessed the minus mating type. For details on morphology and genetics of the mutants see RIZET and ENGELMANN (1949) and ESSER (1956b, 1966, 1969).

Mutant zonata (*z*), linkage group II, 46 map units to the right of the centromere, is a female-sterile mutant with rhythmic growth. Its production of tyrosinase is several times greater than that of the wild-type strain (HERZFELD and ESSER 1969).

Mutant flexuosa (*f-1*), linkage group I, 46 map units to the right of the centromere, is one of four allelic mutants of the *f* locus. Compared to the wild strain, it forms only a few aerial hyphae and fewer fruiting bodies.

Methods: Some methods used in our experiments have been described elsewhere: cultivation of mycelia, the production of extracts, colorimetric determination of activity of the phenoloxidases (ESSER 1963a); purification of the laccase of the wild strain (ESSER *et al.* 1964; MOLITORIS and ESSER 1970); determination of copper, nitrogen and carbohydrate content (ESSER *et al.* 1964;

* *o*-Diphenol: oxygen oxidoreductase (Tyrosinase) EC 1.10.3.1
p-Diphenol: oxygen oxidoreductase (Laccase) EC 1.10.3.2

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in this paper the carbohydrate values have been related to galactose as a standard); analytic disc-electrophoresis and serological methods (ESSER 1966, 1963b); and purification and characterization of tyrosinase (HERZFELD and ESSER 1969).

Cultivation of mycelia. Due to its slow growth, *z* mycelia were cultivated for ten days instead of the usual four days for the wild strain and the mutant flexuosa. This difference in the time of cultivation has no influence on the production of laccase. This has been shown by corresponding control experiments in which we have cultivated *z* for four days and the other two strains for ten days.

Protein determinations: The spectrophotometric method of WARBURG and CHRISTIAN (1942) has been used. The calculation of specific activities during the purification procedures is based on these values. Since the WARBURG-CHRISTIAN method is based on enolase we had to calculate the conversion factors for the laccases. This has been achieved after dry weight determinations of the purified enzymes. The following factors have been found: laccase II from mutant *z*: 0.747; laccase II from mutant *f-1*: 0.720; laccase III from mutant *z*: 0.621. All analytical data are related to these corrected protein values.

For *preparative disc-electrophoresis* the CANALCO apparatus was used; experimental conditions at pH 8.3 correspond to the recommendations of the CANALCO company.

Determination of molecular weight: a) *Gel Filtration:* In a modification of the method proposed by ANDREWS (1964) we used Sephadex G-150, a taller column (2 × 110 cm) and 0.02 M sodium phosphate buffer, pH 7.5, containing 0.1 M sodium chloride. Sodium azide (0.02%) was added to prevent bacterial contamination. Although the sodium azide inactivates the laccases, control experiments have shown that this does not affect the rate of migration of enzyme protein. b) *Ultracentrifugation.* Two methods have been used. (1) Sedimentation constant and diffusion constant (Schlieren optics) determined in the analytical ultracentrifuge. Experimental conditions for sedimentation runs were as follows: speed 59,780 rpm; double sector center piece; four different protein concentrations in the range of 0.4–1.5 mg/ml; buffer, 0.15 M sodium phosphate pH 6; temperature 20 ± 0.1°C. Experimental conditions for diffusion runs were as follows: speed 3,848 rpm; synthetic boundary cell, double sector capillary type; protein concentration, buffer and temperature as for sedimentation determinations. The experimental values have been converted to standard conditions: infinite dilution and water as solvent. The molecular weight has been calculated according to the SVEDBERG equation. (2) Sedimentation equilibrium centrifugation was carried out according to the method of YPHANTIS (1964). Experimental conditions were as follows: UV absorption optics and photoelectric scanner; 6 channel centerpiece; concentration range 0.1–0.7 mg/ml; 0.15 M sodium phosphate buffer, pH 6; temperature 20 ± 0.1°C; equilibrium after 15 hr at a speed of 7,928 rpm for laccase II and at 8,225 rpm for laccase III.

The partial specific volume of the purified laccases has been determined with a digital precision density measuring apparatus (manufacturer: A. Paar K. G., Graz/Österreich) following the method of STABINGER, LEOPOLD and KRATKY (1967). These measurements have been done in the Max Planck Institut für Immunbiologie (Director Prof. Dr. WESTPHAL) at Freiburg, Germany. We thank Dr. B. KICKHÖFEN for advice and helpful discussions.

Serological methods. In a modification of our earlier method (ESSER 1963b), we injected rabbits with 3–5 mg purified laccase mixed with "Freund's adjuvant complete." With a booster of 3–5 mg enzyme with "adjuvant incomplete" after 10–14 days we obtained a remarkable increase (15–25×) in the enzyme-antibody titer.

Determination of the isoelectric point by ampholine electrofocusing has been done with the LKB apparatus 8101 (SVENSSON 1961, 1962a, b). The following pH gradients were used: laccase I at pH 3–5, pH 4–6, pH 3–10; laccase II at pH 3–10; laccase III at pH 3–5. By measurements of activity and determination of the pH values of 1.5 ml fractions, the isoelectric points could be ascertained.

Determination of the carbohydrate portion of the laccases after disc electrophoresis has been done with periodic acid (Schiff reagent) (CLARKE 1964).

Definitions: One *enzyme unit* corresponds to Δ E of 0.2/min at 436 nm (millimicra) and a light path of 1 cm. As a substrate we used DL-3,4-dihydroxyphenylalanine = Dopa (reacts with

laccase and tyrosinase) and potassium ferrocyanide (specific for laccase). For quick testing and staining of the enzyme bands after disc electrophoresis *p*-cresol was used. Upon addition of laccase, *p*-cresol forms a white precipitate of dicresols; whereas upon addition of tyrosinase, red pigments are formed. *Specific activity* is equivalent to the number of enzyme units per milligram of protein. One *unit enzyme-antibody* is equivalent to that amount of antiserum which binds one enzyme unit. The *titer of an antiserum* is equivalent to its content of enzyme-antibody per ml serum.

