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Short Communication

In Vitro Biosynthesis of *Vicia faba* Polyphenoloxidase

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ABSTRACT

Poly A$^+$ mRNA was isolated from *Vicia faba* leaves and translated *in vitro* using a rabbit reticulocyte translation system. From analysis of the total translation products, the major proteins synthesized *in vitro* were 32 kilodaltons and 20 kilodaltons. When antibodies to *Vicia faba* polyphenoloxidase were added, a specific immunoprecipitable protein was observed. This protein's molecular weight was shown to be similar to that of the isolated enzyme (45 kilodaltons). The isolated enzyme successfully competed with the *in vitro* synthesized product for antipolyphenoloxidase. In addition, the *in vitro* synthesized product was not immunoprecipitated with antitomato peroxidase and comigrated with isolated and/or iodinated enzyme in sodium dodecylsulfate-polyacrylamide gel electrophoresis. Using *in vitro* translation and specific immunoprecipitation, a primary translation product corresponding to *Vicia faba* polyphenoloxidase was identified as a 45 kilodaltons protein.

Polyphenoloxidase (EC 1.14.18.1) is localized in the thylakoid membranes of higher plants where it exists in a latent state (17, 18, 23, 24, 27). The latent enzyme can be activated by a variety of methods, one of which is treatment with SDS (1, 18, 27). *In vitro*, the enzyme catalyzes the *o*-hydroxylation of monophenols and oxidation of *o*-diphenols, but the physiological function of this enzyme remains unknown at present (18). Although PPO has been studied extensively, little information is available on the biosynthesis, transport, and integration of this enzyme into the chloroplast thylakoid membranes.

Using enzymic, electrophoretic, and cytochemical analysis, Vaughn and Duke (25, 26) have discovered that tentoxin-treated sorghum and mung beans lack PPO[1] in the chloroplasts and that integration of PPO into the chloroplast was blocked by tentoxin treatment. They reported that tentoxin blocked not only incorporation, but also processing of PPO into *Vicia faba* chloroplasts (28). Using Western blots, they also identified an inactive 40-kD protein which was immunologically related to PPO. This protein was shown by immunocytochemical methods to accumulate at the plastic envelope in tentoxin-treated plants (28). The mol wt of the native and inactive protein appeared to be similar, which suggests that the precursor to the native enzyme may not be synthesized as a pre-protein, like typical nuclear-encoded chloroplast proteins.

If PPO is not synthesized as a larger mol wt precursor, then translation of broad bean mRNA and identification of a product corresponding to PPO synthesized *in vitro* should show a similarity in mol wt between the native enzyme and the *in vitro* synthesized product. Our results indicate that PPO is synthesized as a 45 d kD protein. This mol wt is somewhat larger than reported by Vaughn and Duke (28) but is similar to the partially purified protein we have isolated. This is the first report of the *in vitro* biosynthesis of PPO from higher plants and suggests that the mol wt of the *in vitro*-synthesized protein may not be larger than the native enzyme.

MATERIALS AND METHODS

Broad bean (*Vicia faba* L. Moensch cv Long Pod) seeds were obtained from W. Atlee Burpee Co., Warminster, PA and grown under greenhouse conditions. Leaves were harvested and RNA was isolated as described previously for fungal RNA except that 0.1% ascorbate was included in the initial extraction buffer (10, 19). Messenger RNA was isolated from the total RNA by chromatography on oligo(dT) cellulose (2). The mRNA was translated *in vitro* using a commercial rabbit reticulocyte lysate translation system (New England Nuclear or Promega BioTec, Madison, WI) using established procedures (10, 11). Translation mixtures also contained 1 μg of beef liver tRNA, 300 units of RNaisin, and 1 μg of ε-amino caproic acid and benzamidine. The translations were terminated by the addition of two volumes of 2× PTD buffer (phosphate-buffered saline, 2% TX-100 [v/v], and 2% sodium deoxycholate [w/v]). Unlabeled methionine (100 μg), ε-amino caproic acid (20 μg), benzamidine (20 μg), and 0.2 μg of leupeptin, pepstatin, elastinal, chymostatin, and antipain were also added to each translation mixture. Two volumes of 10% *Staphylococcus aureus* cells were added and the suspension shaken for 1 h at room temperature. After centrifugation, antibodies were added to the supernatant and the suspension shaken an additional 2 h at room temperature. Antibodies to *Vicia faba* PPO were kindly provided by S. Hutcheson and B. B. Buchanan (University of California, Berkeley, CA). *Staphylococcus aureus* cells (10–15 μl) were added and the mixture shaken for 1 h at room temperature. The suspension was centrifuged three times through a 5% sucrose pad (in PTD) and washed once with 1 ml of PTD. The immunoprecipitates were boiled for 5 min in 4× Laemmli sample buffer, centrifuged, and the supernatant subjected to SDS-PAGE (10). Electrophoresis was followed by autoradiography using Kodak XAR-5 film. Alternative methods for immunoprecipitation of the *in vitro* translation samples included the use of Protein A Sepharose, goat antirabbit IgG immunobeads, and swine antirabbit IgG.

