Pergamon 0031-9422(94)00816-7 Phytochemistry, Vol. 38, No. 6, pp. 1355–1360, 1995

0031-9422/95 $9.50 + 0.00

CARBOHYDRATE ASSOCIATED WITH BROAD BEAN POLYPHENOL OXIDASE IS RESISTANT TO ENZYMATIC AND CHEMICAL DEGLYCOSYLATION

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(*Received in revised form* 24 *May* 1994)

Key Word Index—*Vicia faba*; Leguminosae; broad bean; polyphenol oxidase; carbohydrate; glycosidases.

Abstract—Broad been polyphenol oxidase, associated with carbohydrate, was treated with endoglycosidases which remove O- and N-linked sugars. Neither *O*-glycanase, *N*-glycanase F, endoglycosidase F, endoglycosidase D, endoglycosidase H, nor glycopeptidase A were able to alter the apparent M_r of broad bean polyphenol oxidase after SDS–PAGE when stained for protein or Western blotting. Glycosidase treatment followed by SDS–PAGE and glycan staining or Con A lectin immunoblotting indicated no apparent removal of carbohydrate by the glycosidases. Chemical deglycosylation with trifluoromethanesulfonic acid and treatment with other glycosidases also failed to alter the M_r of the broad bean enzyme. Chromatography on jacalin agarose also suggested a lack of O-linked sugars. Although the broad bean enzyme appears to contain carbohydrate material, it seems to be resistant to endoglycosidases and chemical methods normally used to analyse glycoprotein structure.

INTRODUCTION

Polyphenol oxidase (PPO, EC 11.4.18.1, EC 10.3.2) is a copper-containing enzyme that catalyses the hydroxylation of monophenols and oxidation of diphenols to *o*-diquinones. The enzyme has been studied frequently and has been shown to exist in latent forms in many plants, fruits, vegetables and fungi [1–4]. Multiple forms of the latent (partially or totally inactive) and non-latent enzyme exist in crude extracts and in samples that have been purified to apparent homogeneity [1–5]. These multiple forms can arise by a variety of mechanisms including association–dissociation, association with lipids, limited proteolysis, post-translational modifications and carbohydrate attachment [1–5]. Limited proteolysis or abberant processing may also be responsible for different forms of the enzyme in grapes and broad beans [6, 7]. In contrast, the microheterogeneity observed in broad bean PPO was suggested to be related to differences in charge, size and covalently bound carbohydrate [8].

Carbohydrate attachment to, or association with, PPO has been reported in potatoes [9], apples [10], peaches [11], Jerusalem artichokes [12], spinach [13], strawberries [14] and broad beans [8, 15]. In potatoes and apples, this carbohydrate material appeared to be RNA-like using absorption spectroscopy and reactivity with orcinol [9, 10]. In peaches, RNA-like and other polysaccharide materials were apparently associated with PPO [11]. These observations are somewhat questionable considering the more recent observations concerning carbohydrate association with PPO. For example, Zawistowski *et al.* [12] used periodic acid–Schiff base (PAS) staining to show that artichoke PPO contained approximately 20% carbohydrate after isoelectric focusing (IEF). This suggests that the carbohydrate is covalently bound rather than in some type of loose association. Binding of spinach [13] and strawberry PPO [14] to Con A columns also suggests that some of these PPO forms contain covalently bound sugars. Further evidence for covalent bound carbohydrate was reported by Ganesa *et al.* [8] who showed that broad bean PPO contained carbohydrate(s) after sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and IEF. This latter observation was determined using antibody conjugated glycan detection kits and lectin immunoblotting [8]. Although these reports suggest covalent linkages of sugars to PPO, a specific type of linkage attachment (O or N) has not yet been demonstrated.

A variety of methods can be used to determine the type of carbohydrates present and their linkage to proteins. Of these, enzymatic deglycosylation and chemical hydrolysis followed by Western/lectin immunoblotting are most amenable to analysis of carbohydrate linkages when small amounts of enzyme are available. In this report, we

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attempted to determine how the carbohydrates are attached to broad bean PPO using endo- and exoglycosidases, chemical hydrolysis and jacalin chromatography.

