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The Phenoloxidases of the Ascomycete *Podospora anserina*

Structural Differences between Laccases of High and Low Molecular Weight

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In order to investigate the extent of the relationship between the three copper-containing glycoproteins, laccases I, II and III (M_r 70000, 80000 and 390000 respectively) of *Podospora anserina*, the following experiments were carried out on laccases II and III: (a) determination of amino acid composition; (b) determination of N-terminal and C-terminal amino acid; (c) determination of sugar composition; (d) dissociation studies on native and denatured laccases and also after removal of copper from the enzymes; (e) digestion of the carbohydrate moieties with the aid of glycosylhydrolases.

A comparison between the results of these experiments and data previously obtained with laccase I allows the following conclusions to be drawn.

1. Laccases II and III are not identical.
2. Neither of these low molecular weight laccases are as complete molecules subunits of the oligomeric laccase I.
3. The possibility of partial identity of amino acid sequences of laccases I and III can not be excluded.
4. Laccase II possibly consists of subunits of M_r 37000 whereas laccase III does not.
5. Digestion of 50% of the carbohydrate content leads to complete loss of serological specificity (serological reaction and cross reaction). This finding is discussed with regard to the possible role of the carbohydrate moiety as antigenic determinants and thus as the reason for the immunological relationship.

As a consequence, at least three independent structural genes for laccases must be assumed.

As reported in previous papers of this series [1–4] *Podospora anserina* is able to produce three different forms of laccases. There is a high-molecular-weight form: laccase I (M_r 390000) and two low-molecular-weight forms: laccase II (M_r 70000) and laccase III (M_r 80000) respectively [3,5]. All three enzymes are copper-containing glycoproteins. The amount of each enzyme species depends on environmental as well as on genetic conditions [2–4,6,7]. It was postulated from biochemical data and later confirmed by electronmicroscopic studies that laccase I consists of subunits which might be identical either with laccase II or laccase III or with both [5,8]. This working hypothesis was supported by the finding that the three laccases showed a fairly identical substrate specificity [1,9,10]. However, some serological differences and the fact, that the low-molecular-weight laccases also oxidize *m*-diphenolic compounds (substrates which are not accessible to the high-molecular-weight laccase I) questioned this hypothesis. In order to obtain further information, laccases II and III were submitted to a more detailed biochemical analysis of the protein and carbohydrate moiety. The results of these experiments are compared with equivalent data for laccase I [5] and lead to the conclusion that none of the low-molecular-weight laccases II and III can be considered anymore as subunit of laccase I.

Enzymes. Laccase or monophenol, dihydroxyphenylalanine: oxygen oxidoreductase (EC 1.14.18.1), [formerly *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2)]; ribonuclease I (EC 3.1.4.22); trypsin (EC 3.4.21.4); carboxypeptidase A (EC 3.4.12.2); β-glucosidase (EC 3.2.1.21); β-galactosidase (EC 3.2.1.23); α-mannosidase (EC 3.2.1.24); β-*N*-acetylglucosaminidase (EC 3.2.1.30).

This is contribution no XIV of the series.

MATERIALS AND METHODS

Strains

The wild strain *s* and the rhythmic growing mutant *zonata* (*z*) of *Podospora anserina* were used. Mutant *z* is located on the right arm of linkage group II, at a

distance of 46 map units from the centromere. Details concerning the origin, properties and genetics of the strains in Esser [11].

General Procedures

Cultivation of mycelia, preparation of extracts, techniques for purification and characterization of laccases II and III are described or referred to in Esser and Minuth [3].

Protein content of purified laccases was determined spectrophotometrically at 280 nm [12] and converted to dry weight according to Esser and Minuth [3]. Phenoloxidase activity was measured with dihydroxyphenylalanine (Sigma) as substrate. The increase in absorbance of 0.2 units/min at 436 nm and 1-cm light path corresponds to one enzyme unit.

Copper content was determined with the 2,2-bipyridine method as has been described by the authors [3].

Amino Acid Analysis

Lyophilized samples of laccases II and III (25 nmol) were freed from oxygen by repeated evacuation and flushing with nitrogen, and subsequently hydrolyzed under nitrogen atmosphere in 5 ml 6 M HCl at 110 °C for 24, 48, 72 and 96 h. The hydrolysates were analyzed on an NC-1 amino acid analyzer (Technicon, Frankfurt) according to Spackman et al. [13]. 25 nmol norleucine were used as internal standard.

Half-cystine and methionine were determined as cysteic acid and methionine sulfone respectively after performic acid oxidation [14] followed by acid hydrolysis for 24 h. Values for tyrosine, serine and threonine were extrapolated to zero time as a correction for decomposition during hydrolysis.

Values for valine and isoleucine are constant after 72 h of hydrolysis. Therefore only the 72-h and 96-h values were used.

Sulfhydryl assays were performed according to the methods of Boyer [15] and Ellman [16]. Laccases II and III (10–100 nmol), freshly prepared or denatured (15 h in 6 M guanidinium chloride or 9 M urea) were incubated with 5,5′-dithio-bis(2-nitrobenzoic acid) or with chloromercuribenzoic acid (Sigma).

Disulfide assays were carried out following the method of Zahler and Cleland [17]. Reduction was performed with 150 µM dithiothreitol (Sigma) for up to 24 h on samples containing 10–150 nmol of denatured laccases II and III (15 h in 6 M guanidinium chloride or 9 M urea) and was followed by reaction with 5,5′-dithio-bis(2-nitrobenzoic acid). Cystine and ribonuclease A (Sigma) were used as controls.

