Phytochemistry, 1978, Vol. 17, pp 633–636. Pergamon Press. Printed in England.

THE *O*-DIPHENOL-OXYGEN-OXIDOREDUCTASE OF *AGARICUS BISPORUS*: ACTIVITY AND MULTIPLE FORMS DURING AGEING

PETER W. GOODENOUGH

University of Bristol, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, U.K.

(*Revised* 1 *November* 1977)

Key Word Index—*Agaricus bisporus*; mushroom; *o*-diphenolase; multiple forms.

Abstract—Mushroom *o*-diphenol oxidase was separated into multiple forms by isoelectric focusing. Three major bands, as opposed to the four isoenzymes previously found, were separated over the pH range 3.5–9.5. A fourth form was obtained when the pH range was narrowed to 5.0–8.0. Changes in the enzyme activity were investigated during post-harvest ageing at different temperatures. Rapid ageing using tissue discs with or without inhibitors of protein synthesis showed that an increase in activity of the enzyme took place during this time, but was prevented by actinomycin D and 6 methyl purine.

INTRODUCTION

Keilin and Mann showed that *o*-diphenol oxidase (EC 1.14.18.1) acts on *o*-diphenols and monophenols [1]. The enzyme from *Agaricus bisporus* fruiting bodies was separated into four isoenzymes by electrophoresis [2]. Of these only three (β, γ and δ) showed activity towards catechol. In contrast all four acted on dopa, tyrosine and *p*-cresol, with the highest activity using dopa as substrate. Both of the quoted molecular weights (118600 and 118800) and the amino acid composition of the isoenzymes were very similar. Further starch gel and acrylamide electrophoresis indicated that the β isoenzyme protein could undergo aggregation into a polymeric series, showing association dissociation phenomena [3]. The total activity of these isoenzymes has been shown to increase during growth [4]. During this period of increasing enzyme activity *o*-benzoquinone is formed by the enzyme from catechol and reacts further to give three phenols (3,4,3,4; 2,3,3,4 and 2,3,2,3 hydroxydiphenols) in addition to diphenylene-dioxide-2,3-quinone from 2,3,4-trihydroxy-diphenyl ether [5]. These intermediates react to form the dark pigment melanin which accumulates during growth and causes loss of high quality. In another pathway tyrosine is converted to dopa and thence to dopaquinone and eventually melanin [6]. In attempts to minimize this deterioration it is important to determine the response of the enzyme system to environmental conditions after harvest.

The fungal fruiting body may be similar to that of higher plant fruits where there is considerable evidence of changes in enzyme synthesis during ripening [7, 8]. There is clear evidence of an increase in activity of some oxidase enzymes in this fungus during the transition from mycelium to fruiting body but it is not clear whether this involves protein synthesis of a new enzyme system [9, 10].

This paper describes the separation of the multiple forms of the diphenol oxidase during the quantitative changes in their aggregated activity which takes place during continued post-harvest growth. Evidence from experiments preventing correct transcription during rapid ageing in tissue discs is presented, and discussed in relation to senescence in this fungal fruiting body.

RESULTS AND DISCUSSION

Separation of multiple forms

Using acrylamide gel, with added ampholine, showed three different multiple forms which could be separated into two groups at *ca* pI 6.5 and 5.0. All bands in each group were active towards catechol (these were at pI 6.25, 5.41 and 5.12). A band at 5.12 reacted with tyrosine, and all three bands reacted with dopa (Table 1). The depth of stain at each location was estimated by eye and classified as high, medium or low.

The enzyme preparations used were extracted with SDS. As it is known that isoelectric focusing in the presence of SDS will give incorrect results this detergent had to be removed. Dialysis against a large volume of urea and subsequent dilution into buffer gave successful renaturation of the enzyme. After concentration to the original volume 90% of the activity was recovered. This confirmed, with this enzyme, the results of Miller and Elgin [11] as well as those of Weber and Kuter [12] with

Table 1. Separation of *Agarius bisporus* *o*-diphenol oxidase activity on acrylamide gel

		* Activity towards		
pI	Subunit	Dopa	Catechol	Tyrosine
5.12	$n^{\alpha} + n^{\alpha}$	medium	medium	high
5.41	$n^{\alpha} + n^{\beta}$	medium	medium	v. low
6.25	$n^{\beta} + n^{\beta}$	high	high	none

* Activity by visual depth of stain.

other enzymes. In this case the pattern of multiple forms should not be affected as long as all SDS was removed. When a pH gradient of 5–8 was established in polyacrylamide gel a fourth band between that at pI 5.12 and 5.41 could be seen. This was usually visible only after the concentration of SDS was increased in the extractant.