RESULTS

Purified broad bean PPO contains two protein components which differ slightly in size (Fig. 1a) and in charge [8, 16]. The purified enzyme was treated with a variety of endoglycosidases. Treatment of denatured PPO (boiled) with *O*-glycanase, endo F, *N*-glycosidase F, and mixture of endo F and *N*-glycosidase F, endoglycosidase D, or endo H did not change the apparent size of PPO after SDS–PAGE and staining for protein (Fig. 1a). Comparison with an untreated sample showed no change in apparent mobility or the number of protein bands observed. The intense stained upper band in lane 5 of Fig. 1A is due to the presence of BSA in the endoglycosidase D sample and may mask the upper doublet band of PPO. The band in lane 2 at *ca* 35 kD is the *N*-glycosidase F enzyme. Interestingly, and regardless of the source of *O*-glycanase, less intense protein stained bands were consistently observed in samples treated with *O*-glycanase (lane 1). Similar results were obtained using O and N specific glycosidases from either Boehringer Mannheim or Genzyme. PPO that was not denatured before treatment with the glycosidases also showed no change in mobility or number of bands after SDS–PAGE (data not shown).

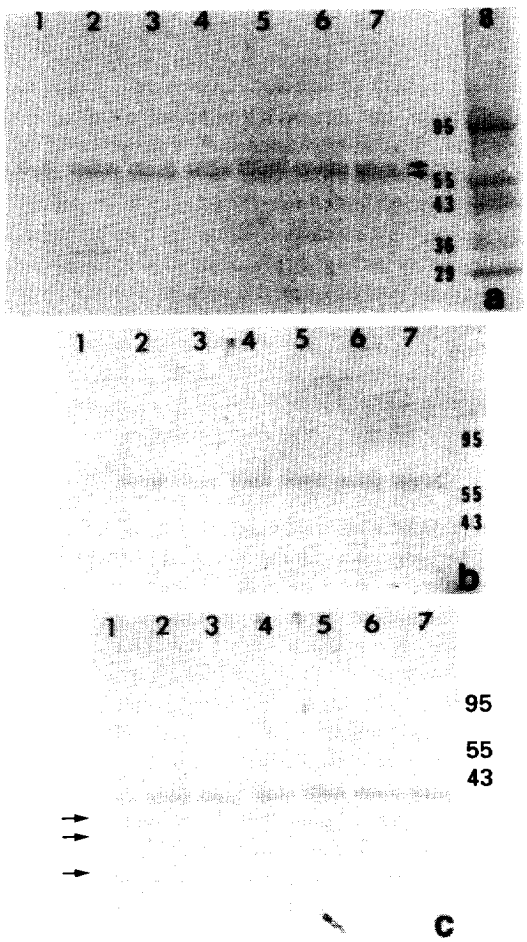

Fig. 1. Endoglycosidase treatment of denatured broad bean PPO. Broad bean PPO (5 μg) was denatured by boiling and incubated overnight with either *O*-glycanase (1), a mix of endoglycosidase F and *N*-glycanase (2), endoglycosidase F (3), *N*-glycanase (4), endoglycosidase D (5), endoglycosidase H (6) or no glycosidase addition (7). Lane 8 contained prestained M_r markers. After glycosidase treatment the samples were subjected to SDS–PAGE and analysed for either protein (a), immunological cross-reactivity by Western blotting (b), or carbohydrate detection using a glycan detection kit (c). For details see the Experimental section. Arrows indicate the two major forms of PPO. (c) Arrows indicate bands that gave a positive glycan stain but could not be photographed well.

Removal of a small amount of carbohydrate might not result in a noticeable difference in mobility using SDS–PAGE. Therefore, samples treated with the above glycosidases were also subjected to Western blotting and glycan detection to determine if there was any loss of carbohydrate. Western blotting showed the appearance of a major, but diffuse and overlapping, doublet band at *ca* 58–60 kD (Fig. 1b). These patterns were essentially identical to protein stained gels and did not result in the detection of any other immunological cross-reacting forms with lower molecular sizes. Samples subjected to blotting and carbohydrate detection showed similar patterns (Fig. 1c) to those above except that the band at *ca* 65 kD was not readily discernible. No apparent change in the mobility or number of bands was observed except in the *O*-glycanase treated samples where faint staining was evident at the 58–60 kD region and three bands with lower M_r were faintly detected after glycan staining.