Tryptophan was determined spectrophotometrically according to the procedure of Edelhoch [18]. Laccases II and III (100 nmol) were denatured in 6 M guanidinium chloride for 15 h. The spectra were scanned with a Cary 15 spectrophotometer (kindly disposed by Prof. Trebst, Bochum). The molar absorption coefficients for tryptophan and tyrosine were determined with *N*-acetyl-L-tryptophanamide and *N*-acetyl-L-tyrosineamide respectively (Sigma) and were calculated to be $\varepsilon_{280} = 5709\ M^{-1}\ cm^{-1}$ and $\varepsilon_{288} = 4865\ M^{-1}\ cm^{-1}$ for tryptophan and $\varepsilon_{280} = 1480\ M^{-1}\ cm^{-1}$ and $\varepsilon_{288} = 3275\ M^{-1}\ cm^{-1}$ for tyrosine.

Coupling with Fluorodinitrobenzene

The coupling reaction was monitored over a period of 2 h in the dark in a temperature-controlled pH-stat on 200 nmol of laccases II or III and 50 nmol of laccase I respectively, at a constant pH of 8.0. This was followed by hydrolysis in 6 M HCl, essentially according to the procedure of Needleman [19]. Aliquots of the final ether extracts were used for separation of the dinitrophenyl derivatives by thin-layer chromatography. Two solvent systems were employed: chloroform/methanol/acetic acid (95/5/1, v/v/v) and benzene/pyridine/acetic acid (50/40/10, v/v/v).

Digestion with Carboxypeptidase A

Digestions over a period of 10 min were carried out at various weight ratios of diisopropyl fluorophosphate-treated carboxypeptidase A (Sigma) to protein substrate (1/20 up to 1/100) in *N*-ethylmorpholine acetate buffer at pH 8.5. Phenoloxidases (100–250 nmol of laccases II or III and 50 nmol of laccase I respectively) had been preliminarily denatured in 1 ml of 10 M urea at 37 °C. Aliquots were removed at periodic intervals into a volume of 0.2 ml acetic acid, and after removal of denatured protein by centrifugation were evaporated to dryness. The residues were analyzed with the NC-1 Technicon amino acid analyzer.

Hydrazinolysis

The phenoloxidases (100 nmol of laccases II or III and 25 nmol of laccase I respectively) were lyophilized and thoroughly dried over phosphorus pentoxide. Water-free hydrazine (0.2 ml) was added to the tubes and these were sealed after freezing in liquid nitrogen. The reaction was maintained for up to 16 h at 80 °C [20]. The hydrazinolysates were analyzed on the NC-1 Technicon amino acid analyzer following the removal of hydrazine over phosphorus pentoxide.

Tryptic Digestion and Peptide Mapping

Laccases II and III (250 nmol) were denatured in 6 M guanidinium chloride in the presence of 25 mmol mercaptoethanol over a period of 15 h at 37 °C under

nitrogen atmosphere. S-aminoethylation was achieved with three portions of 1 mmol ethyleneimine [21]. Reagents were removed by gel-filtration and the eluate was lyophilized. For tryptic digestion the modified laccases were incubated with 1% (w/w) trypsin treated with L-(1-tosylamido-2-phenyl)ethylchloromethylketone (Worthington) in 15 mM ammonium hydroxide at pH 8.0 in a temperature-controlled pH-stat under nitrogen at 37 °C for 2 h. A second portion of trypsin was added and digestion was continued for 4 up to 20 h. Aliquots of the peptide mixture (200–300 µg in 15 mM NH_4OH/pyridine, 1/1, v/v) were dotted on a cellulose thin-layer plate and subjected to electrophoresis at 50 volts cm^{-1} for 45 min at 5 °C in pyridine/acetic acid/water (10/1/89, by vol., pH 6.5). This was followed by thin-layer chromatography in the second direction, in amyl alcohol/isobutyl alcohol/*n*-propyl alcohol/pyridine/water (1/1/1/3/3, by vol.) [22]. Peptides were visualized with ninhydrine reagent.

Arginine-containing peptides were detected after reaction with phenanthrene quinone [23].

Denaturation

Samples of native laccases II and III (100 nmol) were denatured and the rate of inactivation was recorded. Conditions for denaturation were as follows: (a) elevated temperature (60 °C; 20 mM phosphate buffer, pH 6.0); (b) extreme pH-values (pH 3.5; 0.1 M ammonium acetate buffer; pH 10.0; 0.1 M Tris/HCl buffer); (c) in 8 M urea or 6 M guanidinium chloride or 1% sodium dodecylsulfate, containing mercaptoethanol (0.5 mM) for reduction.

Preparation of Copper-Free Laccases

These were prepared from native enzymes by a procedure based on that of Dijk et al. [24]. Formic acid was added to samples of the phenoloxidases (100 nmol) to a final concentration of 70% (v/v). Portions of the reaction mixtures were withdrawn after 2 and 5 h, followed by removal of excess reagents through exhaustive dialysis versus 1 M formic acid (40 h) and finally versus phosphate buffer (20 mM, pH 6.0) for 8 h.

Dissociation of the Apo-Laccases

For dissociation experiments apo-laccases II and III (100 nmol) were exposed for 24 h at 37 °C to a mixture of denaturing solvents consisting of 8 M urea and 1% sodium dodecylsulfate in the presence of 0.5 mM mercaptoethanol as reductant, followed by 12 h dialysis against a solution of 8 M urea, 0.1% dodecylsulfate and 0.05 mM mercaptoethanol.

