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PURIFICATION AND PROPERTIES OF LACCASE FROM *BOTRYTIS CINEREA*

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Abstract—The partial purification of an extracellular laccase from *Botrytis cinerea* is described. Specificity of the enzyme, its K_m for a number of substrates and sensitivity to some inhibitors are described. The enzyme is a typical laccase but has an exceptionally low pI and great stability to acid pH. On gel electrophoresis two isoenzymes could be detected.

INTRODUCTION

Botrytis cinerea infection causes various forms of rot in grapes. Laborde [1] was the first to suggest that oxidases secreted by *Botrytis* into the grape berries might be the cause of oxidative reactions in musts and wines. Bertrand [2], having described the laccase of *Rhus succedanea*, showed that addition of the laccase to wine resulted in oxidation products similar to those observed in the presence of *Botrytis*. Laborde [3] concluded that the function of the two enzymes is similar. Dubernet and Ribereau-Gayon realised the significance of the enzyme, isolated it, and briefly reported on some of its characteristics [4, 5]. In the following we give further details of the purification of the extracellular enzyme of *Botrytis* and describe some of its properties.

RESULTS

The liquid culture of *Botrytis* was carried out under conditions resembling the natural growth of the fungus, to give rapid mycelial growth. During growth, phenolics present in the medium were oxidised. The extracellular enzyme isolated from the medium is not necessarily identical to that present in the mycelium. However, the lacasse present in musts and wine is that secreted by the mycelium.

The substrate specificity of crude preparation of *Botrytis* laccase is shown in Table 1. It can be seen that a wide range of *o*-diphenols are oxidised by the crude preparation of laccase of *Botrytis* (Table 1). Some *m*-diphenols, *p*-diphenols and *p*-phenylenediamine are also oxidised as is the monophenol, *p*-cresol. *p*-hydroxybenzoic acid was not attacked while tyrosine was oxidised very poorly. The oxidation of *p*-cresol by *Botrytis* laccase resulted in formation of a white precipitate as also reported by Graubard [6] and Benfield *et al.* [7]. The wide range of substrates oxidised by this laccase correspond to those described previously for other laccases [8–10].

The apparent K_m of the laccase, determined from Lineweaver–Burk plots [11], was 0.19 mM for quinol, 0.045 mM for 4-methylcatechol, 0.67 mM for *p*-cresol and 0.77 mM ascorbic acid. The K_m of the enzyme did not change during purification.

Table 1. Substrate specificity of laccase from *Botrytis*

Substrate	Relative activity
4-Methylcatechol	100
Catechol	104
3,4-Dihydroxyphenylalanine	97
Protocatechuic acid	119
Caffeic acid	132
Chlorogenic acid	100
(+)-Catechin	100
3-Methylcatechol	110
Gallic acid	109
Phloroglucinol	143
p-Cresol	109
Tyrosine	7
p-Hydroxybenzoic acid	0
Vanillic acid	33
p-Coumaric acid	90
Ferulic acid	109
3,4-Dimethylphenol	100
Quinol	100
p-Phenylenediamine	95
N,N-Dimethyl-*p*-phenylenediamine	95
Ascorbic acid	95
Leucoanthocyanins	84
Anthocyanins	97

Test carried out on crude enzyme extract.

Diethyldithiocarbamate, which has been reported to inhibit laccase from *Rhus* [12] and peaches [13] did not inhibit the *Botrytis* enzyme. As can be seen from Table 2, relatively high concentrations of cyanide or EDTA were required to inhibit the laccase from *Botrytis*.

The study of the dependence of the activity of the laccase on pH shows that while the pH optimum for the oxidation of 4-methylcatechol and quinol is 4.7, the oxidation of a mixture of anthocyanins extracted from

Table 2. Inhibition of *Botrytis* laccase by KCN and EDTA

Inhibitor	Concn. mM	Inhibition %
KCN	0	0
	1	25
	3	50
	10	83
EDTA	0	0
	0.1	5
	0.5	11
	1	16
	2	37
	3	50

wine shows an optimum at pH 4.0, with a rather broad pH optimum (Fig. 1). The dependence of the pH optimum on the substrate has been reported for the phenol oxidase of *Polyporus* [14].

The temperature optimum of the enzyme was between 30° and 55° depending on the substrate. This rather unusual property may be related to the rapid temperature inactivation of the enzyme at slightly elevated temperatures. The time required for 50% inactivation was 250 sec at 40° and 64 sec at 45°. Presumably some protection against thermal inactivation is afforded by some of the substrates e.g. a mixture of anthocyanins extracted from wine.

