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PURIFICATION AND PROPERTIES OF LACCASE AND STELLACYANIN FROM *RHUS VERNICIFERA*

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SUMMARY

1. A new simple and efficient method for the preparation of laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2) and stellacyanin from the latex of the Japanese lacquer tree *Rhus vernicifera* is presented. The two proteins were obtained in increased yields as compared with earlier methods and in a high degree of purity, as judged from ultracentrifugation, gel electrophoresis, and isoelectric-focusing electrophoresis as well as from chemical and spectroscopic properties. Together with the main components of laccase and stellacyanin, other chromatographic forms of both proteins were always obtained in small amounts.

2. The amino acid and elemental compositions of laccase were determined. Only about 55 % of the laccase molecule is made up of amino acid residues, which accounts for the low nitrogen content of 10.4 %. The copper content of laccase is 0.23 %, which corresponds to four copper atoms per protein molecule of a molecular weight of 110000.

INTRODUCTION

Laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2), a copper-containing oxidase obtained from the latex of the Japanese lacquer tree *Rhus vernicifera*, was first discovered in 1883 by YOSHIDA[1] and about 10 years later BERTRAND[2] partially purified the enzyme. More recently laccase has been prepared by different methods by NAKAMURA[3], OMURA[4], PEISACH AND LEVINE[5], and OSAKI AND WALAAS[6].

Another copper protein, Rhus blue protein or stellacyanin, with not yet known physiological role, also obtained from the same latex, was first characterized by OMURA[7] even if it had been observed by KEILIN AND MANN[8] 20 years earlier.

The properties of these two copper proteins have been investigated by several of the above-mentioned investigators. However, the results obtained in different laboratories often show considerable discrepancies. For instance, the reported hydrodynamic properties of laccase vary considerably, with the result that molecular weights from 120000[3] to 141000[4] have been assumed. The claimed number and valence of the copper atoms in laccase also differ. NAKAMURA[3] claims that there are four Cu^{2+} in the molecule. OMURA[4] reports between five and six copper atoms, but their valence state was not determined. BLUMBERG *et al.*[9], on the basis of six copper atoms in

laccase, found four Cu^{2+} detectable by electron paramagnetic resonance (EPR) and two EPR-nondetectable ions. In addition, the reported optical properties of laccase vary between the laboratories[4,10], published EPR spectra are dissimilar[9-11] and sometimes quite different EPR spectra have been published, even from the same laboratory[10,11].

In this laboratory fungal laccase and ceruloplasmin have been investigated for some years (for example, see refs. 12 and 13), and there has been growing interest for performing comparative studies on other laccases and copper proteins. In view of the discrepancies concerning the reported properties of Rhus laccase and stellacyanin, we have found it necessary to reinvestigate certain basic properties of these two proteins as a first step towards a better understanding of their function.

MATERIALS AND METHODS

Purification of laccase and stellacyanin

The purification procedure described below has been used in five preparations of laccase and stellacyanin and the following description presents the observations from a representative preparation together with a few minor differences observed in other preparations. The starting material was an acetone powder prepared from the natural lacquer of *Rhus vernicifera* of Japanese origin, obtained from Saito and Co. Ltd., Tokyo, Japan.

Potassium phosphate buffers (pH 6.0) were used in all steps in the purification procedure.

Step 1: *Extraction.* The acetone powder (165 g), extracted from 2 kg of natural lacquer, was suspended in 3.5 l of 0.01 M buffer and treated for 2 min in a homogenizer. The suspension was stirred overnight and then filtered through a Büchner funnel. The filtrate was turbid and had a greenish color.

Step 2: *Cation-exchange chromatography.* The filtrate was applied to a column (25 cm × 8 cm) of CM-Sephadex C-50 equilibrated with 0.01 M buffer. Fractions of 18 ml were collected. A blue band, about 5 cm in width, was formed at the top of the column, and large amounts of yellow and turbid fractions were eluted during the application. The column was then washed with 0.05 M buffer (usually 1–2 l) until the eluate no longer absorbed at 250 nm, whereafter the buffer concentration was increased to 0.1 M. The blue band, still at the top of the column, now divided. One part of it remained at the top of the column while the other part was eluted in a volume of about 1.5 l. Ahead of the blue band, which was found to be laccase, yellow and greyish-turbid fractions often appeared. Sometimes yellow fractions followed close after laccase. The column was further washed with the same buffer until the absorption at 250 nm of the eluate was zero.

