Phytochemistry, Vol. 27, No. 12, pp. 3735–3737, 1988.
Printed in Great Britain.

0031–9422/88 $3.00+0.00

HISTOCHEMICAL AND IMMUNOCHEMICAL LOCALIZATION OF TYROSINASE IN WHOLE TISSUE SECTIONS OF MUSHROOMS

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(*Received* 3 *March* 1988)

Key Word Index—*Agaricus bisporus*; histochemistry; immunochemistry; tyrosinase; nitrocellulose blotting.

Abstract—Vertical and cross-sectional slices of live mushrooms were blotted onto nitrocellulose. The blotted membranes were stained for tyrosinase activity using L-DOPA in the presence and absence of tyrosinase inhibitors. Histochemically stained areas of tyrosinase were present throughout the entire tissue, but the gills, stalk, and cap epidermis showed darker staining reactions. These staining reactions were eliminated if the blots were preincubated or incubated in tropolone or diethyldithiocarbamate. Immunostaining patterns of cross-sectional slices were similar to that of staining with L-DOPA.

INTRODUCTION

Mushroom tyrosinase (EC 1.14.18.1, monophenol monooxygenase) has been studied frequently with regard to its enzymatic and physical characteristics [1–4]. In recent years, the subunit composition of tyrosinase has been reexamined [5–7], potent inhibitors of the enzyme have been found [8, 9], conformational and hydrodynamic differences have been observed [10–14], and the involvement of tyrosinase in the metabolism of λ-glutamyl-4-hydroxybenzene has been studied [15–18]. While many of these studies have utilized commercial preparations of the enzyme, much less information is available on tyrosinase during mushroom development, ultrastructural localization of the enzyme, and latent forms of the enzyme. We have used a technique developed by Spruce *et al.* [19] for histochemical localization of plant enzymes on nitrocellulose to determine the location of tyrosinase in growing mushrooms. Conformation of the histochemical staining pattern was carried out by incubating blots with specific inhibitors of tyrosinase. Kahn and Andrawis have already reported that these inhibitors are very potent [8] and that one of them, tropolone, can be used to distinguish between peroxidase and tyrosinase [9]. Localization of tyrosinase in the presence and absence of these inhibitors on nitrocellulose was much clearer than incubating tissue slices with appropriate substrates and the results showed that tyrosinase could be localized to specific regions within the mushroom.

RESULTS AND DISCUSSION

Vertical slices of mushroom tissues showed that tyrosinase was present throughout the mushroom tissue. Certain areas, however, showed increased enzyme staining. These areas corresponded to the stalk, gills, and cap epidermis. Because vertical sections were more difficult to blot onto nitrocellulose, only the results obtained with cross-sectional slices will be presented in this report. Cross-sectional slices were made starting at the top of the cap and proceeding downward to the stalk. Blotting of the slices onto nitrocellulose and staining with L-DOPA clearly showed intense staining in the regions of the cap epidermis, gills, and the stalk (Fig. 1 a–e). In this series of blots areas of enzyme staining can be followed from a cut through the cap epidermis (a) to a cut through the cap near the stalk (e). Panel c showed the best and clearest distribution of tyrosinase activity. These results agree with Burton [20, 21], who observed that tyrosinase was distributed in the cap skin (pilei pellis) and the cap flesh (pileus trama) and with Yamaguchi *et al.* [22] who reported that tyrosinase was present in the cap and stalk (stipe).

Using tropolone to differentiate between peroxidase and tyrosinase in fresh tissue slices, Kahn [9] reported that mushrooms possessed no peroxidase activity. In contrast, Stussi and Rast [18] and Rast *et al.* [23] have reported a peroxidase activity associated with mushrooms and mushroom spores. To determine if these staining regions were exclusively a result of tyrosinase activity, several cross-sectional blots were incubated with various inhibitor/substrate combinations. Preliminary data already indicated that the staining pattern could be eliminated if the blots were preincubated in tropolone or diethldithiocarbamate and that incubation in diaminobenzidine or guaiacol plus hydrogen peroxide resulted in a positive staining reaction albeit much weaker than that observed with DOPA. These results suggested the presence of another oxidative enzyme.

