0273-2289/87/1503—0213$02.60

Purification and Properties of Two Laccase Isoenzymes Produced by *Botrytis cinerea*

NABIL ZOUARI,* JEAN-LOUIS ROMETTE, AND DANIEL THOMAS

Laboratoire de Technologie Enzymatique, Université de Technologie de Compiègne, B.P. 233; 60206, Compiègne Cedex, France

Received November 17, 1986; Accepted April 15, 1987

ABSTRACT

Laccases produced by five strains of *Botrytis cinerea* were studied. Extraction and purification have been performed in order to compare the enzymatic characteristics both, physicochemically and kinetically.

Two strains produced isoenzymes of laccase. These two molecular forms of laccase had different isoelectric points (2.6 and 2.8) and sugar content (86 and 91%). The optimum reactional pH was found to be very similar for both enzymes, in contrast to the temperature sensitivity, which is very different.

Index Entries: *Botrytis cinerea;* laccases; phenoloxidases; isoenzymes; production, purification, and characterization of laccase.

INTRODUCTION

Many fungi produce laccase intracellularly or extracellularly to oxidize some phenolic compounds (*1*). It was first suggested by Laborde (*2*) that laccase from *Rhus succedanea* is similar to that from *Botrytis cinerea* in wine. But, it is well known that laccases from fungi and those from higher plants are different in their characteristics.

Dubernet and Ribereau-Gayon (*3*) purified the laccase from *Botrytis cinerea* and briefly characterized its properties. In the literature, the heterogeneity of the laccases from *Botrytis cinerea* is clearly demonstrated.

* Author to whom all correspondence and reprint requests should be addressed.

It has been known for a long time that laccases from *Botrytis cinerea* are involved in the rotting of grapes and its oenological consequences. The study of these laccases is important for the development of technology to control this disease.

Furthermore, laccase activity can be used as a tool for the estimation of phenolic compounds, particularly in waste waters.

The aim of this paper is to study the laccases produced by five strains of *Botrytis cinerea,* to purify them, and to compare the enzymatic characteristics both physicochemically and kinetically. This has permitted us to develop applications (*4*) in the fields described previously.

MATERIALS AND METHODS

Organisms and Culture Conditions

The following strains of *Botrytis cinerea* (*B.c*) were obtained from the Museum National d'Historie Naturelle de Paris:

I. *Botrytis cinerea* n° 3127 isolated from tomato.
II. *Botrytis cinerea* isolated from pumpkin.
III. *Botrytis cinerea* n° 841 isolated from grape.
IV. *Botrytis cinerea* n° 842 isolated from grape.
V. *Botrytis cinerea* n° 844 isolated from grape.

The strains were picked on agar (2% w/v) and malt extract (2% w/v).

The fungi were grown on liquid media consisting of an equal mixture of grape juice prepared from ripe grapes (Muscat) and 2% (w/v) malt extract sterilized at 120°C for 10 min.

The fungus was cultured in .5 L flasks containing 120 mL medium and glass wool to serve as inert support at room temperature. After two weeks, the culture medium was harvested for the purification of laccase.

Preparation of Crude Extract

The enzyme was precipitated from the culture filtrate by acetone (−20°C, 60 v/v) as previously described (*5*) with modifications.

The crude culture was centrifuged at 14,000×g for 15 min at 2°C in order to eliminate the cellular debris, vegetable proteins precipitated by heat, and the glass wool utilized as an inert support for growth of fungus.

The precipitate was collected after centrifugation at 40,000×g for 20 min at 2°C. The resultant clear solution crude enzyme extract was concentrated by ultrafiltration using an Amicon P.M. 10 filter with a cutoff point of about 10,000 d.

Purification of the Laccase

10 mL of the concentrated crude extract were applied onto a DEAE-cellulose DE 52 preswollen (Whatman, Maidstone, England) column (2.5× 15 cm) preequilibrated with 40 mM citrate-phosphate buffer (pH 6.0).

The column was eluted using a NaCl gradient in the same buffer in the following order: 0, .1, .2, and .5 M, respectively. Fractions of 7.5 mL were collected and enzyme activity was measured.

Measurement of Laccase Activity E.C .1.10.3.2

The enzyme activity was determined by measuring the oxygen consumption rate caused by the enzyme reaction using an amperometric oxygen electrode.

