Phytochemistry, Vol. 20, No. 6, pp. 1211–1213, 1981.
Printed in Great Britain.

0031-9422/81/061211-03 $02.00/0
Pergamon Press Ltd.

PROPERTIES OF GALLIC ACID-INDUCED EXTRACELLULAR LACCASE OF *BOTRYTIS CINEREA*

ORITH GIGI, IRITH MARBACH and ALFRED M. MAYER

Botany Department, Hebrew University, Jerusalem, Israel

(*Revised received* 20 *October* 1980)

Key Word Index—*Botrytis cinerea*; laccase; extra- and intracellular; molecular properties.

Abstract—The properties of the extracellular laccase produced by *Botrytis cinerea* induced by gallic acid are compared with the laccase produced by *Botrytis* in the presence of grape juice. The extra- and intracellular laccases are compared and found to be very similar.

INTRODUCTION

In a previous paper [1] we reported that gallic acid was able to induce laccase formation in *Botrytis cinerea* and that the enzyme was excreted into the growth medium. The amounts produced were much greater than those produced in the presence of grape juice [2]. It seemed important to determine whether this enzyme was indeed the same as that previously characterized [3] or whether more than one laccase is excreted, as reported for example for *Pleurotos* laccase [4]. At the same time it was of interest to characterize the intercellular enzyme and compare it to the extracellular enzyme.

RESULTS AND DISCUSSION

Various means of purifying the extracellular laccase were tried. The enzyme was precipitated from solution between 80 and 100% saturation with ammonium sulphate but with poor yield. Precipitation with 60% acetone was more effective [3]. Passage of the enzyme obtained from acetone precipitation through a Sepharose–ConA column followed by elution by α-methyl-D-mannoside resulted in good purification, with 40% yield. However, the methyl mannoside interfered in subsequent analytical procedures. We therefore finally adopted purification by passage through a Bio-Gel P150 column (Table 1).

The purified enzyme moved as a single symmetrical peak in the ultracentrifuge. The purified enzyme had the following physical characteristics as determined by ultracentrifugation: $s_{20,w}$ 2.4S, $D_{20,w}$ 10^7 cm^2/sec. The protein moved as a single sharp peak. The MW of the enzyme was 35 500 assuming a partial specific value of 0.7 [5]. The sugar content was determined using two different methods of estimating protein (Table 2). The sugar content of the enzyme, as given in Table 2, shows very great variability depending on the method used to estimate protein. Such discrepancies are not unusual and are a function both of the sensitivity of the method and composition of the protein and of the sugar of the enzyme.

Table 1. Purification of extracellular laccase from *Botrytis* (enzyme formation induced by gallic acid)

	Specific activity (μl O_2/mg protein/min)	Purification	Yield
Crude growth medium	2.7	1	100
Acetone precipitation	12.6	4.7	71
Ultrafiltration	8.2	3.0	46.6
Bio-Gel P 150	77.5	28.7	37.3

Table 2. Sugar content of extracellular laccase from *Botrytis*

Basis for protein determination	% Sugar determined by Anthrone reagent	GLC
Lowry *et al.* [6]	67	77
UV absorption [7]	26	36

Table 3. Relative amounts of sugars found in acid hydrolysate of extracellular laccase from *Botrytis* (amounts relative to rhamnose)

Sugar	Relative amounts	% of total
Rhamnose	1	4.15
Arabinose	3.63	15.1
Xylose	2.65	11.0
Galactose	14.92	62.0
Glucose	1.84	7.5
Acetyl-*N*-glucose	0.16	

This indicates a sugar content comparable to that of other laccases [5]. Insufficient protein was available in order to allow determination of sugar content on a dry weight basis. The sugar composition of the enzyme was determined qualitatively by TLC. The following sugars were found: rhamnose, xylose, arabinose, mannose, glucose and galactose. Quantitative analysis of the sugars by GLC is shown in Table 3. Mannose was not detected by GLC, probably because its presence was masked by the large excess of galactose.

The sugar content of only a few laccases has been described. The sugar composition is similar to that of *Rhus* and of *Schinus* [5] as well as to the fungal laccases [8]. However, quantitative data on the fungal laccases are scant. The sugar composition suggests that the sugar protein linkage is an N=glycosidic one, but this requires further study [9].