Broad bean PPO shows specific binding to Con A [8]. To determine if this binding capability was lost after glycosidase treatment, and thus loss of glucose or mannose binding, PPO samples were denatured and then treated with the various glycosidases. They were then subjected to SDS–PAGE followed by Western blotting or blotting with biotinylated ConA/avidin conjugated alkaline phosphatase (Fig. 2). As can be seen in Fig. 2a, broad bean PPO was resolved into two bands which tended to overlap after Western blotting. Con A binding showed staining of the 58–60 kD band and perhaps some slight staining of the 60–65 kD band after glycosidase treatment, but the two bands smeared together (Fig. 2b). Similarly to Fig. 1, *O*-glycanase treatment showed less intense staining of PPO with antibodies and with biotinylated Con A.

Glycopeptidase A has also been used to remove carbohydrate from proteins [17, M. Chrispeels personal communication]. Non-denatured or denatured (boiled) broad bean PPO was treated with this enzyme in the presence or absence of sodium perchlorate. After treatment, no apparent differences or alteration in the size of PPO were noted compared to non-enzyme treated samples (Fig. 3). We did, however, notice some loss of material when PPO was treated with this enzyme in the presence of sodium perchlorate under non-denaturing conditions (Fig. 3, lane 3). An additional faint band was noted at *ca* 60 kD (lanes

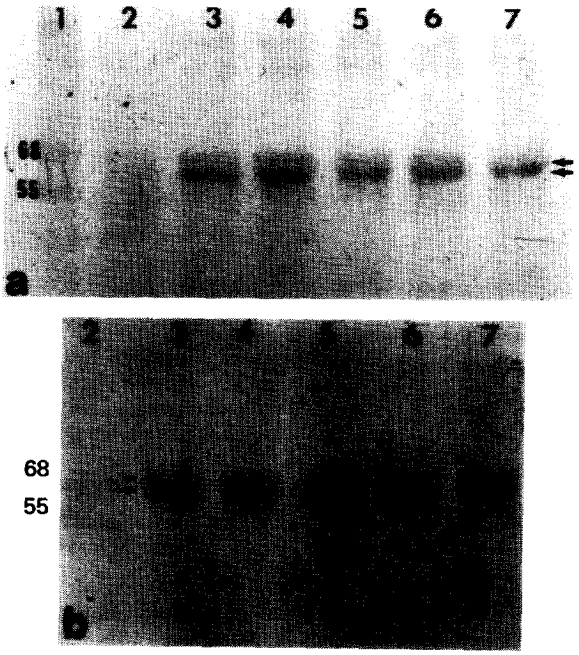

Fig. 2. Western blotting and Con A binding of endoglycosidase treated broad bean PPO. Broad bean PPO (5 μg) was denatured and incubated with either *O*-glycanase (2), a mixture of endoglycosidase F and *N*-glycanase (3), endoglycosidase F (4), *N*-glycanase (5), endoglycosidase H (6), or no glycosidase addition (7). Lane 1 contained prestained M_r markers. After glycosidase treatment, the samples were subjected to SDS–PAGE followed by Western blotting (a) or blotting with biotinylated Con A (b). For details, see the Experimental section. Arrows represent the two major forms of PPO identified by immunostaining and Con A staining.

