Phytochemistry, Vol. 23, No. 12, pp. 2713–2717, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

MOLECULAR PROPERTIES OF EXTRACELLULAR *BOTRYTIS CINEREA* LACCASE

IRITH MARBACH, EITAN HAREL and ALFRED M. MAYER

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

(*Revised received* 1 *June* 1984)

Key Word Index—*Botrytis cinerea*; laccase; induction; amino acid composition; sugar composition; pathogenicity.

Abstract—Two molecular forms of extracellular laccase induced by different phenolics were studied in *Botrytis cinerea*. The enzyme induced by grape juice had a MW of 38 000 and contained 80% sugar while that induced by gallic acid had a MW of 36 000 and contained 70% sugar. Both forms contained arabinose, xylose, mannose, galactose and glucose but differed markedly in the relative content of these sugars. Tunicamycin, which inhibits glycosylation of peptide chains, considerably reduced the level of laccase in both hyphae and medium. The two enzyme forms differed also in their isoelectric focusing pattern and amino acid composition, the grape juice enzyme being richer in basic amino acids and poorer in acidic ones. A third form, induced by *p*-coumaric acid, resembled the laccase induced by gallic acid in many of its properties but was apparently not identical to it. The possible significance of the various forms in relation to the infection process by the fungus is discussed.

INTRODUCTION

In previous papers, we have reported some of the properties of the extracellular laccase from *Botrytis* [1–4] and demonstrated that different enzymes were produced depending on the nature of the inducer present in the growth medium [5]. Two distinct molecular forms of laccase have been described, for example in *Pholiota* [6]. The role of the enzyme in fungal development has been discussed [7] and its function continues to interest plant pathologists. Thus the interaction of phenolic compounds with *Fomes* laccase has been studied [8], and the laccase from *Stereum purpureum* has been investigated in relation to infection of apple wood [8–10]. In *Botrytis*, differences in substrate specificity and molecular properties have also been reported for other extracellular enzymes—two polygalacturonases and two pectinesterases [11]. The apparent importance of laccase in the process of infection and the ability of *Botrytis* to produce and excrete a variety of enzymes similar but not identical in property make a more complete characterization of different extracellular laccases of some importance. It was essential to compare the molecular properties of the enzyme in fungal cultures grown simultaneously in the presence of different inducers because of the well-known variability of *Botrytis* [5] and of fungi in general. In the following we report such a characterization.

RESULTS

The MWs of the enzymes produced in the presence of two different inducers were determined by ultracentrifugation. Enzyme, purified 350-fold, having a specific activity of 45 μl O_2/min per mg protein, was used. The enzyme induced by grape juice had an $S_{20,w}$ value of 2.4, $D_{20,w}$ of 5.4 (10^7 cm^2/sec) and a MW of 38 000, while the corresponding values for the gallic acid induced enzyme were $S_{20,w} = 2.3$, $D_{20,w} = 5.5$ and a MW of 36 000. The standard errors of S and D were between ± 0.03 and ± 0.07. Since we had previously demonstrated that the laccase was a glycoprotein [2, 3, 5], it was important to determine its precise sugar content. Some difficulty arose in this respect, since we had insufficient enzyme to determine sugar content on a dry weight basis. We therefore determined sugar content using the anthrone method and related it to protein as determined by three methods: UV absorption, Folin–Ciocalteu reagent and Nesslerization (see Experimental). The results are shown in Table 1. All methods of calibration showed a very high sugar content, the real value evidently lying at around 80% for the grape-juice-induced enzyme and 70% for the gallic-acid-induced enzyme. Even the UV-based calculations showed a very high sugar content. The two enzymes differed significantly in the relative amount of sugars linked to them (Table 2), linkage to the protein being of the *N*-glycosidic type, frequently reported for plant glycoproteins [12]. The differences between the enzymes were extremely striking in all the sugars present. The significance of the sugar part of the enzyme was indicated from experiments using tunicamycin, which is known to inhibit glycosylation of peptide chains [13].