The amino acid composition of the purified enzyme is shown in Table 4. This result differs significantly from that previously reported by us [3]. The amount of basic amino acids is significantly higher than in our previous preparation. This is accompanied by a significantly lower MW, 35 500 cf. 48 000. The reason for these differences is not immediately obvious. However, it must be recalled that the enzyme dealt with here is produced in much greater amounts, and that its method of purification is totally different from our previous method (DEAE-cellulose column). It is possible that in the presence of the inducing agent, and due to the method of purification used, part of the molecule is lost during excretion from the cell. Alternately a different laccase may be produced under the conditions of growth used. The literature on laccase indicates that such a possibility exists, for example, in *Polyporus* [10] and *Podospora* [11]. Differences between an induced and a constitutive laccase have been reported for *Pholiota* [12]. This difference between the two enzymes is also borne out by their behaviour on electrophoresis on cellulose acetate. The purified enzyme moved as a single band on electrophoresis. This band stained for protein, sugar and enzyme activity. The laccase previously isolated had an isoelectric point determined by electrophoresis of 2.5 [2]. The enzyme described here moved to the positive pole at pH 3.5 and at pH 2.5 it moved slightly towards the negative pole. This would indicate an isoelectric point between 2.5 and 3.5, i.e. higher than we previously recorded. Heat inactivation of the extracellular enzyme was slightly greater than that reported for the extracellular enzyme previously reported [2]. It was stable at 45°, lost 50% of its activity within 220 sec at 53° and within 30 sec at 69°. The differences are probably not very significant.

Table 4. Amino acid composition of purified extracellular laccase from *Botrytis* (amounts relative to arginine or leucine)

Amino acid	Amount relative to Arginine	Amount relative to Leucine	Previous results relative to leucine*
Aspartic acid	5.28	1.72	1.22
Threonine	5.3	1.72	1.86
Serine	5.8	1.89	2.19
Glutamic acid	3.62	1.18	0.65
Proline	2.69	0.88	0.63
Glycine	4.24	1.38	1.0
Alanine	5.39	1.76	1.71
Cystine + cysteine	0.11	0.04	0.04
Valine	3.24	1.06	0.67
Methionine	0.63	0.21	0.08
Isoleucine	2.07	0.68	0.95
Leucine	3.07	1.0	1.0
Tyrosine	1.97	0.64	0.29
Phenylalanine	2.34	0.76	0.26
Histidine	0.74	0.24	0.15
Lysine	0.87	0.28	
Arginine	1.00	0.33	0.06

* Recalculated from ref. [3].

We determined a number of properties of the intracellular enzyme and compared these to the extracellular enzyme in order to determine whether laccase undergoes any significant changes during excretion into the media. The pH optimum and dependence of activity on pH in the two preparations were identical (data not shown) and were similar to that of the laccase previously isolated [2]. The pH optimum was 4.5. The K_m for O_2 of the extracellular enzyme was 0.25 mM and that of intracellular enzyme 0.37 mM in the presence of air. Although some differences in the rate of oxidation of a number of phenolic substrates was observed between the two enzymes, these were not very large and did not point to any very major structural differences. Both oxidized ascorbic acid, quinol, catechol, caffeic acid, chlorogenic acid and ferulic acid at about the same rate. They both oxidized tyrosine very slowly and failed to oxidize *p*-hydroxybenzoic acid. The extracellular enzyme oxidized *p*-cresol rather better than the intracellular enzyme. However, this might be due to the carry-over of small amounts of phenolic compounds during purification of the extracellular enzyme and consequent coupled oxidation. Slight differences in electrophoretic mobility on cellulose acetate were observed between the two enzymes.

It seems, therefore, that no major alteration occurs in enzyme structure or function during its excretion into the media. However, minor changes probably do occur. Our methods were inadequate to determine these with any certainty. The extra- and intracellular laccase of *Neurospora crassa* have been found to be similar [13] as determined by gel filtration and ion exchange chromatography although other properties were not studied.

The main outstanding problem remaining is therefore the major difference in amino acid content and MW of the gallic acid-induced laccase and that previously described by us [3]. This problem requires much more detailed study.

EXPERIMENTAL

Botrytis cinerea was cultured as previously described [1] in 21 flasks containing 500 ml medium. The extracellular enzyme was precipitated from the growth media by addition of ice-cold Me_2CO to 60% v/v after centrifugation of the media for 10 min × 20000 *g* to remove spores and mycelium. The intracellular laccase was prepared by grinding the mycelium with sand in the cold and centrifuging 10 min × 7000 *g*. The enzyme was precipitated by the addition of −20° Me_2CO to 60% v/v. The precipitates of both Me_2CO precipitations were suspended in all cases in cold 0.1 M phosphate–citrate buffer, pH 3.5. In some cases, e.g. substrate affinity experiments, these solns were used as source of enzyme after centrifugation at 13000 *g* × 20 min.