With quinol as substrate, the following reaction occurs

$$\text{quinol} + O_2 \xrightarrow{\text{laccase}} \text{quinone} + H_2O$$

The oxygene electrode was Radiometer E. 5046 (Radiometer, Copenhagen, Denmark) inserted in a thermostated measurement cell. The measurement was carried out at an imposed potential of -630 mV. The electrode response was measured with a pO_2 analyzer manufactured by the Electronic Departement of the University of Compiègne (France).

The slope of the signal at the inflexion point (dpO_2/dt) was obtained and enzyme activity was determined from this slope.

The pO_2 in the reaction medium is directly proportional to the current measured by the analyzer. It can be estimated by the following formula.

$$pO_{2\,(air)} = (P_A - P_V) \times \%\,(O_2)_{air}$$

p_A is the atmospheric pressure (mmHg).

p_V is the saturated vapor pressure at 20°C: $17.6 \times O_{2\,(air)} = 20.93$ mmHg.

If p_A is 760 mmHg, the pO_2 is equal to 157 mmHg.

From Henry's law, the $pO_2 = k[O_2]$, where k is the constant of oxygen solubility in the reaction medium and $[O_2]$ is the oxygen concentration.

In water $[O_2]$ is equal to $2.64\ 10^{-4}$ M.

The diminution of 1 mmHg in the reaction medium corresponds to the variation of $2.64\ 10^{-4}/157 = 1.66\ 10^{-4}$ M of oxygen.

One unit of enzyme activity is defined as the amount of enzyme that consumes one μmole of molecular oxygen/min at 25°C in .1 M citrate-phosphate buffer (pH 4.0) containing quinol 10 mM as substrate.

Protein Determination

The protein content in the successive steps of the enzyme extraction was determined according to the method described by Lowry et al. (*6*).

Determination of Sugar Content

Total sugar was determined using the anthrone reagent (*7*) or using phenol-sulphuric acid (*8*).

Polyacrylamid Gel Electrophoresis

The homogeneity of the enzyme preparation was established on polyacrylamide gel 7.5% (w/v) in .2 M Tris-glycine buffer (pH 8.9), as indicated in the application Note 306 of L.K.B. Company (England) (April 1977).

The molecular weight of the enzyme was estimated similarly at pH 7.1 in the presence of SDS 1% (w/v) in the gel and .5% (w/v) in the electrode buffer using standard proteins from Pharmacia Company (England).

Gradient gel: PAP 4/30 was obtained from Pharmacia Company for the determination of the molecular weight without SDS.

A kit of proteins of standard MW (Pharmacia, Fine chemical) was used to calibrate the gel.

Isoelectric Focusing

Isoelectric focusing was carried out on acrylamide gel 4.85% (w/v) between pH 2.5–5.0 at 1000 V (15 mA, 4W) until no further migration occurred. The electrode solutions were .2 ML-Histidine for the cathode and .1 M sulphuric acid for the anode.

To standardize the gel, the calibration kit of proteins used was in the range pI 2.5–6.5. (Pharmacia, Fine chemical).

Determination of Molecular Weight (mw) by Gel Filtration

The pure enzyme solution was applied onto a Sephacryl S-200 Superfine (Pharmacia, Fine chemical) column (2×50 cm) preequilibrated and eluted with .01 M potassium-phosphate buffer (pH 7.0) containing .5 M NaCl.

Fractions of 1 mL were collected and the elution profile was obtained by measuring the absorbance at 280 mm.

Standard proteins (α-CHY, ADH, HEX, ALD) of known MW were used to calibrate the column.

RESULTS AND DISCUSSION

Production, Extraction, and Purification of Laccases from *Botrytis cinerea*

All organisms were grown in the liquid medium prepared as described in Materials and Methods. The enzyme activities from the strains tested are summarized in Table 1.

Botrytis cinerea strains (III, IV, V) isolated from grape were better producers of laccase. The yield was between 60 and 65%, the purification factors varied with strains.

Laccase extracts from all the strains were passed through the same D.E.A.E. cellulose column. The chromatograms are shown in Fig. 1 and summarized in Table 2.

An isoenzyme separated on DEAE-cellulose was produced only by *Botrytis cinerea* III and IV. After separation, the fractions of each peak were collected and concentrated by ultrafiltration (P.M. 10,000) to determine the specific activity (S.A.).