The buffer concentration was then increased to 0.15 M, often causing yellow fractions and sometimes small amounts of greenish material to be eluted. The greenish fractions showed the same catalytic activity and gave the same EPR spectrum as the main portion of laccase eluted earlier.

The buffer concentration was further increased to 0.2 M when the eluate showed no absorption at 250 nm. The blue band, which had migrated a few cm down the column started to be eluted. It was often preceded by yellow fractions and sometimes by small amounts of greenish fractions having no laccase activity but showing the

same EPR spectrum as the blue band that followed. This blue band represented stellacyanin according to its EPR spectrum and its lack of laccase activity. In Fig. 1 the elution pattern from the CM-Sephadex C-50 column of this representative preparation is shown.

In one preparation there remained a narrow blue band at the very top of the column. This band could be eluted with 0.2 M Na_2HPO_4. It had laccase activity, but the specific activity was only about 10% as compared with that of the main fraction of laccase, and the EPR spectrum was different.

The main fraction of stellacyanin obtained in most preparations was pure at this stage according to the gel electrophoresis test. If found impure, it was rechromatographed on a column (10 cm × 5 cm) of CM-Sephadex C-50 by the same procedure as above. The pure stellacyanin was often very dilute and therefore concentrated by the following procedure. The solution, generally about 1 l, was diluted with the same volume of distilled water and then applied to a column (5 cm × 5 cm) of CM-Sephadex C-50 equilibrated with 0.1 M buffer. The sharp blue band, which was formed at the top of the column during application, was afterwards eluted in a small volume with 0.2 M Na_2HPO_4. Stellacyanin was in most cases desalted on a Sephadex G-25 column and stored in small aliquots at $-30°$.

Step 3: *Anion-exchange chromatography*. The main fraction of laccase was further purified by the following procedure. It was first dialyzed against 0.01 M buffer and then applied to a column (15 cm × 2.5 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. Laccase was eluted unretarded while a yellow band remained at the top of the column. In most preparations laccase was obtained pure by this procedure according to the gel electrophoresis test. If not, it was rechromatographed according to the procedure reported under Step 2.

The volume of laccase solution after the chromatography on DEAE-Sephadex was often 1–2 l, and the enzyme was generally concentrated by application of the solution to a column (5 cm × 5 cm) of CM-Sephadex C-50, equilibrated with 0.01 M buffer. It formed a narrow band at the top of the column and was then eluted in a high concentration by 0.2 M buffer. After desalting as in the case of stellacyanin, laccase was stored in small aliquots at $-30°$ until used. Laccase and stellacyanin solutions were sometimes concentrated by ultrafiltration, but the concentration on ion-exchange columns was preferred as this method was thought to be milder and also automatically included a purification step.

Sometimes laccase solutions contained small amounts of Cu^{2+} of a different form as compared with the other two forms of Cu^{2+} normally found according to EPR spectra. It could be freed from this extra form of copper by passing the enzyme solution through a column (10 cm × 1 cm) of Chelex 100 equilibrated with 0.01 M buffer. After the passage of the enzyme there remained a narrow blue band at the top of the column.

All steps of the purification were performed in a cold room at a temperature of about 5°.

Chemicals

Reagent grade chemicals, without further purification, and glass distilled water were used for making all solutions. Quinol was purchased from May and Baker Ltd., Dagenham, England, and *N*,*N*-dimethyl-*p*-phenylenediamine from Merck, Darmstadt,

Germany. The chelating resin Chelex 100, 200–400 mesh, was obtained from Bio-Rad Laboratories, Richmond, Calif., and the Sephadexes were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Gel electrophoresis

The purity of the main fractions of laccase and stellacyanin was tested by polyacrylamide-gel electrophoresis according to standard methods[14]. Laccase was tested in two different buffer systems, Tris–borate buffer (pH 9.5) or β-alanine–acetate buffer (pH 4.5), whereas stellacyanin was run only in the second buffer.

