Large Scale Production and Purification of Laccase from Cultures of the Fungus *Polyporus versicolor* and some Properties of Laccase A

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The laccase-producing fungus *Polyporus versicolor* was grown in a 100 liter tank. The growth medium was changed, compared to the earlier composition, for maximal production of the enzyme.

The purification method for the enzyme was changed to a very simple and more efficient method. The main steps in the purification are ammonium sulphate precipitation of the enzyme and anion-exchange chromatography on DEAE A 50 Sephadex.

The amino acid composition, carbohydrate content and elemental composition of laccase A were determined, and the partial specific volume and molecular weight of laccase A were calculated from the values for amino acid and carbohydrate composition.

In the past few years, laccase has been used in several studies of the structure and function of copper-containing enzymes.[1] More or less pure preparations have also been used in investigations of the dehydrogenation of phenolic compounds [2-4] and the degradation of lignin.[5] The enzyme has been obtained from two different sources: from the latex of lacquer trees (*Rhus* species) and from fungi.

In 1958, we published two papers [6,7] describing a method for production of laccase from the fungus *Polyporus versicolor Fr.** From a comparison of various basidiomycetes [7] we concluded that *P. versicolor* was the most promising species for the production of laccase.** This is, for instance, due to the fact that it is an easily recognizable species, common in nature on stumps of deciduous wood. From the sporophores monocaryotic and dicaryotic

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* Synonyms widely used are: *Trametes, Coriolus* or *Polystictus versicolor*.

** *Polyporus versicolor* has been used also for production of cellulolytic enzymes.[11] Strains with high cellulase activity are probably not good for laccase production and *vice versa*; further, nutrient media must be chosen to suit the particular production process. Generally, however, growth requirements of the different strains should be similar, and our results may therefore be of some interest also to those working with other enzymes from this or related fungi.

strains can readily be isolated. Most strains grow rapidly on simple media. Laccase-producing strains secrete most of the enzyme from the mycelium into the surrounding medium, which makes extraction of the mycelium super-fluous; this is in contrast with some other laccase sources, for instance the ascomycete *Podospora*, where the enzyme was obtained by extraction of the mycelium.[8] Although a large number of wood-inhabiting basidiomycetes produce laccase, our studies have not revealed any source better than *Polyporus versicolor*. This fungus has also been used by other investigators using our original methods.[4,9] It is recommended for commercial production of laccase in Japan.[10]

In all preparations of the enzyme there have always been two forms of Cu^{2+} according to electron spin resonance (ESR) spectra.[12] As this could be due to heterogeneity of the enzyme preparations it prompted more detailed preparation studies. Though it was not possible to obtain enzyme with only one type of Cu^{2+}, the investigations led to a simple and improved purification method which is reported here together with some modifications for the production of the enzyme.

As a basis for future work on the more detailed structure and function of the enzyme some properties have been determined for laccase A.

EXPERIMENTAL

Culture of Polyporus versicolor. Polyporus versicolor strain No. 11 a and, in later experiments, No. 12 [7] were used throughout this work. Both are dicaryotic and the mycelia have clamp connections (Fig. 1). No morphological or physiological differences between these strains seem to exist. They were originally isolated in 1953, and at least No. 12 has kept its good laccase-producing ability during 14 years of laboratory culture. Stock cultures were maintained on malt extract agar slants (2.5 % Oxoid malt extract, 2 % Difco agar).

Growth medium. For all cultures, a synthetic solution was used. As was discussed in an earlier publication [7] such a medium was preferred, because additions such as yeast

Fig. 1. Mycelium from tank culture of *Polyporus versicolor* showing the characteristic clamp connections. Phase contrast, × 360.

extract complicated the purification of the enzyme, even though the growth of the fungus is greatly stimulated. The basal medium was described earlier,[13] but some modifications have now been made. The main changes are an increase of the copper content and an addition of phenylalanine and adenine, both of which in several experiments stimulated the growth rate considerably. These latter additions may, however, have little significance for other strains than those described here.