The specific activity of the first laccase eluted (laccase 1) from strain III is higher than that of the second laccase (laccase 2). For strain IV, it was the contrary.

Table 1
Extraction of Laccases Produced by *Botrytis cinerea*

Stains	Culture medium		Enzyme extract		Concentrated enzyme extract		Yield, %	Purification factor
	Proteins, mg/mL	S.A., U/mg	Proteins, mg/mL	S.A., U/mg	Proteins, mg/mL	S.A., U/mg		
I	1.19	.019	.72	.032	1.39	.313	67.48	16.50
II	1.28	.021	.81	.176	1.42	.394	60.55	18.76
III	3.07	.054	1.39	.750	3.02	1.135	61.00	21.00
IV	2.84	.048	1.21	.560	2.13	.752	65.70	18.76
V	1.36	.031	.89	.140	1.51	.271	60.00	8.66

Table 2
Purification of Laccases Produced by *Botrytis cinerea*

Stains	Enzyme extract		Pure enzymes				Factor of purification
			Laccase 1		Laccase 2		
	Proteins, mg/mL	S.A., U/mg	Proteins, mg/mL	S.A., U/mg	Proteins, mg/mL	S.A., U/mg	
I	1.44	.30	—	—	2.13	08.30	61.0
II	1.41	.38	—	—	2.27	28.67	75.4
V	1.63	.35	—	—	2.19	17.15	55.3
III	3.46	1.10	3.46	26.17	2.72	19.79	74.5
IV	3.17	.70	1.66	7.84	3.38	10.64	68.3