The three multiple forms of *o*-diphenol oxidase separated by gel electrofocusing correspond to some extent to the four forms described by Bouchilloux *et al.* [2]. The band at pI 5.1 was found to be most active towards tyrosine and would include the α and β isoenzymes mentioned previously (*loc. cit.*). But whereas Bouchilloux *et al.* [2] found greatest activity towards dopa in the α and β forms this occurred at pI 6.25 in this investigation. Constantinides and Bedford [13] separated the four forms further so that some of the original bands were separated into three closely spaced bands. This gave a total of nine bands but activity towards tyrosine was restricted to one area of the gel which had at least two bands close together. These same bands had activity towards dopa. Jolley *et al.* [3] had shown that each of the multiple forms could dissociate into a series consisting of trimer, tetramer and octomer and this situation was presumably visualized in the work of Constantinides and Bedford [13]. Recently the polyphenoxidase from avocado [14] which had been separated into several different isoenzymes by gel electrophoresis give fewer, sharper bands with isoelectric focusing in polyacrylamide gels. In the fungal enzyme considerable dissociation could occur during electrophoresis.

Bouchilloux *et al.* [2] have evidence that α and β forms may be heterogeneous, and have shown that the α protein can be further separated in the presence of SDS. Jolley and Mason [15] concluded that the existence of two unlike subunits may explain the differences among the forms of tyrosinase to mono and diphenols. On the basis of the isoelectric focusing evidence presented here and previous results it is suggested that the enzyme consists of two polypeptide chains (subunits) grouped as two homogeneous and one heterogeneous isoenzymes (Table 1).

During gel electrophoresis, as opposed to isoelectric focusing, it was possible that the bands made up of dissimilar subunits would easily dissociate to each give two bands of activity. When one of the preparations was focused over a narrow pH range after extraction with SDS an extra band appeared between pI 5.1 and 5.4. Nakamura *et al.* [16] separated two subunits from the enzyme with a molecular weight of approximately one half of that previously reported for the enzyme. All other workers have found only two sedimetation peaks for *o*-diphenol oxidase, and from amino acid analysis two not quite identical chains appear to be present. Recent data has shown that at least one multiple form of the enzyme can be separated into two polypeptides with molecular weights of 43000 and 13400 [17].

Quantitative changes in enzyme activity during storage at three temperatures

The enzyme activity during storage was found to depend on temperature. At 25° activity increased with time and was accompanied by an increase in protein content, which became maximal after 3 days and then declined. The specific activity of *o*-diphenol oxidase reached a peak after 24 hr and then declined despite the protein content continuing to increase for another 48 hr. A comparison of the isoenzymes by isoelectric focusing indicated that all three isoenzymes occurred during storage at 25°. At 24 hr after harvest, when enzyme activity was at a maximum, the isoenzymes showed a more intense staining reaction than after 6 days of storage.

During storage at 10° enzyme specific activity started to increase after 5 days and reached a maximum at 7 days. Protein content again increased but much less than at 25°, the maximum being reached after 5 days. All isoenzymes were present and responding most intensely to staining after 7 days. They stained weakly after 11 days but there was a possibility that a fourth band was present. At 2° results were similar, but with the greatest specific activity, staining and protein content after 8 days (Fig. 1).

Changes in mannitol concentration during storage

The role of mannitol as a storage carbohydrate has been discussed by Hammond and Nichols [18]. It is not thought to be used for respiration to any great extent and at lower temperatures it did not decrease significantly. However the rapid decline of mannitol at 25° showed that pathways involved in catabolism have been activated at the same time as general enzyme activity is declining.