About 10 μg of protein were applied to the gel, and the electrophoresis was run for 60 min at a current of 3 mA/gel. Coomassie Brilliant Blue R-250, 0.25 % in 20 % trichloroacetic acid, was used to stain the protein bands whereafter the gel was destained with 7 % acetic acid.

Copper analyses

Determination of copper in solutions of laccase and stellacyanin was in most cases performed by the biquinoline method[15], as described earlier[16], with ascorbic acid as reducing agent. However, ascorbic acid reduces the blue color of laccase very slowly, and thus the reduction of Cu^{2+} in the enzyme could be incomplete, resulting in low values for copper content. To test the validity of the use of the biquinoline method for copper determinations with laccase solutions, two samples of laccase were also analyzed by the atomic absorption method. A Perkin–Elmer atomic absorption spectrophotometer 303 was used. Copper standards contained between 2 and 10 mg of copper per l 0.01 M HCl. Prior to all copper analyses, the protein samples were dialyzed against deionized water. The concentration of laccase was determined by nitrogen analysis of the samples by a micro-Kjeldahl method.

Isoelectric focusing

The isoelectric focusing of laccase and stellacyanin was essentially performed according to the method published earlier[17]. The carrier ampholyte gradient covered a pH range from 3 to 10. Protein from the main fraction and the second chromatographic form of laccase as well as from the main fraction of stellacyanin was mixed and run together. About 5 mg of each protein were used in each run. Samples for enzyme activity measurements were taken from the fractions obtained by emptying the isoelectric-focusing column. These samples thus contained both sucrose and carrier ampholytes besides laccase and stellacyanin.

Enzyme activity measurements

Enzyme activity was measured by a spectrophotometric method described earlier[16] with 0.6 mM *N,N*-dimethyl-*p*-phenylenediamine as substrate with the exception that EDTA was omitted from the substrate medium and the pH was 7.5. The photometer used was a Zeiss PMQ II coupled to a potentiometric recorder. The temperature was maintained at 25°. The enzyme activity is expressed as the increase in absorbance at 323 nm per min per μmole of protein added to 3 ml substrate in a 1-cm cell. An oxidase unit corresponds to an increase in absorbance of 1.

Enzyme activity was also measured by recording the oxygen consumption in substrate solutions with a polarographic method[18]. The oxygen electrode used in these experiments was of Type E 5046 from Radiometer, Copenhagen, Denmark, and it

was coupled to a gas monitor of Type PHA 9276 connected to a pH meter Type 25 from the same manufacturer. The thermostated reaction chamber contained 2.2 ml substrate solution and the oxygen electrode was connected to the reaction chamber in a gas-tight manner to avoid oxygen leakage into the substrate. Solutions of the substrate quinol were prepared immediately before use, to avoid extensive autooxidation, by dissolving various amounts of the substrate in air-equilibrated 0.1 M phosphate buffer (pH 7.5) at 25°. Enzyme solution, 10–20 μl, was introduced into the substrate solution by a Hamilton microsyringe through a small hole in the cap of the reaction chamber. Oxygen leakage through this hole was found to be negligible. The reaction mixture was stirred by a magnetic stirrer and the temperature was maintained at 25°.

Amino acid and elemental composition of laccase

For these determinations the enzyme was first treated in the following way. A solution of laccase in water (about 200 mg in 10 ml) was mixed with 5 ml of 20 % (w/v) trichloroacetic acid which did not precipitate the enzyme but made it colorless. Acetone (35 ml) was added and a precipitate formed. The precipitate was washed twice with acetone, twice with diethyl ether and was then allowed to dry at room temperature.

Part of the protein precipitate (70 mg) was used in the analyses of elemental composition. Nitrogen was determined by a micro-Kjeldahl method and sulfur by a methylene-blue method[19]. The analyses were made in duplicate, and the values were corrected for the moisture content in the samples, on the basis of the weight loss on drying at 110° for 24 h.

