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Antigenic and some kinetic properties of three *p*-diphenol oxidase isoenzymes of *Trametes versicolor*

The fungus *Trametes*[1] *(Polyporus) versicolor* produces large quantities of the exoenzyme *p*-diphenol oxidase[2] (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2, formerly known as 'laccase') which has been shown to exist in two forms, *p*-diphenol oxidase A and *p*-diphenol oxidase B[3,4]. The present communication reports the detection of a third *p*-diphenol oxidase isoenzyme produced by *T. versicolor*. The sequential development of the three isoenzymes has been studied, along with their antigenic, kinetic, and electrophoretic properties.

Three distinct *p*-diphenol oxidase isoenzymes were detected when a number of *T. versicolor* isolates were grown on a peptone–yeast extract medium[5], while only two were detected in the medium used by MALMSTRÖM, FÅHRAEUS AND MOSBACH[6] (Fig. 1).

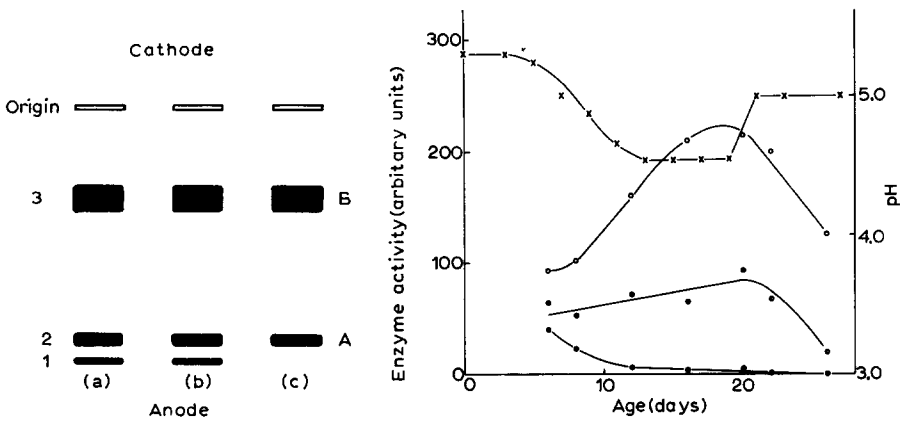

Fig. 1. Polyacrylamide-gel electrophoresis of *p*-diphenol oxidase isoenzymes. Samples (a) from culture filtrate and (b) from mycelial extract of *T. versicolor* D4 grown on a peptone–yeast extract medium[5]. Sample (c) from culture filtrate of the same organism grown on the medium recommended by MALMSTRÖM, FÅHRAEUS AND MOSBACH[6]. 1, 2 and 3 signify *p*-diphenol oxidase 1, 2 and 3, respectively; A and B signify *p*-diphenol oxidase A and B, respectively, as described previously by others[3,4]. Gel consisted of acrylamide monomer 6.5%, *N,N*-methylenebisacrylamide monomer 0.289 g, Tris 2.952 g, 1 M HCl 3.9 ml *N,N,N',N'*-tetramethylethylenediamine 0.075 ml, in water to a volume of 130 ml. Polymerization was effected with ammonium persulphate (0.07%) within 30 min at 20–25° in a gel trough (18 cm × 9 cm × 0.8 cm) sealed with a perspex lid carrying 15 slot formers[10] (each of 0.8 cm × 0.15 cm × 0.75 cm). The gel (pH 8.9) was used within 24 h. Horizontal electrophoresis[10] in Tris–glycine buffer (3.0 g/l Tris, 14.4 g/l glycine, pH 8.5) was performed at 1–2° with 35 mA d.c. until a tracker dye (bromophenol blue) had migrated 5 cm. The gel block was sliced into 4 identical thin pieces. Each piece was equilibrated for 1 h in 2 changes of 100 ml of acetate buffer (0.2 M, pH 5.0). The *p*-diphenol oxidase pattern was developed in 0.02 M *o*-dianisidine in the acetate buffer.

Fig. 2. Sequential production of the *p*-diphenol oxidase isoenzymes by *T. versicolor* D4 on peptone–yeast extract medium, and the changes in pH of the culture medium during growth of the fungus. ●—●, *p*-Diphenol oxidase 1; ◐—◐, 2; ○—○, 3; ×—×, pH. Measures of enzyme activity were obtained using acrylamide-gel (6.5%) electrophoresis of culture filtrates. Transparent positive prints of the isoenzyme patterns were scanned in an E.E.L. electrophoresis scanner. The area bound by the absorbance curve of each band indicated the catalytic activity of the isoenzyme concerned.