The present composition of the medium is as follows:

Glucose, puriss. (Kebo)	20	g
L-Asparagine, purum (Merck)	2.5	g
D,L-Phenylalanine, puriss. (Fluka)	0.15	g
Adenine (Nutr. Bio.-Corp.)	0.0275	g
Thiamine-HCl	50	μg
KH_2PO_4	1.0	g
$Na_2HPO_4 \cdot 2H_2O$	0.1	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$CaCl_2$	0.01	g
$FeSO_4 \cdot 7H_2O$	0.01	g
$MnSO_4 \cdot 4H_2O$	0.001	g
$ZnSO_4 \cdot 7H_2O$	0.001	g
$CuSO_4 \cdot 5H_2O$	0.002	g
Distilled water	1000	ml

pH about 5.0 after sterilisation.
All salts were p.a. quality.

After distribution in the culture vessels the solutions were sterilized in an autoclave at 120° for 20 min. The 100 liter fermentor was sterilized as specified below.

Propagation of mycelium. For laccase production the mycelium was propagated in three steps:

1) A piece of mycelium was transferred to 20 ml growth medium in 125 ml Erlenmeyer flasks, where it formed a mat covering the surface in 5—7 days at 25°.

2) Two such mycelial mats were disintegrated together with the growth medium and 100 ml distilled water by vigorous shaking in a 250 ml glass stoppered bottle with glass beads. This gave a fairly homogeneous mycelial suspension. From this, 10 ml amounts were transferred to 1000 ml Erlenmeyer flasks containing 500 ml growth medium. The flasks were placed on a rotary shaker run at a speed of approximately 120 rev./min. (2.5 cm radius) in a constant temperature room (25°) for 4 days. After this time the mycelium, in the shape of small pellets, amounts to about 500—700 mg per flask.

3) The contents of 10 shake flasks (5 l of liquid) were used as inoculum for the culture vessel, the 100-liter tank containing 60 l of medium.

Laccase induction. To induce laccase formation, 2,5-xylidine (Fluka No. 53371) dissolved in 50 % ethanol was added after 2—4 days growth, giving a concentration in the growth medium of 2×10^{-4} M. In most experiments a second addition of half this amount of xylidine was made a few days later to increase the inducing effect. Under such conditions, an additional amount of glucose (1 %) was also usually given, since the prolonged incubation tended to deplete the medium of sugar.

Tank culture. The most successful experiments were carried out in the 100 liter fermentor containing 60 liters of growth solution. A description of the fermentor has recently been published by Jönsson[14] (p. 5 and Fig. 1). The fermentor, filled with 40 l distilled water, was sterilized by passing steam through the liquid at a pressure of 1.5 atm. for 30—40 min. By this procedure the volume of water increased to about 50 l. After cooling, 10 l of the tenfold concentrated and separately sterilized growth medium was added. A direct sterilization of the fermentor together with the growth medium was not possible because, for some yet unknown reason, such a technique reduced the growth rate of the fungus considerably.

For the production of laccase the following conditions were applied: temperature 25°, agitation rate 120 rpm, aeration 0.1 liter per liter medium per minute. pH was recorded but not controlled automatically. No antifoam was needed.

The laccase formation was followed daily after the addition of xylidine by determination of the oxidation of catechol in an E.E.L. portable colorimeter at approximately

440 mμ (filter No. 303). The reaction mixture contained 110 mg (1 mM) catechol dissolved in 10 ml 0.1 M acetate buffer pH 5.0 and 0.1 ml test solution. Readings were made after 10 min.

At the end of each experiment, the growth medium was separated from the mycelium by filtration through large Büchner funnels. This solution was then used for the purification of the laccase.

Determination of copper in the enzyme solutions was performed with the biquinoline method, as described earlier,[15] with ascorbic acid as reducing agent rather than hydroquinone. Prior to the copper analyses, the enzyme solutions were dialysed for one day against 0.1 M phosphate buffer, pH 6.0, which was freed from heavy metals by dithizone extractions (0.01 % dithizone in CCl_4).