Addition of 10 μg/ml of tunicamycin to the growth medium reduced both extracellular and intracellular laccase activity (Table 3). The suppression of activity of the extracellular enzyme was slightly greater than that in the hyphae. Glycosylation might be related to enzyme secretion but we found no convincing evidence that the tunicamycin changed the percentage of sugar in the extracellular enzyme. When sugar content was determined and based on the Folin reaction for protein, there was a 3% reduction of the sugar content. When based on protein determined by UV absorption, reduction was greater—34%.

In our previous report we indicated that the isoelectric point of the extracellular laccase differed depending on the inducer [5]. In more detailed experiments, this was

Table 1. Sugar content of the extracellular laccase from *Botrytis*

	% Sugar			
	(using anthrone reagent)			(using GLC)
	Protein determined by:			
Inducer	Folin–Ciocalteu	Nessler	UV	Folin–Ciocalteu
Grape juice	81.6	81.6	75	84
Gallic acid	71.4	71.8	55	70

Table 2. Sugar composition in the extracellular laccase from *Botrytis*

	Inducer			
	Grape juice		Gallic acid	
Sugar	% of total	Relative to xylose	% of total	Relative to xylose
Arabinose	2.7	2.1	7.4	1.4
Xylose	1.3	1	5.4	1
Mannose	55.2	43.9	38.8	7.2
Galactose	28	22.2	32.9	6.1
Glucose	13	10.4	23.6	4.3
Acetyl-*N*-glucosamine	trace		trace	

Table 3. Effect of tunicamycin on the laccase activity of *Botrytis cinerea**

	Enzyme activity (μl O_2/min per mg protein)	% Inhibition
In medium – tunicamycin	1.67	
+ tunicamycin	0.54	68
In hyphae – tunicamycin	1.28	
+ tunicamycin	0.48	62.5

*Inducer was gallic acid. Tunicamycin concentration was 10 μg/ml. Enzyme activity was determined after 20 days.

very clearly shown to be the case. Figure 1 shows the isoelectric focusing pattern of the two forms of laccase—induced by gallic acid and by grape juice. The gallic acid induced enzyme showed enzyme activity only at very acid isoelectric points while that of the grape juice induced enzyme showed some bands even at pH 5.0

The difference in isoelectric points of the two enzymes is clearly reflected in their amino acid composition (Table 4). The most striking differences are in the content of total basic and total acidic amino acids. As was to be expected, the grape juice induced enzyme had a markedly higher basic and lower acidic amino acid content than that of the gallic-acid-induced enzyme.

It seemed possible that part of the very low (acid) isoelectric point of the gallic acid induced enzyme was due to phosphorylation of some groups in the enzyme. In order to test this possibility, the fungus was grown in the presence of gallic acid as inducer, in the presence of citrate buffer. After 7 days, when extracellular enzyme activity could not yet be detected, radioactive phosphate (carrier-free) was added to the medium and growth was continued for 10 days. The proteins excreted into the medium were collected and partially purified by acetone fractionation followed by passage through a mini-column of Biogel P-150. The enzyme fraction contained a significant number of counts. However, following electrophoresis on cellulose acetate, radioactive protein was clearly separated from enzyme activity (Fig. 2). Thus, although the fungus did excrete at least one phosphorylated protein, the laccase was not phosphorylated.

Since each inducer resulted in the formation of a distinct enzyme, we wanted to determine whether formation of the two enzyme forms was mutually exclusive. For this purpose, the fungus was grown in the presence of both inducers simultaneously. The laccase was partially purified and its pH dependence, substrate specificity and isoelectric focusing pattern were determined (Table 5 and Figs. 3 and 4). Clearly, a mixture of the two forms was produced since substrate specificity represented both forms, the pH dependence course was broadened, and on isoelectric focusing, enzyme activity over a wide range of isoelectric points was detected.