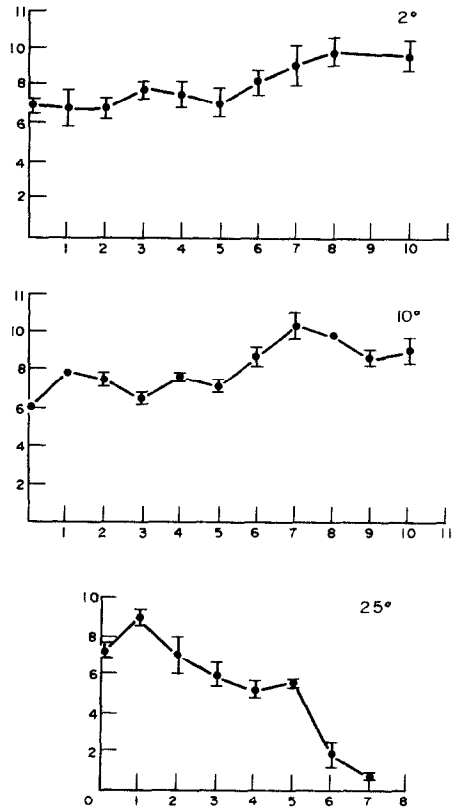

Fig. 1. Activity of *o*-diphenol oxidase/mg protein in basidiocarps at one of three different temperatures. One unit of activity (60 μkat) is that oxidising one micromole of catechol/min/mg protein in the enzyme soln at 30°. Activity measured by increase in *A* at 525 nm of a catechol–proline mixture. Vertical bars represent the standard error of the mean (9 determinations were made for each point).

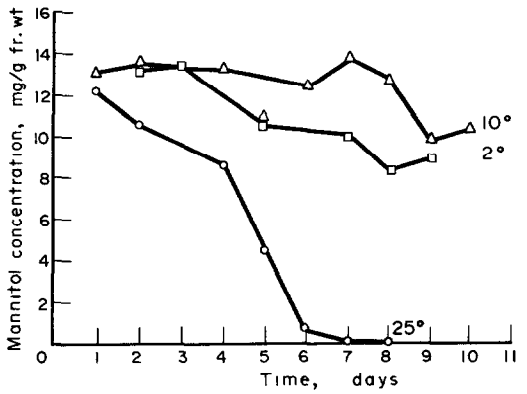

Fig. 2. Mannitol concn in basidiocarps held at either 2 □—□, 10 △—△, or 25° ○—○.

Fig. 2 shows that this concentration of mannitol decreased quickly after 4 days at 25°. In contrast, the decrease at 2 or 10° was slow (Fig. 2).

Effect of actinomycin D or 6-methyl purine on the development of enzyme activity

Each tissue disc had two cut surfaces and these were immersed in the solution of buffer or buffer + inhibitor. After only an hour of incubation there was a significantly larger amount of activity in the controls than in the discs with inhibitor present. All discs had been randomized before the experiment so that a particular sample did not consist of discs all from one or two fruiting bodies. 6-Methyl purine seemed to have an initially larger effect than actinomycin D but the specific activity did not fall more than 28.5% before a steady state seemed to have been reached. During the time the protein content of all of the discs decreased from that amount found after 1 hr but never more than 20%. In the solutions of inhibitor a steady state was reached for both protein and enzyme concentration at 7 hr, but protein content began to fall rapidly after 24 hr with no accompanying fall in enzyme activity. This occurred in all samples and gave an apparent second peak in activity but by this time the tissue was starting to disintegrate. It was found that streptomycin

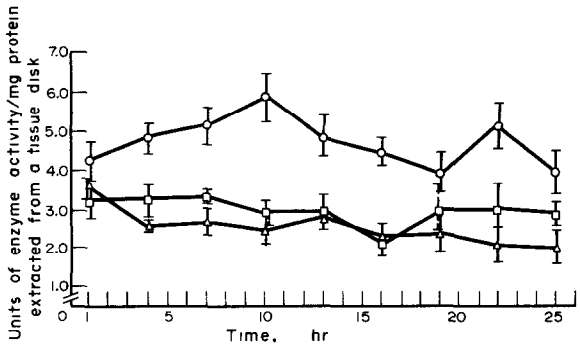

Fig. 3. Activity of *o*-diphenol oxidase/mg protein in tissue discs with time and either in the presence or not of inhibitors of correct transcription. Vertical bars represent the standard error of the mean. 5 replicates of each treatment were assayed in duplicate at each sampling time. ○—○ Phosphate buffer pH 5.99. △—△ 6-Methyl purine (0.1 mM) + phosphate buffer pH 5.99. □—□ Actinomycin D (0.1 mM) + phosphate buffer pH 5.99.

sulphate at the concentration used gave data which was lower in protein and enzyme than the controls. This may have been due to precipitation of nucleotide linked protein but the specific activities were still similar to the control tissue and increased in the same way.

The experiment could not be repeated when there was only one cut surface to the discs, in this case enzyme activity rose in the discs exposed to inhibitors as rapidly as it rose in the controls. In a further attempt to discover whether the rise in specific activity was due entirely to *de novo* protein synthesis, rather than the lifting of inhibition on the enzyme, rates of activity were plotted against enzyme concentration. There was no deviation from the reponse found at the first sampling time.