Another part of the protein precipitate (60 mg) was dissolved in 12 ml of formic acid, and eight 1-ml samples were transferred to Pyrex ignition tubes and dried on a rotary evaporator. Four samples were subjected to oxidation and hydrolysis according to MOORE[20] for the determination of methionine and half-cystine. Two samples were hydrolyzed in 6 M HCl at 110° for 72 h and two samples for 24 h. All hydrolysis tubes were carefully evacuated to 0.2 mm Hg and then sealed by a flame.

Determinations of tryptophan were performed on native enzyme samples according to "Procedure K" in the method of SPIES AND CHAMBERS[21].

Amino acid analyses were performed with a Beckman 120 B automatic amino acid analyzer according to the standard procedure[22] with the exception that the second buffer used on the long column in some experiments was 0.185 M (pH 4.0). This buffer gave a better separation of amino sugars appearing just ahead of tyrosine.

Total nitrogen in the hydrolysates and the native enzyme samples used for the determination of tryptophan was determined by a micro-Kjeldahl method.

Optical spectra of laccase and stellacyanin

Visible and ultraviolet absorption spectra of laccase and stellacyanin were recorded at 25° in a Zeiss M4Q II or a Cary 15 recording spectrophotometer with a 1-cm light path. The protein solutions contained 0.1 M potassium phosphate buffer (pH 6.0).

Partial specific volume of laccase

The partial specific volume of laccase was calculated from the amino acid composition on the assumption that the rest of the laccase molecule consists of carbo-

hydrates with a partial specific volume of 0.62, which is a common value for the carbohydrates in related glycoproteins, *e.g.* fungal laccase[23]. The values for the partail specific volumes of the amino acid residues were taken from COHN AND EDSALL[24] and the amide groups were distributed in proportion to the ratio of glutamic to aspartic acid residues found in the analyses.

RESULTS

Preparation of laccase and stellacyanin

Fig. 1 shows the elution pattern from the CM-Sephadex C-50 column. About 100 fractions, containing brown and yellow pigments, have been eluted before the buffer concentration was increased to 0.1 M. The first peak in Fig. 1, designated A, is the main fraction of laccase and contains at least 90 % of the total enzyme. Peak B is a greenish-yellow fraction containing pigments and often a second chromatographic form of laccase. Peak C contains yellow pigments with no laccase activity. Peak D represents yellow pigments and in some preparations small amounts of a second chromatographic form of stellacyanin. Peak E contains the main fraction of stellacyanin, which generally is pure after this first step. It contains no laccase, since measurements with the substrate *N,N*-dimethyl-*p*-phenylenediamine gave less than 0.01 % activity compared to the same molar concentration of laccase.

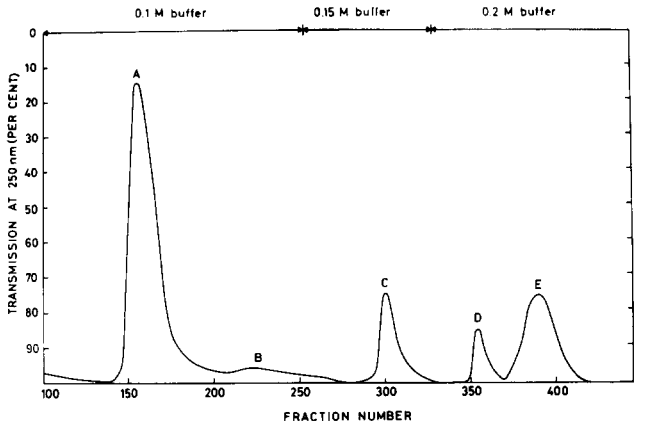

Fig. 1. Ultraviolet transmission (250 nm) elution pattern of the CM-Sephadex C-50 column. A represents the main fraction of laccase. B, C, and D are pigment fractions sometimes mixed with small amounts of laccase and stellacyanin. Peak E represents the main fraction of stellacyanin.