Vicia faba PPO was partially purified from fresh leaves or acetone powders using a protocol similar to that described by Hutcheson and Buchanan (15), except that the enzyme was rechromatographed on the DEAE cellulose and hydroxylapatite columns. The partially purified protein was analyzed by SDS-PAGE under denaturing (boiled enzyme) and partially denaturing (sample applied directly to gel) conditions. The preparation was also labeled with [^{14}C]acetic anhydride as described by Flurkey *et al.* (11). The enzyme was also iodinated with [^{125}I] iodine as described by Thorell and Johansson (22). Enzyme

[1] Abbreviations: PPO, polyphenoloxidase; Con A, concanavalin A.

activity was measured spectrophotometrically at 410 nm in 2 ml of 50 mM sodium phosphate buffer (6.0) containing 0.05% SDS and 10 mM catechol (1, 8). One unit of activity was defined as one absorbance unit change per min (9). Protein content was estimated using the BioRad protein assay.

RESULTS AND DISCUSSION

Polyphenoloxidase was partially purified from broad bean leaves. The isolated enzyme contained one major band (mol wt 45 kD) and several minor bands when analyzed by SDS-PAGE under denaturing conditions (Fig. 1A) Angleton and Flurkey (1) showed that when PPO was subjected to electrophoresis in the presence of SDS (partially denaturing conditions), the enzyme became activated and the enzyme-stained bands tended to become less diffuse. Under these conditions two major proteins were observed with approximate mol wt of 45 and 53 kD, respectively (Fig. 1A). Only the 45-kD protein stained for enzyme activity using catechol or dopa as substrates. When this active 45-kD protein was cut out of the gel and run in a second gel under denaturing conditions, a protein band of 45 kD and a band of 60 to 62 kD was detected (data not shown). This suggests that the active enzyme has a subunit mol wt of 45 kD or possibly is associated with a larger mol wt protein. The partially purified enzyme also cross-reacted with antibodies against PPO (prepared by Hutcheson and Buchanan against membrane-bound *Vicia faba* PPO) in Ouchterlony double diffusion analysis and after immunoblotting.

Poly A mRNA was isolated from broad bean leaves and translated in a rabbit reticulocyte translation system. The translation mixtures were reacted with anti-PPO, and immunoprecipitable products corresponding to PPO were analyzed by SDS-PAGE and autoradiography. In earlier translations, an immunoprecipitable product of 45 kD was tentatively identified as the produce for PPO. This protein showed a similar mol wt as ^{14}C-labeled and iodinated PPO. Other proteins (mol wt 30–32, 22–24 kD) were present in the ^{35}S-immunoprecipitate and contained a substantial amount of label. These same proteins were the major products synthesized from translations primed with untreated or heat-treated mRNA when compared to translations with no added RNA (Fig. 1B, lanes 1 and 2). These proteins were found to be nonspecifically adsorbed to Protein A Sepharose, IgG immunobeads, and *S. aureus* cells and could be removed by treating the translation mixtures with excessive amounts of *S. aureus* cells before adding anti-PPO (Fig. 1B, lane 9; data not shown). This nonspecific binding was estimated to account for at least 75% of the total immunoprecipitable counts. The reason for this nonspecific binding is not known but when it was eliminated or reduced substantially, a single immunoprecipitable product could be detected (Fig. 1B, lane 6). This product was not observed from translations primed with no added RNA but with added anti-PPO. Nor was it found in translations with added mRNA but no anti-PPO (Fig. 1B, lanes 4 and 5). No product was found in translations primed with poly A mRNA and containing antitomato peroxidase antibodies (Fig. 1B, lane 8). In addition, the *in vitro*-synthesized produce and isolated enzyme showed similar mobilities in various percentage acrylamide gels.