Disc-Electrophoresis

For 7.5% standard gels the original method of Ornstein and Davis [25] was followed for laccase III; for laccase II the buffer formula of Williams and Reisfeld [26] of pH 4.5 was chosen. For separation of the dissociation products of apo-laccases and molecular weight determination copper-free laccases II and III (10–100 µg) were applied to dodecylsulfate-acrylamide gels containing 7.5% cross-linker, 8 M urea and 0.1% sodium dodecylsulfate, according to Weber and Osborn [27] in the variation of Swank and Munkres [28]. Bovine serum albumin, ovalbumin and cytochrome *c* (Boehringer, Mannheim) were added as marker proteins. The gels were fixed in sulfosalicylic acid and stained with Coomassie brilliant blue [29] and destained overnight in 7% acetic acid.

Carbohydrate Analysis

Qualitative and quantitative carbohydrate analysis by thin-layer chromatography and by gas-liquid chromatography respectively were performed as described earlier [30].

Enzymatic Degradation of the Carbohydrate Moiety

Samples of freshly prepared native laccases II and III (500 nmol in 10 mM citrate buffer, pH 4.3) were incubated with a mixture of the following enzymes (all Boehringer): β-galactosidase, β-glucosidase, α-mannosidase and *N*-acetyl-β-D-glucosaminidase. Digestions were run over a period of 100 h at 10 °C. (The glycosylhydrolases were still active at the end of incubation as was determined with the corresponding *p*-nitrophenylglycosides.) At periodic intervals phenoloxidase activity was determined. Additional aliquots were transferred to 20% trichloroacetic acid; after removal of precipitated proteins by centrifugation the supernatants were analyzed for total carbohydrate content by the orcinol method [3] and for sugar composition by thin-layer chromatography. Controls were run under similar conditions without hydrolases. At the end of digestion, heat inactivation, the Michaelis constant for dihydroxyphenylalanine, disc-electrophoresis and immuno-electrophoresis were performed as described in Esser and Minuth [3].

RESULTS

The biochemical data were obtained from purified laccases isolated either from the wild strain (laccase I) or the mutant *zonata* (laccases II and III). According to Esser and Minuth [3] ultracentrifugation, disc-electrophoresis and immuno-electrophoresis were used

Table 1. *Amino acid composition of laccases I, II and III*
Results are the mean of 4 analyses of different hydrolysates rounded to nearest integers

Amino acid	Amino acid in		
	laccase I[a], M_r 97500	laccase II, M_r 70000	laccase III, M_r 80000
	mol/mol enzyme		
Arginine	22	17	17
Isoleucine	32	20	24
Histidine	18	16	17
Alanine	27	30	29
Tyrosine	13	14	15
Tyrosine[b]		12	14
Phenylalanine	18	16	19
Serine	22	25	32
Glycine	39	38	45
Lysine	17	13	8
Proline	35	34	30
Half-cystine	19	11	18
Tryptophan[b]	24	13	19
Methionine	18	9	7
Leucine	28	29	39
Threonine	28	36	48
Glutamic acid	45	25	48
Valine	22	34	28
Aspartic acid	57	60	60
Total residues	484	439	503
M_r[c]	63979	56096	64623

[a] The number of amino acid residues was calculated on the basis of one subunit of laccase I (M_r monomer: 390000/4 = 97500).
[b] Determined spectrophotometrically.
[c] Molecular weight of the protein moiety calculated from the amino acid composition.

as criteria for purity. With exception of the data concerning end group determination, the figures for laccase I were taken from Molitoris [5].

Amino Acid Composition

The data of the amino acid analyses of laccases I, II and III are summarized in Table 1 and require the following additional comments.

Firstly, as expected, the determination of the tyrosine content caused problems. Although these values were corrected for degradation during hydrolysis by extrapolation to zero time, they were rather high compared with those calculated from the spectrophotometric tryptophan determination. Therefore a mean of both values was used for the calculation of the molecular weights on the basis of amino acid composition.

Furthermore the determination of cystine-cysteine also caused difficulties, because it was not possible to calculate from the half-cystine values the amount of both amino acids, as will be explained briefly.

The amino acid analysis revealed a comparatively high amount of cysteic acid corresponding to 11 and 18 mol of half-cystine residues for laccases II and III respectively. No free sulfhydryl groups could be detected upon reaction with −SH reagents [5,5′-dithio-bis(2-nitrobenzoic acid and chloromercuribenzoic acid] in contrast to one sulfhydryl group in each, laccases A and B, of *Trametes versicolor* [31]. Thus the cysteic acid should have originated from cystine residues. Surprisingly enough, titration of reduced laccases II and III with 5,5′-dithio-bis(2-nitrobenzoic acid), however, yielded no *p*-thiobenzoic acid, apparently demonstrating the absence of cystine in both enzymes.

The reliability of this method could be proved in a control experiment with ribonuclease I: 4 mol of disulfide were found for this enzyme [32]. However, this reaction was completely inhibited if laccase II or III was added in equimolar amounts to the assay.

The failure of the methods in the presence of laccases may be due to the copper content of these enzymes, particularly because upon denaturation in guanidinium chloride or urea as a preliminary treatment for determination of sulfhydryl or disulfide groups, only 1 of the 4 copper atoms per mol of laccase was removed. Thus it remains uncertain whether the cysteic acid is an oxidation product of cystine or of cysteine.

For a comparison of the three laccases the data of the amino acid compositions were converted to a percentage (w/w) (Fig. 1).