Purification of extracellular laccase. The soln in 0.1 M phosphate–citrate, pH 3.5, was centrifuged at 10000 *g* × 10 min and stored at 4°. The result of a number of preparations was combined and concd by ultrafiltration using an Amicon PM10 filter with cut-off point of about 10000 daltons. The enzyme preparation was applied to a Concanavalin A–Sepharose column equilibrated with 0.1 M phosphate–citrate buffer, pH 5.5, containing 1 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$. The enzyme was eluted off the column with 10% α-methyl-D-mannoside in 0.1 M phosphate–citrate buffer, pH 5.5. The second method of purification was on a Bio Gel-P150 column. The column was equilibrated with 0.1 M phosphate–citrate buffer, pH 3.5 and enzyme eluted with the same buffer. In all cases fractions were collected with a fraction collector and the columns maintained at 0–4°.

Estimation of enzyme activity was with an oxygen electrode using quinol 10 mM as substrate [1, 3].

Electrophoresis on cellulose acetate. The enzyme was run on cellulose acetate strips (Macherey–Nagel) and normally the runs were in 50 mM Tris–citrate buffer, pH 5.25, at 300 V for 2 hr. Enzyme was detected on the strips by incubation with 10 mM quinol and colour amplified by addition of 1% *p*-phenylenediamine. Protein was located by staining with Amido black, and glycoprotein detected by the reaction with basic fuchsin [14].

Thin layer chromatography. Purified enzyme was exhaustively dialysed. Protein (10 mg) was hydrolysed with 0.5 ml 2 N H_2SO_4 at 100° for 4 hr. The acid was neutralized with solid $BaCO_3$ and the soln centrifuged. The hydrolysate was passed through two 1 × 1 cm columns, first Dowex 50 W and then Dowex 1 W, to remove amino acids and the sugars eluted off with water. The soln was applied to cellulose plates, 0.1 mm (Merck). The plates were developed in one direction with pyridine–EtOAc–HOAc–H_2O (36:36:7:21) and in the second direction with EtOAc–pyridine–H_2O (2:1:2, upper phase). The sugars were detected using *p*-anisidine phthalate [15]. The sugar content of the enzyme was determined using the anthrone reagent with glucose as standard [16]. For this purpose the glycoprotein was precipitated using 20% trichloroacetic acid and 60% Me_2CO.

Ultracentrifugation, amino acid composition, MW were determined by the methods quoted in a previous paper [5].

Acknowledgements—Our thanks are due to Mr. P. Yanai for running the sedimentation analyses, to Mrs. S. Rogozinsky for the GLC analyses and to Professor N. Sharon for valuable discussions.

REFERENCES

1. Gigi, O., Marbach, I. and Mayer, A. M. (1980) *Phytochemistry* **19,** 2273.
2. Dubernet, M., Ribereau-Gayon, P., Lerner, H. R., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16,** 191.
3. Mayer, A. M., Marbach, I., Marbach, A. and Sharon, A. (1977) *Phytochemistry* **16,** 1051.
4. Prillinger, H. and Molitoris, H. P. (1979) *Physiol. Plant.* **46,** 265.
5. Bar-Nun, N., Mayer, A. M. and Sharon, N. (1981) *Phytochemistry* **20,** 407.
6. Lowry, O. H., Rosebrough, N. J., Farr, L. A. and Randall, A. J. (1951) *J. Biol. Chem.* **193,** 265.
7. Layne, E. (1955) in *Methods of Enzymology* (Colowick, S. D. and Kaplan, N. O., eds.) Vol. 3, p. 451. Academic Press, New York.
8. Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18,** 193.
9. Sharon, N. and Lis, H. (1979) *Trans. Biochem. Soc.* **7,** 783.
10. Cheung, D. S. M. and Marshall, K. C. (1969) *Biochim. Biophys. Acta* **178,** 172.
11. Molitoris, H. P., van Breemen, J. F. L., van Bruggen, E. F. J. and Esser, K. (1972) *Biochim. Biophys. Acta* **271,** 286.
12. Leonowicz, A., Grzywnowicz, K. and Malinowska, M. (1979) *Acta Biochim. Pol.* **26,** 431.
13. Froehner, S. C. and Erikssen, K.-K. (1974) *J. Bacteriol.* **120,** 458.
14. Zaccharius, R. M., Zell, T. E., Morrison, J. H. and Woodlock, J. J. (1969) *Analyt. Biochem.* **30,** 148.
15. Schweiger, A. (1962) *J. Chromatogr.* **9,** 374.
16. Ashwell, G. (1957) in *Methods of Enzymology*, Vol. 3, p. 84. Academic Press, New York.