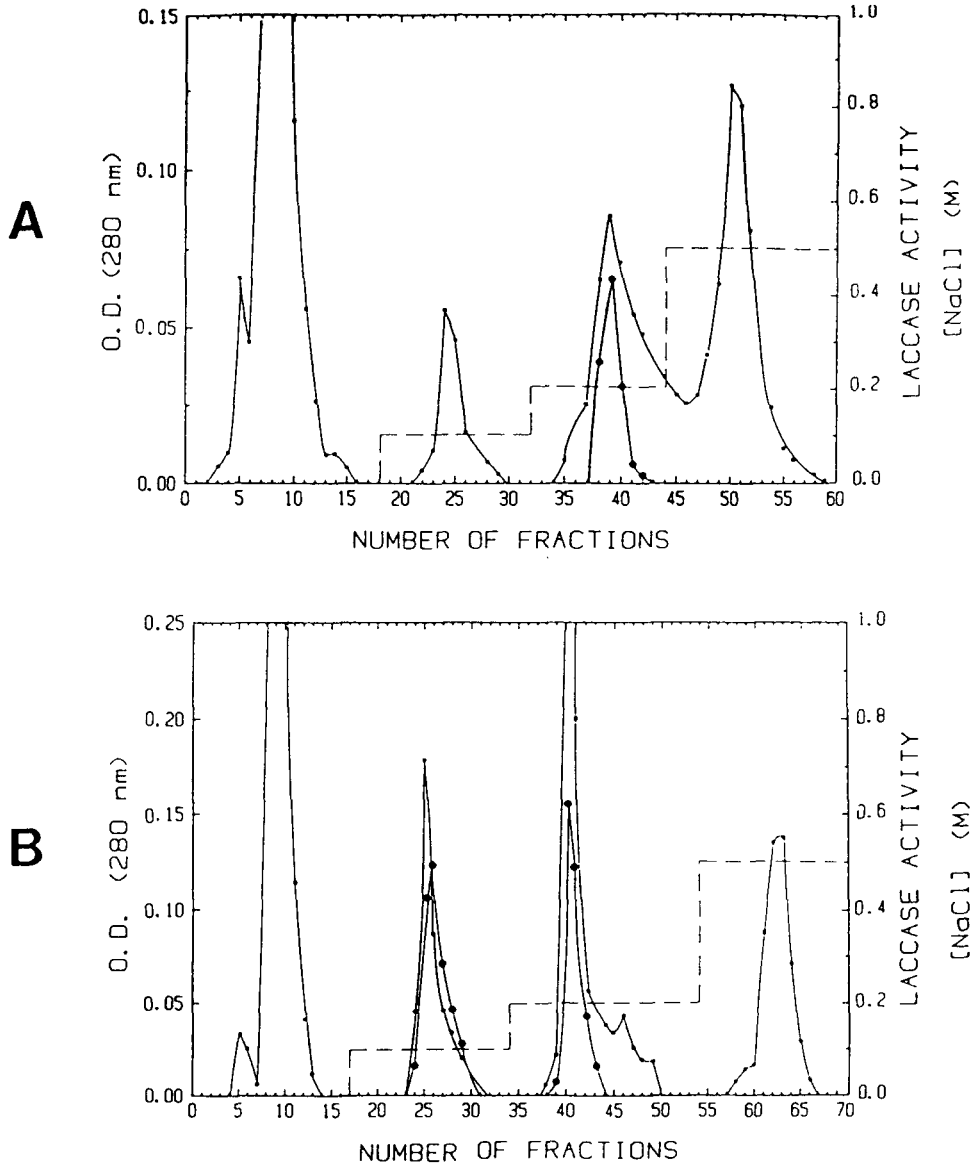

Fig. 1. Elution profiles of laccase purification on DEAE-Cellulose column: A: example of chromatograms with laccases from *B. cinerea* I, II, and V. B: example of chromatograms with laccases from *B. cinerea* III and IV. ■, Optical Density (O.D.); ●, laccase activity; — —, NaCl concentration.

The pure enzymes were stocked at −20°C for months without loss of activity. The homogeneity of the pure enzymes obtained from all the strains was proven by electrophoresis in polyacrylamide gel; only one band was obtained for each enzyme. It is clear that the strains of *Botrytis cinerea* do not produce many enzymes other than laccases in the culture medium and that the method of purification used for the extracellular laccase is sufficient to obtain the pure enzyme.

Our following experiments were carried out using enzymes produced by *Botrytis cinerea* III because of the high specific activity, yield, and purity.

Properties of Laccases from *Botrytis cinerea* III

Determination of Sugar Content

Gigi et al. (*9,10*), Marbach et al. (*11*), and Esser and Minuth (*12*) have shown that laccase is a glycoprotein. Hence, it is important to determine the sugar content in the two forms of laccase produced by *Botrytis cinerea.* Two methods were used: the anthrone method to estimate the hexose content and the phenol-sulphuric acid method to estimate the total sugar content.

The two methods showed high sugar content in laccase: 82 to 86% in laccase 1 and 88 to 91% in laccase 2.

Previous studies have shown that laccases from *B. cinerea* have high carbohydrate-content: 81.6% (*13*) and 77% (*10*). Our results are comparable to those of Esser and Minuth (*12*) on laccases from *Podospora anserina.* In effect, these authors found microheterogeneities in sugar content and they attribute this to the differences in the chain lengths of the carbohydrates.

Isoelectric Focusing

The isoelectric focusing pattern of the two forms of extracellular laccase were different. They showed some bands between pI 2.5 and 3.0, but the most intense bands were obtained at pH 2.8 and 2.6, respectively, for laccase 1 and laccase 2. (Fig. 2). Comparable results have been reported by Mayer et al. (*14*) for laccases from *B. cinerea.*

Molecular Weight (mw) Determination

The molecular weight of the two different forms of laccase was determined by SDS-PAGE, or by gradient gel PAA 4/30, and by gel filtration on sephacryl S-200.

No differences were observed between the two forms of laccase, but the results obtained from electrophoresis (MW 72 400) and those from gel filtration (MW 102000) show great differences.

The difference obtained in MW between SDS-PAGE and gel filtration could be attributed to anomalous behavior of glycoproteins in SDS-gel electrophoresis (*15*).

Nevertheless, MW values obtained by electrophoresis are comparable to those reported by Mayer et al. (*14*) for laccases of *B. cinerea.*

Effect of pH

The pH dependence of the enzyme activity was determined for the crude extract and for the purified preparations with quinol as substrate in .1 M phosphate-citrate buffer at different pH values.

Laccase 2 showed maximum reactivity at pH 4.0, but the activities of laccase 1 and crude extract were the same at pH between 2.5 and 3.0. The reaction rate of the three laccase activities declined markedly after pH 6.0.