It may be seen from Fig. 1 that firstly, the three laccases show quantitative correspondence for only two amino acids (class I). Secondly, eight amino acids are present in laccases II and III in similar amounts (classes I and II). Thirdly, laccase I shows a quantitative agreement with laccase II for 3 amino acids (classes I and III). The appropriate correlation with laccase III is 5 amino acids (classes I and IV). Fourthly, the three laccases show no agreement at all for six amino acids (class V). Therefore it is most unlikely that the two low-molecular-weight laccases have identical polypeptide chains and, furthermore, that these might be subunits of the high-molecular-weight laccase I.

End Group Determination

The experimental data of the determination of N-terminal and C-terminal amino acids are compiled in Table 2; they allow the following statements: serine was found to be the N-terminal amino acid of all three laccases; valine, leucine and isoleucine were found to be the C-terminal residues of laccases I, II and III respectively after hydrazinolysis.

The fact that all three C-terminal values deviate from integer figures for mol amino acids per mol enzyme is in agreement with literature data. According

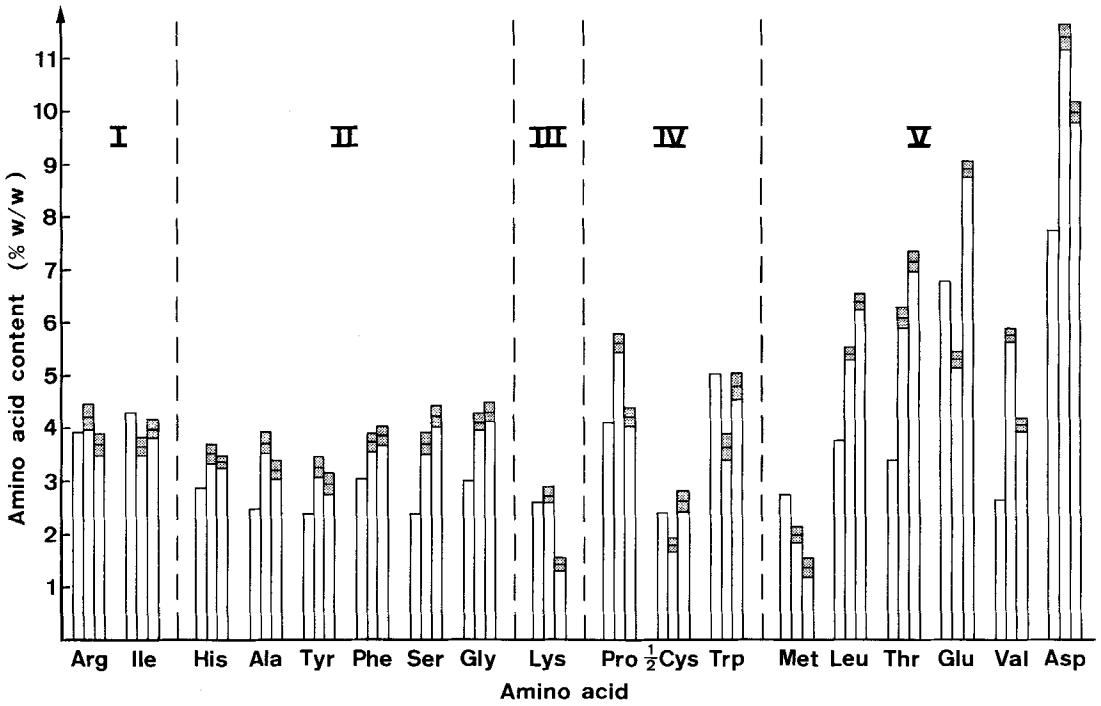

Fig. 1. *Amino acid composition of laccases I, II and III.* For classification according to similarities and dissimilarities respectively, the values of Table 1 were converted to a percentage (w/w). The sequence of laccases in the columns is I, II, III. In each bar for laccases II and III, the dotted areas represent three times the standard error of the mean. Unfortunately comparable values were not available for laccase I, since these figures were taken from another source [5]

to Niu and Fraenkel-Conrat [33] and to Narita [34] the experimental values 0.7–0.8 (valine), 0.5–0.6 (leucine) and 0.5 (isoleucine) were found. This means that for laccases II and III one single polypeptide chain and for laccase I, at least two polypeptide chains have to be postulated.

In addition, the C-terminal residues were also assayed after digestion with carboxypeptidase A. These data confirmed that valine, leucine and isoleucine respectively are the end groups.

Table 2. *N-terminal and C-terminal amino acids of laccases I, II and III*
For details see Materials and Methods. N-terminal residue was determined by coupling with fluorodinitrobenzene; C-terminal residue was determined by hydrazinolysis

Enzyme	Amino acid of	
	N-terminal residue	C-terminal residue
		mol/mol enzyme
Laccase I	serine	valine: 1.57
Laccase II	serine	leucine: 0.52
Laccase III	serine	isoleucine: 0.66

Peptide Mapping

20 tryptic digestions from each enzyme were fingerprinted. For laccase II 28 peptides were found, 16 of which contained arginine and 12 lysine. The corresponding values for laccase III are 24 peptides (15 arginine and 9 lysine) (Fig. 2A, B).

In all experiments the number and position of peptides showed no variation with respect to time of digestion (4–20 h), type of denaturing agent (8 M urea, 6 M guanidinium chloride) or method used for modification of –SH groups (S-carboxymethylation, S-aminomethylation).

Provided that both enzymes are composed of single polypeptide chains or of nonidentical subunits, these results are in fairly good agreement with peptide number of 31 for laccase II and 26 for laccase III to be expected from the results of amino acid analysis (see Table 1).

