Phytochemistry, 1980, Vol. 19, pp. 2273–2275. © Pergamon Press Ltd. Printed in England. 0031-9422/80/1101-2273 $02.00/0

INDUCTION OF LACCASE FORMATION IN *BOTRYTIS*

ORITH GIGI, IRITH MARBACH and ALFRED M. MAYER

Botany Department, Hebrew University, Jerusalem, Israel

(*Revised received* 9 *March* 1980)

Key Word Index—*Botrytis cinerea*; Ascomycetes; laccase; gallic acid.

Abstract—Optimal conditions for laccase excretion by *Botrytis cinerea* were determined. Addition of gallic acid to the culture medium induced maximal enzyme production and excretion. Other inducers previously reported for other organisms were ineffective. The relationships between internal and external laccase formation were determined.

INTRODUCTION

The presence of laccase in many fungi is well known, the enzyme is sometimes cellular, but it is frequently extra-cellular [1]. Some of the properties of *Botrytis* laccase have been described [2–5]. These indicated that the enzyme from *Botrytis* has unusual properties, including a very low p*K*. We wanted to study this enzyme further and sought ways to improve production of the extra-cellular enzyme. A number of workers have studied conditions favourable for laccase production. Fahraeus *et al.* [6] found that in *Polyporus*, laccase formation is stimulated by addition of 2,5-xylidine to the culture medium 3–4 days after sowing. In *Neurospora*, addition of Cu^{2+} or inhibitors of protein synthesis favoured enzyme production [7, 8] while promotion of inhibition of extra-cellular laccase formation by phenolic compounds in various other fungi has also been reported [8–11]. A specific effect of ferulic acid in laccase production by a number of Basidiomycetes has been reported by Leonowicz and Trojanowski [12]. In *Botrytis* the problem has been studied only by Kovac [13] who in a preliminary report showed great differences in laccase production depending on the strain of *Botrytis* and/or the growth medium. He found that in a defined medium, in some cases, chlorogenic acid or caffeic acid promoted laccase excretion after 30 days. With the exception of the work on *Polyporus*, no time course of laccase production has been reported in detail. We now report on laccase formation and excretion by *B. cinerea*.

RESULTS AND DISCUSSION

When the fungus was grown on malt with the addition of wine juice, laccase was produced. However, on malt alone there was little or no laccase production. Following the earlier reports on other fungi, we attempted to promote formation of extra-cellular laccase by the addition of Cu^{2+} or cycloheximide. Addition after 3 weeks of culture had little effect on enzyme production whether the fungus was grown in the standard medium (malt, see Experimental) or malt with the addition of wine juice [4]. Addition of a number of phenolic compounds was then attempted (Table 1). It is clear that induction of enzyme excretion is specific and that of the compounds tested, gallic acid was by far the most effective. The effect depended on the time of addition of the gallic acid. Addition on sowing or after 2 days was equally effective, but addition after 4 days resulted in much lower extra-cellular enzyme formation (1.96 μl

Table 1. Effect of addition of phenolic compounds on production of extra-cellular laccase (phenol concentration in all cases 1 g/l. medium added at zero time)

Phenolic compound added	Enzyme activity	
	μl O_2/ml medium/min	μl O_2/mg protein/min
Gallic acid	1.96	5.9
Tannic acid	1.5	2.6
Quinol	0	0
2,5-Xylidine* †	0	0
3,4-diOH phenylalanine	0	0
Caffeic acid	1.0	0.9
Ferulic acid	0	0
4-Methylcatechol	0	0

* Addition 4 days after sowing, final concentration 2×10^{-4} M.
† Addition in 50% alcohol.

Table 2. Effect of time of addition of tannic acid on production of extracellular laccase (1 g tannic acid/l. added)

Addition of phenol	Enzyme activity after 12 days a	b	14 days a	b	22 days a	b
Zero time	1.5	3.4	1.1	2.6	0.7	2.2
2 days	1.5	4.5	1.2	3.1	0.6	1.9
4 days	0.4	1.1	0.5	1.4	0.4	1.2
6 days	0.6	1.7	0.5	1.6	0.4	1.4

(a) μl O_2/ml medium/min.
(b) μl O_2/mg protein/min.

