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COPPER CONTENT AND OTHER CHARACTERISTICS OF PURIFIED PEACH LACCASE

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Abstract—The laccase from peaches was purified 750×. The enzyme is a glycoprotein of mol. wt 73500 containing 2 atm Cu/mol. It is inhibited by diethyldithiocarbamate. Some other characteristics of the enzyme, including its amino acid composition, are described.

INTRODUCTION

PREVIOUS work on the laccase-like enzyme from peaches led to partial purification of the enzyme.[1] However, the presence of the pectins in the extract necessitated the use of Pectinol in the purification.[2] Moreover, although the enzyme could be eluted from a DEAE column after pectinol treatment, the purified enzyme so obtained was extremely unstable and lost activity rapidly on storage.

There are few detailed reports on laccases from higher plants, although the enzyme from Rhus has been purified and characterized.[3–5] There is still some uncertainty about the copper content of the Rhus enzyme and even about its molecular weight. The enzyme from the fungus Podospora differs greatly from that of Rhus,[6] while that of Polyporus again shows different features.[7] We therefore attempted to further characterise the enzyme from peaches. In the following we will report on its purification, using the isoelectric focussing technique, and on some properties of the purified enzyme.

RESULTS AND DISCUSSION

A typical result of purification, by isoelectric focussing, of the laccase from peaches is shown in Fig. 1(a). Recovery from the column was somewhat variable but in the better runs was 66%. The elution pattern shows two peaks, only one of which had activity towards quinol. The purification could be further improved by rerunning the purified preparation at a narrower pH range, 4–6 (Fig. 1b).

The results of the purification are summarized in Table 1.

[1] HAREL, E. and MAYER, A. M. (1968) *Phytochemistry* **7**, 1253.
[2] HAREL, E. and MAYER, A. M. (1970) *Phytochemistry* **9**, 2447.
[3] MALMSTROM, B. G., REINHAMMER, B. and VANNGARD, T. L. (1970) *Biochim. Biophys. Acta* **205**, 48.
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[6] MOLITORIS, H. P., VAN BREEMEN, J. F. L., VON BRUGGEN, E. F. J. and ESSER, K. (1972) *Biochim. Biophys. Acta* **271**, 286.
[7] MALKIN, R., MALMSTROM, B. G. and VANNGARD, T. L. (1969) *European J. Biochem.* **10**, 324.

The highly purified preparation from the isoelectric focussing were run on acrylamide gels. The gels were stained for enzyme activity, with 4-methylcatechol or quinol as substrates and also for carbohydrates. The band showing laccase activity (Fig. 2) also stained for protein and for carbohydrates. The highly purified laccase appears to be a glycoprotein.

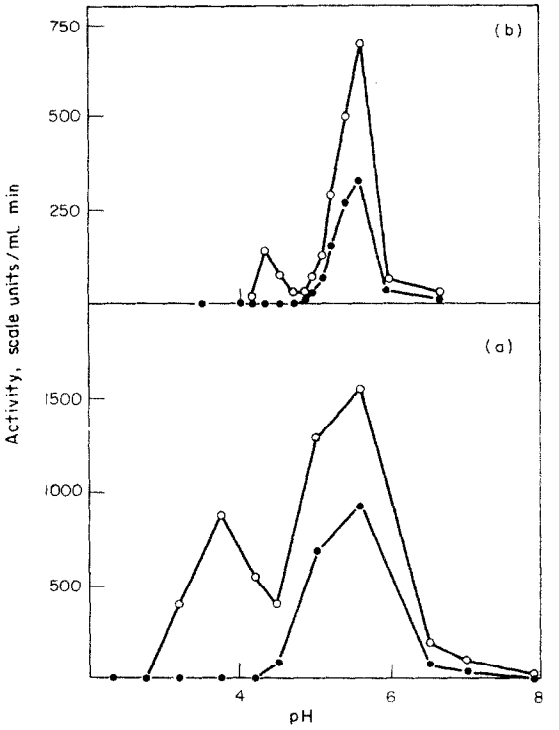

FIG. 1. PURIFICATION OF PEACH LACCASE ON AN EMPHOLINE COLUMN. Activity towards two substrates is given against pH of the fraction. (a) First run, pH 3–10. (b) Fractions active towards quinol rerun at pH 4–10. ○ 4-methylcatechol; ● quinol.