RESULTS

1. *Purification of laccases of mutant zonata:* Since zonata exhibits a different spectrum of laccases than the wild strain and also forms abundant yellow-brown pigments, the purification procedures worked out for the wild strain (ESSER *et al.* 1964; MOLITORIS and ESSER 1970) had to be considerably altered. Unless otherwise stated, 0.02 M sodium phosphate buffer, pH 7.5, was used. The first steps in the purification of *z* have already been described (HERZFELD and ESSER 1969) because this mutant was used for the isolation of tyrosinase. Therefore, we can start with the description of the DEAE-chromatography which leads to separation of the laccases and to the removal of most of the tyrosinase.

The dark brown clear protein solution is layered on DEAE-Sephadex (A-50). From this column (5 × 50 cm) fractions of 6 ml each are eluted with buffer. In fractions 125–175 one obtains a faintly blue-colored laccase which we have called *laccase II*. After using a saline gradient (0–0.5 M) at a concentration of 0.2 M, additional proteins with laccase properties leave the column along with a large amount of tyrosinase and yellow-brown pigments. The former have been called *laccase III* and like laccase II they have been subjected to further purification.

Purification of laccase II: Laccase II is precipitated from the enzyme-containing fractions by means of ammonium sulfate at a saturation of 85%. The precipitate is dissolved in buffer and dialyzed for fifteen hours against the same buffer. Small amounts of denatured protein which are present after dialysis are eliminated by centrifugation (20 min at 20,000 × *g*). By two subsequent gel-filtrations on a Sephadex G-150 column (3 × 120 cm), the remaining protein impurities are removed. After each step, concentration of laccase is accomplished by salting-out and subsequent dialysis.

Purification of laccase III: The proteins of the laccase III fractions, after precipitation by ammonium sulfate (85% saturation), were dissolved in 0.015 M phosphate-citrate buffer (MCILVAINE 1921) pH 3.8, dialyzed for fifteen hours against the same buffer and centrifuged as usual. Thereafter we put the enzyme solution on a SE-Sephadex column (4 × 40 cm) and eluted step-wise with phosphate-citrate buffer of successively decreasing hydrogen ion concentrations. The first two steps (pH 3.8 and 4.1) removed a part of the pigment. At pH 4.4 laccase III, contaminated with some yellow pigment, is eluted. The pigment can be separated from the enzyme by preparative disc electrophoresis. The blue-colored laccase III leaves the gel column after six hours (at a current of 8 mA). The pigments coming off after ten hours contain only traces of activity.

Determination of laccase I: Under the conditions used for elution of laccase III on the SE-column there remains, in addition to a portion of the tyrosinase and most of the pigments, a heat-labile laccase. This enzyme (see Table 2) cannot be distinguished from the high molecular weight laccase of the wild strain. In accordance with the nomenclature used in ESSER (1966) we have called this enzyme laccase I. Since laccase I is present only in small amounts in zonata, a further purification has not been possible. Nevertheless since laccase I has already previously been detected in partially purified extracts following disc electrophoresis (ESSER 1966), there is no doubt that the enzyme is produced by *z*. Furthermore it has been possible by apatite chromatography, using the purification procedures for wild-type laccase (ESSER, DICK and GIELEN 1964) to separate laccase I from laccase III.

TABLE 1

A resumé of the experimental steps which are necessary for a purification of the laccases from the mutants zonata and flexuosa

No.	Purification step	Mutant *z* Units/kg (wet weight)	Specific activity unit/mg protein	Percent recovery	Purification	Mutant *f-1* Units/kg (wet weight)	Specific activity	Percent recovery	Purification
1	Crude extract	9,550	0.41	100	1.0	720	0.035	100	1.0
2	Ammonium sulfate precipitation	10,100	5.7	105.8	13.9	664	0.38	92.2	10.9
Laccase		II III	II III	II III	II III			II	
3	DEAE-Sephadex	810 1830	20.3 13.1	8.5 19.2	49.5 32.0	198	19.9	27.5	568
4a	1. Sephadex G-150	370	38.1	3.9	92.9	153	31.2	21.3	891
5a	2. Sephadex G-150	310	41.3	3.2	100.7	105	42.0	14.6	1200
4b	SE-Sephadex	510	17.6	5.3	42.9				
5b	Prep. disc-electrophoresis	320	28.6	3.4	69.8				

From the total of thirteen purifications for zonata and eight purifications of flexuosa, the data of one representative experiment of each are given. The crude extract has been prepared from 3 kg (*z*) and 5 kg (*f-1*) mycelial wet weight, respectively. Dopa has been used as substrate for the determination of enzyme activity.

In the left half of Table 1 we have summarized the results of a typical purification of the laccases from the mutant *z*. From these data one may deduce: (1) About 80% of the phenoloxidase activity is due to the presence of tyrosinase. The greatest part of this enzyme is removed by DEAE-chromatography (compare line 2 with line 3 and see also HERZFELD and ESSER (1969)). The remaining tyrosinase contamination is separated from laccase III after SE-chromatography. The presence of tyrosinase also explains the apparent augmentation of enzyme activity after ammonium sulfate treatment, because the tyrosinase is activated during these procedures (HERZFELD and ESSER 1969). (2) Laccases II and III are recovered in about equal amounts (compare line 5a with 5b). Both of them are relatively stable. It will be recalled that because of the assay procedure (see METHODS) tyrosinase contributes to the activity of laccase in the extract. Thus, the apparent loss of enzyme activity is due to a great extent to the separation of the laccases from tyrosinase. An indication that this loss of activity is not due to instability of the laccases, is shown by the fact that after tyrosine has been removed there is no further significant loss in enzyme activity. A second effect of this tyrosinase complication is the difficulty in estimating the extent of purification. The tyrosinase causes the estimation of enzyme levels in crude extracts to be high and hence the extent of purification (100 × for laccase II and 70 × for laccase III) is (superficially) small. In summary: *In addition to forming small amounts of a laccase similar to the wild-type laccase I, the mutant zonata forms two more laccases (II and III), which can be purified chromatographically.* The purification of the tyrosinase which comprises the greater part of phenoloxidase of *z* has been reported in HERZFELD and ESSER (1969).