To resolve this problem, we used a similar substrate/inhibitor combination scheme to the one reported by Kahn [9]. Cross-sectional slices were blotted onto nitrocellulose and incubated with tropolone and hydrogen peroxide to detect peroxidase (Fig. 2 a). No detectable enzyme staining was present, suggesting the absence of peroxidase. Replicate blots were preincubated in tropolone or L-DOPA (Fig. 2 b,c). Tropolone completely eliminated the staining reaction observed with L-DOPA confirming the inhibitory effect of tropolone on mush-

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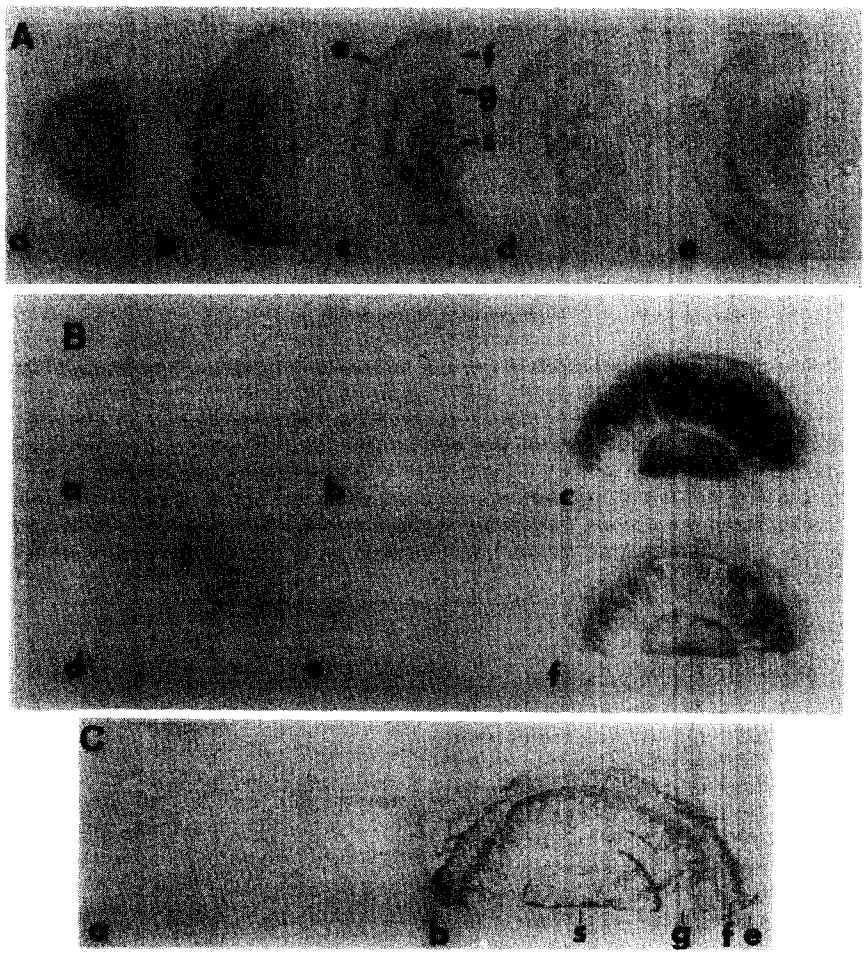

Fig. 1. A Histochemical staining of tyrosinase in cross-sectional slices from the mushroom cap. Blots were incubated in the presence of L-DOPA and catalase (100 Keilin units). Slices were taken at intervals from the top of the cap (a) to the bottom of the cap (e). In panel c, the letters refer to the cap epidermis or skin (e), cap flesh (f), gill tissue (g), and the stalk (s). B. Histochemical staining of tyrosinase in mushroom blots incubated in various substrate/inhibitor combinations. The blots were incubated in the following solutions: a, 0.5 mM tropolone plus 0.01% hydrogen peroxide; b, 0.5 mM tropolone; c, 5 mM L-DOPA; d, 0.5 mM tropolone, 5 mM L-DOPA, and 0.01% hydrogen peroxide; e, 0.5 mM tropolone plus 5 mM L-DOPA; f, 5 mM L-DOPA plus 0.01% hydrogen peroxide. Staining conditions were as described in the Experimental section. C. Immunochemical staining of tyrosinase in mushroom cross sectional tissue slices. A control blot was incubated with mouse antibodies (a) while the blot for localization of tyrosinase was incubated with mouse antibodies and rabbit anti-mushroom tyrosinase (b). Letters refer to the same morphological regions as in A. See Experimental section for staining methods.

room tyrosinase reported by Kahn [9]. DOPA staining was intense in the cap epidermis, gills, and stalk. In contrast, incubation of replicate blots in tropolone/DOPA/hydrogen peroxide and tropolone/DOPA solutions showed some enzyme staining although at a much lower level compared to staining with DOPA alone (Fig. 2 d, e). These results suggest the presence of an enzyme which can utilize DOPA even in the presence of tropolone. Enzyme staining was also observed when the blots were incubated in a DOPA/hydrogen peroxide solution (Fig. 2 f). These observations were not related to a loss of enzyme transfer during sequential blotting because even the last blots stained with DOPA/hydrogen peroxide (Fig. 2 f). A blot incubated in a DOPA/catalase solution (data not shown) showed a similar histochemical staining pattern and intensity as with DOPA alone. Preincubation of the blots in *O*-phenanthroline eliminated the oxidative activity associated with guaiacol/hydrogen peroxide utilization but did not decrease staining with L-DOPA (data not shown). In spite of the apparent presence of another oxidative enzyme which could utilize DOPA as a substrate, our results strongly suggest that the major histochemical staining reaction was due to the presence of tyrosinase.

