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CROSS-REACTIVITY OF POLYCLONAL AND MONOCLONAL ANTIBODIES TO POLYPHENOLOXIDASE IN HIGHER PLANTS

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Key Word Index—Polyphenoloxidase; polyclonal; monoclonal; antibodies; Western blotting.

Abstract—Polyclonal and monoclonal antibodies against broad bean polyphenoloxidase were used to examine the cross reactivity of the enzyme in higher plants after Western blotting. Both the polyclonal and monoclonal antibodies recognized similar enzyme forms in broad beans and mung beans, but only the polyclonal antibody identified polyphenoloxidase in pea and soybean extracts. The polyclonal antibodies were able to identify inactive and/or active enzyme forms of polyphenoloxidase in the various plant sources under partially denaturing SDS-PAGE. The polyclonal antibody was also used to identify several cross-reacting proteins in five other plant species after denaturing SDS-PAGE. Under denaturing SDS-PAGE, the number and types of polyphenoloxidase identified were surprisingly similar among the eight species and were localized to proteins with M_rs of 60–63000, 43–45000, and 34–36000.

INTRODUCTION

Polyphenoloxidase (E.C. 1.10.3.2; PPO) is an enigmatic enzyme widely distributed in plant species [1, 2]. Considerable progress has been made in recent years toward understanding the molecular biology, mechanism of the enzymatic reaction, and biosynthesis of the enzyme [3–7]. For example, the broad bean enzyme has been synthesized *in vitro* and immunoprecipitated with antibodies against the broad bean enzyme [6]. The M_r of the *in vitro* synthesized product appeared to be similar, if not identical, to the M_r of the native enzyme [6]. Flurkey [7] also reported that eight different plant species cross-reacted with anti-broad bean PPO antibodies in Ouchterlony double diffusion tests. The M_rs of the *in vitro* synthesized PPO from these plant species were of the same apparent size even though all species showed different isoenzyme staining patterns on PAGE [7]. These results suggest that the enzyme may be synthesized with a similar M_r in many plant species but that some type of post-translational modification or association occurs after synthesis. Antibodies can be useful in identifying these isoenzyme forms, determining their cross-reactivity among different plant species, and in isolation of the enzyme from different species. In this report, polyclonal and monoclonal antibodies against broad bean PPO were used in conjunction with Western blotting to identify and examine the cross-reactivity of the enzyme from broad beans, mung beans, peas and soybeans, and a variety of other plant species.

RESULTS AND DISCUSSION

Native electrophoresis has been and is commonly used to detect PPO isoenzyme composition. Using native electrophoresis, detection of PPO isoenzymes in some plant species was difficult because of streaking and band broadening [8]. Transfer of PPO from native gels by electroblotting to nitrocellulose also had its limitations and transfer appeared to be incomplete [9, data not shown]. On the other hand, partially denaturing SDS-PAGE has been shown to minimize streaking, increase resolution, and allow detection of latent PPO [8]. These conditions have been used frequently in recent years for analysis of PPO isoenzymes [8–15]. Thus, analysis of cross-reactivity among various PPO isoenzymes needs to be carried out under partially denaturing or denaturing SDS-PAGE, and if possible native PAGE conditions, to identify active and inactive enzyme forms.

Broad bean, mung bean, pea, and soybean species were initially chosen because of their PPO content [7], isoenzyme patterns [7], and cross-reactivities to anti-broad bean PPO [7, 8, 13–15]. Using partially denaturing SDS-PAGE and L-DOPA as the substrate, isoenzyme staining of these extracts showed an intense staining band at M_r 43–45000 and 36–39000 in broad beans and mung beans respectively (Fig. 1). No observable isoenzyme form was stained in pea extracts and soybeans extracts showed a very faint stained band between 43000 and 45000.

To compare enzymatically active isoenzyme forms of PPO with immunologically cross-reactive forms of PPO, the above extracts were subjected to partially denaturing SDS-PAGE followed by Western blotting. Cross-reactivity of the polyclonal and monoclonal anti-PPO to the enzyme was examined in each. No immunostaining was present in control blots treated with preimmune rabbit or mouse sera (Fig. 2). At least three cross-reactive species were detected in broad bean samples using either the polyclonal or monolconal antibody. A similar pattern was observed by Lanker *et al.* [14] although Vaughn and Duke [2, 13] identified a single active and a single inactive broad bean PPO of similar M_r which cross-reacted with polyclonal anti-PPO. The immunostained band we observed at M_r 43–45000 appeared to be the same size as that present in enzyme stained gels (Fig. 1) and therefore probably represents the active form of the enzyme. Using either the polyclonal or monoclonal antibody, a single form of PPO was also observed in mung bean extracts (Fig. 2). With regard to broad bean and mung beans, these