The concentration of mannitol in the discs did not change to a greater extent in the controls than in the discs treated with inhibitor.

The changes in enzyme activity in fruiting body tissue may be an indication of a switch from growth to senescence. A possible change in the phenolic metabolism may occur as growth reaches a maximum and there is a greater capacity for conversion of mono and diphenols. Whether this is a direct induction effect on enzyme synthesis of greater amounts of the phenolic substrates, or a programmed process of senscence under direct genetic control is not known. However the process is a direct part of ageing, and the possibility that ageing as well as protein synthesis may be regulated at the translational step [7, 19] has led to several studies on ageing in plant tissue [20]. Recent work has indicated that senescence linked activation of genes and expression of the same genes may be separated in time [21, 22]. In *A. bisporus* the use of two compounds to prevent normal transcription has shown that the rise in activity depends directly on transcription. In some cases actinomycin D does not completely prevent protein synthesis [23, 24] and 6-methyl purine is more effective. The analogue 6-methyl purine was more quickly effective in these experiments but after an initial fall in enzyme activity there was no further fall in activity over 36 hr. In these experiments there was no change in the multiple forms of *o*-diphenol oxygen oxidoreductase during maturation and senescence in this fungus but considerable quantitative changes in total activity. This enzyme is important in the maintenance of quality in this fruiting body, and it would be important to determine whether other enzymes of phenolic metabolism are also synthesized during ageing. This could be a response to increased concentration of substrates or a predetermined change in existing compounds. The activity of dioxygenase concerned with fission of the aromatic nucleus has been detected in this tissue, *p*-hydroxyphenyl pyruvate oxidase (Goodenough, unpublished work), and further studies of the metabolism of these phenolic compounds during ageing are needed.

EXPERIMENTAL

Prepn of enzyme extracts. The source of extracts was either complete immature fungal bodies at Stage 2, as designated in ref. [18], or 5 mm thick tissue discs with two cut surfaces. From each of the former experimental treatments 3 equal tissue samples were individually frozen in liquid N_2, homogenized with Me_2CO at −35° and filtered through GFA glass fibre paper. In the case of the tissue discs, 5 discs were taken per treatment at each sampling time and each treated as previously mentioned. Me_2CO powders were kept below 0° during filtration and subsequent lyophilization. Dry powders could be stored at −30°

for a month without losing *o*-diphenol oxidase activity. Samples of dry powder were homogenized with 0.1% dodecyl sulphate (BDH purified) +0.2 M Na phosphate pH 6 (phosphate buffer 0.2 M monobasic/0.2 M dibasic Na phosphate dil. 1:1 with H_2O) at 0° in a tissue grinder, and centrifuged at 8–10 000 *g*. The clear supernatant was used for enzyme assay. Samples for isoelectric focussing were dialysed against 10 M urea before use [11].

Enzyme assay. The enzyme first reacts with catechol to give *o*-benzoquinone as the primary product. Commercial *o*-benzoquinone was purified by resubliming twice and then immediately reacted with proline. The two compounds were mixed at 0.01 M concn while being continuously oxygenated. The reaction, which gave a red complex, reached 89.4% of completion after 8 hr. The molar absorptivity (ε) of this complex at maximum absorption (525 nm) was 1.79×10^3, compared to 5×10^3 quoted in ref. [4]. As the method of computing the latter figure was not stated, the former value was used throughout. The reaction mixture consisted of 3 ml of a catechol–proline mixture (both 0.01 M) in Pi buffer pH 6 (0.1 M) and 100 μl of enzyme. One unit of enzyme activity (60 μ Kat) was taken as that oxidising one mmole of catechol/min/mg protein in the soln.

Mannitol estimation. Mushroom tissue was extracted with EtOH–H_2O (4:1), and aliquots of extract evapd to dryness. The TMSi derivatives were prepared and 1 μl samples were injected into a GC equipped with dual FID. Columns containing 3% silicone gum rubber with N_2 at 30 ml/min as carrier gas were used. Injector and detector temps were 250° and the column temp. was programmed from 150° with a subsequent 8° rise to 250° [18]. The RR_t of mannitol to inositol (int. stand.) was 1.20 [25].

Estimation of protein content of extracts. TCA was added to extracts prepared as for enzyme analysis (final concn 5% w/v) ppt. redissolved and protein determined [26]. Nitrogen content was estimated using an Auto Analyser [27]. BSA was used as a standard.

