

The position of the oligosaccharide side-chains of phytohemagglutinin and their accessibility to glycosidases determines their subsequent processing in the Golgi

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Phytohemagglutinin (PHA), the glycoprotein lectin of *Phaseolus vulgaris* has two types of asparagine-linked oligosaccharides per polypeptide: a high-mannose chain with the formula $(\text{Man})_8-9(\text{GlcNAc})_2$ on Asn¹² and a modified chain with fewer mannose residues and additional fucose and xylose residues on Asn⁶⁰. Glycosylation of PHA is a cotranslational process, which occurs in the endoplasmic reticulum, and newly synthesized PHA has two high-mannose chains. Transport of PHA to the protein bodies via the Golgi complex is accompanied by the modification of one of the two high-mannose chains. Why is only one chain modified, while the other remains in the high-mannose configuration? By determining the effect of digestion with various glycosidases (α -mannosidase, endo- β -N-acetylglucosaminidase H and endo- β -N-acetylglucosaminidase F) on native and denatured PHA we obtained evidence consistent with the interpretation that the accessibility of oligosaccharide chains to modifying enzymes is of major importance in determining whether a high-mannose chain becomes modified or not. The high-mannose chain of mature undenatured PHA is only partially accessible to glycosidases, while PHA obtained from the endoplasmic reticulum has one high-mannose chain, which is readily accessible to α -mannosidase and endoglycosidases H and F. We show that this readily accessible chain is in the same position on the polypeptide (Asn⁶⁰) as the modified oligosaccharide on mature PHA. Thus, accessibility of the oligosaccharide side-chains to processing enzymes in the Golgi determines whether a particular oligosaccharide side-chain is processed or not.

Glycoproteins contain two types of asparagine-linked oligosaccharide side-chains termed high-mannose chains and modified chains. The high-mannose chains have the composition $(\text{Man})_5-9(\text{GlcNAc})_2$ and bind strongly to concanavalin A (ConA). The modified chains generally have fewer mannose residues and contain other glycosyl residues. Modified chains are derived from high-mannose chains by modifications which occur primarily in the Golgi apparatus, after the proteins have assumed a globular configuration in the endoplasmic reticulum (ER). Modifying enzymes include two different α -mannosidases and various glycosyltransferases, which are localized in the Golgi complex. The sequence of processing events, which occur in the Golgi complex, has been in large part elucidated for animal and viral glycoproteins and is described in several recent reviews [1–3]. Only a few plant glycoproteins carrying modified N-linked chains have been described so far [4–9]. The modified chains

of these plant glycoproteins are similar in carbohydrate composition, having been trimmed to 3–4 mannose residues and having acquired fucose and/or xylose residues. These oligosaccharide chains are different from those commonly found in animal and viral glycoproteins, which are usually more complex as well as heterogeneous.

Phytohemagglutinin (PHA), the glycoprotein lectin of the common bean *Phaseolus vulgaris*, is a tetramer formed by different combination of PHA-E and PHA-L subunits [10] with M_r 34000 and 32000 respectively. Sequence analysis of a genomic PHA-E and PHA-L gene shows that the derived amino acid sequence contains three and two glycosylation sites respectively [11]. PHA is particularly useful for the study of oligosaccharide side-chain modification in plants because each polypeptide has one high-mannose chain $(\text{Man})_8-9(\text{GlcNAc})_2$ and one modified chain in which N-acetylglucosamine, mannose, fucose and xylose occur in the molar ratios 2:3.8:0.6:0.5 [7]. The modified chain is formed by processing of one of the two original high-mannose chains present on newly synthesized precursor of PHA associated with the ER [12]. This processing occurs for the most part in the Golgi complex and is completed after PHA reaches its site of accumulation, the protein bodies [13]. Recent results show that the high-mannose chain is attached to Asn¹² and the modified chain to Asn⁶⁰ of the PHA polypeptide [14]. The existence of two different chains on one polypeptide allows one to ask questions about the regulatory mechanism, which determines why a high-mannose chain becomes modified or

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Abbreviations. ER, endoplasmic reticulum; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecylsulfate/polyacrylamide gel electrophoresis; endo H, endo- β -N-acetylglucosaminidase H; endo F, endo- β -N-acetylglucosaminidase F; ConA, concanavalin A; HPLC, high-performance liquid chromatography.