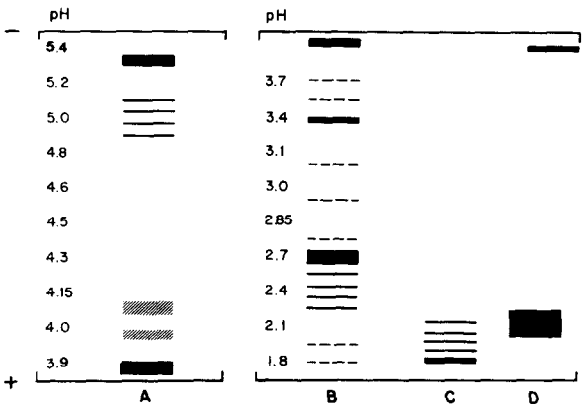

Fig. 1. Isoelectric focusing pattern of the three forms of extracellular laccase from *Botrytis* induced by grape juice, gallic acid and *p*-coumaric acid, respectively. (■) Strong activity; (▨) weak activity; (– – –) very weak activity. (A and B) Purified grape juice induced enzyme; (C) purified gallic acid induced enzyme; (D) partially purified *p*-coumaric acid induced enzyme. No activity was detected between pH 4 and 6 for the gallic acid induced enzyme.

We previously indicated [5] that *p*-coumaric acid also acted as an inducer of enzyme formation and that its addition resulted in a cloudy white culture medium due to the secretion of some compounds, possibly polysaccharide. It was necessary to characterize the laccase found by this inducer, too. The substrate specificity closely resembled that of the gallic acid induced enzyme; its K_m was 0.22 mM for O_2, and 2.22 mM towards quinol. Its sensitivity to heat inactivation was similar to that of the gallic acid induced enzyme. At 60°, its activity decreased to 50% after 4 min. Its isoelectric focusing pattern was similar to that for the gallic acid induced enzyme (Fig. 1) but it showed some bands also found in the grape-juice-induced enzyme; however, its pH dependence was distinctly different (Fig. 3).

DISCUSSION

We previously suggested that variability in the properties of extracellular laccase from *Botrytis* might be related to the phytopathogenic process [5]. In the present paper, we have characterized two distinct extracellular laccases more fully and report some of the properties of a third one. It seems that we should not refer to these as isoenzymes since the two forms differ in many important parameters—pH optimum, amino acid and sugar composition, isoelectric point and substrate specificity. Both show some heterogeneity and appear to contain isoenzymes as shown by the isoelectric focusing pattern. The formation of each enzyme form is switched on specifically by a well-defined inducer, or more probably a group of inducers. Production of the two distinct forms is not mutually exclusive. Clearly each inducer is capable of switching on a specific protein-synthesizing system, in all probability a specific gene or group of genes. While a variety of laccases are apparently formed constitutively, e.g. *Agaricus* [14, 15], *Fomes* [8] and *Stereum* [9, 10], they are inducible in other cases, e.g. *Polyporus versicolor* [16], *P. anceps* [17] *Neurospora* [18] and *Pholiota* [6]. Fungi

Table 4. Amino acid composition of the purified extracellular laccase from *Botrytis**

	Amount relative to leucine		% of total	
Amino acid	Grape juice	Gallic acid	Grape juice	Gallic acid
Aspartic acid + asparagine	1.56	1.75	9.68	13.03
Threonine	2.01	1.47	12.47	10.91
Serine	2.35	1.35	14.63	10.01
Glutamic acid + glutamine	1.05	0.99	6.55	7.40
Proline	0.86	0.85	5.36	6.29
Glycine	1.46	1.25	9.09	9.30
Alanine	1.83	1.44	11.35	10.70
Cysteine + cysteic acid	0.28	0.06	1.73	0.48
Valine	0.95	0.81	5.93	5.94
Isoleucine	0.68	0.66	4.24	4.94
Leucine	1.0	1.0	6.22	7.44
Tyrosine	0.26	0.46	1.64	3.40
Phenylalanine	0.55	0.55	3.40	4.09
Histidine	0.17	0.27	1.09	1.94
Lysine	0.43	0.27	2.66	1.97
Arginine	0.64	0.28	3.96	2.09

	Grape juice	Gallic acid
Basic amino acids	7.7%	6.0%
Acidic amino acids	16.0%	20.4%

*Enzyme formation was induced with either gallic acid or grape juice and the enzyme was purified as described in Experimental.