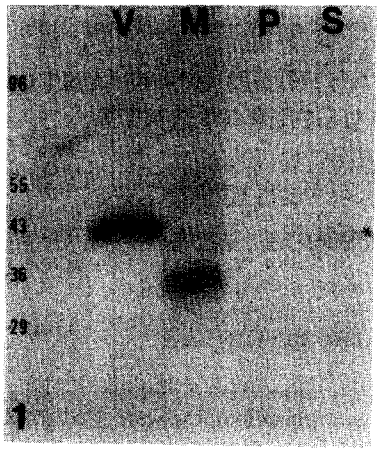

Fig. 1. Isoenzyme staining pattern of polyphenoloxidase. Crude extracts of broad bean (V), mung bean (M), pea (P), and soybean (S) were subjected to partially denaturing SDS-PAGE and stained for PPO activity using L-DOPA. Numbers refer to prestained M_r marker (pmw) in kD (Diversified Biotech, Newton Centre, MA). Asterisk designates faint staining band.

results showed that the specificity of the monoclonal was similar to that of the polyclonal antibody and that both antibodies recognize PPO in different species. In addition, both antibodies identified higher M_r forms of PPO in broad beans which did not appear to be active (Fig. 1 vs 2).

After partially denaturing SDS-PAGE and Western blotting, concentrated pea and soybean extracts contained a single PPO form which cross-reacted with the polyclonal antibody but not the monoclonal antibody (Fig. 2). This was not entirely unexpected since the monoclonal antibody would have specificity for a single epitope while the polyclonal would recognize many epitopes. The appearance of an immunostained band in peas (M_r 33–35000) was striking in light of the apparent absence of an active pea PPO in gels stained for isoenzymes (compare Fig. 1 vs 2). Thus, the polyclonal antibody appeared to recognize an inactive or less active pea PPO. Inactivation of the enzyme during extraction or a low specificity for L-DOPA, however, could account for this apparent anomaly. A very faint immunocross-reacting PPO form (M_r 35–37000) was observed in concentrated soybean extracts using the polyclonal antibody (Fig. 2). The M_r of this form was dissimilar to that observed after isoenzyme staining, suggesting the presence of an immunological cross reactive but enzymatically inactive soybean PPO. Alternatively, the immunoreactive soybean enzyme could have a weak specificity for L-DOPA or the enzymatically active enzyme could be present below the detection limits of Western blotting methods.

Western blotting of denatured PPO can show not only specificity of antibodies but also cross-reactivity among PPO subunits within the same species as well as in different species. To examine this, plant extracts were boiled and subjected to denaturing SDS-PAGE before Western blotting (Fig. 3). Similar immunostaining patterns were observed in mung and broad bean extracts using either the polyclonal or monoclonal antibody. In addition, a higher M_r (60–65000) form of PPO was present in all samples probed with the polyclonal antibody. With the exception of broad beans, this band was not detected using the monoclonal antibody. In general, the patterns of immunostaining after denaturing SDS-PAGE were very similar, but not identical, to those obtained after partially denaturing SDS-PAGE for all plant extracts examined. Even under denaturing conditions, the antigenic site remained intact for polyclonal and monoclonal antibody recognition of PPO in these sources. Thus, the polyclonal antibody can recognize a variety of PPO forms in partially denaturing and denaturing conditions.

Except for broad and mung beans, detection of PPO in other plant species by Western blotting proved to be difficult. Based upon enzyme activity, broad beans and mung beans contained substantially more PPO than bush beans, peas, soybeans, spinach, tobacco, and tom-