The effect of inhibitors on the purified enzyme was studied (Table 2). It can be seen that the only really effective inhibitor was diethyldithiocarbamate, in agreement with our previous results using a partially purified preparation.[1] In order to prepare the enzyme in amounts sufficient for ultracentrifuge analysis, the purification procedure was further modified.

TABLE 1. PURIFICATION OF PEACH LACCASE BY REPEATED ISOELECTRIC FOCUSSING. Data for most active fraction collected at each run

	Purification	
	Laccase	Catechol oxidase
20000 *g* Supernatant for peaches	0·33	0·33
Freeze dried powder	1·0	1·0
Isoelectric focussing		
1st run pH 3–10	31·0	15·3
2nd run pH 4–6	9	7·3
Total purification	839	333
Recovery	52%	27%

50 g of freeze dried powder from peaches was dissolved and treated with Pectinol (see Methods). The solution was immediately treated with $(NH_4)_2SO_4$ and the fraction precipitating between 65 and 100% saturation collected, dissolved in 5 mM phosphate buffer pH 6 and dialysed against the same buffer. 70 ml of the dialysed solution, containing 10·7 mg protein/ml were applied to the isoelectric focussing column. The active fractions from two successive runs were pooled. The purification was 255-fold compared to the freeze dried powder, which correspons to 750-fold for the 20000 *g* supernatant fraction of entire peaches. The copper content and molecular weight of the purified fraction were determined (Table 3). The enzyme has a mol wt of *ca* 73500. The minimum copper content of the enzyme is about 2 atm of copper per molecular weight unit of about 73500, assuming that the enzyme is composed only of protein. If the carbohydrate moiety is an appreciable part of the molecular weight, say 25%, then the copper content would be only 1·5 atm/mol. This value is lower than usually reported for laccase in the literature. However, it is possible that part of the copper is lost during the purification procedure.

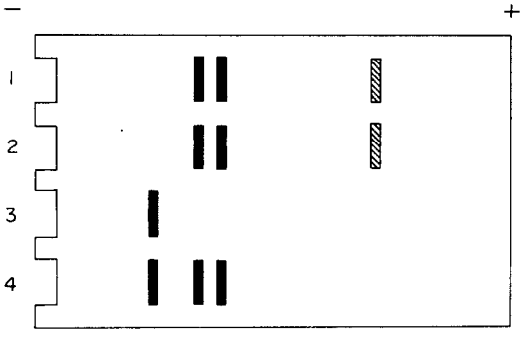

FIG. 2. ACRYLAMIDE GEL ELECTROPHORESIS OF PARTIALLY PURIFIED PEACH LACCASE OBTAINED FROM AMPHOLINE COLUMN pH 3–10. (1) Fraction obtained at pH 4·3. (2) Fraction obtained at pH 4·7. (3) Fraction obtained at pH 5·7 (laccase purified 27×). (4) Original preparation. Activity developed with 4-methylcatechol. The single band in run (3) was active also towards quinol, and stained with basic fuchsin reagent for carbohydrates.

The amino acid composition of the purified enzyme is given in Table 4. This composition is distinguished by the apparent absence of the sulphur containing amino acid and the relatively high threonine and aspartic acid contents. Also note worthy is the presence of both lysine and histidine in relatively large amounts. The presence of glucosamine provides further evidence for the linkage of carbohydrate to the protein. The amino acid composition shows considerable differences from that of the laccase from Podospora.[8]

TABLE 2. INHIBITION OF PURIFIED PEACH LACCASE

Inhibitor	Conc. giving 50% inhibition	Inhibitor	Conc. giving 50% inhibition
Phenylthiourea	$3{\cdot}2 \times 10^{-3}$ M	Neocuprein	5×10^{-3} M
p-Nitrophenol	5×10^{-3} M	KCN	6×10^{-4} M
Na-diethyldithio-carbamate	$2{\cdot}2 \times 10^{-5}$ M	4,5-Methylortho-phenylecdiamine	5×10^{-3} M

Phenylhydrazine, *p*-tolylthiourea, salicylaldoxime, orcinol, were all tested and found to be less effective than the above compounds. (Source of enzyme was preparation purified by isoelectric focussing on ampholine column pH 3–10.)

[8] ESSER, K. and MINUTH, W. (1971) *European J. Biochem.* **23**, 484.

The absorption spectrum of the purified enzyme showed no bands in the visible part of the spectrum, but only characteristic protein absorption at 280 nm. These results therefore clarify the nature of the laccase-like enzyme from peaches and show it to be a copper-containing glycoprotein.