Electrophoresis of extracts of varying degree of purification always yielded two bands showing laccase activity. The laccase from *Botrytis* was unusually stable at acid pH, but was rapidly inactivated above pH 7.0. 50% activity was lost after 10 min at pH and 90% after 40 min. The enzyme was quite stable at pH 3.4 which is also the pH of must and wine. Below pH 2.5, however, the laccase was rapidly and irreversably inactivated. Inactivation above pH 7.0 also occurred in the presence of inhibitors of proteases such as phenyl methylsulfonylfluoride. The inactivation occurred both in crude and partially purified extracts. It is therefore unlikely that inactivation can be ascribed to proteolytic breakdown of the enzyme. The extreme stability of the laccase to acid pH prompted us to study its isoelectric point (pI). The enzyme did not migrate on cellulose acetate at pH 2.5. This result was confirmed by isoelectric focussing.

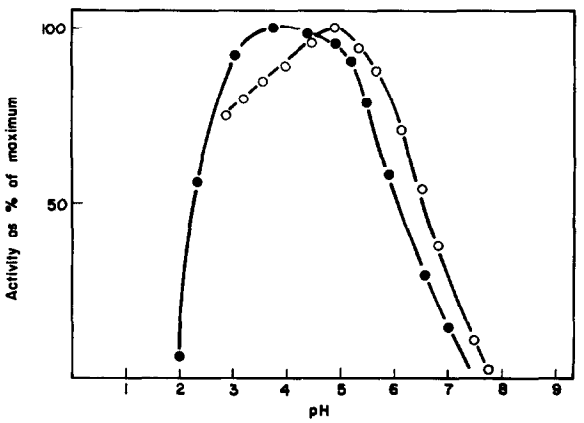

Fig. 1. Effect of pH on the activity of *Botrytis* laccase. o——o Quinol or 4-methylcatechol as substrate. ●——● Mixture of anthocyanins as substrate.

For this purpose we devised a mixture of amino acids as the ampholyte, since commercial ampholines are not available below pH 3.0 [5]. The enzyme activity was recovered in a sharp peak, at pH 2.5. The very low pI indicated that purification of the enzyme might be achieved using a DEAE-cellulose column. On passage through a DEAE column at pH 6 the enzyme was very tightly bound. However, it could be eluted with 0.2 M NaCl with two fold purification. The eluted enzyme was distinctly blue in colour, as has been reported for other laccases [9, 12].

Neurospora laccase [15] has much lower affinities for *p*-cresol and ascorbic acid than the laccase described here. The extracellular laccase from *Polyporus* has been investigated in considerable detail. It appears to contain two or three isoenzymes, one of which is a molecular hybrid [16, 17], which differ in their stability above pH 7.3, but are all inactivated above pH 8.4 or below pH 2.6. The *Polyporus* laccase has a MW of 62000 and contains 4 atoms Cu [18] which exists in two forms [16]. The extracellular laccase from *Botrytis* appears to be bound much more tightly to DEAE-cellulose than either the *Polyporus* or *Neurospora* laccase or the mycelial laccase of *Podospora* [19] and is characterised by its very low pH optimum and pI and stability to acid pH. Some of these unusual characteristics of the *Botrytis* laccase justify a more detailed study of its structure and molecular weight. Such a study is presently being undertaken.

EXPERIMENTAL

Botrytis cinerea was grown on liquid media consisting of an equal mixture of grape juice, prepared from ripe grapes 'Merlot' and 2% malt sterilised at 120° for 10 min. The fungus was grown in 1 l. Erlenmeyer flasks containing 200 ml medium and glass wool to serve as an inert support. After 15 days at 20° the culture medium was harvested.

Preparation of crude extract. The culture medium was centrifuged for 10 min at 12000 *g*. Me_2CO (−20°), was added to the medium, 3 ml per 2 ml culture. The ppt. was collected by centrifugation (40000 *g* for 12 min) and redissolved in Pi–citrate buffer, 0.1 M, pH 3.4. After centrifugation, the resultant clear soln (the crude enzyme extract) retained its activity for several weeks at 4°.

Measurement of enzyme activity. Enzyme activity was determined by following O_2 uptake using a polarographic O_2 electrode. The substrate was 10 mM quinol, final conc., and activity was measured at pH 4.75 at 25°.

Electrophoresis. Acrylamide gel electrophoresis was carried out essentially as described in ref [20] at pH 5.2 using as buffer 20 mM citrate–20 mM Tris (1:2). Electrophoresis was carried out for 2 h at 80 V and 20 mA. Enzyme was detected by immersing the gels in 10 mM *p*-phenylenediamine dissolved in Pi–citrate buffer. Electrophoresis on cellulose acetate was carried out in the presence of glycine–HCl buffer, 50 mM at pH values between 2.5 and 3.6. Migration was for 30 min at 200 V and at 25°. Enzyme activity was detected as above.

Isoelectric focussing was carried out as described in ref. [5].

Column chromatography on DEAE-cellulose was as described in ref. [21]. The column was 2.5 × 15 cm. Elution was stepwise with 40 mM Pi–citrate buffer pH 6.6 containing 20, 50, 100 and 200 mM NaCl. Protein in the eluate was estimated from absorption at 280 nm.

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