O_2/ml/min on days zero and 2, cf. to 0.87 after 4 days) when activity was estimated 14 days after sowing. Similar effects were noted using tannic acid. Sporulation began after 3 days. The results in Table 2 show that addition of tannic acid at zero time results in a peak of both specific and total activity after 12 days of culture. Addition later does not result in greater total activity. This means that the inducing effect becomes operative immediately on sowing. If it had merely accelerated enzyme production then its later addition should have caused a delayed increase in enzyme formation, which was not observed. Apparently addition after 2 days has some effect on production of other proteins as a transient increase of sp. act. was observed compared to addition at zero time. The inductive effect is therefore real and cumulative and is not simply an accelerated enzyme production. Moreover, enzyme production clearly has a peak and thereafter the enzyme already excreted appears to undergo inactivation. Incandescent light had no effect on growth or laccase production by *Botrytis*. When the cultures were grown in the presence of fluorescent light, growth and consequently laccase production were somewhat depressed. Routinely the fungus was grown in weak incandescent light.

The effect of the concentration of gallic acid was determined (Fig. 1). Optimal enzyme concentration was obtained using 2 g gallic acid/l., *ca* 30% more activity than at 1 g/1. (Fig. 1A). However, the sp. act. was 25–40% higher at the lower concentration of gallic acid. This greatly facilitates purification of the enzyme and for further work the lower concentration was therefore selected (Fig. 1B).

To study the course of laccase production further we followed enzyme production with time, both in the medium and in the mycelium (Fig. 2). Protein formation in the mycelium steadily increases till *ca* 20 days, after which there is a drop. The presence of gallic acid promotes this protein production. In the presence of gallic acid, much more protein is secreted into the medium. This protein excretion reaches a maximum long before maximal growth has been attained (Fig. 2A). Total extra-cellular laccase activity was followed in the absence and presence of gallic acid (Fig. 2B). In its absence no laccase was excreted. In its presence laccase excretion reached a peak after 12–14 days and thereafter the level in the medium declined. The intracellular laccase rose steadily up to *ca* 12 days and thereafter remained at a constant level for a long period. There was no peak prior to excretion. Essentially the same picture is also shown by enzyme sp. act. Laccase production was not related to sporulation. Under the conditions used in these experiments, sporulation of the mycelium began 3 days after sowing and continued thereafter as the mycelium developed. Extra-cellular production of laccase by *Botrytis* appears to require the continual presence of a phenolic substrate. In agreement with Kovac [13], caffeic acid was effective but gallic acid was a much better medium for growth and laccase production. Laccase production and excretion proceed in parallel. There is no major build-up in the mycelium, followed by a sudden excretion. Nevertheless, excretion of laccase is not part of general

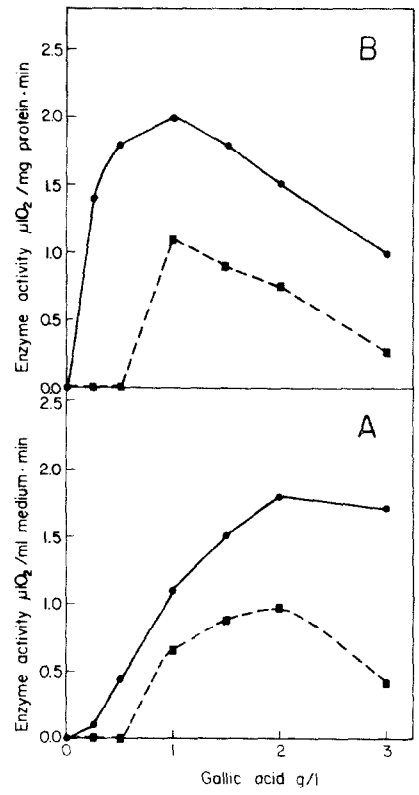

Fig. 1. (A) Effect of gallic acid concentration on amount of laccase in culture medium. Results as μl O_2/ml medium/min. (B) Effect of gallic acid concentration on specific activity of laccase in culture medium. Results as μl O_2/mg protein/min. (Gallic acid was added at time of sowing.) (●—●) After 15 days of culture, (■---■) after 12 days of culture.