TABLE 3. COPPER CONTENT AND MOL. WT OF PURIFIED LACCASE

Cu content (on protein basis)	0·17%
$D_{20,w}(10^7\ cm^2\ S^{-1})$	5·3
$S_{20,w}$	4·8 S
Molecular weight	73 500
Approximate number of Cu atoms/enzyme molecule	2

EXPERIMENTAL

Freeze dried powder, prepared from peaches,[2] served as enzyme source. This was dissolved in distilled water, 20 mg/ml, and the soln treated with Pectinol R-10 as previously described.[2] The Pectinol R-10 in this batch was low in proteolytic activity and the final step in its purification (chromatography on DEAE-cellulose) could be omitted. 17 ml Soln of peach enzyme were treated with 3 ml pectinol soln for 0·5 hr at room temp. This treatment reduced viscosity caused by pectins sufficiently to permit the use of the soln, without further treatment, in the column. This soln was placed in an ice bath and immediately run on the isoelectric focussing column (LKB Ampholine column 8101). The following solutions were used: Anode compartment—0·05 ml conc. H_2SO_4· 15 ml H_2O and 11 g sucrose. Cathode compartment—5 ml 0·25 N NaOH. The gradient was formed from 2 solns: (a) 1·9 ml ampholine, 37 ml H_2O and 26 g sucrose; and (b) 0·6 ml ampholine and 51·5 ml H_2O, containing the enzyme preparation after Pectinol treatment. The current was maintained at 3 W and the voltage slowly raised to 900 V. The column was run for 24 hr at this voltage. After the cessation of the run, the column was discharged through a fraction collector. Most of the partially degraded pectins precipitated at the anode and come off the column well before the enzyme. Interference of pectins in the purification was thereby completely prevented.

TABLE 4. AMINO ACID COMPOSITION OF PURIFIED LACCASE. (a) as relative amounts of amino acid residues (b) as % of total amino acids (without glucosamine and tryptophane)

	(a)	(b)
Lysine	1·55	5·85
Histidine	1·37	5·17
Arginine	0·56	2·11
Tryptophane	present*	
Aspartic acid	3·9	14·71
Threonine	2·52	9·51
Serine	2·08	7·85
Glutamic acid	1·94	7·32
Proline	1·73	6·53
Glycine	2·52	9·51
Alanine	2·10	7·92
Half cystine	—	—
Valine	1·61	6·07
Methionine	—	—
Iso leucine	1·0	3·77
Leucine	2·06	7·77
Tyrosine	0·49	1·85
Phenylalanine	1·08	4·07
Cysteic acid	trace	—
Glucosamine	1·39	

* Significant amount of tryptophane (0·45 residues) was found. This is a minimal amount not corrected for destruction of tryptophane during hydrolysis, which is increased in the presence of sugars. Tryptophane and glucosamine coincide exactly on the column for basic amino acids and tryptophane was estimated only.

Enzyme activity of the fractions was determined using a Clarke type O_2 electrode as described by Harel *et al.*[9] using either quinol 10^{-2} M or 4-methylcatechol 5×10^{-3} M as the substrate. Activity was determined in the presence of the ampholine buffer and sucrose, which at the concentration used did not interfere with the reaction.

Protein in the fractions was determined by the Folin–Ciocalteu reaction, after exhaustive dialysis (72 hr) to remove the ampholine which interfered in the reaction.

Ultracentrifugation. Material taken off the column was dialysed against 5 mM phosphate buffer containing 100 mM NaCl and then conc. by freeze drying to 1/10 its vol. The soln was run in a Beckman–Spinco Ultracentrifuge.

Copper determination. Some of the material prepared for ultracentrifugation was digested with sulphuric–perchloric acid mixture, diluted and diethyldithiocarbamate and EDTA added. The complexed copper was extracted into methyl isobutyl ketone and the copper content of the soln was determined with an atomic absorption spectrophotometer Perkin–Elmer Model 403, using a hollow cathode lamp 3036024. The results were calculated from a calibration curve prepared using $CuSO_4$. Suitable reagent controls were run.

Electrophoresis was carried out according to Harel and Mayer[2] and staining for carbohydrates according to Zacharius *et al.*[10]

Amino acid analysis. 1 mg of enzyme preparation were hydrolysed for 24 hr at 110° under N_2 with 50% HCl (V/V). The material was dried and dissolved 2·5 ml citrate buffer. The amino acid analysis was performed on a Beckman/Spinco Model 120 amino-acid analyser.

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