Activity measurements during enzyme purification. The enzyme activity was measured spectrophotometrically at 25° with N,N-dimethyl-p-phenylenediamine as substrate, as described earlier,[15] with the exception that EDTA was excluded from the substrate medium. The reaction was followed in a Zeiss PMQII spectrophotometer coupled to a potentiometric recorder. The specific activity of the enzyme is expressed as the increase in absorbancy at 323 mμ per cm and minute divided by the absorbancy at 280 mμ per cm of the enzyme solution used.

Visible and ultraviolet spectra of enzyme and pigment forms in 0.1 M phosphate buffer, pH 6.0, were recorded with a Zeiss RPQ 20A recording spectrophotometer.

Purification of the enzyme

Step 1. Ammonium sulphate precipitation. The filtrate from the culture of the fungus was saturated with ammonium sulphate (750 g/l) in large cylinders and cooled in a refrigerator overnight. The precipitate, which had collected on the surface of the liquid, was separated from excess liquid by centrifugation. The precipitate was cooled and transferred to a Dewar for transport from Uppsala to Göteborg for further purification.

The precipitate was dispersed in some 0.1 M phosphate buffer, pH 6.0, in a Waring blendour for 2 min; the suspension was transferred to a dialysis tubing and dialysed for 1—2 days against 10 l of the same buffer. At the end of the dialysis, the suspension often contained a lot of gelatinous material, which entrapped enzyme. To change the gel, in order to free most of the enzyme, the suspension was frozen at —20° for 1 day,[16] then thawed. After thawing, the suspension was filtered through a fluted filter.

The filtrate was concentrated by another ammonium sulphate precipitation, and the precipitate dissolved in minimal amount of 0.1 M phosphate buffer, pH 6.0. The solution was centrifuged for 15 min at 14 600 g. The dark brown precipitate was discarded. Just a little more ammonium sulphate was added to the supernatant, until the enzyme precipitated. After centrifugation as above, a yellow supernatant, which was rich in pigments, was discarded. Due to this extra precipitation, considerable amounts of pigments were excluded and the precipitate could be dissolved in 25—50 ml of the phosphate buffer. This solution was desalted on a 25 × 3 cm column of Sephadex G 25 (in bead form) equilibrated with the same phosphate buffer. Large amounts of low molecular weight pigments were retarded on the column while the enzyme and pigments of high molecular weight were eluted with the void volume. The fraction containing the enzyme was blue-green in colour.

Step 2. Anion exchange chromatography. The blue-green fraction from Sephadex G 25 was then applied to a 25 × 3 cm column of DEAE A50 Sephadex equilibrated with the phosphate buffer used in step 1, and the column was eluted with the same buffer. The flow from the column was set to one drop per two seconds and 10 ml fractions were collected. A blue fraction containing laccase B was eluted unretarded followed by retarded yellow fractions over a course of several hours.

After about 10 h, no more yellow pigments were eluted, which was tested by measuring the fractions in a spectrophotometer at 250 mμ, and another blue band, containing laccase A, had migrated a few centimeters down the column followed by a yellow and a brown zone. The flow was then increased to one drop per second and 25 ml fractions were collected. Laccase A was then eluted in a rather large volume followed by yellow pigments. The last fractions of the two enzyme forms were often contaminated by pigments and must be re-chromatographed. Sometimes the first fractions of both enzyme forms

were turbid and contained a high molecular weight impurity, which was removed by passage through a millipore filter with a pore size of 0.8 μ.