Isoelectric focusing and electrophoresis. The enzyme was focused in a flat plate of 5% polyacrylamide gel containing 4% ampholine. The percentage of cross linking in the gel was 3, and the pH range of the ampholine was 3.5–9.5 (LKG PAG plate). Using the pH range 5–8 and 4–6 it was found that less distortion of the gel was caused by an acrylamide concn of 4.4% and 2.96% ampholine. During electrofocusing the applied current did not exceed 8 mA. nor the temp. 2°. After electrofocusing the gels were stained with either catechol + proline (1.0 mM), dopa (1.5 mM) or tyrosine (1.5 mM).

Experimental treatment. Packs of *A. bisporus* were kept at 2, 10 or 25° under perforated plastic film. Samples were taken at 24 hr intervals and divided into 4 fractions. Three were used for enzyme and one for mannitol analysis. When slices of fungal tissue were 'aged' the soln used was one of Pi buffer pH 5.99, actinomycin D (0.1 mM), 6-methyl purine (0.1 mM) or streptomycin sulphate (0.1 mM). The slices of fungal tissue with only one cut surface were used, whereas in others cap slices had two cut surfaces. All solns were held at 22°.

REFERENCES

1. Keilin, D. and Mann, T. (1938) *Proc. Roy. Soc. Ser. B* **125**, 187.
2. Bouchilloux, S., McMahill, P. and Mason, H. S. (1963) *J. Biol. Chem.* **238**, 1699.
3. Jolley, R. L., Robb, D. A. and Mason, H. S. (1969) *J. Biol. Chem.* **244**, 1593.
4. Yamaguchi, M., Hwang, P. M. and Campbell, J. D. (1970) *Can. J. Biochem.* **48**, 198.
5. Dawson, C. R. and Tarpley, W. B. (1963) *Ann. N.Y. Acad. Sci.* **100**, 937.
6. Hearing, V. J. and Ekel, T. M. (1976) *Biochem. J.* **157**, 549.
7. Mettler, I. J. and Romani, R. J. (1976) *Phytochemistry* **15**, 25.
8. Rattanapanone, N., Grierson, D. and Stein, M. C. (1977) *Phytochemistry* **16**, 629.
9. Turner, E. M. (1964) *Trans. Brit. Mycol. Soc.* **63**, 541.
10. Wood, D. A. W. and Goodenough, P. W. (1977) *Arch. Microbiol.* **114**, 161.
11. Miller, D. W. and Elgin, S. C. R. (1974) *Anal. Biochem.* **60**, 142.
12. Weber, K. and Kuter, D. J. (1971) *J. Biol. Chem.* **246**, 4504.
13. Constantinides, S. M. and Bedford, C. L. (1967) *J. Food Sci.* **32**, 446.
14. Kahn, V. (1976) *Phytochemistry* **15**, 267.
15. Jolley, R. L. and Mason, H. S. (1965) *J. Biol. Chem.* **240**, 1489.
16. Nakamura, T., Sho, S. and Ocura, Y. (1966) *J. Biochem.* **59**, 481.
17. Strothkamp, K., Jolley, R. and Mason, H. S. (1976) *Biochem. Biophys. Res. Commun.* **70**, 519.
18. Hammond, J. B. W. and Nichols, R. (1976) *J. Gen. Microbiol.* **93**, 309.
19. Strehler, B. L., Hendley, D. D. and Hirsch, G. P. (1967) *Proc. Natl. Acad. Sci. US* **57**, 1751.
20. Bick, M. D., Liebke, H., Cherry, J. H. and Strehler, B. L. (1970) *Biochim. Biophys. Acta* **204**, 175.
21. Thomas, H. and Stoddart, J. L. (1977) *Proc. Ann. Appl. Biol.* **85**, 461.
22. Thomas, H. (1976) *Plant Sci. Letters* **6**, 369.
23. Kende, H. and Shen, T. C. (1972) *Biochim. Biophys. Acta* **286**, 118.
24. Oaks, A., Wallace, W. and Stevens, D. (1972) *Plant Physiol.* **50**, 649.
25. Sawardekar, J. S., Slonekar, J. R. and Jeanes, A. (1965) *Anal. Chem.* **37**, 1602.
26. Potty, V. H. (1969) *Anal. Biochem.* **29**, 535.
27. O'Neill, J. V. and Webb, R. A. (1970) *J. Sci. Food Agr.* **21**, 217.