The amounts of the two proteins obtained in the representative preparation reported in the preparation section were 1.7 g of laccase and 0.45 g of stellacyanin. Moreover, small amounts of other chromatographic forms of the two proteins were produced. In another preparation as much as 5.2 g of laccase and 0.60 g of stellacyanin were obtained from 180 g of acetone powder made from 3 kg of lacquer. The oxidase activity of the purified laccase with the substrate *N,N*-dimethyl-*p*-phenylenediamine was $2.8 \cdot 10^4$ units.

Criteria of purity

Both laccase and stellacyanin from their main fractions were found to migrate as sharp single bands in the gel electrophoresis at pH 4.5 (see Fig. 2) and the proteins therefore seem to be homogeneous. Gel electrophoresis of laccase at pH 9.5 resulted in two bands containing about the same amount of material and two other very weak bands. This heterogeneity at high pH might be based on changes in conformation of laccase.

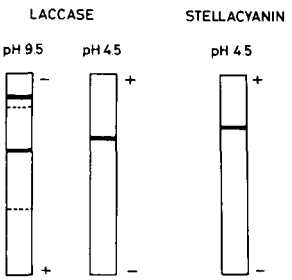

Fig. 2. Polyacrylamide-gel disc electrophoresis of laccase and stellacyanin. The gels were made in 70 mm × 5 mm Pyrex tubes and the concentration of the "running" gel was 5%. In the runs with the β-alanine–acetate buffer (pH 4.5), a 2.5% "spacer" gel was applied on top of the "running" gel. Only laccase was run in the Tris–borate buffer (pH 9.5). The polarity of the electrodes are given as + or − signs in the figure.

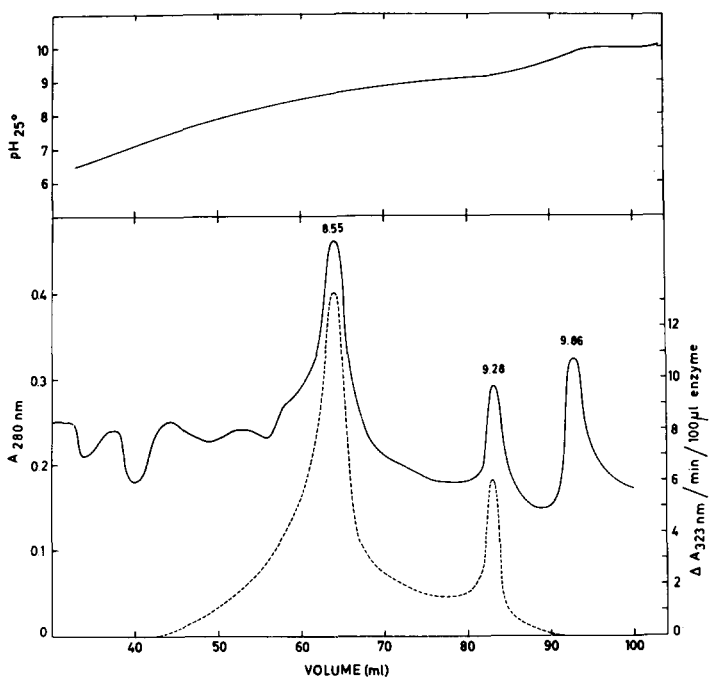

Fig. 3. Isoelectric focusing of laccase and stellacyanin. pH (25°, upper solid curve), ultraviolet absorption (280 nm, lower solid curve), and enzyme activity (dashed curve) of the column solution after isoelectric separation of Rhus laccase and stellacyanin. The numbers above the peaks give the p*I* values of the protein components.

The results from the isoelectric-focusing experiments with the two chromatographic fractions of laccase and the main fraction of stellacyanin show that they are very homogeneous. Fig. 3 shows the absorbance at 280 nm, enzyme activity, and pH as functions of the level in the isoelectric-focusing column. In the ultraviolet absorption curve there are only three sharp peaks, containing laccase or stellacyanin as judged from the blue color and the enzyme activity measurements. The other ultraviolet-absorbing components and the background absorption are due to the carrier ampholytes, as found from separate experiments with no protein but with the same amounts of carrier ampholytes. In the activity curve of the main fraction of laccase a certain asymmetry is observed. This asymmetry may depend on incomplete equilibrium of the protein in the pH gradient. The isoelectric points found for these three protein fractions are high: 8.55 for the main fraction of laccase, 9.28 for the second chromatographic form, and 9.86 for the main fraction of stellacyanin.