Competition experiments were carried out to determine if the isolated enzyme was related to the tentative *in vitro* product for PPO. The *in vitro* translation sample was added to a preincubation mixture containing anti-PPO and the partially purified PPO (Fig. 1B, lane 7). The *in vitro* product was not immunoprecipitated to the same extent as control samples minus an antibody antigen preincubation mix; thus, the partially purified enzyme successfully competed with the *in vitro* product corresponding to the PPO for antibody (Fig. 1B, lanes 6 and 7). A small amount of ^{35}S-labeled PPO was present even when excessive amounts of antigen were added. This may be due to antigenic differences in the *in vitro* product and the isolated enzyme. Alternatively, the ^{35}S-labeled PPO may be slowly exchanged with the unlabeled PPO in the antigen-antibody complex. Further proof for the authenticity of the *in vitro* translation product was determined by mixing the iodinated PPO with the immunoprecipitated *in vitro* translation product. Both the iodinated PPO and *in vitro* product showed a similar mol wt in SDS-PAGE (Fig. 1C). In addition, mixing experiments indicated that the *in vitro* product and the iodinated PPO comigrated with the isolated 45 kD enzyme stained for protein with Coomassie blue (data not shown). Thus, the *in vitro* synthesized product corresponding to PPO had a similar mol wt a the iodinated and isolated protein.

Many proteins destined to be incorporated into organelles are synthesized as precursors to the native proteins and processed posttranslationally during passage into the organelle (6). One example, the small subunit of ribulose bisphosphate carboxylase/oxygenase is synthesized as an 18 to 20 kD protein and processed to a 14-kD protein (4, 5, 7, 14). This process was found to be energy dependent by Grossman *et al.* (12). Two polypeptides of the Chl *a/b* protein complex were also found to be synthesized as precursors and processed posttranslationally (6). Several other proteins, including plastocyanin, ferredoxin-NADP oxidoreductase, malate dehydrogenase, isocitrate lyase, and malate synthase are processed in a similar manner (13, 20). These proteins were synthesized as a larger mol wt precursor and could be converted to the native protein by loss of the signal peptide and/or attachment of carbohydrate.

Our partially purified *V. faba* PPO was characterized by a mol wt of 45 kD. The *in vitro* synthesized product corresponding to PPO had a similar mol wt as the isolated enzyme. This would suggest that the enzyme was not synthesized as a precursor, as are many nuclear encoded chloroplast proteins. Since Lax *et al.* (16) have already shown that tobacco PPO is nuclear encoded, an alternative explanation may be that the *V. faba* enzyme has had the transit peptide removed and has been posttranslationally glycosylated. By coincidence, the isolated PPO enzyme could migrate with the same apparent mol wt as the *in vitro* synthesized product. This suggestion is not without precedent as Riezman *et al.* (20) have reported that the glyoxysomal glycoprotein malate synthase has a similar *in vitro* and *in vivo* mol wt. There is also evidence for glycosylation of PPO. Biehl *et al.* have shown that spinach PPO contains carbohydrate, could be bound to Con A, and that the carbohydrate could be removed by treatment β-glycosidases (3; personal communication). Earlier studies by Flurkey and Jen (8, 9) suggested that peach PPO had carbohydrate associated with it, while Robb *et al.* (21) reported that *V. faba* PPO contained 3 to 4% carbohydrate. We have observed that a portion of iodinated *V. faba* PPO binds to Con A which also suggests that the enzyme may be glycosylated (unpublished data). Thus, it may be likely that this alternative explanation is possible. Because the broad bean enzyme is rather difficult to isolate, further experimentation will be needed to determine if the above possibility is correct. In any case, the *in vitro* translation product corresponding to *V. faba* PPO was synthesized as a single protein with an apparent mol wt of 45 kD. In light of the fact that these results represent the first attempt at identifying the primary translation product of PPO from higher plants, further studies will be needed in other species to confirm these observations.