In order to check identity, 10 fingerprints of an equimolar mixture of both laccases were made. As may be seen from Fig. 2C there are, instead of the 52 peptides to be expected in case of complete nonidentity, 40 peptides, 24 of which contain arginine, thus leaving the rest of 16 to the lysine type. Furthermore the 12 peptides of identical position also show agreement in arginine and lysine content respectively. Thus from peptide mapping the existence of partially identical amino acid sequences can not be excluded for laccases II and III.

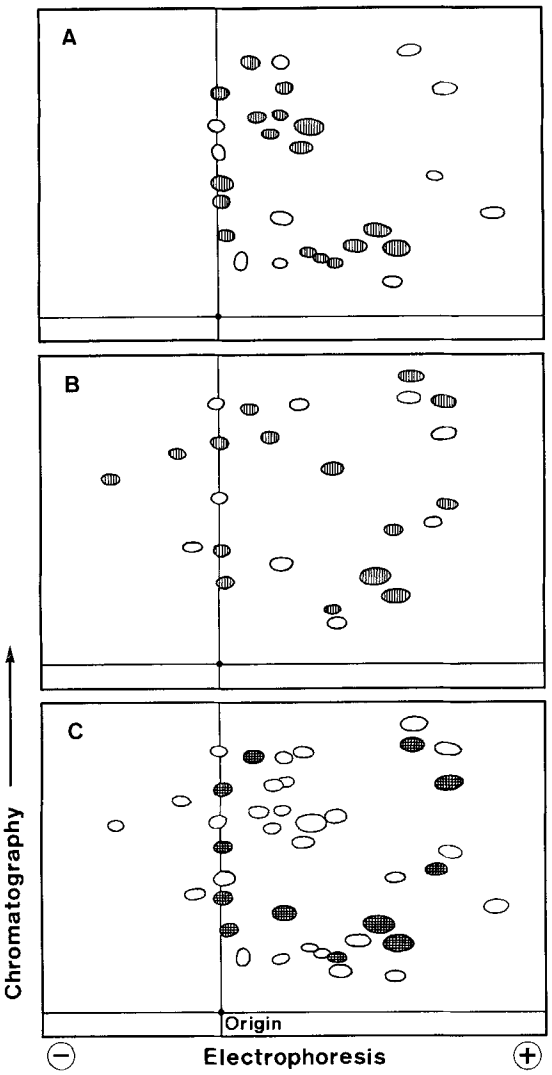

Fig. 2. *Maps of the tryptic peptides of aminoethylated laccases II (A), III (B) and of an equimolar mixture of laccases II and III (C).* Peptides revealed by ninhydrine are circled, vertical hatching designates arginyl peptides, cross-hatching indicates identical position of peptides of laccases II and III. (Experimental details see text)

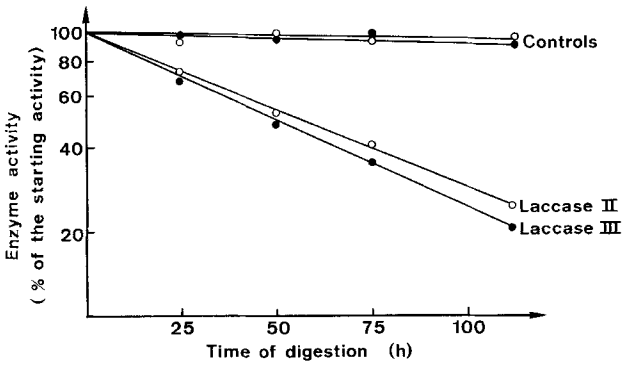

Fig. 3. *Inactivation of laccases by glycosylhydrolases.* Digestion by a mixture of β-galactosidase, β-glucosidase, α-mannosidase and *N*-acetylglucosaminidase (4 units each, in 10 mM citrate buffer, ph 4.3). Phenoloxidase activity was measured with dihydroxyphenylalanine. Controls were untreated laccases

Table 3. *Carbohydrate composition of laccases I, II and III after gas-liquid chromatography*
The data represent the mean of quadruplicate analyses. The standard deviations are given. Numbers in parentheses are nearest integers of mol sugar per mol laccase

Monosaccharide	Carbohydrate in		
	laccase I[a], M_r 97500	laccase II, M_r 70000	laccase III, M_r 80000
	% (w/w)		
Mannose	14.3 (77)	11.7 ± 0.4 (45)	16.8 ± 0.4 (75)
Rhamnose	2.8 (15)	4.7 ± 0.2 (20)	3.1 ± 0.2 (15)
Galactose	1.1 (6)	4.3 ± 0.2 (17)	1.2 ± 0.2 (5)
Glucose	0.4 (2)	1.2 ± 0.1 (5)	0.5 ± 0.1 (2)
N-Acetylglucosamine	1.5 (7)	0.8 ± 0.2 (3)	1.6 ± 0.2 (6)
Total (Glc)	20.0 (107)	22.7 (90)	23.1 (103)
Total (orcinol)[b]	23.7	24.9	22.7
M_r[c]	19598	16021	18560

[a] The number of sugar residues have been calculated on the basis of one subunit of laccase I (M_r monomer: 390000/4 = 97500).
[b] From Esser and Minuth [3].
[c] Molecular weight of the carbohydrate moiety calculated from integers of each monosaccharide.

Carbohydrate Composition

In a previous communication [3], it was shown that laccases II and III are glycoproteins. Thin-layer chromatography had revealed for both enzymes the presence of the following five sugars: mannose, rhamnose, galactose, glucose and *N*-acetylglucosamine.