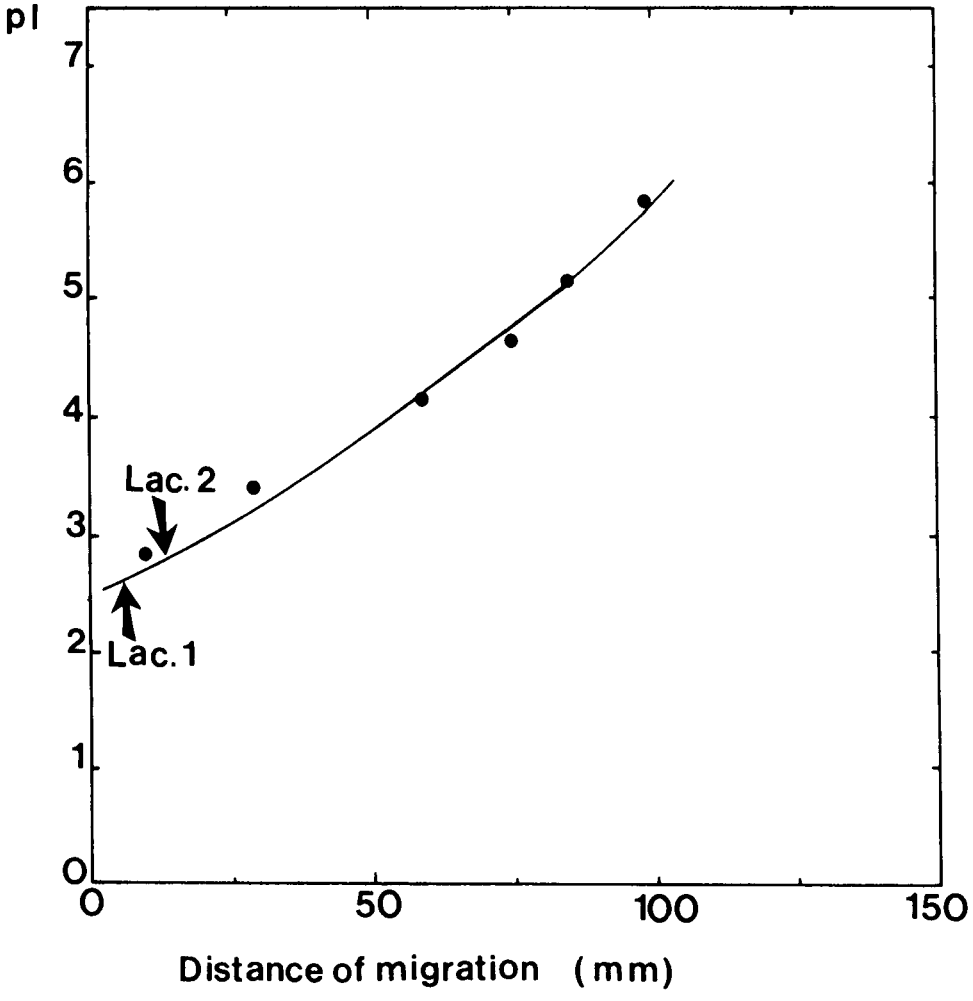

Fig. 2. Isoelectric point estimation of the two forms of laccase.

Effect of Temperature

The influence of temperature of the reaction medium on laccase activity was studied in .1 M phosphate-citrate (pH 4.0).

The optimum temperature for maximal laccase activity was found to be 55°C for the crude extract and laccase 1 and 60°C for laccase 2.

Half-life time plots indicate that the kinetics of thermal inactivation are biphasic and that the two laccases have different thermostability.

The effect of temperature on the enzyme activity may be a result of several causes. The optimal temperature is determined by the balance between the effect of temperature on the rate of the enzyme reaction and its effect on the rate of inactivation of the enzyme.

However, an additional factor must be taken into account, the change in concentration of dissolved oxygen, caused by a change of solubility as a function of temperature.

Therefore, the enzyme activity values were relative and not absolute. Surprisingly, few accurate determinations of the K_m for oxygen have been made. However, copper-containing oxidases generally show a relatively low affinity for oxygen (*1*).

Activation Energy

The temperature dependence of the rate constant, at temperatures below the temperature of inactivation, can be described by the Arrhenius equation

$$k = A \exp(-Ea/RT) \quad \text{or} \quad \log k = (-Ea/2.303\,RT) + \log A$$

Where K = the rate constant
A = the preexponential factor
Ea = the activation energy
R = the gas constant
T = the temperature in Kelvin

To determine *Ea*, the method was applied directly to the rates at the inflexion point, expressed in logarithmic activity, instead of rate constants, since any constant factor will affect the position and not the slope of the line.