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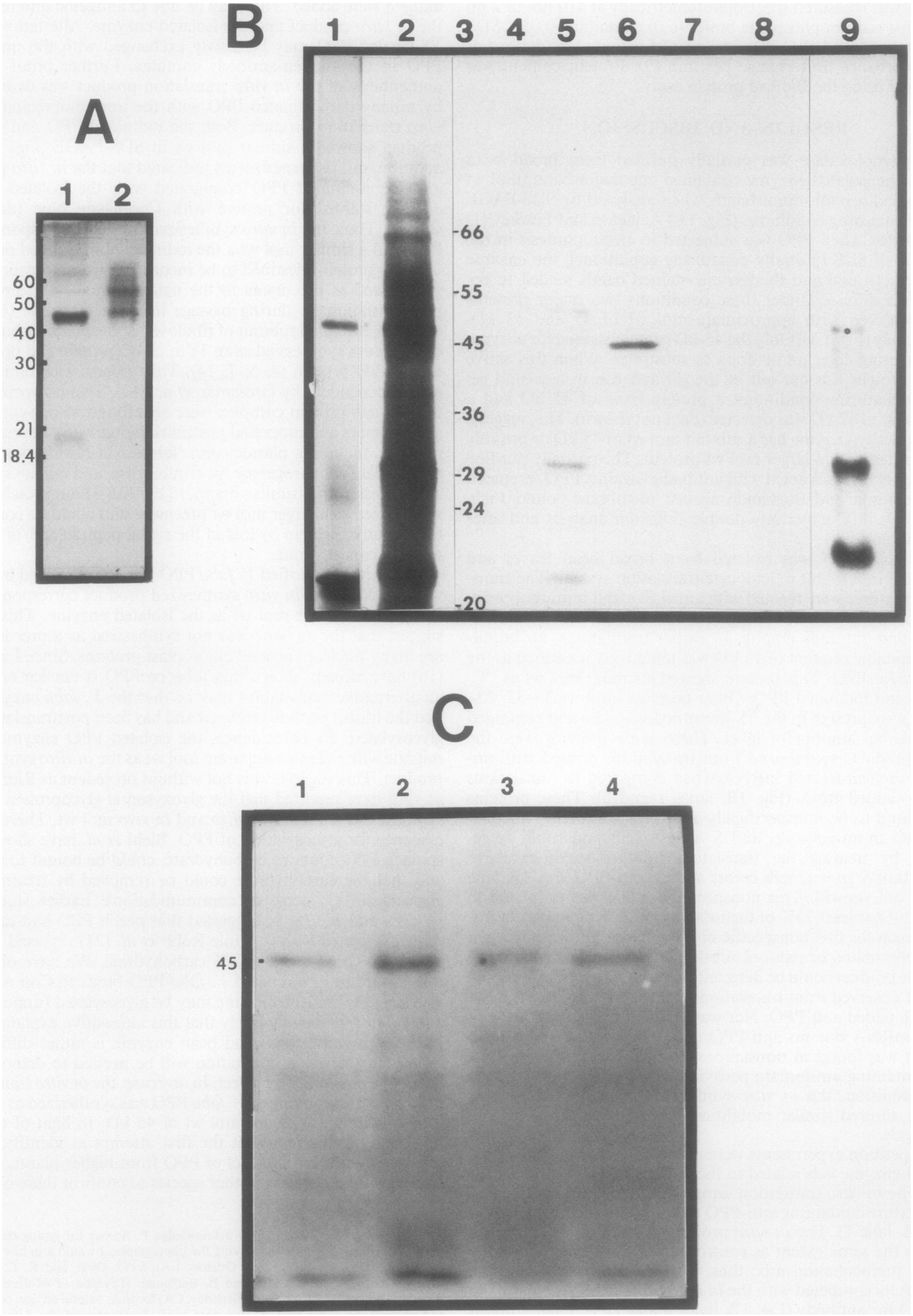
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FIG. 1. A, SDS-PAGE of partially purified *V. faba* PPO stained for protein with Coomassie blue R-250. Proteins (10 μg) in lane 1 were boiled in 2× Laemmli sample buffer containing mercaptoethanol while those in lane 2 (10 μg) were not boiled or treated with SDS and mercaptoethanol. B, Autoradiogram of total and immunoprecipitable *in vitro* synthesized translation products. Lanes 1 and 2 were the total translation products from translations with no added mRNA and with 2 μg of heat-treated poly A^+ mRNA (65°C for 5 min), respectively. Lane 3 shows mol wt markers. Lane 4 contained products from translations with no added mRNA and immunoprecipitated with anti-PPO. Lane 5 contained products from translations with added mRNA but no anti-PPO was added during the immunoprecipitations. Lane 6 showed products from translations primed with added mRNA and immunoprecipitated with anti-PPO. Lane 7 showed products from translations primed with added mRNA and immunoprecipitated in the presence of an antigen antibody complex (anti-PPO, 3 μg; isolated PPO, 6 μg). Lane 8 showed products from translation primed with mRNA but immunoprecipitated with antitomato fruit peroxidase. Lane 9 showed translation products preadsorbed and nonspecifically bound to *S. aureus* before addition of antibodies. Autoradiography was carried out for 7 d. C, Autoradiogram of the *in vitro*-synthesized PPO mixed with isolated iodinated PPO. *In vitro* translations and immunoprecipitations were carried out as described in the methods. Lanes 1 and 4 contained the *in vitro*-synthesized and immunoprecipitated product (15,000 cpm). Lane 2 contained a mixture of the immunoprecipitated product (15,000 cpm) and the iodinated PPO (17,000 cpm). Lane 3 contained the iodinated enzyme (17,000 cpm). The ^{35}S-labeled product was counted by liquid scintillation. The amount of iodinated sample chosen was determined from band intensities on previous gels. The top portion of the autoradiogram was not shown but no radioactive bands were present in the missing portion. Autoradiography was carried out for 4 d.