The absorbance of the fractions containing the two enzyme forms was measured in a spectrophotometer at 280 mμ and 250 mμ. Fractions having an absorbance ratio 280 mμ/250 mμ of 2.0 or more were pooled, and the enzyme was concentrated by ammonium sulphate precipitation followed by desalting on Sephadex G 25 equilibrated by the phosphate buffer. The enzyme was stored in 2 ml fractions, containing about 24 mg enzyme, in plastic bottles at $-20°$ until used. The fractions having an absorbance ratio 280 mμ/250 mμ less than 2.0 were re-chromatographed on the same anion exchanger. All preparation steps were performed at $+ 5°$.

Preparation of laccase A samples for elemental composition, amino acid analysis and carbohydrate composition. Two different preparations of laccase A were used in these investigations. The two protein samples to be analysed were desalted by dialysis against distilled water. The copper was removed by precipitation of the protein with 10 % w/v (final concentration) trichloroacetic acid, followed by washing the precipitate three times with 10 % trichloroacetic acid, three times with 80 % v/v ethanol, twice with absolute ethanol, twice with acetone and three times with diethyl ether. The samples were allowed to dry at room temperature.

Elemental composition. Total nitrogen was determined by a micro-Kjeldahl method and total sulphur by a methylene-blue method.[17] Both protein samples were analysed in duplicate. The nitrogen and sulphur values were corrected for the moisture content of the samples after determination of the weight loss on drying at 110° for 24 h.*

The carbohydrate content of the enzyme was determined both on trichloroacetic acid precipitated samples and on native enzyme samples dialysed against distilled water. Sialic acid, hexoseamine, hexoses, and pentoses were determined according to methods reported by Svennerholm.[18-21],**

Amino acid analysis was performed on a Beckman 120 B automatic amino acid analyser according to the procedure of Spackman, Stein and Moore.[22] For hydrolysis about 5 mg of each protein sample was mixed with 2 ml 6 M HCl in a Pyrex tube (25 mm × 65 mm), which was then cooled in ethanol dry ice bath and evacuated with an oil pump. The contents were allowed to melt under vacuum and the tube was sealed with a flame. After 20 h or 70 h hydrolysis at 110°, the acid was removed in a rotary evaporator. The hydrolysates were dissolved in 5 ml starting buffer for the amino acid analyser. Total nitrogen in the hydrolysates was determined by a micro Kjeldahl method.

Cysteine and cystine were determined as cysteic acid after oxidation of the enzyme with performic acid according to Moore.[23] After 18 h hydrolysis, as above, the samples were analysed with the amino acid analyser.

Determinations of tryptophan were performed on native protein samples according to the colorimetric method of Spies and Chambers,[24] "Procedure K", using 5 h or 18 h time for "Reaction I".

Partial specific volume of laccase A was calculated from the amino acid composition and the carbohydrate content. The values for the partial specific volumes of the amino acid residues were taken from Cohn and Edsall.[25] In these calculations the amide groups were distributed according to the ratio found for glutamic acid to aspartic acid. The value for the partial specific volume of the carbohydrate part was estimated by use of earlier reported values for polysaccharides with essentially the same composition as the carbohydrate in the enzyme.[26]

Molecular weight. An attempt to calculate the molecular weight of the enzyme based upon the known amino acid content, was carried out according to the procedure of Nyman and Lindskog.[27] The values for lysine, tyrosine, and ammonia were omitted in these calculations because of their poor reproducibility. The values for tyrosine were disturbed by hexoseamine appearing just before tyrosine in the chromatograms. The recovery of lysine might be influenced by a reaction between this amino acid and carbohydrates or decomposition products of carbohydrates formed during hydrolysis, or depend on possible bonding of carbohydrate to this amino acid in the native enzyme.

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* The determinations of nitrogen, sulphur, and moisture content were performed by Dr. L. Gustafsson at the Department of Analytical Chemistry, University of Uppsala.

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RESULTS

Culture of the fungus. Results obtained in some typical tank culture experiments are collected in Table 1.