The average values are calculated for *Ea*. The *Ea* values for the three enzyme preparations were different: 20.587 kj/mol for laccase 1. 31.388 kj/mol for laccase 2, and 25.422 kj/mol for the crude extract. The low *Ea* values obtained for laccase prove that the oxydation of quinol was a result of the enzyme activity and not a result of inorganic catalysts.

To establish the inactivation of laccase at high temperatures, the laccase from all the preparations were incubated for different times at 60 and 80°C, then laccase activity was determined (Fig. 3).

It is clear that the laccase 2 is more thermostable than the laccase 1 or the crude extract. The thermostability could be caused by the sugar content, which is greater in laccase 2 than in laccase 1. However, the impurities in the crude extract may facilitate the inactivation of enzyme by heat.

Effect of Substrate Concentration

The effect of substrate concentration on the rate of oxidation was determined for quinol in the range of concentration between .125 and 5 mM.

The kinetic constants were determined by the linear transformation of the Hanes equation (Fig. 4).

The K_m values obtained are .133 m*M* for laccase 1, .533 m*M* for laccase 2, and .25 m*M* for the crude extract.

Laccase shows high affinity to quinol, the K_m value for laccase 2 is four times that of laccase 1.

Substrate Specificity

The substrate specificity of crude laccase, laccase 1, and laccase 2 was studied with some phenolic compounds at 10 m*M* in .1 M phosphate-citrate buffer (pH 4.0).