In order to obtain information about the quantitative contribution of each of these sugars to the carbohydrate moieties, the laccases were analyzed by gas-liquid chromatography following methanolysis and silylation.

Table 3 summarizes the monosaccharide composition of all three laccases. The carbohydrate content in all laccases amounts to one-fifth up to one-fourth of the molecular weight, with mannose being the main component (50–75%). A comparison of the results for the two low-molecular-weight laccases indicates statistical significant differences on a 99.7% level in all five sugars. On the other hand, the similarities between laccases I and III are striking, thus pointing to a close relationship in the carbohydrate fractions.

Enzymatic Degradation of the Carbohydrate Moieties

The low-molecular-weight laccases II and III were subjected to a mixture of glycosylhydrolases for a period of 100 h. During this time, the phenoloxidase activity was monitored with dihydroxyphenylalanine as the substrate. Fig. 3 shows that the laccases were inacti-

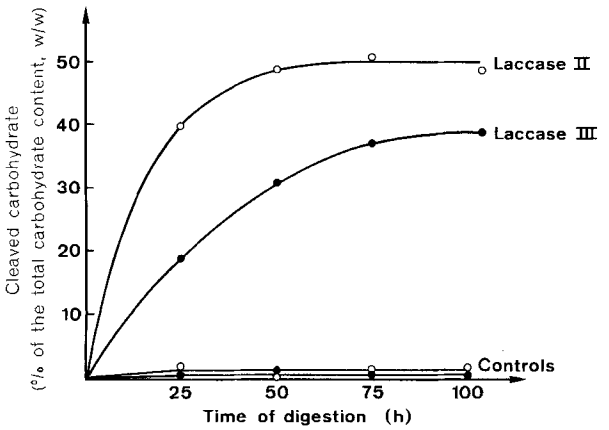

Fig. 4. *Liberation of sugars from laccases by glycosylhydrolases.* For conditions of digestion see Fig. 3. Sugar content in the hydrolysates was determined by the orcinol reaction after precipitation of the proteins with trichloroacetic acid. Controls were untreated laccases

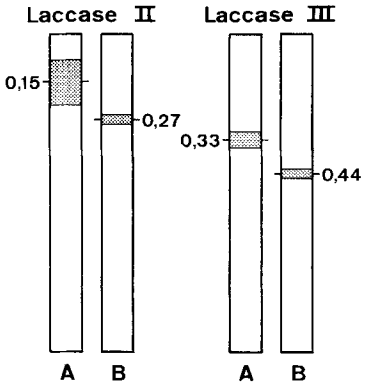

Fig. 5. *Disc-electrophoresis of laccases after treatment with glycosylhydrolases.* For method see text. Staining for phenoloxidase activity with dihydroxyphenylalanine. (A) Before, (B) after hydrolysis of carbohydrate moiety

vated under the influence of the hydrolases, indicating a similar reaction for both enzymes. After 100 h the remaining activities were 30 and 20% respectively. The liberation of sugars was followed by the orcinol method [3]. As can be seen in Fig. 4 nearly 50% of the carbohydrate content of laccase II was hydrolyzed within 50 h. There was no further increase during the next 50 h. Degradation of the carbohydrate portion of laccase III was much slower during the first 25 h and after 100 h a final value of 40% was obtained, a result similar to that of laccase II.

The sugar composition of the hydrolysates was determined by thin-layer chromatography without further acid treatment. After 25 and 50 h only mannose had been liberated; after 75 h galactose appeared in the reaction mixture but only with laccase II; after 100 h small amounts of rhamnose had been split off from both laccases. No free glucose nor *N*-acetylglucosamine could be detected in the hydrolysates. This may be expected if these sugars are located in the initial part of the carbohydrate fractions. This supports the assumption that the carbohydrate is attached to the polypeptide chain by an *N*-glycosidic bond via *N*-acetylglucosamine, as has been discussed earlier for laccase II [30]. This would mean that the carbohydrate moiety of laccase II is composed of 2 or 3 heteropolysaccharide chains of the mannane-type in contrast to 6 and 7 chains of laccase III and the subunits of laccase I respectively.

Properties of Laccases after Degradation of the Carbohydrate Moieties

After treatment of the laccases II and III with glycosylhydrolases, the phenoloxidases were still active. However, they had obtained several new properties.

Alteration of Electrophoretic Mobility. Disc-electrophoresis revealed one single band of laccase II after staining with 3,4-dihydroxyphenylalanine (Fig. 5). The R_F value had shifted from 0.15 to 0.27. A similar alteration, from 0.33 to 0.44, was observed for laccase III. Staining for protein with Coomassie brilliant blue also revealed only one laccase band thus indicating that inactivation is not due to proteolysis.

Loss of Microheterogeneity of Laccase II. A preparation of freshly purified laccase II consists of a population of molecules with constant composition of polypeptide chains, but differs in quantitative sugar composition leading to a microheterogeneity which accounts for the broad and diffuse band in disc-electrophoresis [30]. Digestion with the hydrolases, however, revealed a sharp and narrow band (Fig. 5); cleavage of 50% of the heteropolysaccharide obviously had given a homogenous population of laccase molecules with similar carbohydrate content.

Decrease of Substrate Affinity. From the hydrolysates, the Michaelis constant for dihydroxyphenylalanine was determined. The affinity of the laccases to this substrate was reduced by one order of magnitude (laccase II from 0.65×10^{-3} to 1.35×10^{-2} M and laccase III from 1.75×10^{-3} to 3.05×10^{-2} M).