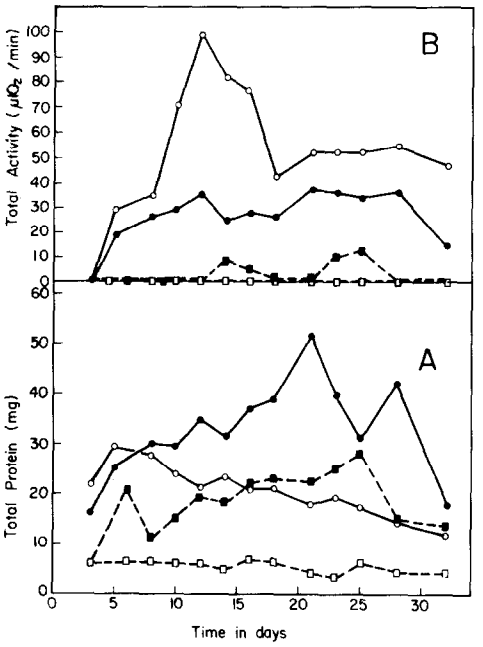

Fig. 2. (A) Mycelial and extra-cellular protein production by *Botrytis* in the absence and presence of gallic acid, 1 g/l. (Gallic acid was added at time of sowing.) (●—●) Mycelial protein + gallic acid, (■---■) mycelial protein − gallic acid, (○—○) protein in media + gallic acid, (□---□) protein in media − gallic acid. (B) Intra-cellular and extra-cellular laccase production by *Botrytis* in the absence and presence of gallic acid, 1 g/l. Results as total activity. (Gallic acid was added at time of sowing.) (●—●) Laccase in mycelium + gallic acid, (○—○) laccase in medium + gallic acid, (■---■) laccase in mycelium − gallic acid, (□---□) laccase in medium − gallic acid.

protein excretion since the enzyme sp. act. in the medium shows a distinct peak. The excretion of laccase seems clearly to respond to environmental conditions. Various factors inducing laccase production in other fungi were ineffective as regards *Botrytis*. Since the phenolic substance must be present continuously, no true induction phenomenon is involved. It might be speculated that laccase production and excretion is a response developed by the fungi to aid in the detoxication of possible toxic compounds. However, total mycelial protein produced by the fungus also increased in the presence of gallic acid, making this interpretation somewhat doubtful.

EXPERIMENTAL

B. cinerea was grown in 1l. conical flasks containing 200 ml medium at 22°. The medium contained 20 g/l. Bacto malt extract (Difco) and 0.1 M KPi/citrate buffer, pH 3.5. The phenolic substances were added at time of sowing. The medium was autoclaved in the absence of the phenolics, since they become oxidized under conditions of autoclaving.

Enzyme activity in the medium was determined in suitable aliquots after centrifugation at 20000 g for 10 min to remove all debris, spores, etc. Enzyme activity in the mycelium was determined in crude homogenates. The mycelium was first disintegrated in a blender with 0.1 M KPi/citrate buffer, pH 3.5. The extract was homogenized and then suitable aliquots used for determination of activity.

Laccase activity was determined using an O_2 electrode in the presence of KPi/citrate buffer, pH 4.6 and 10 mM quinol as substrate [5]. Protein was determined using the Folin–Ciocalteu reagent. All other experimental procedures have been described previously [4].

REFERENCES

1. Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18**, 193.
2. Dubernet, M. and Ribereau-Gayon, P. (1973) *C. R. Acad. Sci. Ser. D* **277**, 975.
3. Dubernet, M. and Ribereau-Gayon, P. (1975) *C. R. Acad. Sci. Ser. D* **280**, 1313.
4. Dubernet, M., Ribereau-Gayon, P., Lerner, H. R., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16**, 191.
5. Mayer, A. M., Marbach, I., Marbach, A. and Sharon, A. (1977) *Phytochemistry* **16**, 1050.
6. Fahraeus, G., Tullander, V. and Ljunggren, H. (1956) *Physiol. Plant.* **11**, 631.
7. Froehner, S. C. and Eriksson, K.-E. (1974) *J. Bacteriol.* **120**, 450.
8. Froehner, S. C. and Eriksson, K.-E. (1974) *J. Bacteriol.* **120**, 458.
9. Johansson, M. and Hagerby, E. (1974) *Physiol. Plant.* **32**, 23.
10. Grabbe, K., Koenig, R. and Haido, K. (1968) *Arch. Mikrobiol.* **63**, 133.
11. Bocks, S. M. (1967) *Phytochemistry* **6**, 77.
12. Leonowicz, A. and Trojanowski, J. (1975) *Acta Biochim. Polonica* **22**, 291.
13. Kovac, V. (1979) Assemblée annuelle de Groupe Polyphénol, Logrono, 1979.