The ratio between the absorbance at 280 nm and at the blue maximum at about 600 nm as well as the absorbance ratio between 280 nm and the minimum at 250 nm of the two proteins has some relation to the purity, as it is found that the main impurities are yellow and brown pigments. These pigments have a small absorbance only at about 600 nm, but it increases as the wavelength decreases. The $A_{280\text{ nm}}/A_{250\text{ nm}}$ ratio obtained in this laboratory is 2.3 for laccase and 3.0 for stellacyanin. $A_{280\text{ nm}}/A_{614\text{ nm}}$ for laccase and $A_{280\text{ nm}}/A_{604\text{ nm}}$ for stellacyanin are 15.2 and 5.6, respectively.

The optical properties of laccase and stellacyanin prepared by the present method are comparable to earlier published data by three other laboratories[3,4,25], but there are some minor differences in the extinction coefficients of the various absorption bands. The optical data for laccase and stellacyanin are presented together with the other spectroscopic properties in the accompanying paper[26].

Amino acid analysis of laccase

The results from the determination of the amino acid composition of laccase are given in Table I. The low content of amino acids, about 55 %, is unusual among proteins. The rest of the protein molecule is probably carbohydrates but no analyses for carbohydrates have yet been performed in this laboratory. However, other investigators[3,4] have reported high contents of carbohydrates in their preparations of laccase, and we observed relatively large amounts of amino sugars in the amino acid analyzer chromatograms.

The recovery of sulfur-containing amino acids is somewhat lower than expected from the sulfur analysis (see Table III) which corresponds to about 19 sulfur atoms per molecule if the molecular weight is 110000. This low recovery may depend on the high content of carbohydrates.

In laccase there is an excess of 38 basic amino acid residues over acidic residues (arginine + histidine + lysine = 56, aspartic acid + glutamic acid $-NH_3$ = 18). This result is in accordance with the high isoelectric point mentioned above.

The values obtained for the various amino acids in the samples hydrolyzed for 18, 24, and 72 h were averaged with the exception that only the values obtained after 72-h hydrolysis are reported for valine, leucine and isoleucine. Shorter hydrolysis time gave considerably lower values for these amino acids. Furthermore, the values reported for serine and threonine are obtained after extrapolation to zero time of hydrolysis, assuming first-order kinetics.

TABLE I

AMINO ACID COMPOSITION OF *R. vernicifera* LACCASE

For details about the technique used, see the text.

Amino acid residue	*Residue (g/100 g protein)*	*Residue (moles/110000 g protein)*	*Nitrogen (g/100 g protein)*
Aspartic acid	6.28 ± 0.20	60.0 ± 1.9	0.764 ± 0.024
Threonine	4.57 ± 0.10	49.7 ± 1.1	0.633 ± 0.014
Serine	2.93 ± 0.02	37.0 ± 0.3	0.471 ± 0.003
Glutamic acid	4.30 ± 0.18	36.6 ± 1.5	0.467 ± 0.020
Proline	3.31 ± 0.10	37.5 ± 1.1	0.478 ± 0.014
Glycine	1.87 ± 0.10	36.0 ± 1.9	0.459 ± 0.025
Alanine	2.52 ± 0.11	39.0 ± 1.7	0.496 ± 0.022
Valine	3.87 ± 0.08	43.0 ± 0.9	0.547 ± 0.011
Half-cystine	0.64 ± 0.03	6.9 ± 0.3	0.088 ± 0.004
Methionine	1.28 ± 0.03	10.7 ± 0.2	0.137 ± 0.003
Isoleucine	3.20 ± 0.06	31.1 ± 0.6	0.396 ± 0.007
Leucine	3.53 ± 0.06	34.3 ± 0.6	0.437 ± 0.007
Tyrosine	3.62	24.4	0.311
Phenylalanine	4.04 ± 0.20	30.2 ± 1.5	0.385 ± 0.017
Amide ammonia	1.01	79.3	0.831
Lysine	3.03 ± 0.06	26.0 ± 0.5	0.662 ± 0.013
Histidine	2.10 ± 0.04	16.8 ± 0.3	0.643 ± 0.012
Arginine	1.78 ± 0.08	12.5 ± 0.6	0.638 ± 0.029
Tryptophan	1.06 ± 0.06	6.3 ± 0.4	0.159 ± 0.009
Copper	0.232 ± 0.005	4.0 ± 0.1	
Total for residues	54.94		9.002 = 86.93 of total nitrogen