The use of a range of phenolic compounds as substrates[7] revealed that all three isoenzymes were of the *p*-diphenol oxidase type. It is obvious that the slowest-moving band is identical to the so-called *p*-diphenol oxidase B while the intermediate band is *p*-diphenol oxidase A (Fig. 1). These isoenzymes have been renamed *p*-diphenol oxidases 1, 2 and 3 according to the system recommended by the Standing Committee on Enzymes[8]. *p*-Diphenol oxidase 1 was produced during the early stage of growth of the fungus when the pH of the medium was relatively high (Fig. 2). As the medium became more acidic, the activity of *p*-diphenol oxidase 1 decreased rapidly. During this period, the production of *p*-diphenol oxidase 3 increased rapidly and reached a maximum when the culture was 18–20 days old, while the production of *p*-diphenol oxidase 2 increased very gradually (Fig. 2). The absence of *p*-diphenol oxidase 1 in preparations of other workers could have resulted from the addition to the culture medium of inducer substances which might affect the physiological age of the mycelium.

Initial separation of the *p*-diphenol oxidases from brown material present in crude preparations[6] was achieved by electrophoresis using acrylamide gel (8.0% acrylamide monomer, pH 8.9), the *p*-diphenol oxidase isoenzymes being at least 1 cm behind the brown material. The gel containing the brown material was removed and the remaining gel was granulated, packed in a vertical column and the proteins eluted electrophoretically (10 mA d.c. for 12 h at 1–2°, Tris–glycine buffer at pH 8.5) into a dialysis bag. Dialysis and subsequent separation of the isoenzymes on a DEAE-Sephadex (A50, medium) column was made using Tris–HCl buffer (0.0025 M, pH 9.25). Gradient elution was performed with NaCl (0–1 M) in the starting buffer. The isoenzymes were located using D-catechin (0.01 M) as substrate in acetate buffer (0.2 M, pH 5.0) at 25°. Despite the fact that all the isoenzymes are inhibited at pH values above 8.4 (see below), satisfactory separation was obtained at pH 9.25, and any inhibition must have been reversible. Separation of all 3 isoenzymes was confirmed using acrylamidegel electrophoresis (6.5% gel) of selected fractions.

The Michaelis constants of the 3 *p*-diphenol oxidase isoenzymes using D-catechin as substrate at pH 5.0 were identical (approx. 10^{-4} M) but the v_{max} values varied in the order 1 > 2 > 3. When tested directly in acrylamide gel, *p*-diphenol oxidases 2 and 3, but not 1, were inactivated at pH 7.4. At pH 3.0, the reverse was true. At pH values above 8.4 and below 2.6, all 3 isoenzymes were inactivated. The *p*-diphenol oxidase isoenzymes exhibited differential inhibition by 0.02 M sodium diethyldithiocarbamate, with *p*-diphenol oxidase 3 being completely inhibited, 2 partially so, and 1 not inhibited.

The activity of the *p*-diphenol oxidases was not inhibited in the antigen–antibody complex, the precipitin arcs being readily characterized. Antigenic relationships between the *p*-diphenol oxidase isoenzymes were demonstrated with the double-diffusion technique, using an antiserum (A4)[5] developed against *T. versicolor* isolate D4. It is clear that *p*-diphenol oxidases 1 and 2 are antigenically distinct (Fig. 3). A spur was formed between *p*-diphenol oxidases 1 and 3 indicating that these 2 isoenzymes are antigenically related but not identical. Spur formation was also observed between *p*-diphenol oxidases 2 and 3. When a mixture of *p*-diphenol oxidases 1 and 2 was tested against *p*-diphenol oxidase 3, a spur was still produced by the latter isoenzyme (Fig. 4).

In immunoelectrophoretic analysis, the precipitin arcs of *p*-diphenol oxidases 1

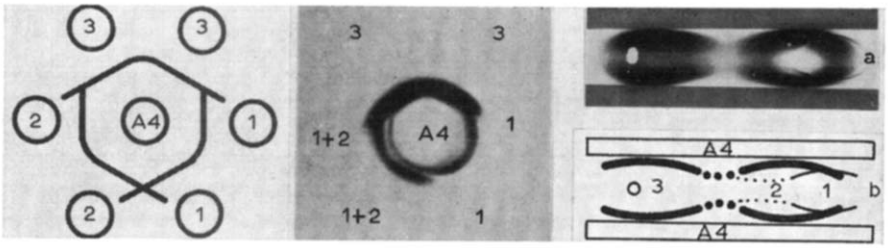

Fig. 3. Diagrammatic representation of the antigenic relationships between *p*-diphenol oxidases 1, 2 and 3 obtained using Ouchterlony double-diffusion tests in agar gel[5]. 1, 2 and 3 signify *p*-diphenol oxidases 1, 2 and 3, respectively; A4, antiserum A4 developed against *T. versicolor* D4. *p*-Diphenol oxidase activity was characterised using 0.02 M *o*-dianisidine in acetate buffer (0.2 M, pH 5.0).