Decrease of Heat Stability. The half life times at 60 °C of hydrolase-treated laccases II and III were determined as 22 min and 46 min respectively compared with 58 min and 110 min of untreated enzymes [3].

Alteration of Immunological Specificity. Electrophoresis in agarose and subsequent staining of the digested laccases II and III and of untreated controls, showed identical positions for the corresponding enzymes (Fig. 6). The liberation of neutral sugars had diminished the molecular weights to about 15% but obviously had no effect on the electrical charge and as a consequence, on the electrophoretic mobility in a

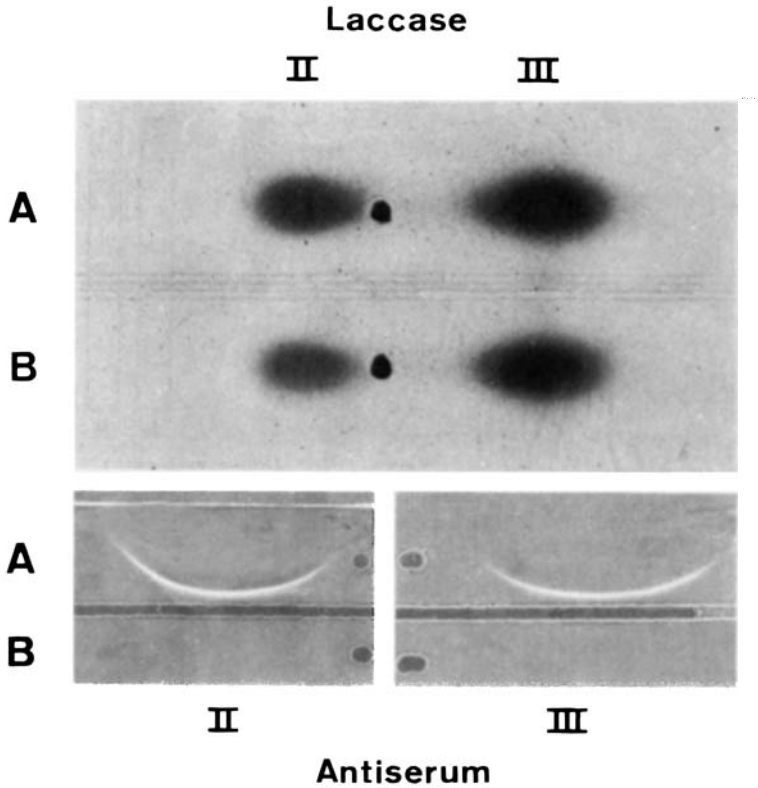

Fig. 6. *Agar gel-electrophoresis and immuno-electrophoresis of laccases II and III.* (A) Freshly prepared native laccases; (B) laccases after digestion with glycosylhydrolases. Staining for phenoloxidase activity with 3,4-dihydroxyphenylalanine

medium without sieve effect. Incubation of the laccases upon agarose-electrophoresis with the isoantibodies gave no immuno precipitation bands with the digested phenoloxidases, whereas the control showed the specific single lines (Fig. 6).

Dissociation

If laccases II and III were exposed to several different experimental conditions such as elevated temperature, extreme pH-values or high concentrations of dissociating solvents such as urea, guanidinium chloride and dodecylsulfate in the absence or in the presence of mercaptoethanol as a reducing agent, native enzymes were completely inactivated but dissociation was not observed. Under similar conditions Butzow [35] found two to four subunits in laccase A of *Trametes versicolor*. However, from one laccase II preparation, an additional faint band was obtained in dodecylsulfate disc-electrophoresis following incubation in a mixture of urea, sodium dodecylsulfate and mercaptoethanol, indicating the possible existence of a quaternary structure at least in this laccase.

In a comparable case, Dijk et al. [24] obtained subunits of a minimal molecular weight of 450000 from the copper protein hemocyanin and smaller fragments only when copper was removed.

Of the 4 copper atoms per mol of native laccases II and III, one was set free under the above mentioned conditions and three remained tightly bound. Complete removal of copper is achieved only by treatment with 70% formic acid.

Exposition of these apo-laccases II and III to a combination of urea, dodecylsulfate and mercaptoethanol gave a new band in dodecylsulfate disc-electrophoresis from laccase II only (Fig. 7) thus demonstrating a possible subunit structure of this enzyme. The cleavage was incomplete: approximately one fourth of the starting material was dissociated. The molecular weight of this new component was computed from the dodecylsulfate disc-electrophoresis to 37000 (35000 – 39000) corresponding to half the size of the native laccase II. So far it has not been possible to achieve dissociation into subunits from laccase III under the same conditions.