2. *Purification of the laccase of mutant flexuosa:* The crude extract of this mutant has been subjected to the same purification procedures which have been described above for zonata. The unfractionated extract contains fewer melanin pigments than the extract of zonata, but is contaminated with considerable amounts of high molecular weight carbohydrates. These can be removed by two subsequent centrifugations (each for two hours at 100,000 × *g*) from the concentrate after ammonium sulfate precipitation. Extracts of *f-1* contain only 7–8% of the phenoloxidase activity which is present in zonata (Table 1, line 1). The only phenoloxidase present in amounts sufficient for purification cannot be distinguished from laccase II with the criteria we have used. During the purification procedure, we have indeed found small amounts of laccases I and III and of tyrosinase at corresponding steps of the purification procedures used for the other strains. However, these amounts are not sufficient for purification. The results of a typical purification of laccase II are presented in the right half of Table 1. Laccase II of *f-1* has about the same specific activity as laccase II of *z*, but the amount formed is only 30–40% of that produced by *z*. The total enzyme yield is apparently greater than in *z*. This is due to the fact that *f-1* forms considerably less tyrosinase and therefore the total activity of the crude extract is very small. With respect to the crude extract, the degree of purification of laccase II is about a factor of 10 greater than in *z*. This is due not only to the small amounts of tyro-

sinase, but also to the turbidity of the crude extract caused by carbohydrates which interfere with the spectrophotometric protein determinations. In summary: *The mutant flexuosa produces much lower amounts of phenoloxidases than does the mutant zonata. In flexuosa, only laccase II is produced in sufficient amounts for purification. Laccases I, III and tyrosinase are present in levels too low to be purified.*

3. *Criteria for purity of the laccases:* For each sample of laccase which was obtained after the conclusion of a purification sequence, we determined the absorption spectra in UV and in the visible range, the electrophoretic migration (disc electrophoresis) and the sedimentation behavior (analytical ultracentrifuge). These experiments have led to the following results:

(1) Small amounts of yellow-brown impurities in laccase samples result in increased absorption at 400–450 nm. All purified samples of laccase II and III exhibit no peak in this range. Their absorption maximum is at 605 nm, as could be expected from their blue color. In the UV range, there are no differences between laccase II and III and laccase I (peak at 280 nm, shoulder at 290 nm).

(2) In contrast to laccase III and laccase I from wild type, which both migrate to the anode at pH 8.3, laccase II from *z* and *f-1* moves toward the cathode (Figure 2). Laccase II is not identical to the enzyme which has been described in ESSER (1966) as laccase II of the wild-type strain. This anodally migrating laccase (*Rf* value 0.6) has not been detected again during the purification procedures.

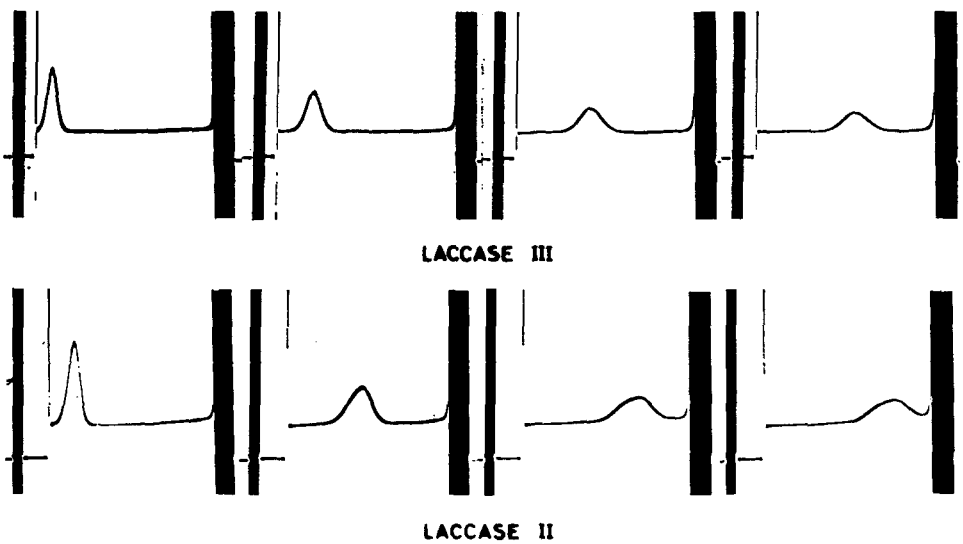

FIGURE 1.—Sedimentation of purified laccases in 0.15 M phosphate buffer at pH 6.0 at 20 ± 0.1°C; 67,770 rpm. Top row: purified laccase III; protein concentration 6.0 mg/ml; photographs are taken at 4, 12, 24, 32 min, respectively, after reaching maximum speed. Bottom row: purified laccase II which is not molecularly homogenous; protein concentration 8.5 mg/ml; photographs are taken at 8, 24, 48, 56 min, respectively, after attainment of maximum speed.

(3) In electrophoresis laccase III shows a single band with an *Rf* value of 0.3 (see also ESSER 1966, Figure 6). In the ultracentrifuge one symmetrical peak is always observed (Figure 1, above). Laccase III is free of both protein and pigment contaminations and can be regarded as *molecularly homogeneous.*

(4) The two laccase II species cannot be distinguished with the aid of these criteria. Therefore, we will omit mentioning their origins in the following account. After migration in the electric field at pH 4.3, a broad band with an *Rf* value of 0.08–0.22 is obtained for laccase II. A similar phenomenon can also be observed after electrofocusing: laccase II cannot be concentrated, as proteins usually can, in a distinct, narrow pH range which corresponds to the isoelectric point, but rather it forms a broad band between pH 7 and 10 (Table 2, line 6). Protein impurities have not been detected. Immediately after the ultracentrifuge reaches maximum speed, a symmetrical peak which flattens during the sedimentation more than normal and which becomes asymmetric can be observed (Figure 1, below). Therefore, *laccase II exhibits molecular heterogeneity.*

We have tried to remove any possible impurities from the laccase II samples with the following methods: 1.) rechromatography a) on molecular sieves, Sephadex G-100, G-150, G-200; polyacrylamide gels, BioGel P-100, P-150, P-300 (we used the latter substances in order to eliminate any possible interaction between Sephadex and enzyme carbohydrates); b) on DEAE- and SE-Sephadex; 2.) preparative ultracentrifugation in a sucrose gradient; 3.) preparative disc electrophoresis. In each case we have found only one peak after several variations of the separating conditions.