Tryptophan and methionine were not detected presumably due to decomposition during hydrolysis.

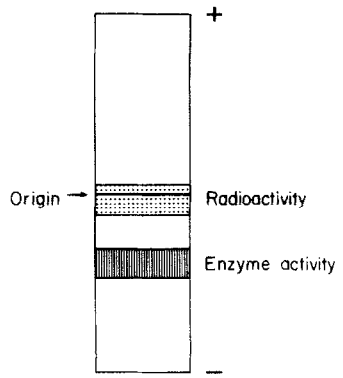

Fig. 2. Electrophoresis on cellulose acetate of the partially purified extracellular protein fraction from *Botrytis* fed with $^{32}PO_4^{3-}$ just before the onset of laccase formation.

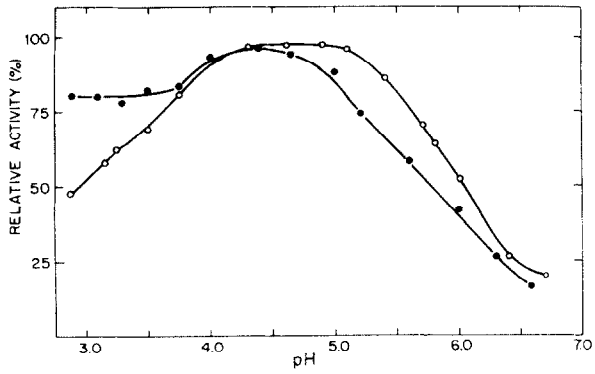

Fig. 3. pH dependence of the extracellular laccase from *Botrytis* formed in the presence of both gallic acid and grape juice, or in the presence of *p*-coumaric acid. (○—○) Inducer: grape juice + gallic acid; (●—●) inducer: *p*-coumaric acid.

containing the constitutive enzyme may also form inducible laccase. Blaich and Esser [19] claimed that in a wide variety of races of *Pleurotus* only slight differences in enzyme property could be brought about, but later work by Prillinger and Molitoris [20] using genetic analysis, brought clear-cut evidence of biochemical differences in the races of this fungus. In a very recent report [21], it has been shown that in *Fomes* exposure to lignosulfonate results in *de novo* synthesis of extracellular laccase, and that only this enzyme is excreted, although an intracellular laccase was present in the absence of inducer. The apparent contradictions in the reports on inducibility versus constitutiveness of the enzyme must be related to the very large variety in host plants and the mode of nutrition of the different fungi.

It is not unlikely that those fungi which must attack a lignified substrate for growth contain, in the first instance, a constitutive enzyme, while those which are more widespread in their attack, such as *Botrytis* or *Neurospora*, will also contain an inducible enzyme. What characterizes *Botrytis* is that under normal conditions and in a variety of growth media the enzyme is totally absent at all phases

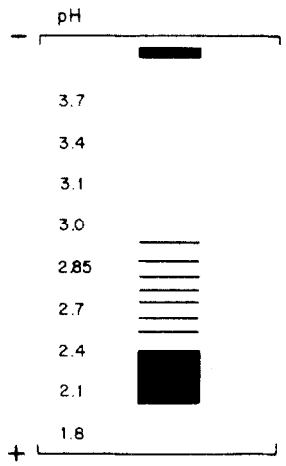

Fig. 4. Isoelectric focusing pattern of the extracellular laccase from *Botrytis* formed in the presence of both gallic acid and grape juice. Symbols as in Fig. 1.