The purification of the enzyme from a typical culture (the last culture in Table 1) is reported in Table 2. As mentioned in the experimental section, the enzyme is divided into two fractions when chromatographed on DEAE A 50 Sephadex. These two fractions are identified as the A and B forms of laccase according to earlier nomenclature, by the finding that the A form is more strongly bound to the anion exchange groups than the B form, as found earlier [16] when DEAE-cellulose was used. The two forms differ with respect to specific activity and relative amounts consistent with earlier results.[16] The specific activity increases considerably when the enzyme is purified, which is due to the removal of large amounts of pigments absorbing at 280 mμ. As earlier found,[6] essentially no other proteins than laccase exist in the culture medium.

Table 1. Culture of *Polyporus versicolor* in the 100 liter fermentor.

Expt. No.	Additions (days after start)		Duration of culture (days)	Laccase activity in colorimeter units after days					Remarks	Final yield (mg pure laccase A)
	xylidine	glucose		4	5	6	7	8		
1	3, —	—	6	9	22	23				
2	3, —	3	5		41					800
3	3, 6	5	7	6	11	15	23			
4	3, 4, 5	5	6		0				Contaminated by *Aspergillus*	
5	3, 6	3	8				8	28 37	Large temp. fluctuations (8−40°) }	1.600
6	2, 3	3	6			37				
7	3, 5	5	7	13	29		70		Larger inoculum	1.200 (+ 440 laccase B)

Table 2. Purification of laccase. Total protein is expressed as the absorbance at 280 mμ in 1 cm light path multiplied by the volume in milliliters. The specific activity is expressed as defined in the experimental section. The total activity is specific activity multiplied by total protein.

Fraction	Total protein	Specific activity	Total activity	Activity yield in per cent
Filtrate of culture medium	122 000	27.2	3 320 000	100
Solution after dialysis of first ammonium sulphate precipitate	12 000	275.9	3 320 000	100
Solutions from chromatography on DEAE A 50 Sephadex.				
Laccase A	1580	1338.9	2 120 000	64 } 74 %
Laccase B	568	592.2	336 000	10 }

As all the pigment forms that might contaminate the enzyme, when this purification method is used, were found to have spectral properties different from the enzyme (for example, they all have an absorbance ratio of 280 mμ/250 mμ less than 1) it is easy to test the purity of the enzyme just by measuring the absorbance at these wave lengths. The different forms of the pigments were found to contain no copper by chemical analysis.

The recovery in this typical preparation was 75 % and the total amount of the enzyme was 1.20 g of laccase A and 0.44 g of laccase B. The amount of enzyme varies somewhat in different cultures (see Table 1).

The absorbance at 280 mμ of a 0.1 % solution of laccase A (1 cm light path) was calculated from seven preparations of the enzyme. The enzyme concentration was estimated from the copper content and assuming 4 copper atoms per enzyme molecule and a molecular weight of 64 400 (see later section for molecular weight). The mean value was 1.16. A value of 1.17 was obtained for one preparation of laccase A when the enzyme concentration was determined by nitrogen analyses and the mean value for total nitrogen reported in Table 3 was used.

The mean value of absorbance at 610 mμ (1 cm light path) per mole of copper, of 11 preparations of laccase A and B, was found to be 1230.

Elemental composition of laccase A. In Table 3 the results of the analyses for total nitrogen and total sulphur are reported. In comparison with earlier data,[16] the nitrogen value is now about 5 % higher. The nitrogen content of laccase is lower than in most proteins, due to the high content of carbohydrates, the nitrogen content of which is much lower than that of amino acids (see Table 5).

The sulphur content might give an estimation of the molecular weight of the enzyme. If there are 10 sulphur containing amino acids in the enzyme, the molecular weight should be 64 650 g per mole. That value is consistent with the earlier reported value 62 000 g $\pm$ 3000 g obtained from ultracentrifugation data.[16] It is also consistent with the value calculated from the amino acid composition by the method of Nyman and Lindskog;[27] that calculation gave a marked minimum around 64 000 g.

Table 3. Elemental composition of laccase A.