TABLE II

PARTIAL SPECIFIC VOLUME OF *R. vernicifera* LACCASE

The partial specific volume ($\bar{v}$) of laccase was calculated as follows: 67.61/100 = 0.676 ml/g protein.

	Wt. %/100 g laccase	$\bar{v}$	$\bar{v} \times$ *wt. %*
Amino acid residues	54.94	0.723	39.71
Carbohydrate (assumed)	45	0.62	27.90
Total	100		67.61

Methionine was determined as methionine sulfone in the oxidized samples, and as the values obtained for methionine in the unoxidized samples were quite similar, the reported value is an average of all eight hydrolyses.

The value for tryptophan is the average of six determinations on native enzyme samples.

Tyrosine was most difficult to determine as amino sugars were often eluted close to this amino acid. The value reported is obtained from the chromatogram giving the best separation.

Partial specific volume of laccase

The partial specific volume of the protein part of laccase calculated from the amino acid composition is 0.723 (see Table II). If there is 45 % carbohydrates in the laccase molecule, and if the partial specific volume of the carbohydrates is 0.62 (a value common for several carbohydrates[27]), the partial specific volume of laccase is 0.68. A value of 0.70 has been obtained by pycnometric measurements in another laboratory[4].

Determination of copper

Both methods for the determination of total copper mentioned above gave the same amount of copper in two preparations of laccase. As the biquinoline method is less enzyme-consuming and also very rapid and reproducible, it is recommended for copper determinations on laccase solutions. In Table III the results from two carefully performed copper analyses with both methods are reported. The copper content corresponds to a minimal molecular weight of laccase of 27400, or, on the basis of four copper atoms per enzyme molecule[26], a true molecular weight of about 110000 (*cf.* DISCUSSION). The value for copper content is comparable to earlier reported values from two other laboratories[3,4].

Elemental composition of laccase

The elemental composition of laccase is given in Table III. The unusually low nitrogen content is certainly related to the low amount of amino acids in this protein (*cf.* Table I). The value for nitrogen is somewhat higher compared with earlier published values[3,4].

TABLE III

ELEMENTAL COMPOSITION OF *R. vernicifera* LACCASE

The values for nitrogen, sulphur, and copper are expressed as weight %. Copper was determined on native enzyme samples, the concentrations of which were determined by nitrogen analysis according to Kjeldahl.

Analysis	*Nitrogen*	*Sulphur*	*Copper*
1	10.34	0.56	0.226
2	10.37	0.57	0.237

DISCUSSION

The purification of laccase and stellacyanin by the method described was found to be very easy and reproducible and large amounts of the two proteins can be produced in a very high state of purity in about 3 days.

The yields of laccase and stellacyanin are higher than those obtained by other preparation methods[3,4,7]. This is certainly based on the fact that precipitation with $(NH_4)_2SO_4$ is used in these other methods, and neither laccase nor stellacyanin are completely precipitated with satd. $(NH_4)_2SO_4$.