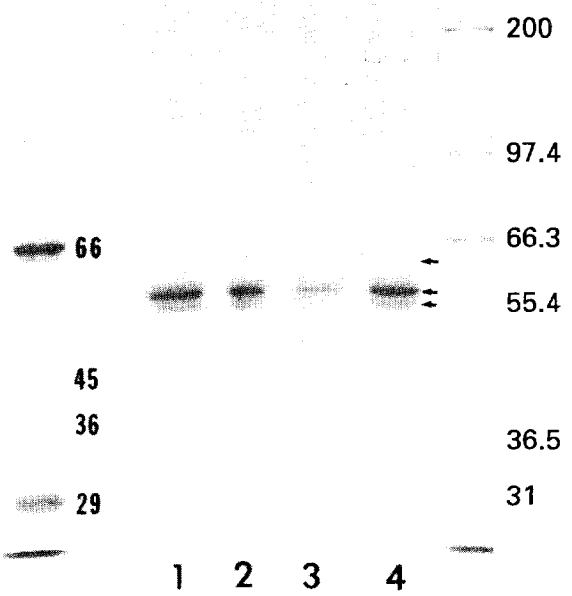

Fig. 3. Glycopeptidase A treatment of broad bean PPO. Broad bean PPO (5 μg) was either denatured (boiled, lanes 1, 2) or not denatured (not boiled, lanes 3,4) and incubated with glycopeptidase A overnight in the presence (lanes 2, 3, 4) or absence (lane 1) of enzyme. Lane 3 also contained 0.1 M sodium perchlorate. Molecular weight markers are in the outer two lanes and lanes were stained for protein. Arrows indicate major and minor bands observed after protein staining.

2 and 3) but we do not know if this was derived from PPO or glycopeptidase A.

Even though many proteins can be deglycosylated by endoglycosidases, perhaps the carbohydrate associated with PPO was not recognized by the variety of endoglycosidases chosen above. A chemical deglycosylation approach, using trifluoromethanesulfonic acid (TFMS) under anhydrous conditions, was used to try and remove O- and N-linked carbohydrates non-discriminately from PPO. Because of the limited amount of PPO available and possible degradation of protein during this chemical treatment, the enzyme was subjected to Western blotting after treatment. As shown in Fig. 4, no apparent change in the size of PPO was noted after chemical deglycosylation. This would suggest that either the amount of carbohydrate removed was so small that it had little effect on the size estimation of PPO by SDS–PAGE or the sugar linkage to PPO was something other than an acid labile O- or N-linkage.

Broad bean PPO was also treated with other glycosidases. These glycosidases included a beta glycosidase from *Hevea*, a mixed glycosidase from *Charonia lampas*, an α-fucosidase from bovine epididymus, an α-galactosidase from coffee beans, a β-galactosidase from Jack bean meal, a β-*N*-acetylhexosaminidase from Jack bean meal, an α-mannosidase from Jack bean meal, and a β-mannosidase from *Helix pomatia*. None of these enzyme treatments appeared to affect the apparent size of non-denatured or denatured PPO after SDS–PAGE (data not shown).

Because we observed some difficulty with interpreting *O*-glycanase treatment of PPO, such as loss of material and generation of lower M_r bands, affinity chromatography of the enzyme was carried out using Jacalin agarose. Jacalin agarose has been reported to bind to D-galactose in O-linked polypeptides [18]. Non-denatured broad bean PPO showed no binding to Jacalin agarose (Fig. 5) when assayed for enzyme activity. SDS–PAGE and Western blotting showed that if any PPO was bound to the column, it could not be eluted with typical reagents to remove bound O-linked proteins (i.e. 100 mM melibiose. Fig. 5 inset). Except for fraction three, enzyme activity appeared to correlate with the location of PPO by immunostaining.

DISCUSSION

Carbohydrate attachment to proteins usually occurs at Ser/Thr residues in O-linked glycoproteins and at Asn residues in N-linked glycoproteins. In N-linked oligosaccharides the polypeptide backbone is linked to carbohydrate through *N*-acetylglucosamine–Asn bonds. N-Linked carbohydrates in plant proteins are composed of

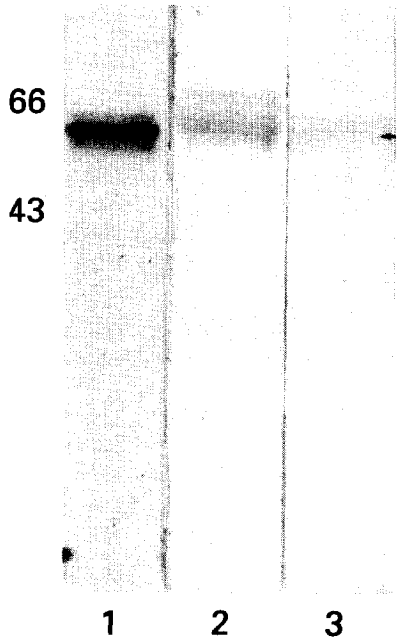

Fig. 4. Chemical deglycosylation of broad bean PPO. Broad bean PPO (5 μg) was subjected to chemical deglycosylation in the presence or absence of TFMS. The samples were lyophilized and subjected to SDS–PAGE followed by staining for protein or followed by Western blotting for the localization of PPO. Lane 1 contained 5 μg of PPO not treated with TFMS and stained for protein. Lane 2 contained 5 μg of PPO not treated with TFMS and subjected to Western blotting. Lane 3 contained 5 μg of PPO treated with TFMS and subjected to Western blotting. Arrow indicates faint immuno-cross-reacting material.