Protein sample	Nitrogen [a]	Sulphur [a]
Preparation 1	15.01	0.496
	15.05	0.486
Preparation 2	15.24	0.505
	15.23	0.498
Mean value	15.13	0.496

[a] The values for total nitrogen and sulphur refer to trichloroacetic acid precipitated protein samples corrected for moisture after drying at 110° for 24 h; the values are expressed as weight per cent.

Table 4. Carbohydrate analysis of laccase A.

Protein sample	Hexoseamine [a]	Hexose [a]	Galactose/Mannose	Sialic acid
Native enzyme [b]	2.88 [c]	7.20	$\cong 3/1$	— — —
Trichloroacetic acid precipitated protein	2.95 [c]	7.20	$\cong 3/1$	— — —
Average	2.92	7.20		
Total [a]		10.12		

[a] In weight per cent.
[b] Dialysed against distilled water.
[c] Expressed as N-acetylglucoseamine.

The carbohydrate analyses are reported in Table 4. It can be noticed that essentially the same results were obtained whether the sample to be analysed was precipitated with trichloroacetic acid or if native enzyme was used directly. This result strongly favours the view that the carbohydrate is covalently bonded to the protein, and not a contaminating impurity. No sialic acid was detected in either sample. Among neutral sugars, only galactose and mannose could be found after hydrolysis and chromatography of the samples.

The amino acid composition of the enzyme is recorded in Table 5. The values obtained for the various amino acids in the analyses performed with four 20-h hydrolysates were averaged, as were the values obtained with four 70-h hydrolysates. The values from four analyses in which the enzyme was subjected to performic acid oxidation followed by 18 h hydrolysis were also averaged. If the difference between the 18-h, 20-h, and 70-h average values for a particular amino acid was less than 3 %, the values from all twelve amino acid analyser runs were averaged.

The value for half cystine is only from oxidized samples; the values from the other hydrolysates were much smaller, amounting to only 56 % in 20-h hydrolysates and 72 % in 70-h hydrolysates, compared to the oxidized samples. The value reported in the table corresponds to six residues of half cystine; together with the four residues of methionine, this makes ten sulphur containing amino acid residues, an expected amount for a molecular weight of 64 400 and a sulphur content of 0.496 % (see Table 3).

The value for tryptophan is the average of five determinations. No significant differences in the values were found whether 5 h or 18 h were used for "Reaction I".[24]

Table 5 contains the following data: In column 1 the weight percentages of the individual amino acid residues and carbohydrates are reported. Minimal molecular weights (column 2) were calculated from the weight percentages by dividing the molecular weight of the individual amino acid residue by the weight percentage of that amino acid residue. Values for the amino acid residues (column 3) were then obtained for 64 000 g, the molecular weight of the enzyme as estimated by the methods described in the previous section.

Table 5. Amino acid composition of laccase A.