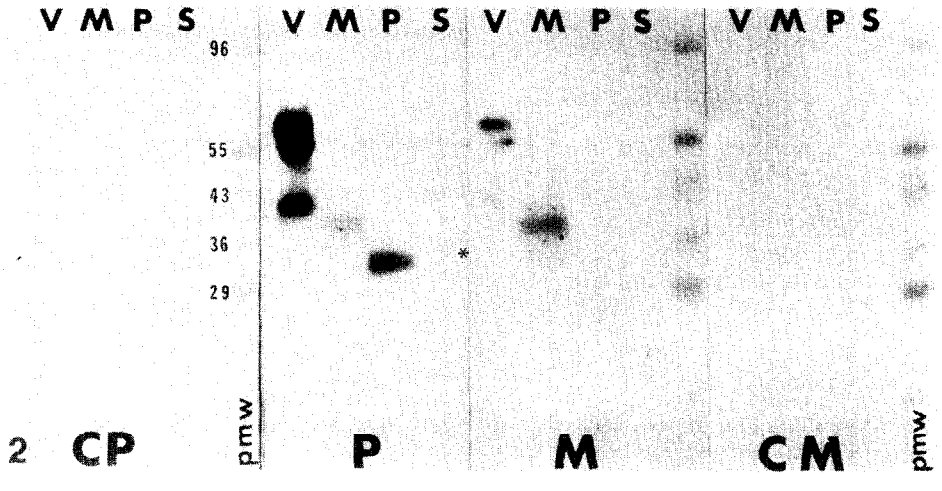

Fig. 2. Western blot of PPO in broad bean, mung bean, pea, and soybean extracts after partially denaturing SDS-PAGE. Blots were probed with polyclonal (P) and monoclonal (M) anti-PPO antibodies. C-P and C-M represent control blots probed with preimmune sera. Plants species are designated as above. Asterisk designates very faint immunostained band.

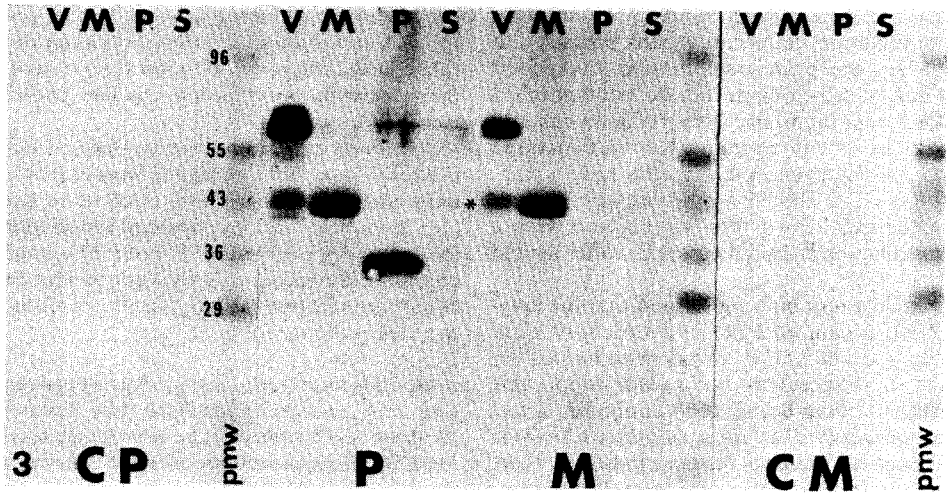

Fig. 3. Western blot of PPO in broad bean, mung bean, pea, and soybean extracts after denaturing SDS-PAGE. Designations are as above.

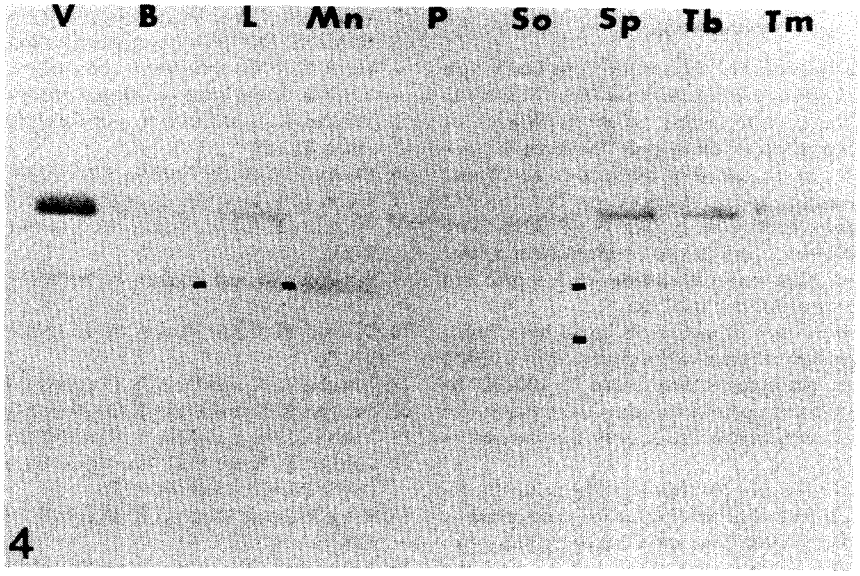

Fig. 4. Western blot of denatured PPO in various plant species. Crude extracts from broad bean (V), bush bean (B), lettuce (L), mung bean (M), pea (P), soybean (So), spinach (Sp), tobacco (Tb), and tomato (Tm) were concentrated and precipitated with TCA and subjected to denaturing SDS-PAGE. After transfer to nitrocellulose, Immunocross-reactive forms were located using polyclonal anti-PPO. Hash marks designate very weak immunostained bands.

atoes [7]. The pea and soybean extracts used in Fig. 1 were routinely concentrated *ca* 100–200-fold for blotting while crude extracts of broad and mung beans were commonly used. This would seem to indicate that not only are the enzyme activities lower in pea and soybean extracts, but also that the mass amount of PPO may be lower compared to broad and mung beans.

