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INDUCER AND CULTURE MEDIUM DEPENDENT PROPERTIES OF EXTRACELLULAR LACCASE FROM *BOTRYTIS CINEREA*

IRITH MARBACH, EITAN HAREL and ALFRED M. MAYER

Department of Botany, The Hebrew University of Jerusalem, 91904, Jerusalem, Israel

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Key Word Index—*Botrytis cinerea*; fungus; extracellular laccase; enzyme induction; gallic acid; grape juice.

Abstract—Both the composition of the culture medium and the nature of the phenolic inducer determine the amount, the rate of formation and the molecular properties of extracellular laccase formed by *Botrytis cinerea*. Coumaric acid is shown to act as inducer in addition to gallic acid and grape juice. It is suggested that the fungus adapts to different environments by excreting different laccases. These laccases differ in pK, heat stability and substrate specificity but not in K_m values to quinol and oxygen.

INTRODUCTION

Previous reports on extracellular laccase from *Botrytis* [1, 2] indicated differences in the properties of the enzyme, depending on conditions prevailing during fungal growth. At the time it seemed possible that this was related either to the nature of the inducer, a component of grape juice or gallic acid, or to changes after prolonged subculture. It has been reported that the infectivity of *Botrytis* depends on the composition of its nutrient media [3]. Differences in level of production of laccase by *Botrytis* have also been reported [4, 5] depending on the presence of other fungi in the culture media.

Heterogeneity of laccase has been reported for fungi, e.g. *Podospora* [6–9]. In *Trametes* the relative amounts of three isoenzymes differing in electrophoretic mobility, appeared to be dependent on the composition of the culture medium [10]. Clearly defined factors in the culture medium are able to modify the formation of laccase. This must depend on the ability of the fungus to sense signals from the host plant or culture media and its ability to respond to such changes. Such properties would permit the organism to adapt its secretory activities to changing conditions and needs.

In the present paper we report on factors changing the amount of extracellular laccase formed by *Botrytis*, the effect of inducers on the nature of the enzymes formed and on some of the differences in properties of laccases formed in the presence of the different inducers. Since there appeared to be certain discrepancies between the reports on *Botrytis* extracellular laccase [1, 2, 11] these studies should help to resolve these.

RESULTS AND DISCUSSION

Gallic acid, which was used as inducer in our previous work [1, 12], caused browning of the growth medium, due to its oxidation. This browning causes difficulties during purification of the enzyme due to tanning reactions. We tried to reduce the browning effect by shortening the time of exposure to gallic acid.

The fungus was grown in the presence of gallic acid, and the medium containing the acid replaced by growth medium at different periods after sowing (Table 1).

Table 1. The effect of length of exposure to gallic acid on the level of extracellular laccase activity of *Botrytis*

Period of exposure to gallic acid	Enzyme activity	
	$\mu l\ O_2$/min · ml medium	$\mu l\ O_2$/min mg protein
0	0	0
First 3 days	0.76	4.9
First 6 days	0.70	4.0
13 days	2.0	3.5

In order to obtain maximal levels of extracellular enzyme in the growth medium the continuous presence of gallic acid is required. Lower levels of enzyme activity are obtained if gallic acid is present for the first 3 days only, despite the fact that enzyme secretion begins only after 1 week of fungal growth, when the inducers have already been removed from the growth medium. The exposure of the fungus to the inducer for 6 days did not increase the level of the enzyme beyond that achieved after 3 days. The processes which occur in the first 2–3 days of fungus growth are important for enzyme induction. This is in accord with our previous results [12] that in order to achieve a high level of enzyme secretion, the inducer must be added to the growth medium in the first 2 days of growth.

The specific activity of the enzyme is higher when the fungus is exposed to the inducer for a short time and the growth medium showed far less browning. However, the total recovery of enzyme during short periods of induction was too low to make this method of culture useful.