Amino acid residue	1 Residue in g per 100 g of protein	2 Minimal molecular weight	3 Residues per 100 g of protein	4 Nearest integral number × minimal molecular weight	5 Residues in moles per 64 400 g of protein	6 Integral numbers of residues per 64 400 g of protein	7 Nitrogen in g per 100 g of protein
Aspartic acid	14.09 ± 0.12	816.9	78.34	63 718	78.7 ± 0.7	79	1.713 ± 0.015
Threonine	6.50 ± 0.08 [a]	1555.4	41.15	63 771	41.4 ± 0.5	41	0.900 ± 0.011
Serine	4.77 ± 0.10 [a]	1826.0	35.05	63 910	35.3 ± 0.7	35	0.767 ± 0.016
Glutamic acid	5.37 ± 0.10 [b]	2404.1	26.62	64 911	26.8 ± 0.5	27	0.583 ± 0.011
Proline	6.35 ± 0.04	1529.1	41.85	64 222	42.1 ± 0.3	42	0.916 ± 0.006
Glycine	3.80 ± 0.02 [b]	1502.6	42.59	64 612	42.9 ± 0.2	43	0.932 ± 0.005
Alanine	6.29 ± 0.05 [b]	1130.4	56.62	64 433	57.0 ± 0.5	57	1.239 ± 0.010
Valine	5.44 ± 0.09 [c]	1821.7	35.13	63 760	35.4 ± 0.6	35	0.769 ± 0.013
Half-cystine	0.92 ± 0.02 [b]	11108.7	5.76	66 652	5.8 ± 0.1	6	0.126 ± 0.003
Methionine	0.82 ± 0.02	16000.0	4.00	64 000	4.0 ± 0.1	4	0.088 ± 0.002
Isoleucine	5.33 ± 0.06 [c]	2123.8	30.13	63 714	30.3 ± 0.3	30	0.659 ± 0.007
Leucine	6.13 ± 0.04	1846.7	34.66	64 635	34.9 ± 0.2	35	0.758 ± 0.005
Tyrosine	3.69 ± 0.28	4422.8	14.47	61 919	14.6 ± 1.1	15	0.317 ± 0.024
Phenylalanine	7.09 ± 0.10	2076.2	30.83	64 362	31.0 ± 0.4	31	0.675 ± 0.009
Amide ammonia	1.46 ± 0.05	1164.4	54.93	64 042	55.3 ± 1.9	55	1.201 ± 0.041
Lysine	1.10 ± 0.05	11654.6	5.49	58 283	5.5 ± 0.3	6	0.240 ± 0.011
Histidine	3.62 ± 0.06	3790.1	16.89	64 432	17.0 ± 0.3	17	1.108 ± 0.018
Arginine	3.39 ± 0.09	4607.7	13.89	64 508	14.0 ± 0.4	14	1.216 ± 0.032
Tryptophan	1.15 ± 0.04 [d]	16191.3	3.95	64 765	4.0 ± 0.1	4	0.173 ± 0.006
Total for residues	85.85 ± 1.37 [e]			64 400 [f]		521	14.380 ± 0.245
Carbohydrates	10.12 [g]						0.188
Total	95.97 ± 1.37						14.568 ± 0.245 = 96.29 ± 1.62 %

The deviations given are the average values of the absolute deviations from the mean.

[a] Values obtained by extrapolating to zero time of hydrolysis, assuming first-order kinetics as recommended by Moore et al.[29]
[b] Values from oxidized samples included.
[c] Values from 70-h hydrolysates only.
[d] Determined colorimetrically on unhydrolysed protein samples.[24]
[e] The value for amide ammonia is omitted from the total.
[f] The values for lysine, tyrosine, and amide ammonia are omitted.
[g] The value is taken from Table 4.

Table 6. Partial specific volume of laccase A.

		$\bar{v}$	$\bar{v} \times$ wt %
Total for amino acid residues per 100 g protein (in weight per cent)	87.31 [a]	0.715	62.448
Carbohydrates	10.12	0.62	6.281
Total	97.43		68.729

[a] This value has been obtained after including the amide groups (see Table 5, column 1).

Values for $\bar{v}$ for the amino acid residues were taken from Cohn *et al.*[25] Value for $\bar{v}$ for the carbohydrate part was taken from Greenwood.[26] The partial specific volume of laccase A was calculated as follows: $68.729/97.43 = 0.705$ ml per g.

A molecular weight of 64 400 could then be computed from these numbers by multiplying the nearest integral number for each amino acid residue and the minimal molecular weight for the respective amino acid residue (column 4), and then the moles of each amino acid residue per mole of enzyme was calculated (column 5) for this molecular weight. Nearest integral numbers of residues are reported in column 6.

The recovery of nitrogen from the amino acids and carbohydrates is given in column 7; compared to the recovery in per cent of amino acids and carbohydrates there is no significant difference.

