may indicate the presence of a stable dimer. However, the low content of Cu does not support such a view. It was not possible to dissociate the enzyme (e.g. by gel electrophoresis in sodium dedecylsulphate) while retaining its activity. Potato catechol oxidase was reported to have a MW of 72800 with 50% content of an RNA-like component and about one atom of Cu per molecule. We found no indication of a non-protein constituent in the enzyme from grapes, though this possibility cannot be ruled out.

We have previously reported the partial purification and some of the properties of catechol oxidase from table grapes [7,9]. The MW estimated by gel filtration through Sephadex was 55–58000. Partial degradation of the enzyme [9] and underestimation by the gel filtration method may account for the discrepancy between the two values. It is unlikely that the difference is due to the difference in grape varieties used.

The amino acid composition of grape catechol oxidase shows considerable similarities with that reported for the enzymes from potato [1] and spinach beet [2]. The grape enzyme is richer in proline and tyrosine but has relatively less leucine than the potato enzyme. Compared with the spinach beet enzyme, the catechol oxidase from grapes has relatively more glutamate and tyrosine and less valine and leucine. The differences between the grape enzyme and fungal tyrosinases [3–6] are somewhat greater.

EXPERIMENTAL

Chloroplasts were prepared from grapes, *Vitis vinifera*, cv. Clairette (Bourbaulenc) as previously described [7,8]. Isolated, freeze-dried chloroplasts were pre-extracted with cold 80% Me_2CO as described by Lerner *et al.* [8]. The extract in 1 mM Pi buffer pH was applied to a hydroxyl-apatite column (Serva) equilibrated with the same buffer. 'Inactive' protein was washed-off the column with 50 ml mM Pi buffer pH 7 and the enzyme was collected by eluting with 75 ml 30 mM buffer. The purified enzyme was stabilized by the addition of 0.05 ml Ampholine pH 3.5–10.0 and kept in the cold. Determination of Cu content, ultracentrifugation and amino acid analysis were performed as previously described [14]. The estimate of the partial specific volume of the enzyme was calculated from the partial specific volumes of the constituent amino acids. Catechol oxidase activity was determined by the use of a polarographic oxygen electrode, in 0.1 M citrate buffer pH 4.8 with 5 mM 4-methylcatechol as substrate. Protein was determined according to Lowry *et al.* [15].

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AMINO ACID COMPOSITION AND MOLECULAR WEIGHT OF *BOTRYTIS CINEREA* LACCASE

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Key Word Index—*Botrytis cinerea*; laccase; glycoprotein; amino acid composition; copper content.

Abstract—The extracellular laccase from *Botrytis cinerea* is shown to be a glycoprotein, of MW 56000, containing at least one Cu atom/molecule. Its amino acid conposition shows an exceptionally low content of basic amino acid and very high content of threonine and serine.

INTRODUCTION

In a previous paper [1] we reported on some properties of laccase partially purified from the fungus *Botrytis cinerea*. An unusual feature of the enzyme was its very low isoelectric point, pH 2.5. We therefore decided to purify and characterize the enzyme further.

RESULTS AND DISCUSSION

The extracellular laccase from *Botrytis* was precipitated from its culture medium with acetone and redissolved in buffer. Following ultrafiltration it was applied to a DEAE cellulose column and eluted with 0.04M phosphate citrate buffer, pH 6. The final purification was 30 × com-

Table 1. Amino acid composition of purified *Botrytis* laccase

	Relative amounts of amino acids	No. of residues/ MW of 56 000*
Lysine, Histidine	2.4	6
Arginine	1	2
Aspartic acid	19.3	47
Threonine	29.6	72
Serine	34.8	85
Glutamic acid	10.4	25
Proline	10.1	25
Glycine	15.8	39
Alanine	27.2	66
Half cystine	0.58	1
Valine	10.7	26
Methionine	1.3	3
Iso-leucine	9.3	23
Leucine	15.9	39
Tyrosine	4.6	11
Phenylalanine	4.10	10
Glucosamine	6.4	

* Not corrected for tryptophane, sugar content not taken into account. Tryptophane was apparently destroyed during hydrolysis. Lysine and histdine separated poorly and are therefore given together.

pared to activity present in the growth medium. On acrylamide gel electrophoresis [1] at 300 volts and 10 mA, for 2 hr, the enzyme moved as a single band of activity, 5.5 cm from the origin.

The enzyme was subjected to ultracentrifugation. A single clear, symmetrical peak was obtained. The $S_{20,w}$ was 3.2 S and $D_{20,w}$ was 5.4 (10^7 cm^2/S). The *ca* MW, assuming V = 0.74, is at most 56 000, depending on the amount of carbohydrate present. The MW of fungal laccase has been reported as about 60–70 000 [2–4], while that of *Rhus* is between 120 000 and 140 000 [5, 6]. The Cu content was determined by atomic absorption spectroscopy and was found to be 0.043% Cu/g dry weight equivalent to half an atom of Cu per molecule, indicating a minimal Cu content of 1 atom per molecule. The low Cu content is presumably the result of loss during purification, particularly on the DEAE cellulose column.

The purified enzyme was hydrolysed and its amino acid composition determined (Table 1). The amino acid composition is quite unusual, and strikingly different from that reported for other laccases from fungi [2–4] and from peaches [7] and *Rhus* [8]. As expected from an enzyme with such a low isoelectric point, the number of basic amino acid residues is exceptionally low, 8 compared to 55 in *Rhus*, total MW 110 000 [8], 56 in *Neurospora* laccase, MW of amino acids 58 000 [3], and 37 in *Polyporus* laccase, MW of amino acids 64 400 [4]. Equally surprising is the relatively low aspartic acid and glutamic acid content. On the other hand, the serine, threonine and alanine content is very high. Tyrosine and phenylalanine are also rather low. Half cystine content is also low.

The presence of glucosamine in the acid hydrolysate indicates that the *Botrytis* enzyme, as other laccases, is a glycoprotein. We have no estimate of the total sugar content of the enzyme, which is 45% in *Rhus* laccase and 13% in *Podospora*. These features make this enzyme one of considerable interest, which should be studied further. If indeed Cu binding is through a basic amino acid, probably histidine, then the low content of basic amino acids makes it particularly suitable for studying this aspect of its chemistry.

EXPERIMENTAL

Botrytis cinerea was cultured in 2 l. flasks containing 500 ml of medium, at 22°. The culture medium was as previously described [1, 9] except that commercially bottled grape juice, 'Tirosh' (Carmel Wine growers cooperative), was substituted for juice prepared freshly from grapes. Enzyme activity in the growth medium was periodically tested, after concn of the medium by ultrafiltration. When growth had ceased and the enzyme content of the medium no longer increased, after about 3 weeks, the medium, 5 l. was collected. The enzyme was pptd with Me_2CO, −20° [1, 9]. The ppt was dissolved in citrate buffer, 0.1 M, pH 3.4, the extract centrifuged at 20 000 g for 10 min. The clear extract was concnd by ultrafiltration, dialysed against 0.04 M citrate buffer, pH 3.4 and the soln applied to a DEAE cellulose column equilibrated with Pi-citrate buffer, pH 6, 0.04 M. The enzyme was eluted off the column with 0.04 M citrate buffer, pH 6. The most active fractions, 42 ml, were combined and concnd by ultrafiltration. The purified enzyme (purification 30 ×) had a specific activity of 21 $\mu l O_2$/mg protein/min. Ultracentrifugation, protein hydrolysis, amino acid composition and Cu content were all determined as previously described [7]. Laccase activity was determined as previously described [1] and protein according to ref. [10].

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