DISCUSSION

Three isomeric forms of laccases (laccase I, II and III) of nearly identical substrate specificity were isolated from extracts of *Podospora anserina*. From biochemical studies, the isozymes were differentiated mainly by the following criteria [3, 36]: molecular

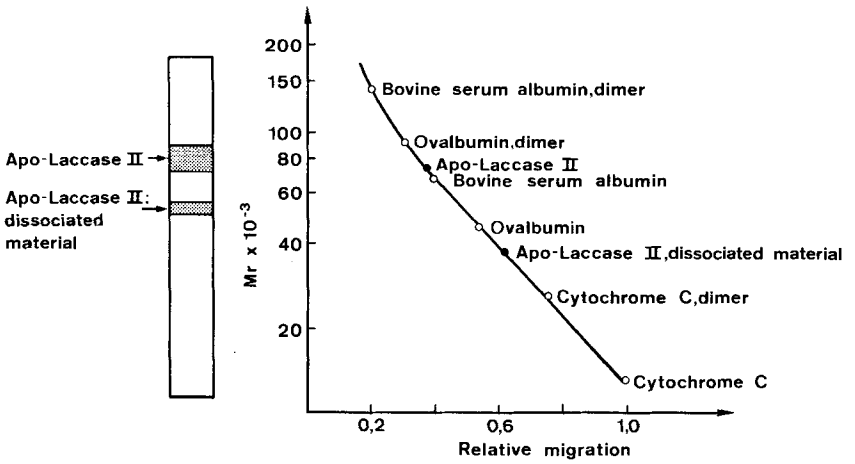

Fig. 7. *Molecular weight determination by dodecylsulfate gel-electrophoresis of apo-laccase II after dissociation.* Left: separation of dissociated laccase II by disc-electrophoresis; right: calibration curve obtained by disc-electrophoresis. For experimental conditions see text

weight; isoelectric point and electrophoretic mobility; kinetic data; serological cross reactions.

A comparison of the biochemical data, however, revealed several remarkable similarities [3, 36, 37]. Firstly, all three laccases are copper proteins with 4 copper atoms per mol (or the four-fold number in case of the tetrameric laccase I). Secondly, the laccases are glycoproteins with comparable amounts of carbohydrates which are composed of the same five sugars. Thirdly, the absorption spectra and the electron paramagnetic resonance spectra are nearly identical. Fourthly, serological cross reactions showed an immunological relationship between all three laccases. Fifthly, the molecular weights of laccases II and III are of similar size.

From these similarities two questions arose. Firstly, are the low-molecular-weight laccases II and III identical, at least in their protein moiety? Secondly, are laccases II and III subunits of laccase I, or are these low-molecular-weight laccases again composed of subunits which contribute to laccase I?

The data presented in this paper support the hypothesis that the three laccases of *Podospora anserina* are coded by different structural genes. This may be concluded for laccases II and III from the following results. The amino acid analyses of laccases II and III showed agreement in 8 amino acids only, but deviated significantly in values for 10 amino acids. Fingerprints of both laccases revealed identical position for only 12 out of 40 peptides. Two different amino acids, leucine and isoleucine, were found terminating the polypeptide chains of laccases II and III respectively at the carboxyl end. On the basis of these facts, the possibility of identity between the polypeptide chains of laccases I and II must be denied.

A comparison of the amino acid values of the low-molecular-weight laccases and laccase I exhibits a similar degree of disagreement. Laccases I and II correspond only in three amino acids whereas laccases I and III shows comparable contents only in five residues. The only C-terminal residue found for laccase I (valine) is different from those of laccases II and III (leucine, isoleucine respectively). When a calculation was made by computer, no combination of laccases II and III could be found, which agreed, on the basis of amino acid composition, with the assumption that these enzymes as complete molecules could be subunits of laccase I. Thus the tetrameric laccase I is composed of subunits neither identical with laccase II nor with laccase III. Further support comes from genetic studies on laccase mutants [6] at least for laccase II. Strains with no laccase II protein have been shown to produce laccase I indistinguishable from wild strain laccase I. These findings together with earlier results: the completely different isoelectric point and electrophoretic mobility and the microheterogeneity of laccase II [3, 30] allow the conclusion that laccase II can not be part of laccase I; neither a subunit of laccase II, nor this enzyme as a whole.

It must be questionable whether the dissociated material obtained from apo-laccase II is a true subunit or an artefact as is supposed in case of haemocyanin [38]. Proteolysis during purification can be excluded since the purified laccases are molecular homogeneous [3].

The genetic analysis of laccases I and III has revealed a more complicated situation. A laccase mutant *incolora* has been shown to produce laccases I and III but these enzymes are both qualitatively altered. Another mutant strain completely lacking laccase activity produces inactive laccase I and III protein.

The coincidental influence of monogenic mutants on two laccases (I and III) led to the suggestion that laccase III as a whole might be a subunit of laccase I. Since this possibility must be excluded from amino

acid analysis there is a possibility that laccase III consists of subunits at least one of which might be a subunit of laccase I monomers. This hypothesis cannot be decided by amino acid analysis, but it could possibly be explained by a general function of the carbohydrate moiety for the laccases. Firstly, all alterations in laccase mutants were found by serological methods only. Secondly, the serological reactions of laccases II and III were completely lost after digestion of 50% of their carbohydrate moiety. Since the laccases with reduced carbohydrate content were still active, the tertiary structure did not seem to be seriously altered. This could be expected if the carbohydrate moiety is responsible for antigenic specificity. Thirdly, this would explain the strong cross reaction between laccases I and III in both antigen-antibody combinations, due to the striking similarity in sugar composition; and also the weak cross reaction of laccase II with laccases I and III, on the other hand, as the result of the poor agreement in sugar composition. Fourthly, the qualitative alterations of laccases I and III in mutant strains could be due to a genetic defect in biosynthesis of the carbohydrate part influencing the characteristics of two or three laccases simultaneously. This could also explain the decrease in activity and in heat stability of laccase I in a mutant strain.

In conclusion the two questions concerning the relationship of the three laccases may be answered as follows. Laccases II and III are not identical. Laccase II is not a subunit of laccase I and shares no subunit with laccase I. Laccase III is not a subunit of laccase I and most probably does not share a subunit with laccase I. Thus the hypothesis of three different structural genes for laccases I, II and III is highly probable.

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