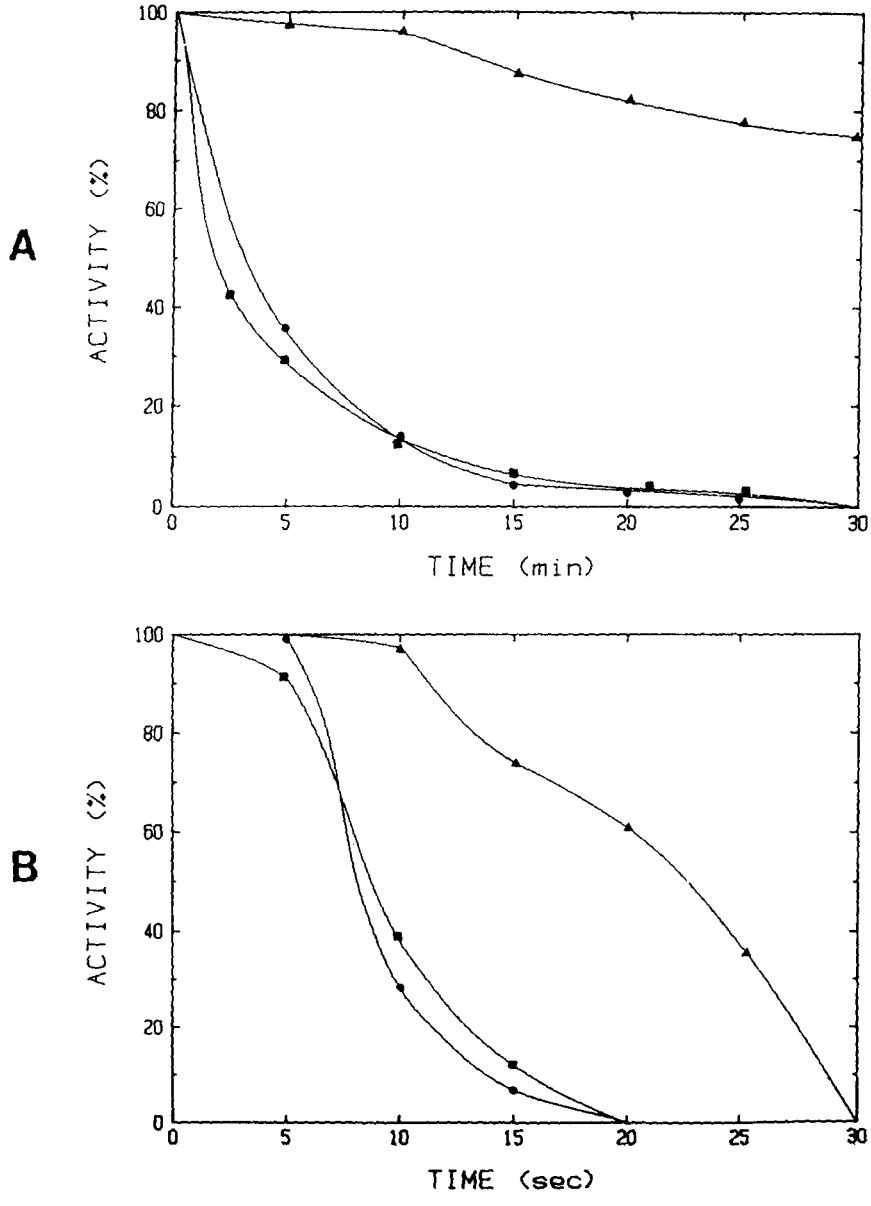

Fig. 3. Inactivation of laccases by heat: A: at 60°C; B: at 80°C. ●, Laccase 1: ▲, Laccase 2; ■, Crude extract.

As shown in Table 3, a wide range of *o*-diphenols, *o*-triphenols, *p*-diphenols, monophenols, some amines, and other phenolics were oxidized. However, phenol and its derivatives, such as nitrophenols and chlorophenols, are not oxidized by laccase. Laccase specificity was different for the two molecular forms in some cases. The wide range of substrates oxidized by these laccases correspond to that described previously for other laccases (*16*).

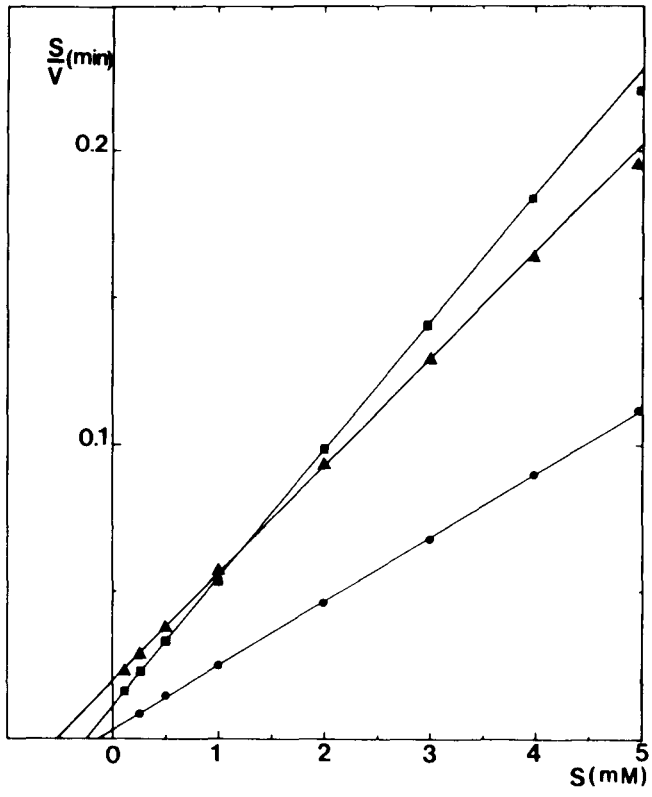

Fig. 4. Hanes plot for the K_m determination. ●, Laccase 1: ▲, Laccase 2; ■, Crude extract.

Inhibition of Laccase from Botrytis cinerea

Some copper chelating agents that have been reported to inhibit laccase from *Rhus*, peaches (*17*), and *Botrytis cinerea* (*1*) were tested for laccase 1, laccase 2, and crude extract.

The ethylenediamine tetracetic acid did not inhibit the laccases up to a concentration of 20 m*M*.

The diethyldithiocarbamate (DIECA) that has been reported not to inhibit the laccase from *Botrytis cinerea* was required to inhibit the laccase from *Botrytis cinerea* III at a quinol concentrations of .5 m*M*. No inhibition occured at a quinol concentration of .2 m*M* (results not shown).

The laccases from *Botrytis cinerea* III were inhibited by sodium azide and cyanide even at low concentrations (results not shown).

Surprisingly, a 4-5-dimethyl *o*-phenylenediamine, which is known as an inhibitor of laccase from peach, was a substrate of laccase from *Botrytis cinerea* III (Table 3). Finally, some phenolics, such as *p*-nitrophenol, *o*-nitrophenol, *p*-chlorophenol, and *p*-nitroaniline known as non-inhibitors of tyrosinase, were tested for laccases from *B.c.* III, however, no inhibition was found.