Table 5. Substrate specificity of the extracellular laccase from *Botrytis* found in the presence of gallic acid, *p*-coumaric acid and commercial grape juice*

	Relative activity			
			Inducer present:	
Substrate	Gallic acid	Grape juice	*p*-Coumaric acid	Gallic acid + grape juice
Quinol	100	100	100	100
4-Methylcatechol	100	81	100	94
Gallic acid	125	99	100	104
p-Cresol	87	12	84	72
p-Coumaric acid (*trans* + *cis*)	50	4		25
Vanillic acid	80	6	82	49
Caffeic acid	100	93	114	
trans p-Coumaric acid			43	
cis p-Coumaric acid			29	

*Activity is relative to quinol. Substrate specificity of laccase activity was induced separately by each inducer according to ref. [5].

of growth. This contrasts with *Agaricus* where the extracellular enzyme is low at the fruiting stage but high during vegetative growth. Despite the extensive literature on laccase, its precise function in the life of the fungi producing it is still uncertain. This is particularly true in the case for laccase in *Botrytis*. The presence of other fungi, such as *Penicillium*, in the growth medium causes an augmentation of laccase production [22], and phenols may have a role in pathogenicity [23], fungal enzyme apparently oxidizing inhibitory phenols, at least in *Botrytis allii*. It has also been shown that bean plants infected by *Botrytis cinerea* respond by phytoalexin formation, production being greatest in the less susceptible cultivars and greatest in lesions produced by the least virulent isolates of *Botrytis* [24]. Among the phytoalexins found was phaseollin. These observations indicate that *Botrytis* evokes a plant response but the more virulent races are able to overcome this response. A similar situation exists in *Vicia faba* infected with *B. fabae* [25]. We would like to suggest that inductive laccase production by *Botrytis* plays an important role in the infective process. The laccase is produced in order to inactivate defence mechanisms produced by the host, be they phenolic compounds present in the host or phytoalexin produced in response to infection. Furthermore, the great versatility of *Botrytis* in producing such varied laccases accords well with its versatility in infecting a wide range of plant species. Preliminary experiments in our laboratory have indicated that when *Botrytis* infects cucumber or carrot, part of the invasion process is laccase production by the fungus, and is accompanied by laccase secretion into the host tissue. This system is now under detailed investigation.

EXPERIMENTAL

The fungus was grown as previously described [5] on malt extract to which was added either 1 g/l. gallic acid or 50% grape juice (Tirosh-Carmel Wine Growers Cooperative, Israel) as inducer. Laccase activity was routinely assayed using an oxygen electrode as described in refs. [2] and [3].

Purification of extracellular laccase. The growth medium was clarified by centrifugation at 13 000 *g* for 10 min. To the supernatant, 1.5 vol. of cold −20° Me_2CO was added and the ppt. collected. The ppt. was dissolved in 0.1 M KP_i–citrate buffer, pH 3.5. The enzyme soln was concd using ultrafiltration and an Amicon PM10 filter with nominal exclusion of 10 000 Daltons. The conc. enzyme soln was applied to a Biogel P150 column (1.6 × 20 cm) and eluted from the column using 0.1 M, KP_i–citrate buffer, pH 3.5. The sp. act. in the fractions was determined and the most active fraction was used for the experiments. Protein was determined by three methods: Nesslerization [26], Folin–Ciocalteu's reagent [27] or UV absorption [28]. Total sugar was determined using the anthrone reagent [29], or using GLC [30]. The composition of the sugars in the protein was determined using GLC. Electrophoresis in cellulose acetate was as previously described [2, 3]. Isoelectric focusing was on acrylamide gel (0.5 mm) between pH 2–4 and 4–6 at 1000 V, for 20 hr, when no further migration occurred. The electrode solns were: for pH 2–4, 1 M H_3PO_4 for the anode and 0.4 M Hepes for the cathode; and for pH 4–6, 0.5 M HOAc for the anode and 0.5 M NaOH for the cathode. Activity in the gel was determined as described for electrophoresis [2, 3]. The pH of the gel was determined using an LKB electrode.