The preparation method presented in this paper is somewhat similar to the method published by OSAKI AND WALAAS[6], as they make use of the same ion ex-

changers. However, they use these ion exchangers in a reversed order as compared to our method. We have observed that if the anion-exchange step is used before the cation one, the enzyme often has to be rechromatographed on the anion-exchange column to obtain satisfactory purity. This is due to the fact that the anion-exchange column is easily overloaded if the filtrate from Step 1 is first applied to it, with the result that pigments having about the same chromatographic properties as laccase run through the column unretarded together with laccase and stellacyanin. However, if the filtrate is first applied to the cation-exchange column, most pigments are eluted while both laccase and stellacyanin are strongly bound. When the laccase-containing fraction is afterwards applied to the anion-exchange column, there is no risk of overloading the column and this results in strong binding of the small amounts of pigments still contaminating the laccase on the top of the column, while laccase runs through the column unretarded.

The purity of Rhus laccase and stellacyanin obtained by the method described here seems to be very good according to the gel electrophoresis and the isoelectric-focusing experiments. As with fungal laccase the main impurities are the yellow and brown pigments of different molecular weights and chromatographic properties. The $A_{280\ nm}/A_{250\ nm}$ ratio of laccase and stellacyanin is affected by the presence of these pigments in the protein preparations. This ratio for the laccase obtained in our laboratory is greater than those obtained in two other laboratories[3,4] indicating a higher purity from pigments of our laccase. On the other hand, stellacyanin obtained in several laboratories[3,7,25] shows the same ratio as we have found. Stellacyanin prepared by some investigators[7,25] has some laccase activity. This activity probably depends on contamination by laccase, as the stellacyanin prepared by the present method shows no laccase activity. Moreover, one laboratory[25] has found that the laccase activity of their preparation of stellacyanin was separable from stellacyanin on a Sephadex G-75 column indicating a higher molecular weight of the component responsible for the enzyme activity than of the stellacyanin. As mentioned in the preparation part, there are often several chromatographic components of laccase in the acetone powder used as starting material. Thus, especially the isoenzyme which is eluted with 0.2 M Na_2HPO_4 might be responsible for the laccase activity in stellacyanin prepared by methods in which 0.2 M Na_2HPO_4 is used to elute stellacyanin from the cation exchangers used[7,25].

No extensive studies of the hydrodynamic properties of laccase have as yet been performed in this laboratory. However, two preparations have been subjected to sedimentation velocity centrifugation according to standard methods[28]. The sedimentation coefficient obtained was 5.3 S for both preparations (B. REINHAMMAR, unpublished results). The enzyme concentration was 0.35 % in 0.1 M phosphate buffer (pH 6.5). This value is close to the 5.4 S reported earlier, corresponding to a molecular weight of 120000[3]. Another laboratory reports a value of 6.25 S and a molecular weight of 141000[4]. However, if the number of copper atoms in the laccase molecule is known, the molecular weight of the enzyme could be calculated from the copper content. The minimum molecular weight of the enzyme calculated from the copper analysis in this laboratory is 27400. OMURA[4] reports copper contents corresponding to a value between 25000 and 27600. The copper content published by NAKAMURA[3] corresponds to a value of 29000. As will be reported in another publication[26] from this laboratory, Rhus laccase, like fungal laccase[12], contains three different forms of

copper. Like fungal laccase there is 50% EPR-detectable copper in Rhus laccase, and of the EPR-detectable copper there are two different forms. These findings suggest that the Rhus laccase molecule must contain a multiple of 4 copper atoms. Then with the above-mentioned minimum molecular weights Rhus laccase will have a molecular weight of a multiple of about 110000. The sedimentation coefficient of about 5.3 S found in this laboratory and by NAKAMURA[3] leads to the assumption that the molecular weight is about 110000 and not a multiple of this value.

The kinetics of the oxidation of quinol by laccase, measured by a recording of the oxygen consumption as mentioned above, shows that this system does not seem to follow simple Michaelis–Menten kinetics at substrate concentrations below 40 mM. Over that concentration there is a marked decrease of the initial rate of oxygen consumption, probably caused by substrate inhibition. The limited experimental results obtained in this laboratory so far do not allow any extensive conclusions about the kinetics. However, the Michaelis constant (K_m) seems to be very high, possibly even higher than the values reported earlier[4,5]. The high K_m values make an accurate determination impossible. Furthermore, possible substrate inhibition makes the apparent K_m values depend on the concentration range used. This factor may explain the large discrepancies in the reported values for this constant.

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