Therefore, one can assume that *laccase II is free of protein contaminations and pigment.* Before proceeding to a further analysis of the abnormal behavior of laccase II during electrophoresis and ultracentrifugation, we will first mention additional data on the characterization of the laccases.

4. *Properties of the purified laccases II and III:* Table 2 contains a resumé of the characteristics of the purified laccases II and III from the mutants *z* and *f-1*. The corresponding data for the other lacasses from the wild strain and from the mutants (as much as we have obtained) are mentioned in the table as reference values.

From Table 2 we may observe:

(1) The laccases I of the wild-type strain and of the mutants cannot be distinguished from one another by the present criteria. The same holds true for laccase II (line 3–6). Only in mutant *z* is laccase III produced in amounts sufficient for quantitative analysis. However, it can be shown to be present in the wild strain and in the mutant *f-1* with the aid of disc electrophoresis (line 7). Therefore, one can assume that all strains, the wild strain as well as mutants, form the same three species of laccase, albeit in different amounts (line 1). *The genes* z *and* f-1 *thus have a quantitative influence on laccase formation.*

(2) Laccases II and III differ from laccase I, not only in the size of their molecules, which are about 5 times as small (lines 8–12), but also by their greater heat stability (laccase II, 10 ×; laccase III, 20 ×).

(3) Heat stability, isoelectric point and Michaelis constants (lines 3–6) are the essential parameters for distinguishing between laccases II and III.

TABLE 2

Properties of laccases from the wild strain and from the mutants zonata and flexuosa

No.	Strain / Laccase	Wild strain I	Wild strain II*	Wild strain III*	Mutant *z* I*	Mutant *z* II	Mutant *z* III	Mutant *f-1* I*	Mutant *f-1* II	Mutant *f-1* III*
1	Units/kg wet weight	68,000±4,000	40	4	5	810±60	750±40	15	198±25	5
2	Specific activity	150–180	6	..	35	41.1±1.9	30.0±2.1	31	39.7±2.4	2
3	Half-life time at 60°C in min	5.4±0.5	75	..	2.8±0.3	60±4	107±6	3	66±10	90
4	$km_{DOPA} \times 10^{-3}$ M	3.03±0.12	0.59	..	3.17	0.55±0.03	1.53±0.05	2.97	0.55±0.05	..
5	$km_{KFC} \times 10^{-3}$ M	1.0±0.2	11.8	..	0.65	12.3±0.7	2.70±0.07	0.69	10.9±0.8	..
6	Isoelectric point	4.9±0.03	..	..	4.9	7–10	4.0±0.03	4.9	7–10	..
7	Disc electrophoresis R_f value	0.10±0.01	0.10–0.20	0.33	0.10	0.08–0.22	0.33±0.02	0.10	0.08–0.22	0.33
8	Sedimentation constant (S_0)	14.47±0.06	..	..	..	5.37±0.08	5.26±0.08	14.8	5.51±0.16	..
9	Diffusion constant (D_0)	3.06±0.04	..	..	..	.....	5.50±0.10	..	.....	..
10	Molecular weight (Sephadex G-150)	.....	..	..	..	70,000±3,000	79,500±3,000	..	70,000±3,000	..
11	Molecular weight (S_0/D_0)	390,000±3,000	..	..	..	.....	82,000±2,000	..	.....	..
12	Molecular weight (sed.equil.)	383,000±6,000	..	..	..	71,000±2,000	78,000±2,000	..	72,000±2,000	..
13	Partial specific volume	0.701±0.001	..	..	..	0.680	0.726	..	0.680	..
14	Copper content (percent)	0.298±0.013	..	..	..	0.298±0.006	0.310±0.001	..	0.305±0.010	..
15	Copper atoms/molecule	18.3±1.0	..	..	..	3.3±0.1	3.9±0.1	..	3.5±0.1	..
16	Nitrogen content (percent)	13.6±0.4	..	..	..	11.58±0.12	12.32±0.36	..	11.85	..
17	Carbohydrate content (percent)	23.7±0.5	..	..	..	24.9±0.8	22.7±0.6	..	23.9	..

All figures which are given with a standard error are each based at least on five measurements. The enzyme species marked with * are formed in small amounts and therefore could be only partially purified. Their specific activity (line 2) is not comparable with the other values. For the same reason there are no data in lines 8–17 for these enzymes. The still smaller amounts of laccase III in the wild strain and the mutant *f-1* were insufficient for even a partial purification. Therefore, for these enzymes only a few data are presented. The values for laccase I of the wild-type strain come from MOLITORIS and ESSER (1970). The figures for the numbers of copper atoms per molecule (line 15) are based on the mean values of the molecular weight determinations done with the ultracentrifuge (lines 11 and 12). For further explanations see text.

(4) The conformity of the values for relative copper content (line 14) and of the relative nitrogen content (line 16) suggests a similarity in the molecular structure of laccases I, II and III. Despite this, the specific activities of the low molecular weight laccases are four times less than that of the high molecular weight laccase I.

(5) All laccases contain relatively large amounts of carbohydrates (line 17). For further characterization of the laccases, serological methods have been used. We have determined the qualitative relationships between laccases I, II and III and their respective enzyme-antibodies with the aid of immunoelectrophoresis. Typical reactions are shown in Figure 2.

From this figure it may be seen: (1) that enzyme-antibody I reacts with all three laccases. (2) Although enzyme-antibody II exhibits a strong reaction with its isoantigen, a cross-reaction with the two alloantigens (laccases I and III) cannot be detected. (3) Enzyme-antibody III behaves like enzyme-antibody II: it forms a heavy precipitation band with the isoantigen and only faint bands with the alloantigens.

Tests with the unpurified laccases (Table 2) have led to comparable results. We will try to interpret these results in conjunction with the discussion of the following quantitative serological tests. In Table 3 are found the CRM values which have been obtained after iso- and alloreactions between the different laccases and their enzyme-antibodies. The nonpurified laccases could not be used in this experiment, because, even after partial purification, the amounts of enzyme were not sufficient for the CRM test.