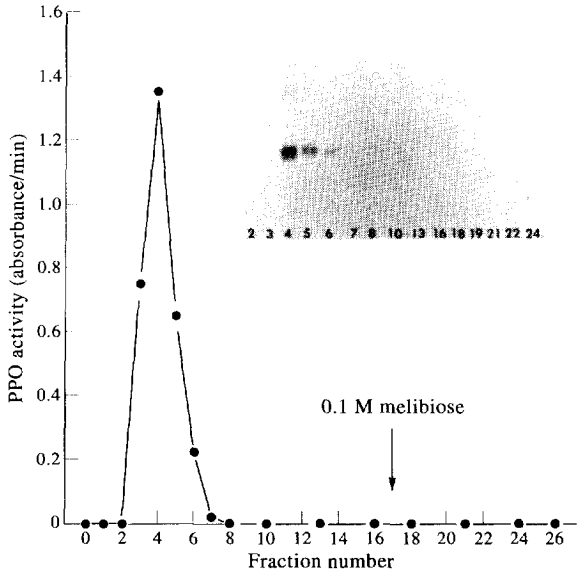

Fig. 5. Chromatography of broad bean PPO on Jacalin agarose. Broad bean PPO (5–10 μg) was passed over a Jacalin agarose column (1 ml) equilibrated in 175 mM Tris (7.5). The column was washed extensively with buffer before eluting with buffer containing 0.1 M melibiose. The insert shows fractions from the column that were subjected to SDS–PAGE followed by Western blotting.

a high mannose and a complex glycan core structure similar to other eukaryotic glycoproteins [19–21]. Because of this similarity to the core structures of mammalian glycoproteins, plant glycoproteins should be amenable to characterization using endo- and exoglycosidases. This method is especially useful if small amounts of protein are available for characterization.

If broad bean PPO contains carbohydrate core structures similar to the high mannose or complex glycans, then it should be susceptible to enzymatic and chemical hydrolysis to release those carbohydrates. None of the endoglycosidases, which are commonly used to remove N-linked sugars in other eukaryotes, were effective in changing the apparent size of PPO after SDS–PAGE. Treatment of PPO with a variety of exoglycosidases and glycopeptidase A also showed no apparent change in the size of PPO after SDS–PAGE. Neither of the endo- or exoglycosidases resulted in a noticeable change in the PPO forms when analysed for protein composition by protein staining, immunolocalization by Western blotting, glucose by Con A binding or general carbohydrate detection. These results would suggest that the carbohydrate(s) associated with PPO are not N-linked and agree with the apparent lack of a Asn-x-Ser/Thr consensus sequence for N-glycosylation sites in cloned broad bean PPO [22]. In contrast, mammalian tyrosinase, the plant phenol oxidase homologue, is localized in the melanosomes and is glycosylated with at least five N-linked sites [23]. The glycosylation of mammalian tyrosinase has also been reported to be important for stabilization and maintenance of tyrosinase activity [24].

Carbohydrates can also be attached through O-glycosidic linkages to the polypeptide backbone. Broad bean PPO showed no apparent change in M_r after *O*-glycanase treatment, a treatment which has been used successfully for mammalian O-linked glycoproteins. This suggests that PPO has no O-glycosidic attachment. Likewise, chemical deglycosylation, which should release O-linked sugars, showed no apparent changes in the M_r of PPO. We did, however, observe partial degradation of PPO treated with a variety of O-glycanases and after chemical treatment. This was presumably the result of proteolysis or the harsh acid treatment. In addition, PPO showed no binding to Jacalin agarose, a resin known to bind O-glycosidic linked glycoproteins.