Partial specific volume of laccase A. In Table 6 the specific volume of the enzyme is reported. The low specific volume, 0.705, is due not only to the low specific volume of the carbohydrate part, the estimated value of which is 0.62, but also to the amino acid composition. Amino acids of low specific volume are in the majority; the specific volume for the amino acid part is only 0.715. The value of 0.705 is lower than the earlier reported value of 0.73;[16] this earlier value must be to high, as it exceeds even the value for the amino acid part of the molecule.

DISCUSSION

The production of laccase as described above takes advantage of the fact that the enzyme formation is strongly influenced by the addition of inducing substances. On the basis of earlier results, 2,5-xylidine was selected as an inducer. Owing to the specificity of the induction process, and the fact that laccase is completely secreted into the growth medium, the protein fraction of the medium is almost exclusively laccase.

Before the addition of xylidine, the laccase production is insignificant. However, because compounds of this type are rather toxic to the fungus, the addition was delayed until sufficient mycelium had formed in the culture vessels. A difficulty may here lie in choosing the right moment, since the synthesis of laccase is certainly dependent on the mycelial development and other conditions during the initial phases of growth. The somewhat irregular yields in the different batches (Table 1) can probably be explained by slight variations in these respects. The process does not, however, seem to be very

sensitive to temperature fluctuations (Table 1, expt. 5). Some cases of complete failure of the laccase production are certainly due to contamination of the cultures by foreign microorganisms, usually yeasts or moulds. This can be verified by microscopic examination, since the mycelium of *Polyporus versicolor* is characterized by typical clamp connections (see Fig. 1).

The purification method reported here has the great advantage of being very simple as well as of employing very mild procedures. The preparation time is decreased to about half the time compared to the earlier method,[16] and the enzyme is apparently more pure as judged by the decrease in absorbance at 280 mμ and the absorbance ratio 280 mμ/250 mμ. The impurities before purification consists mainly of pigments which seem to be associated to the enzyme molecule; for example, they cannot be completely removed in zone electrophoresis or gel filtration; the anion exchanger, however, apparently can absorb and remove them.

Despite the very mild purification conditions, the enzyme still contains two forms of Cu^{2+} by ESR analyses.[28] It is not possible to obtain ESR spectra of the enzyme in the dilute culture medium and in that way determine whether the enzyme contains both forms of Cu^{2+} before any preparation step is introduced. It is open to doubt, however, that all the different preparation methods used up to now should alter the environment of some of the Cu^{2+} similarly in both laccase A and B so as to produce the two forms of Cu^{2+} in the ratio 1:1 found in both isoenzymes.[28] Furthermore it has not been possible to alter this ratio by repeated ammonium sulphate precipitations or re-chromatography on DEAE A 50 Sephadex of both isoenzymes. It is thus most probable that the two Cu^{2+} atoms in the enzyme molecule have different environment in the native enzyme.

There seems to be no contaminating amino acids or short-chain peptides in the preparations of the enzyme used in this investigation, as judged by the fact that no amino acids or peptides were detected when the supernatant after precipitation of the enzyme with trichloroacetic acid was chromatographed in the amino acid analyser.

Proline and glycine show very small deviations in recovery for all amino acid analysis runs, and there is no difference in recovery between 20-h and 70-h hydrolysates. These amino acids, then, might serve as an "internal standard" in hydrolysates of enzyme for experiments in which certain amino acid residues are to be reacted with specific reagents, for example in order to get information on amino acids in the active site.

The enzyme has an excess of acidic amino acids (aspartic acid + glutamic acid — amide groups) over basic amino acids (arginine + histidine + lysine) of 14. This is consistent with the findings that the enzyme (laccase A) is rather strongly bonded to a DEAE A 50 Sephadex at pH 6.0, and is also consistent with preliminary data from determination of the isoelectric point of laccase A.

The amino acids present in low amount, *e.g.* tryptophan, lysine, methionine, and half-cystine, are all present in a multiple of two. It is thus tempting to assume that the enzyme might consist of two identical subunits. The finding of two Cu^{2+} and two Cu^+ also favours this hypothesis. The presence of subunits in the enzyme is now being investigated in this laboratory.

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