To confirm the presence and localization of tyrosinase in mushroom slices, we used identical cross-sectional blots in a Western blotting protocol [24]. Preliminary data suggested that non-specific binding of antibody was a problem. Therefore, control blots were incubated with mouse antibodies and sample blots were incubated with

mouse antibodies and mushroom tyrosinase antibodies [25]. Alkaline phosphatase conjugated goat anti-rabbit antibodies were used as the secondary probe. As shown in Fig. 3 we have not been able to completely reduce the nonspecific binding, but we have been able to reduce it considerably using the mouse antibodies as a blocking agent rather than pre-immune rabbit serum. Mushroom tyrosinase was clearly localized to the stalk, gills, and cap epidermis in the cross sections immunologically. Lesser amounts of immunostaining were present in the cap flesh. These results provide addditional evidence that the major L-DOPA staining regions were due to tyrosinase activity.

It is evident from the histochemical localization of tyrosinase that the enzyme is present throughout the mushroom tissue but that more enzyme is localized in the stalk, gills, and cap epidermis. Although we have not attempted to do so, areas of latent enzyme activity might be detectable using modification of this histochemical technique..Furthermore, cytochemical and immunocytochemical studies at the cellullar level are needed to localize the enzyme within the cells of each of these different tissues.

EXPERIMENTAL

Materials. Mushrooms were obtained from the Campbell Institute for Research and Technology (Napoleon, OH). These mushrooms were grown to the first break on a premixed compost medium. Commercial mushrooms from local supermarkets were also used but they did not blot as well as growing mushrooms.

Blotting and staining. Mushrooms were sliced and blotted onto nitrocellulose as described by Spruce *et al.* [19]. Several consecutive blots (up to 7 or 8) could be made from the same mushroom slice without apparent loss of enzyme during transfer. The membranes were placed blot side up on filter paper soaked in 100 mM Na-Pi buffer (pH 6.0) containing the appropriate substrate/inhibitor combinations. Inhibitors solns consisted of either diethyldithiocarbamate (10 mM), tropolone (1 mM), or *o*-phenanthroline (0.5 mM). Substrate solns contained either L-DOPA (5 mM), diaminobenzidine (2.3 mM) and H_2O_2 (0.01% v/v), or guaiacol (0.1% v/v) and hydrogen peroxide (0.01% v/v). Substrate/inhibitor solns contained 5 mM L-DOPA with either 0.5 mM tropolone and/or 0.01% H_2O_2 (v/v). All substrates and inhibitors were dissolved in 100 mM Na-Pi buffer (pH 6.0). All blots were left in contact with the substrate/inhibitor soln for at least 15 min. They were then allowed to dry for 30 min before rinsing them in H_2O to remove unreacted substrates and nonpolymerized products. Although the histochemical reactions began as orange coloured stains, air-drying the blots resulted in the appearance of permanent gray-black histochemically stained arcas. Control blots soaked in buffer instead of substrate showed no visible staining, indicating that nonenzymatic oxidation or endogeneous oxidation of phenolic materials was of no consequence.

Western blotting. Mushroom slices were blotted onto nitrocellulose and incubated in nonfat dry milk as described by Lanker *et al.* [24]. $(NH_4)_2SO_4$ pptd mouse ascites fluid (200 μl) was added to control blots. Sample blots contained the same amount of mouse ascites fluid plus mushroom tyrosinase antibodies (made in rabbits, 1 μl per ml). Blots were blocked overnight as described by Lanker *et al.* [24]. Areas of immunological cross reaction were located by incubating the blots in alkaline phosphatase conjugated goat anti-rabbit antibodies (1 μl per ml) for 4 hr. The blots were stained for alkaline phosphatase using nitroblue tetrazolium and bromo-chloro-indoylphosphate according to the manufacturer's instructions (Bio Rad Laboratories, Richmond, CA). Alkaline phosphatase conjugated second antibodies were used in preference to peroxidase conjugated second antibodies because an endogeneous oxidase utilized the substrates, diaminobenzidine and H_2O_2, for the peroxidase conjugated antibodies.

Acknowledgements—Supported in part from a grant from the Campbell Institute for Research and Technology (Napoleon, OH). We also appreciate the secretarial assistance of K. Divan and P. Archer.

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