These studies suggest that either (1) broad bean PPO does not contain O- or N-linked carbohydrate, (2) the amount of carbohydrate attached is small and release of the sugars does not result in a substantial and detectable change in the size of PPO, (3) the linkage of carbohydrate to protein is resistant to these glycosidases, or (4) that the carbohydrates are not linked to PPO through an Asn or Ser/Thr residue as found in other plant glycoproteins. How then can the enzyme be associated with carbohydrate in what appears to be a covalent manner? Specula-

tively, broad bean PPO could contain endo- and exo-glycosidase-resistant and chemical-resistant carbohydrate linkages to the polypeptide backbone. The enzyme could also be modified during the isolation procedure by covalent attachment of glycosides to PPO through an undefined linkage. Oxidation of phenolic glycosides and attachment to PPO through the phenol group might also occur. Either of these could account for the small amount of carbohydrate detected using Con A binding, lectin binding and general glycan staining. Although the manufacturers' indicated there is little chance of detecting false positives with the immuno-based glycan general detection kits, we have not ruled out this possibility with regard to broad bean PPO.

EXPERIMENTAL

Materials. *O*-Glycanase, endoglycanase H, endoglycanase F, *N*-glycanase, *N*-acetyl-D-hexosaminidase, Lectin Link kits, and UniLink-N kits (for removal of N-linked carbohydrates) were obtained from Genzyme Corporation (Boston, MA). *N*-Glycosidase F, endoglycosidase F/*N*-glycosidase F-free, a mixture of endoglycosidase F/*N*-glycosidase F, endoglycosidase D, endoglycosidase H, *O*-glycosidase, Glycan Differentiation kits, and Glycan Detection kits were purchased from Boehringer Mannheim Biochemical (Indianapolis, IN). Endoglycosidase D, glycopeptidase A, and a mixed glycosidase preparation were obtained from Seikagaku Kogyo C. Ltd (Tokyo, Japan). GlycoTrack Carbohydrate Detection kits and GlycoFree Chemical Deglycosylation kits were procured from Oxford GlycoSystems Inc. (Rosedale, NY). Jacalin agarose was acquired from Vector Laboratories (Burlingame, CA). A non-specific β-glycosidase from *Helvea brasiliensis* was a gift from Bole Biehl, Botanisches Institut, Technisches Universitat, Braunschweig, Germany.

One unit of *O*-glycanase was equivalent to 1 μmol of Gal-GalNAc released from asialofetuin per min at 37°. One unit of endoF/*N*-glycanase free was equivalent to the hydrolysis (1 μmol) of dansyl-Asn$(GlcNAc)_2(Man)_5$ per 60 min at 37°. One unit of endo F/*N*-glycanase F was equal to the hydrolysis of 1 μmol of dansyl-Asn$(GlcNAc)_2(Man)_5$ per 60 min at 37°. One unit of *N*-glycanase F was equal to the hydrolysis of 1 nmol of dansyl fetuin glycopeptide within at 37°. One unit of endoglycosidase D hydrolysed 1 μmol of dansyl-Asn-$(GlcNAc)_2(Man)_5$ per min at 37° and one unit of endo H released 1 μmol of [^{3}H]dansyl-AsnGlcNAc from [^{3}H]dansyl-Asn-$(GlcNac)_4(MAN)_6$ per min at 37°.

Samples. Two prepns of broad bean PPO were examined. One prepn contained PPO with two different M_r classes of PPO, two forms in the range of 58–65 kD and two forms in the range of 40–45 kD [16]. The 40–45 kD forms in this prepn were most likely the result of C-terminal proteolytic cleavage [7, 16]. The other prepn contained two bands in the 58–65 kD range [8]. Both prepns showed positive identification of carbohydrate associated with PPO using any of the commercial carbohydrate detection and differentiation kits following the manufacturers' instructions and gave similar results in the studies reported here. Only the latter prepn [8], which contained less degradative products, was reported here. Approximately 1–5 μg of PPO was incubated with each glycosidase overnight (*ca* 18 hr) at 37°.