The data of Table 3 essentially confirm the results of the qualitative tests (Figure 2). The only exception is that one may see that enzyme-antibody II reacts faintly with both alloenzymes. The great differences between the CRM values based on activity (line a) and on protein (line b) are a reflection of the differences in specific activity of the high molecular weight laccase, on the one hand, and of the low molecular weight laccases, on the other hand. In summary:

TABLE 3

Quantitative serological tests of the purified laccases from the wild strain and from the mutants z *and* f-1

Antibody form	CRM values Wild strain Laccase I	Mutant *z* Laccase II	Mutant *z* Laccase III	Mutant *f-1* Laccase II
Laccase I a	1	0.16	0.27	0.15
Laccase I b	1	0.81	1.08	0.84
Laccase II a	0.01–0.05	1	0.09	0.96
Laccase II b	0.01–0.05	1	0.07	1.08
Laccase III a	0.53	0.10	1	0.09
Laccase III b	0.13	0.12	1	0.13

The CRM values are relative figures based on a value of 1.0 for the homologous enzyme-antibody combination. a) CRM values based on the quotient: units of enzyme activity/units of enzyme-antibody; b) CRM values based on the quotient: mg enzyme protein/units of enzyme-antibody.

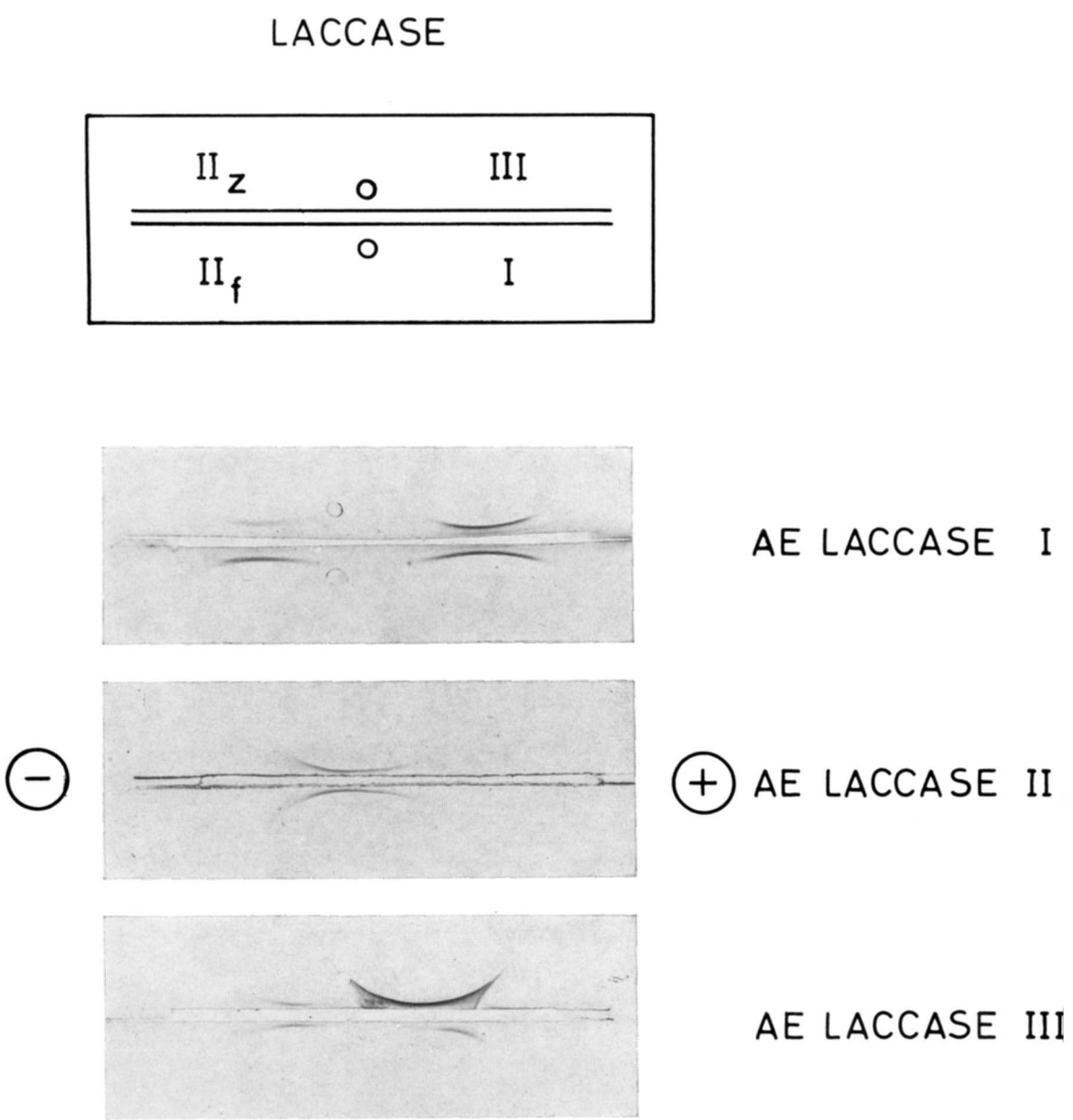

FIGURE 2.—Immunoelectrophoresis of purified laccases against different antisera: a) enzyme-antibody (EA) against laccase I of wild strain; b) c) enzyme-antibody against laccases II and III, respectively, of the mutant *z*. Cathode to the left, anode to the right.

(1) Laccases II and III are serologically very closely related to laccase I, because laccase I-antibody reacts very strongly in the allocombinations. (2) In contrast, laccases II and III seem to be only slightly related (see alloreactions).