When the fungus was grown in the presence of gallic acid oxidized by the addition of sodium hydroxide no enzyme was secreted into the growth medium. Apparently, the quinones which are formed during fungal growth do not cause induction. These quinones may even inhibit further enzyme production or at least inactivate enzymes already present.

The induction of extracellular laccase formation in

various fungi, including *Botrytis*, by phenolic compounds has been reported [4, 13–15]. In order to improve the production of the extracellular laccase or to get a less coloured medium we tried a number of phenolic compounds as inducers (Table 2), in addition to those already tested previously [12].

Table 2. The effect of addition of phenolic compounds on the production of extracellular laccase

Phenolic compound added	Enzyme activity: $\mu l\ O_2$/min·ml medium	Enzyme activity: $\mu l\ O_2$/min·mg protein	Growth media colour
Gallic acid	2.0	3.8	brown
p-Hydroxybenzoic acid	0	0	clear
2,4-Dihydroxybenzoic acid	0	0	clear
Vanilic acid	0	0	clear
p-Hydroxycinnamic acid	1.9	5.6	cloudy white

Enzyme activity measured after 15 days of culture.

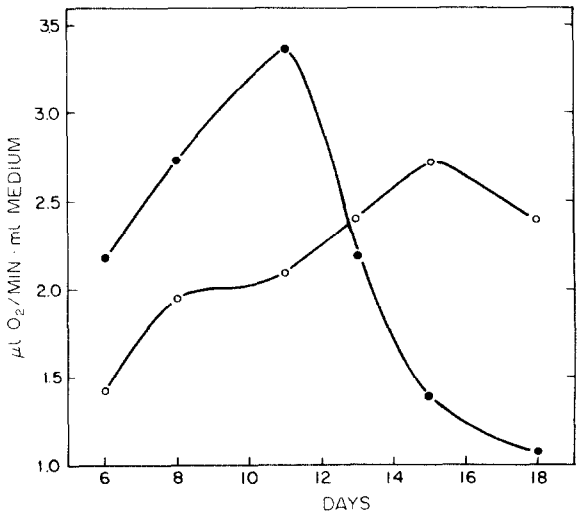

Fig. 1. The course of enzyme production by *Botrytis* in defined artificial medium or medium with malt extract, gallic acid as inducer in both cases. (●) Growth on synthetic medium. (○) Growth on medium with malt extract.

Table 3. The effect of different amounts of glucose in the growth medium on laccase production by *Botrytis cinerea*

Glucose concn (g/l.)	Days after sowing: 6	11	15	6	11	15
	$\mu l\ O_2$/min·ml medium			$\mu l\ O_2$/min·mg protein		
5	1.3	1.1	0.4	2.1	3.2	1.3
10	1.7	1.5	0.8	4.0	5.5	2.2
20	1.7	2.3	2.1	3.6	7.2	2.2
30	2.2	2.5	1.7	4.7	7.4	2.9
40	2.2	3.4	2.2	4.2	9.7	2.5

It is also possible to induce laccase formation with gallic acid as already mentioned or with *p*-hydroxycinnamic acid. Both *cis*- and *trans*-*p*-hydroxycinnamic acids were effective as inducers when the fungus was grown in incandescent light (200 lx). This might have caused some *cis–trans* isomerization, although UV light and alkaline medium are most effective in causing this [16]. Nevertheless, some differences in the effect of the *cis* and the *trans* acid were observed. Clearly, induction of extracellular laccase formation by *Botrytis cinerea* in our system is specific for certain substances (see also ref. [12]). Probably laccases from different fungal strains are induced by different phenols [1, 2, 4, 15, 17].

Additional attempts to increase enzyme production were made using the synthetic, defined medium of Kovak *et al.* [18] with the addition of gallic acid as inducer.

The course of enzyme formation in this artificial growth medium was compared to that of the medium with malt extract (Fig. 1). The maximal level of laccase secreted into the medium is higher in the artificial growth medium and the rise in enzyme activity is more rapid than in the media with malt. However, in the medium containing malt the level of activity remained fairly steady in the latter part of the growth period, while on the defined medium activity fell rapidly after reaching its peak level. The artificial medium contains a fairly large amount of glucose and this might account for the high level of activity. The effect of the amount of glucose in the artificial growth medium on enzyme production was determined. The results are summarized in Table 3.