Fig. 4. Photographic record of the precipitin arcs of the isoenzymes. 1 and 3 signify *p*-diphenol oxidases 1 and 3; 1 + 2, a mixture of *p*-diphenol oxidases 1 and 2; A4, antiserum A4 against *T. versicolor* D4.

Fig. 5a. Immunoelectrophoresis of *p*-diphenol oxidase isoenzymes present in the concentrated culture filtrate of *T. versicolor* D4.

Fig. 5b. A diagrammatic interpretation of the precipitin patterns obtained in Fig. 5a. A4, troughs containing antiserum A4 against *T. versicolor* D4; 1, 2 and 3 signify the precipitin arcs of *p*-diphenol oxidases 1, 2 and 3, respectively. Immunoelectrophoresis was performed in 0.3% agarose gel (18 cm × 9 cm × 0.2 cm) in Veronal buffer of ionic strength 0.033, pH 8.6. 10 μl of the crude *p*-diphenol oxidase preparation was applied to a circular well[10] cut in the gel. Electrophoresis was performed as described (Fig. 1). The antiserum A4 was then introduced into the troughs[10] in the gel, which was then incubated in a humidity chamber for 4 days at 1–2°. The precipitin arcs of the *p*-diphenol oxidase isoenzymes were characterised using *o*-dianisidine.

and 2 overlapped, but the 2 arcs were always distinct (Fig. 5). This demonstrates further the lack of a common antigenic structure between these 2 isoenzymes. Connections between the precipitin arc of *p*-diphenol oxidase 3 and those of 1 and 2 were obvious. Absorption[5] of antiserum A4 with *p*-diphenol oxidase 2 removed all antibodies against this isoenzyme. The *p*-diphenol oxidase 3 precipitin arc was displaced towards the trough containing the absorbed antiserum, indicating the removal of some antibodies against *p*-diphenol oxidase 3 in the antiserum absorbed by *p*-diphenol oxidase 2. The *p*-diphenol oxidase 1 precipitin arc was apparently unaffected in intensity and position. Complete removal of antibodies against *p*-diphenol oxidase 3 was obtained by absorbing the antiserum A4 with this isoenzyme. The *p*-diphenol oxidase 2 precipitin arc was displaced towards the trough containing the absorbed antiserum. Only a very faint precipitin arc of *p*-diphenol oxidase 1 was observed. Absorption of antiserum A4 with *p*-diphenol oxidase 1 was not performed because of the small amounts of the isoenzyme available.

These immunological results suggest that *p*-diphenol oxidase 3 possesses certain unique antigenic properties in addition to those antigenic determinants shared with the other isoenzymes. *p*-Diphenol oxidases 1 and 2 also possess unique antigenic determinants since absorption of the antiserum A4 by *p*-diphenol oxidase 3 did not remove all the antibodies against these 2 isoenzymes. A proposal for the distribution of a minimal number of antigenic determinants among the 3 isoenzymes is presented in Table I.

Thus, all 3 enzymes possess specific antigenic properties, but *p*-diphenol oxidases 1 and 2 also share different antigenic determinants with *p*-diphenol oxidase 3.

TABLE I

	Specific	*Shared*
p-Diphenol oxidase 1	a	d
p-Diphenol oxidase 2	b	e
p-Diphenol oxidase 3	c	d e

If the *p*-diphenol oxidase isoenzymes were derived from various degrees of polymerization of similar subunits, treatment of the isoenzymes with urea followed by dialysis may give rise to some of the other forms of the enzyme through random recombination of the polypeptide chains. When the purified isoenzymes were treated with 8 M urea and then analysed in acrylamide-gel electrophoresis (6.5% gel), *p*-diphenol oxidases 1 and 3 were inactivated completely, while *p*-diphenol oxidase 2 retained considerable activity. The activity of *p*-diphenol oxidase 1, but not of 3, recovered to a certain extent after dialysis. High activity of *p*-diphenol oxidase 2 was observed after removal of urea. When the concentration of urea in the incubation mixture was reduced, the activity of *p*-diphenol oxidase 3 was recovered after dialysis. None of the individual isoenzymes, nor a mixture of *p*-diphenol oxidases 1 and 2, on treatment with urea followed by dialysis, gave rise to other forms of laccase as has been observed by JOLLEY AND MASON[9] with the tyrosinase isoenzymes of mushroom. These findings, when they are considered along with those obtained from serological tests, suggest that *p*-diphenol oxidases 1 and 2 are distinct molecular species of the enzyme, each having its own specific polypeptide chains. *p*-Diphenol oxidase 3 is a molecular hybrid between *p*-diphenol oxidases 1 and 2, in the sense that it shares antigenic structures with these isoenzymes, but structures unique to *p*-diphenol oxidase 3 must be present to account for its specific antigenic and electrophoretic properties.

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