Table 3
Substrate Specificity of Laccases from *Botrytis cinerea*[a]

	Phenolic compound	Crude extract	Laccase 1	Laccase 2
	Caffeic acid	97	135.8	113.2
o-diphenols	Catechol	100	107.0	109.0
	4-methyl catechol	111	100.0	104.0
p-diphenol	Quinol	100	100	100
	Gallic acid	129.7	173.3	129.7
o-triphenols	Pyrogallol	150.0	141.0	148.7
	1-2-4, Benzenetriol (hydroxyquinol)	72.1	168.2	69.3
	p-kresol	72.1	57.7	28.8
	o-kresol	64.4	66.5	28.9
	Gaïcol	82.5	122.2	104.8
	3-5, dimethoxy-hydroxy-benzaldzaine	60.0	109.1	80.8
Monophenols	Phenol	0	0	0
	2-nitrophenol	0	0	0
	4-nitrophenol	0	0	0
	4-chlorophenol	0	0	0
	2-4 dichlorophenol	0	0	0
	2-4-5 trichlorophenol	0	0	0
Diamines	*p*-phenylenediamine	50.7	59.4	53.0
	4-5 dimethyl *o*-phenylenediamine	46.2	65.0	51.0
Monoamine	4- amino-N, N′ dimethylaniline	42.0	35.7	42.4
	Acide L-ascorbique	113.0	129.4	110.2
Autres produits	1-Naphtol	52.4	58.9	47.6
phénoliques	2-Naphtol	47.6	51.1	28.9

[a] The activity was determined relative to quinol (100%).

CONCLUSIONS

Our studies on *B. cinerea* have shown two types of strains producing only one or two laccases.

The aim of the present investigation was to purify the laccases excreted by *B. cinerea,* and to determine some of their physicochemical characteristics.

When compared physicochemically, two isoenzymes showed different properties, particularly in thermostability, specificity, and sugar content. However, they had the same mw and some similar properties—great

thermostability, high sugar content, and very broad specificity. Therefore, using these results, further experiments were performed (*4,18*) in the industrial field.

REFERENCES

1. Mayer, A. M., and Harel, E. (1979), *Phytochemistry* **18,** 193.
2. Laborde, J. (1897), *C. R. Acad. Sci. Paris* **125,** 248.
3. Dubernet, M., and Ribereau-Gayon, P. (1973), *C. R. Acad. Sci. Paris* **277 D,** 975.
4. Zouari, N. (1986), *doctoral thesis,* University of Compiègne, France.
5. Dubernet, M. Ribereau-Gayon, P., Leiner, H. R., Harel, E., and Mayer, A. M. (1977), *Phytochemistry* **16,** 191.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265.
7. Ashwell, G. (1957), in *Methods in Enzymology,* vol. #3, Colowick, S. D., and Kaplan, N. O., eds., Academic Press, New York, p. 84.
8. Ashwell, G. (1966), in *Methods in Enzymology,* vol. #8, Colowick, S. D., and Kaplan, N. O., eds., Academic Press, New York, p. 85.
9. Gigi, O., Marbach, I., and Mayer, A. M. (1980), *Phytochemistry* **19,** 2273.
10. Gigi, O., Marbach, I., and Mayer, A. M. (1981), *Phytochemistry* **20,** 1211.
11. Marbach, I., Harel, E., and Mayer, A. M. (1983), *Phytochemistry* **22,** 1535.
12. Esser, K., and Minuth, W. (1971), *Eur. J. Biochem.* **23,** 484.
13. Marbach, I., Harel, E., and Mayer, A. M. (1984), *Phytochemistry* **23,** 2713.
14. Mayer, A. M., Marbach, I., Marbach, A., and Sharon, A. (1977), *Phytochemistry* **16,** 1051.
15. Segrest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T. (1971), *Biochem. Biophys. Res. Commun.* **44,** 390.
16. Fahreus, G., and Ljunggren, H. (1961), *Biochim. Biophys. Acta.* **46,** 22.
17. Harel, E., and Mayer, A. M. (1970), *Phytochemistry* **9,** 2447.
18. Zouari, N., Romette, J. L., and Thomas D., *Biotechnol. Lett.* (in press).