5. *Further analysis of laccase II:* In order to get evidence concerning the ab-

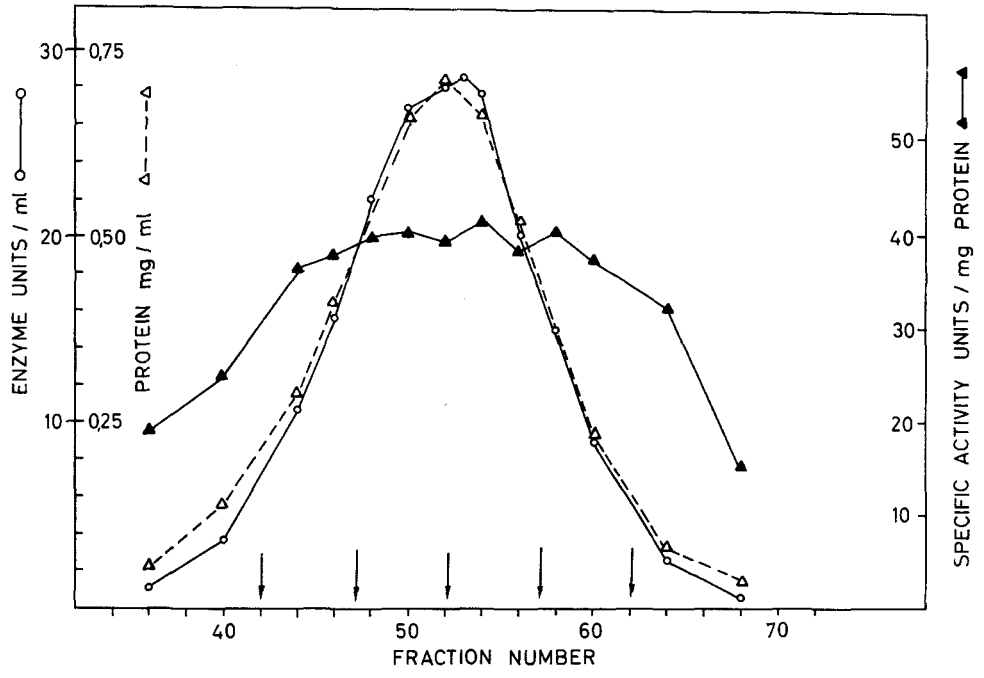

FIGURE 3.—Rechromatography of purified laccase II on Sephadex G-150. Elution with 0.02 M sodium phosphate buffer, pH 6, volume of a single fraction 6 ml. The fractions marked with an arrow have been used for disc electrophoresis (see Figure 4).

normal behavior of laccase II during sedimentation in the gravity field and during migration in the electric field, we have undertaken a rechromatography of purified enzymes on Sephadex G-150. From each fraction, enzyme activity and protein content have been determined (Figure 3).

The slight shift between the protein and activity peaks, which are both symmetrical, points to a heterogeneity of laccase II. As we have already mentioned above, this heterogeneity is not due to the presence of contaminating proteins, since the specific activity curve forms a plateau in the range of the peaks. Three repetitions with other enzyme batches have given comparable results.

In order to elucidate the causes of this phenomenon, we have taken single fractions from five different sites of the Sephadex peak and have determined with the aid of disc electrophoresis the *Rf* values for protein and enzyme activity, respectively (Figure 4). Since the enzyme content of the single fractions is not sufficient for a quantitative determination of carbohydrates, we have assayed the gel columns for carbohydrates in parallel experiments with Schiff's reagent.

From these experiments we may conclude:

(1) Within each fraction the enzyme seems to be largely molecularly homogeneous, since protein content and activity form a sharp band with indistinguishable *Rf* values.

(2) In contrast, the *Rf* values for protein of different fractions are unlike and

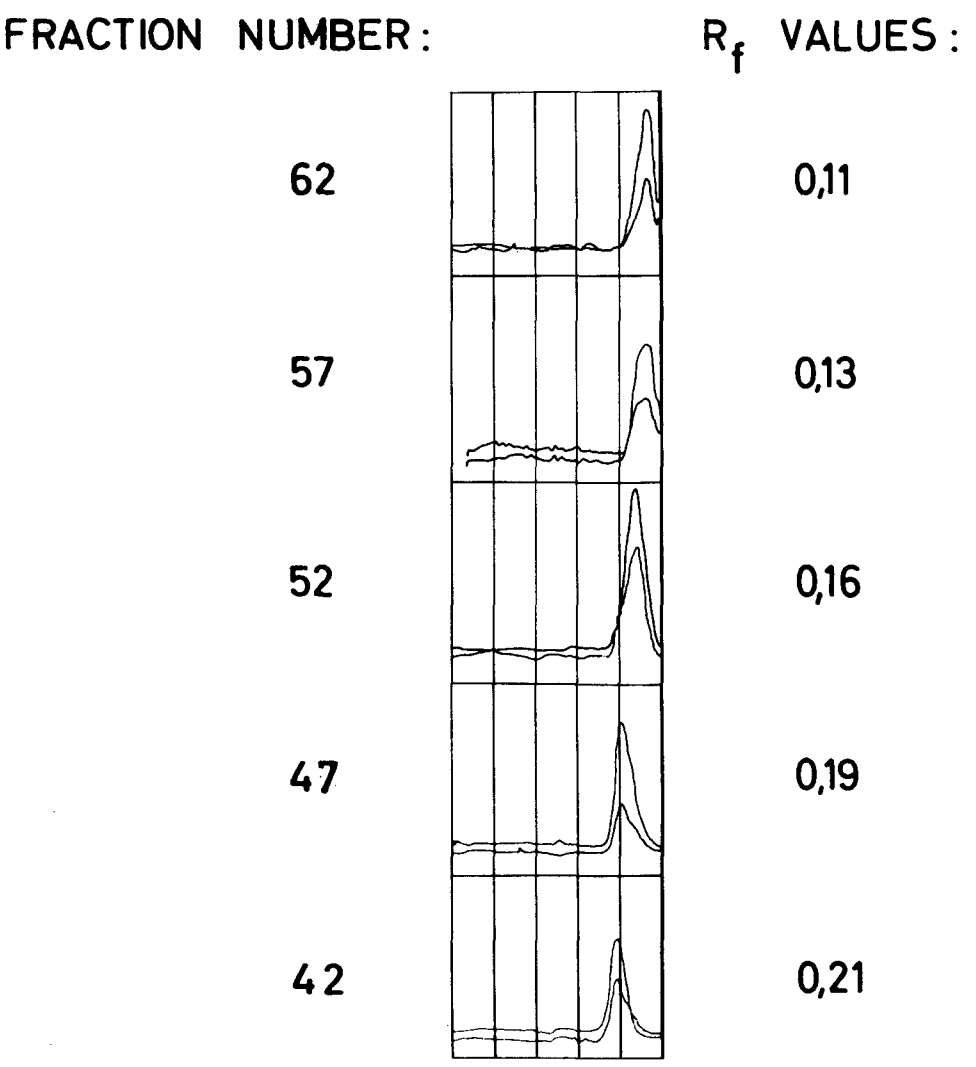

FIGURE 4.—Disc electrophoresis at pH 4.5 of single fractions from the "Sephadex peak" of laccase II (see Figure 3). The quantitative evaluation of pherograms has been done with the aid of a special carriage for the Eppendorf-Photometer which was constructed in the lab. The top curve shows the protein content (stained with amido black) and the bottom curve shows the enzyme activity (stained with *p*-cresol).

spread over the same range which was found in the first experiments (Table 2).