Enzymes. Endo- β -N-acetylglucosaminidase F (EC 3.2.1.–); endo- β -N-acetylglucosaminidase H (EC 3.2.1.96); α -mannosidase (EC 3.2.1.24); pronase (EC 3.4.24.4).

stays in the high-mannose form. Experiments with viral glycoproteins [15] and with yeast glycoproteins [16] indicate that the physical accessibility of the oligosaccharide chains to processing enzymes may be the primary factor in determining whether a side-chain is modified or not. To gain further insight into the regulation of processing in plant cells we treated PHA, which has mature oligosaccharides, and newly synthesized PHA obtained from the ER with a variety of glycosidases (α -mannosidase, endo-*N*-acetylglucosaminidases H and F) and determined which of the two side-chains is accessible to these enzymes when PHA is in a native or a denatured configuration. The results obtained are consistent with the interpretation that side-chains which will be modified *in vivo* are readily accessible *in vitro* to glycosidases down to the chitobiose core, while side-chains which remain unmodified are not susceptible to removal by endo H or endo F, and are only partially susceptible to α -mannosidase.

MATERIALS AND METHODS

Materials

Plants of *Phaseolus vulgaris* L. cv. Greensleeves were grown in a greenhouse. Organic chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated. D-[6-³H]Glucosamine hydrochloride (22 Ci/mmol) was purchased from Amersham Co. (Arlington Heights, IL, USA); L-[6-³H]fucose (30 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA, USA); L-[3,4,5-³H]leucine (142 Ci/mmol) was obtained from NEN (Boston, MA, USA).

Tissue labeling and cell fractionation

Cotyledons from developing seeds were incubated with [³H]glucosamine, [³H]fucose or [³H]leucine (10 μ Ci/cotyledon), as described by Spencer et al. [17], for 2–3 h or 24 h at 20°C. After the labeling period, a 1-mm slice from the radiolabeled surface of each cotyledon was cut with a razor blade, and such radioactive slices from typically 4–8 cotyledons were combined and ground in a small mortar with 3 ml 100 mM Tris/HCl, pH 7.8, containing 1 mM EDTA and 12% (w/w) sucrose. This homogenization procedure disrupts the cell's protein bodies, whose stored proteins (mostly phaseolin and PHA) thus become part of the soluble fraction of the homogenate. The homogenate was centrifuged for 5 min at 1000 $\times g$ to remove cell walls, and the supernatant was layered onto a discontinuous sucrose gradient consisting of 1 ml 35% (w/w) sucrose under 8 ml 16% (w/w) sucrose in the same Tris/EDTA buffer noted above. After centrifugation for 90 min at 150000 $\times g$, the sample layer (3 ml) plus the uppermost 2 ml of the 16% sucrose layer were collected as the soluble protein fraction. The organelle fraction was manifest as an opaque band at the interface of the 16% and 35% sucrose layers; it was collected and adjusted to 1% Triton X 100, then incubated for 1 h at 5°C to ensure solubilization of the membranes. This organelle fraction contains a mixture of membranes and subcellular organelles, but most of the PHA is in the ER [18].

Isolation of rough ER

A crude organelle fraction was obtained by passing the homogenate (in 100 mM Tris/HCl pH 7.8 containing 12% sucrose and 2 mM MgCl₂) over a Sepharose 4B column, as

described [19]. The organelle fraction (in the void volume of the column) was then layered on a continuous 16–54% (w/w) sucrose gradient, which was centrifuged for 2 h at 150000 $\times g$. The zone containing the rough ER, identified by the presence of NADH:cytochrome *c* reductase, was collected, and used for the purification of PHA [18].

Isolation of PHA and fractionation of PHA glycopeptides

The affinity procedure of Felsted et al. [20] was used to isolate PHA from the organelle or soluble protein fractions. Affinity-purified PHA, obtained by this procedure, was either incubated with glycosidases or digested with 1 mg pronase for 48 h at 37°C in 500 μ l 100 mM Tris/HCl, pH 8.5. The digest was boiled for 2 min and chromatographed on a 1-ml column of ConA-agarose to separate the glycopeptides with high-mannose chains from the glycopeptides with modified oligosaccharide chains. The latter do not bind to ConA. The retained glycopeptides were eluted with buffer (20 mM Tris/HCl, pH 7.4, containing 500 mM NaCl and 0.1% Tween 20) containing 500 mM methyl α -mannoside as described [21]. Both glycopeptide fractions were desalted on a short (1.5 $\times$ 18 cm) Bio-Gel P4 (minus 400 mesh, Bio-Rad, Richmond, CA, USA) column equilibrated with 100 mM acetic acid. The glycopeptide fractions were lyophilized and analyzed by gel filtration on a long (1.0 $\times$ 100 cm) Bio-Gel P4 column as described [13]. The positions of glycopeptides with oligosaccharide chains of known size are indicated on the elution profiles.