The level of the extracellular laccase is increased with the rise in glucose level in the growth medium (Table 3). The peak of enzyme production is dependent on the level of glucose in the growth medium and the decrease in enzyme activity after 11 days of fungal growth in part be due to the decrease of the glucose level.

In our previous work [1, 2, 11, 12], different inducers, grape juice from fresh Merlot grapes [11] or commercially bottled grape juice [2] or gallic acid [1], were used to induce laccase formation. We concluded that different enzymes were produced, as judged by their amino acid composition in the presence of grape juice or gallic acid, but no other properties were investigated in detail. It now appears that the nature of the grape juice is also very important (cf. results reported here with those reported in ref. [11]).

Therefore, it became essential to characterize more clearly enzyme produced in the presence of different inducers. Enzyme was partially purified from two lots of culture medium, one with gallic acid as inducer and the other with commercially bottled grape juice. A number of properties of the enzymes were determined. The enzymes from the two growth media have the same affinity for oxygen and quinol but differ in their substrate specificity (Table 4). The difference in ability to oxidize monophenols is particularly striking, the grape juice induced enzyme having only low activity towards them.

The dependence of activity on pH of the two isoenzymes also differs (Fig. 2). They also showed marked differences in their heat inactivation curve (Figs. 3 and 4) and their p*K*.

Table 4. Substrate specificity of laccase from *Botrytis cinerea* induced by different inducers

Substrate	Relative activity	
	Enzyme induced by gallic acid	Enzyme induced by grape juice
[illegible]	[illegible]	[illegible]
Methylcatechol	100	81
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
p-Cresol	87	12
p-Coumaric acid	[illegible]	[illegible]
Vanillic acid	80	6

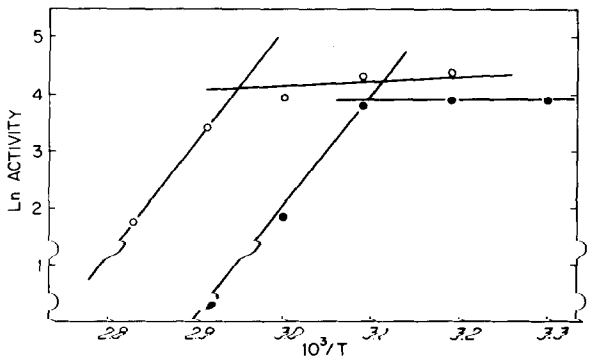

Fig. 4. Heat inactivation of laccase produced by *Botrytis* grown with gallic acid or grape juice as inducers. (Symbols as in Fig. 2.)

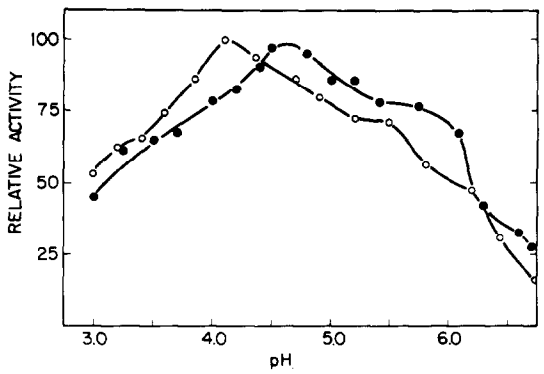

Fig. 2. pH profile of laccase produced by *Botrytis* grown in the presence of grape juice or gallic acid. (○) Grape juice as inducer. (●) Gallic acid as inducer.