(3) In all fractions, the carbohydrate bands have the same *Rf* values as the enzyme bands. Due to the strong coloration of the gel itself, a densitometric evaluation of the columns was not possible. However, the intensity of the carbohydrate band diminished continuously from fraction 42 to fraction 62.

In order to obtain some information on the quantitative distribution of the carbohydrate within the different fractions of laccase II, we have rechroma-

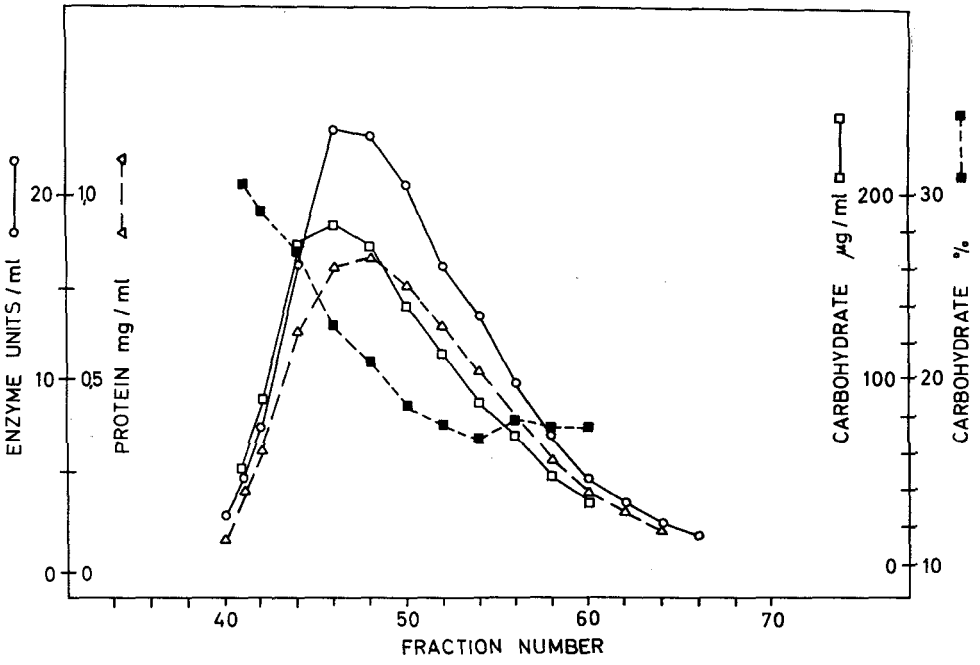

FIGURE 5.—Rechromatography of purified laccase II on Bio-Gel P-150. Experimental conditions the same as for Figure 3.

tographed this enzyme on Bio-Gel P-150 and determined, from each second fraction, in addition to enzyme activity and protein content, the carbohydrate content by the orcein method (Figure 5). We used Bio-Gel instead of Sephadex to exclude carbohydrate contaminants which might originate from the Sephadex. As one may see from Figure 5, the data of this experiment confirm the results represented in Figures 3 and 4. In addition, the carbohydrate content decreases from 30 to 17% within the enzyme peak.

The results of the experiments suggest that the *purified laccase II is a mixture of multiple forms*. In order to explain this microheterogeneity, we may offer the following hypothesis: laccase II consists of homogeneous protein molecules which are identical with respect to their functional properties (e.g., specific activity and heat stability of individual fractions). However, the proteins have bound carbohydrates in different amounts. The amount of carbohydrate bound seems to be distributed continuously between its upper and lower limits, since neither by column chromatography, nor by electrophoresis, nor by ultracentrifugation, can the total laccase II be split into subfractions. PLAPP and COLE (1967) and OSHIRO and EYLAR (1968) have obtained similar results after analysis of β-glucuronidase from bovine liver and fetuin from fetal calf serum.

DISCUSSION

In order to interpret the results of this paper on a genetic basis, we have considered the following facts:

(1) The wild-type strain and the mutants zonata (*z*) and flexuosa (*f-1*) are able to produce three different forms of laccase in widely different amounts. The wild strain produces mostly the high molecular weight laccase I, but only very small amounts of laccases II and III, the molecular weights of which are about 1/5 that of laccase I. In contrast, the laccase spectrum of both mutants is determined by the presence of the low molecular weight enzyme species (i.e., in *z* by laccases II and III; in *f-1* by laccase II only). Both mutants form only traces of laccase I. This is also true for laccase III in mutant *f-1*.

(2) Both of the unlinked genes responsible for these enzyme differences have a pleiotropic effect which is manifest not only on an enzymatic level (increased tyrosinase production, HERZFELD and ESSER 1969) but also on the morphological level. (ESSER 1966). This is especially true for zonata which exhibits no formation of female sex organs, reduced and zonated growth, reduced melanin formation, and altered morphology of the hyphae. The appearance of flexuosa also differs from wild type by a reduced amount of perithecia and lack of aerial hyphae. A pleiotropic mutation affecting phenoloxidases, as well as morphological characters is also known in Drosophila (PEEPLES, BARNETT and OLIVER 1968).

From this condensed presentation of our experimental results, it follows that the loci *z* and *f-1* do not influence the primary structure of the laccases, since only quantitative differences for each of the three enzyme species in the wild-type strain and in the mutants can be demonstrated. Therefore, *neither gene* can be a structural gene for the laccases and instead, they have to be considered as *regulatory genes.*

By the term regulatory gene, in the widest sense of the word, we mean genes whose products intervene in the regulation of protein synthesis. These genetically controlled regulations can occur at four different levels of protein synthesis: transcription, translation, folding, and aggregation of the polypeptides to a native protein (EGELHAAF 1966; GIERER 1967; URSPRUNG 1967).