Acknowledgement—This work was supported by a grant from the National Council for Research and Development of Israel.

REFERENCES

1. Dubernet, M., Ribereau-Gayon, P., Lerner, H. R., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16**, 191.
2. Gigi, O., Marbach, I. and Mayer, A. M. (1980) *Phytochemistry* **19**, 2273.
3. Gigi, O., Marbach, I. and Mayer, A. M. (1981) *Phytochemistry* **20**, 1211.
4. Mayer, A. M., Marbach, I., Marbach, A. and Sharon, A. (1977) *Phytochemistry* **16**, 1051.
5. Marbach, I., Harel, E. and Mayer, A. M. (1983) *Phytochemistry* **22**, 1535.
6. Leonowicz, A. and Malinowska, M. (1982) *Acta Biochim. Polon.* **29**, 214.
7. Herman, T. E., Kurtz, M. B. and Champe, S. P. (1983) *J. Bacteriol.* **154**, 955.
8. Haars, A., Chet, I. and Hutterman, A. (1981) *Eur. J. Forest Pathol.* **11**, 67.
9. Miyairi, K., Shinya, M., Okuno, T. and Sawai, K. (1982) *Bull. Fac. Agric. Hirosuki Univ.* **37**, 11.
10. Miyairi, K., Murakami, A., Okuno, T. and Sawai, K. (1982) *Ann. Phytopathol. Soc. Jpn.* **48**, 177.
11. Marcus, L. and Schejter, A. (1983) *Physiol. Plant Pathol.* **23**, 1.
12. Sharon, N. and Lis, H. (1979) *Biochem. Soc. Trans.* **7**, 783.
13. Cabib, E. and Roberts, R. (1982) *Annu. Rev. Biochem.* **51**, 763.
14. Wood, D. A. (1980) *J. Gen. Microbiol.* **117**, 327.
15. Wood, D. A. (1980) *J. Gen. Microbiol.* **117**, 339.
16. Fahraeus, C. and Reinhammar, B. (1967) *Acta Chem. Scand.* **21**, 2367.
17. Petroski, R. J., Peczynska-Czoch, W. and Rosazza, J. P. (1980) *Appl. Environ. Microbiol.* **40**, 1003.
18. Froehner, S. C. and Eriksson, K. E. (1974) *J. Bacteriol.* **120**, 458.
19. Blaich, R. and Esser, K. (1975) *Arch. Microbiol.* **103**, 271.
20. Prillinger, H. and Molitoris, H. P. (1979) *Physiol. Plant.* **46**, 265.
21. Haars, A. and Hütterman, A. (1983) *Arch. Microbiol.* **134**, 309.
22. Kovac, V. (1979) *Ann. Technol. Agric.* **28**, 341.
23. Kritzma, G. and Chet, I. (1980) *Phytoparasitia* **8**, 27.
24. Fraile, A., Garcia-Arenal, F. and Sagasta, E. M. (1980) *Physiol. Plant Pathol.* **16**, 9.
25. Rossall, S., Mansfield, J. W. and Hutson, R. A. (1980) *Physiol. Plant Pathol.* **16**, 135.
26. Umbreit, W. W., Burris, R. H. and Stauffer, J. E. (1972) *Manometric and Biochemical Techniques*, 5th edn, p. 254. Burgess, Minneapolis.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
28. Layne, E. (1957) in *Methods in Enzymology* (Colowick, S. D. and Kaplan, N. O., eds.), Vol. 3, p. 451. Academic Press, New York.
29. Ashwell, G. (1957) in *Methods in Enzymology* (Colowick, S. D. and Kaplan, N. O., eds.), Vol. 3, p. 84. Academic Press, New York.
30. Clamp, J. R., Bhatti, T. and Chambers, R. E. (1971) in *Methods of Biochemical Analysis* (Blick, D., ed.), Vol. 19, pp. 229–344. John Wiley, New York.