Enzymatic deglycosylation. Native and denatured (boiled) PPO were treated with various glycosidases. Denatured samples were made by boiling the enzyme, adding SDS to a final concentration of 1%, boiling again and adding a non-ionic detergent (i.e. octyl glucoside) in a 10-fold excess over the SDS concentration. The final concentration of SDS in the enzyme treatments was less than 0.1% as suggested by the manufacturer. Using enzymes from Boehringer Mannheim, *O*-glycanase, endoglycosidase D, and endoglycosidase H were incubated with PPO in 20 mM phosphate (pH 7.0). Endoglycosidase F/*N*-glycanase F and *N*-glycanase F were incubated in 20 mM phosphate (pH 7.0) containing 1 mM EDTA. Incubations with *N*-glycosidase F were carried out in 20 mM sodium acetate (pH 5.0) containing 1 mM EDTA. Various modifications of the basic protocols were used without affecting the final results. These modifications included changing the buffer and incubation conditions, varying the type of non-ionic detergent, adding DTE in the reaction mixt., adding protease inhibitors [phenylmethylsulfonyl fluoride (PMSF), *o*-phenanthroline], varying the time of incubation and varying the amount of glycosidase added.

The following deglycosylation procedures were employed using glycosidases from Genzyme Corp. Deglycosylation with *N*-glycosidase F was carried out in 25 mM phosphate (pH 7.5) containing 1 mM dithioerythreitol (DTE), 1.3% Nonidet P-40, 0.17% sodium dodecyl sulfate (SDS), 1 mM PMSF, 1 mM *o*-phenanthroline, and 0.15 U of enzyme. Treatment with endoglycosidase F was carried out in 10 mM NaOAc (pH 6.0) containing 3 mM DTE, 1.3% NP-40, 0.17% SDS, 1 mM PMSF, 1 mM *o*-phenanthroline, and 0.17 mU of enzyme. *O*-Glycanase reactions were carried out in 10 mM phosphate (pH 6.0) or in 25 mM Tris–maleate (pH 7.5) containing 1 mM $Ca(OAc)_2$, 0.1% SDS, 0.75% NP-40, 1 mM PMSF, 1 mM *o*-phenanthroline and 2 mU of enzyme. PPO treated with endoglycosidase H was carried out in 10 mM citrate (pH 6.0) or in 20 mM potassium acetate (pH 5.5) containing 0.07% SDS, 1 mM PMSF and 0.2 mU of enzyme. *N*-Acetyl-α-D-hexosaminidase was incubated with PPO in 30 mM phosphate (pH 6.0) containing 0.3 U of enzyme.

PPO was treated with endoglycosidase D (Seikagaku) for 18 hr at 37° in 50 mM phosphate (pH 7.5) containing 4 mM PMSF. Treatment of PPO with glycopeptidase A (0.2 mU) was carried out in 50 mM citrate–phosphate (pH 5.0) in the presence and absence of 0.1 M Na perchlorate for 18 hr at 37°.

All glycosidases were checked periodically for loss of activity using fetuin, transferrin and ovalbumin as standard glycosylated proteins. Loss of functionality in these glycosidases was determined by observing mobility changes in the glycosylated protein standards by SDS–PAGE before and after glycosidase treatment.

Chemical deglycosylation. Denatured or native PPO (5–10 μg) was treated with TFMS under anhydrous conditions according to the manufacturer's instructions (Oxford GlycoSystems). Samples were then subjected to SDS–PAGE and Western blotting.

Jacalin chromatography. Approximately 1 ml of Jacalin agarose was equilibrated with 0.175 M Tris (7.5) containing 0.5 M NaCl. PPO (5–10 μg) was resuspended in this buffer and applied to the column. The column was washed with the equilibrating buffer to remove non-bound material. Bound material was eluted from the column in equilibration buffer containing either 0.1 M melibiose, 0.8 M galactose or 20 mM α-methylgalactopyranoside.

Electrophoresis. After incubation with the glycosidases the samples were either boiled or precipitated with trichloroacetic acid and then subjected to denaturing SDS–PAGE under reducing conditions [8, 18]. The gels were either stained for protein using Coomassie Blue R250, transferred to nitrocellulose and subjected to Western blotting using polyclonal rabbit anti-PPO [8] or transferred to nitrocellulose and subjected to carbohydrate/lectin staining using one of several commercial glycan detection kits.

Acknowledgements—The work was supported in part by a DAD grant to Gisela Raffert and by the University Research Committee at Indiana State University to William Flurkey.

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