To determine if PPO can be detected in species in which it is less abundant, we prepared crude extracts from the nine plant species reported earlier [7]. These extracts were concentrated by ammonium sulphate precipitation and ultrafiltration, precipitated by trichloroacetic acid to concentrate them even further, and subjected to denaturing SDS-PAGE. The final solutions, except broad and mung beans, were concentrated at least 200-fold. Western blots were probed with the polyclonal anti-PPO because of its greater capacity for species recognition. Several bands of immunological cross reactivity were present in each plant species (Fig. 4). Each species showed a characteristic pattern representing the number and intensity of cross-reacting PPO forms. Some bands were quite intense while others were very weak in intensity. The slower moving band in all species was characterized with a M_r of 60–63000. The faster moving band in peas and soybeans corresponded to a M_r of 34–36000. An immunostained band at M_r 43–45000 was located be-

tween the fast and slow moving bands in broad bean, bush beans, mung beans, lettuce, soybeans, spinach and tobacco. Soybeans and bush beans showed a very faint band in this region which could not be photographed well. Under these conditions, the pattern of immunostaining was remarkably similar suggesting that the denatured forms of PPO among diverse plant species have a common size. Perhaps, this should not be unexpected since the *in vitro* synthesized PPO in these same species were of similar size and also showed similar peptides after limited proteolysis [7].

In this report, Western blotting showed that the polyclonal anti-PPO recognized PPO in a variety of plant species. The monoclonal anti-PPO has more limited use because of its narrow specificity, but is readily applicable to broad bean and mung beans. Both antibodies detect active enzyme forms as well as apparent inactive forms in several plant species. Western blotting indicated there was considerable similarity between plant PPO isoenzyme forms with regard to subunit composition, but they are not identical. Lastly, antibodies to PPO can be a useful tool to examine how the different isoenzyme forms are generated.

EXPERIMENTAL

Plant material. Broad bean (*Vicia faba* L cv 'Long Pod'), bush bean (*Phaseolus lunatus* cv 'Blue Lake'), lettuce (*Lactuca sativa* L. cv 'Salad Bowl'), mung bean (*Vigna radiata* L. Wilczek cv 'Berken'), pea (*Pisum sativa* cv 'Snow peas'), soybean (*Glycine max* L. Moensch), spinach (*Spinacia oleracea* L. cv 'Winter Bloomsdale'), tobacco (*Nicotiana tabacum* L. cv 'Turkish'), and tomato (*Lycopersicum esculentum* cv 'Big Boy') plants were grown under greenhouse conditions or obtained from a local garden. Various leaf sizes were picked from each source and either used fresh or stored frozen until use.

Enzyme extraction. Crude extracts from broad bean, mung bean, pea and soybean were prepared as described by Flurkey [6, 7]. Soybean and pea extracts were concd *ca* 100-fold by $(NH_4)_2SO_4$ pptn and Centricon concentrators. All extracts were frozen until used. Polyphenoloxidase activity was assayed as reported earlier [6, 7].

Electrophoresis and Western blotting. Partially or totally denaturing SDS-PAGE conditions were performed as described by Angleton and Flurkey [8] and King and Flurkey [15] using the Laemmli system [16]. Samples were mixed with glycerol and applied directly (partially denatured) to the gel. Other samples were boiled or TCA pptd (denatured) and then mixed with two-fold concd Laemmli sample buffer before application. Samples were electroblotted onto nitrocellulose and probed with the appropriate antibody as described in refs [14, 15] using alkaline phosphatase conjugated goat anti-rabbit or goat anti-mouse IgG. Alkaline phosphatase activity was localized using bromochloro-indoyl-phosphate and nitro blue tetrazolium following the manufacturer's instructions (BioRad Laboratories, Richmond, CA).

Monoclonal antibody production. Female CB6F1 mice were injected with purified PPO at four week intervals. Spleens were removed and hybridomas were produced as described in ref. [18]. Monoclonal lines were produced and screened with iodinated PPO as described in ref. [19]. From the hybridoma screening process three monoclonal antibodies were identified. All monoclonal lines were typed as IgG_{2a} and the 7d3-f8 monoclonal line was used in this report.

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