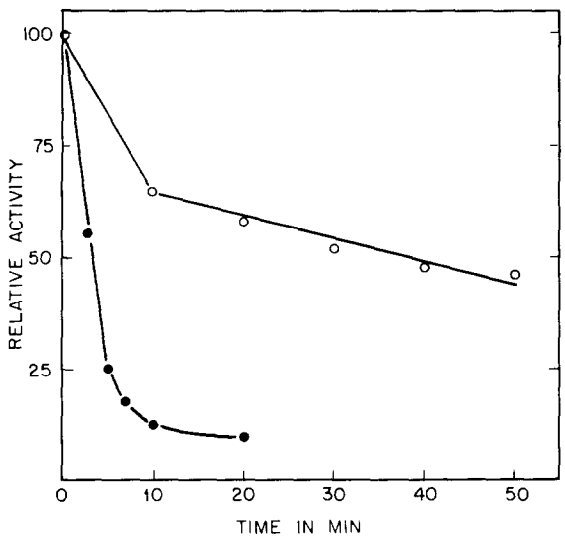

Fig. 3. The change in activity of laccase after different periods at 60°. (Symbols as in Fig. 2.)

In the curve showing dependence of activity on pH there is a slight shift toward acidic pH when grape juice is used as inducer in comparison to the enzyme induced by gallic acid (Fig. 2).

The enzyme induced by gallic acid is more sensitive to heat inactivation. At 60° it's activity decreases to 50% after 4 min while that of the enzyme induced by grape juice decreases to 50% only after 38 min (Fig. 3). The break in the curve of activity against $10^3/T$ in the gallic acid induced enzyme is at 50° while in the grape juice induced enzyme is at 66° (Fig. 4).

Activity towards monophenols is generally less stable than that towards diphenols. However, lack of monophenol oxidation is not accompanied by lower heat stability and, therefore, generally reduced stability of the grape juice induced enzyme is not involved.

The electrophoretic pattern of the two isoenzymes on cellulose acetate at pH 5.25 differed. They both show one band moving to the anode but the gallic acid induced enzyme had a band at the distance of 3.5–4.5 cm from the origin while the grape juice induced enzyme had a band at the distance of 0.5–1.5 cm from the origin.

Preliminary experiments using isoelectric focusing of the enzyme on polyacrylamide gels indicated marked differences in p*I*. The p*I* of gallic acid induced enzyme lies between 2.6–3.0 while that of the grape juice induced enzyme lies between 4.3–4.9.

These results show clearly that different inducers cause the formation of distinctly different extracellular laccases. It is not yet clear what are the molecular differences between the different forms. As indicated by our previous work they probably differ in their amino acid composition and in addition their sugar content may differ both quantitatively and qualitatively.

Some Basidiomycetes form a constitutive and an inducible laccase, which differ in their molecular properties [19, 20]. In the work described here the enzymes induced by different phenolics differ in their properties.

The differences in properties of the extracellular laccase from *Botrytis*, which is probably accompanied by a changed composition suggests that the fungus can adapt not only the amount but also the type of enzyme produced to conditions prevailing during its growth. This may permit the fungus to produce different laccases depending on the host plant or even on the particular stage of development of a given host. It also may allow the fungus to adapt to changing conditions in the growth medium.

Although the physiological function of fungal laccases is still unclear [21], great adaptability might be expected in a fungus with as wide a range of host plants as *Botrytis* [22].

EXPERIMENTAL

Botrytis cinerea was cultured as previously described [2, 6] at pH 3.5 on a media containing malt extract with grape juice or gallic acid 1 g/l. as inducer. In some expts the gallic acid was replaced by other compounds. In expts with defined synthetic culture medium, the medium used was that of ref. [18], also at pH 3.5. The fungi was grown in 2-l. flasks containing 500 ml medium for bulk enzyme preparation or in 500-ml flasks containing 130 ml medium for induction expts.

The extracellular laccase was partially purified by pptn with cold ($-20°$) Me_2CO as previously described [1].

Estimation of enzyme activity was with an oxygen electrode using 10 mM quinol as substrate [2].

Electrophoresis on cellulose acetate was according to ref. [1] at pH 5.25 for 50 min at 350 V.

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