Under the stimulus of the first papers of JACOB and MONOD (1961a, b), there has emerged extensive experimental evidence which has provided an understanding of the action of regulatory genes during transcription. Our knowledge of genetic regulation of the remaining steps of enzyme synthesis has many gaps and it consists mostly of isolated results (e.g., for enzyme aggregation in *E. coli* —BÖCK 1967, 1968; SCHLESINGER 1967; and in the tobacco mosaic virus— JOKUSCH 1966). Furthermore, it is not yet clear whether and to what extent the ideas obtained by these experiments on prokaryotic organisms can be transferred to eukaryotic organisms.

In order to develop some idea concerning the mode of action of the regulatory genes *z* and *f-1*, we may begin with a few of the experiments which have been described elsewhere (MOLITORIS and ESSER 1970).

The high molecular weight laccase I can, for instance, be split into active subunits by repeated freezing and thawing or by treatment with sodium dodecylsulfate (as has been proven by ultracentrifugation in a sucrose gradient and by disc electrophoresis). Due to the small amount of enzyme in these informative

experiments, an analysis and characterization of the dissociated products was not possible.

Therefore, it seems possible that laccase I has a polymeric configuration and consists of subunits which are identical with laccases II and III. A comparison of the molecular weights (Table 2) would be compatible with the assumption that altogether five molecules of II and of III aggregate to form one pentameric molecule of laccase I. Polymeric enzymes with odd numbers of monomers do not occur frequently. However, some cases of pentameric proteins have been described (BOEKER and SNELL 1968; KONINGS 1969). Taking the copper content into consideration one might suggest that three molecules of laccase II and two molecules of laccase III aggregate to form one molecule of laccase I. Further indications which seem to confirm this hypothesis come from the serological experiments (Figure 2, Table 3) and from the work of MOLITORIS and ESSER (1970). Further support comes from the fact that the low molecular weight laccases are far more heat stable than the high molecular weight laccase.

In this connection it seems necessary to point out that the monomeric laccases II and III, although indistinguishable from laccase I by their substrate specificity, have a specific activity which is four times smaller than that of the polymeric laccase I. Evidently, for optimal catalytic capacity of the laccase, the presence of an aggregated molecule is necessary. The literature contains many examples of enzymes with quarternary structures. However, in these cases either the monomers are inactive (tyrosinase of insects, MITCHELL 1967) or are capable of catalyzing only part of the reaction (tryptophan synthetase of *E. coli*, YANOFSKY 1967).

The final proof of the correctness of our assumption is still lacking and can be achieved only by an analysis of the dissociated laccase I. It cannot be ignored that the genes *z* and *f-1*, aside from their other effects, *regulate the aggregation of laccase.* Thus, these data would offer an example of the genetic regulation of the last step of enzyme synthesis. The elucidation of this regulatory mechanism, which requires further experiments, would not only contribute to our understanding of regulation effects during metabolism in general, but also, due to the pleiotropic effect of the mutation, afford ideas concerning the genetic control of morphogenesis.

It is also imaginable that the steps of morphogenesis are influenced directly or indirectly by particular equilibrium points for the different forms of laccase. The mycelia of Podospora are normally grown at a pH of 6.5. However, if wild mycelia are cultured at a pH of 4.5, one may simulate some of the properties of mutant *z*, e.g., reduction in growth, zonation of the mycelium, low production of laccase I, and enhanced production of laccases II and III (ESSER 1969). It must be added that the reverse is not possible–i.e., induction of the mutant *z*, by alteration of external conditions, to phenocopy the properties of the wild strain. These regulatory effects of pH might also hold true for tyrosinase, since the mutant *z* produces this enzyme in considerably larger quantities than the wild type (HERZFELD and ESSER 1969). Several authors have pointed out the possibility of a corre-

lation between phenoloxidase activity and morphogenesis from their experiments with fungi, insects and higher plants (literature in ESSER 1969 and HESS 1968).

SUMMARY

In *Podospora anserina* three different forms of laccase are produced in unequal amounts. In the wild strain, laccase I predominates and laccases II and III are formed only in trace amounts. In the morphological mutants *zonata* (*z*, linkage group II) and *flexuosa* (*f-1*, linkage group I), laccase I is present in small amounts. The mutant *z* yields high amounts of both laccases II and III; in *f-1* only laccase II is formed extensively, with laccase III being formed in traces.—In both strains the mutations have a pleiotropic effect. Apart from the alteration of the laccase spectra, *z* exhibits high amounts of tyrosinase (compared to the wild strain), leaky and rhythmic growth on a complete medium, no aerial hyphae, no formation of ascogonia, and reduced formation of melanin pigments; *f-1* has no aerial hyphae and forms a small number of perithecia.—Laccases II and III of *z* and laccase II of *f-1* have been purified by a number of procedures, principally column chromatography. (The purification of wild-type laccase I has been described in ESSER *et al.* (1964) and in MOLITORIS and ESSER (1970).) Due to the small amounts of the minor laccase components, both in the wild strain and in the mutants, a purification of these was not possible.—In a comparison of some of the characteristics of the purified and unpurified enzymes (see Table 2 and 3), no distinction could be made between laccase I from different sources. The same holds true for laccases II and III, respectively.—In contrast to the relatively high molecular weight (390,000) of the heat-labile laccase I, laccases II and III have molecular weights of 70–75,000 and 80,000, respectively and are heat stable. Since laccase I can be split into active subunits, one can tentatively assume that laccase I is a polymer consisting of monomers identical with laccases II and III.—From these findings it is concluded that the loci *z* and *f-1* quantitatively control the production of laccases. In order to understand the mechanism of this genetic regulation of enzyme synthesis, a working hypothesis is stated and discussed. It is suggested that both loci *z* and *f-1* regulate the aggregation of laccase I indirectly and in different manners. With regard to the pleiotropic action of these genes, this genetic regulation of enzyme formation might be correlated with the control of morphogenesis.

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