Digestion of PHA and PHA glycopeptides with glycosidases

In some experiments labeled or unlabeled PHA either in the native conformation or after denaturation (2 min at 100°C) obtained from organelle or soluble fractions was digested with glycosidases before sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis or glycopeptide preparation. For digestion with α -mannosidase (Sigma) PHA or PHA glycopeptides were diluted in 0.5 ml 50 mM sodium acetate, pH 5.8, containing 5 mM ZnSO₄, and incubated for 48 h at 37°C with 10 U jack-bean α -mannosidase under a toluene atmosphere.

Endo- β -*N*-acetylglucosaminidase H (endo H) (from *Streptomyces griseus*; Miles Laboratories, Elkhart, IN, USA) digestion of native or denatured PHA was performed by incubation at 37°C for 24 h in 500 μ l 100 mM sodium acetate, pH 5.8, with 10 mU enzyme. Endo- β -*N*-acetylglucosaminidase F (endo F) from *Flavobacterium meningosepticum* [22] (a gift from J. H. Elder, Scripps Clinic and Research Foundation, La Jolla, CA, USA), also described as a mixture of peptide *N*-glycosidase F and endo F [23], was used for PHA digestion. In our hands, assayed at pH 4.5 or at pH 9.4, this glycosidase preparation exclusively cleaved high-mannose and biantennary complex oligosaccharides from ovalbumin, α -mannosidase and human transferrin at pH 9.4 consistent with peptide *N*-glycosidase F activity described [23]. Consequently PHA, either native or denatured, was incubated at 37°C for 24 h in 500 μ l 100 mM Tris/HCl, pH 8.4 (pH optimum for peptide *N*-glycosidase F [23]) and 200 mU of this glycosidase preparation. Controls for glycosidase digestion were incubated in the same conditions but without enzymes. Each incubation was performed under a toluene atmosphere. After digestion with α -mannosidase, PHA glycopeptides were analysed by gel filtration on a long Bio-Gel P4 column as described. PHA, digested with

glycosidases, was either used for glycopeptides preparation and analysis as described before or analyzed by SDS-PAGE. For SDS-PAGE analysis of PHA digested with glycosidases, the protein was precipitated with cold trichloroacetic acid (12.5% final concentration), washed with 90% aqueous acetone at 0°C, and resolubilized overnight at 37°C in a small volume of SDS-PAGE sample buffer (20 mM Tris/HCl, pH 8.6, with 1% SDS, 0.24% 2-mercaptoethanol and 8% glycerol).

Polyacrylamide gel electrophoresis, fluorography, immunoblotting and affino blotting

Separation of polypeptides by SDS-PAGE was performed on 15% polyacrylamide gels as described [24]. Fluorographs were prepared according to Bonner and Laskey [25]. Affino blotting with the ConA/peroxidase procedure has been described [26]. PHA immunodetection after electrophoretic transfer from polyacrylamide gels to Trans-Blot nitrocellulose paper (Bio-Rad) was performed according to a procedure described in the Bio-Rad information booklet using an immune serum specific for PHA [27].

Trypsin digestion

Trypsin digestion was done as described by Hsieh et al. [15]. 1.5 mg radiolabeled PHA were dissolved in 1 ml 50 mM ammonium carbonate, pH 8.0 and heated at 100°C for 3 min. PHA was then digested with two aliquots of 150 µg each of trypsin (TosPheCH₂Cl-treated). After addition of each aliquot of enzyme, samples were incubated at 37°C for 2 h. Prior to HPLC analysis, lyophilized tryptic peptides were dissolved in 0.5 ml 0.1 M sodium phosphate, pH 2.2, centrifuged to remove particulate matter and filtered through a 0.2-µm nylon filter.

High-performance liquid chromatography (HPLC)

Separation of the tryptic peptides was carried out by reverse-phase HPLC on a Beckman 342 gradient liquid chromatograph equipped with a Vydac C-18 column (5 µm, 4.6 × 250 mm). The peptide separation was carried out at room temperature at a flow rate of 1 ml/min. The gradient is described in detail in the legends of the figure. Fractions of 1 ml were collected. Radioactivity in the fractions was measured by liquid scintillation counting of a 25-µl aliquot.

RESULTS

Effect of glycosidases on the M_r of PHA

When PHA is digested with pronase, the resulting glycopeptides contain two types of oligosaccharide chain: a high-mannose chain, which is sensitive to endo H, and a modified, fucose-containing chain, which is resistant to endo H digestion [7]. Chromatography on ConA-agarose of [³H]fucose-labeled glycopeptides showed that 95–98% of the radioactivity was not retained by the affinity gel; a similar experiment with [³H]glucosamine-labeled glycopeptides showed that 53–55% of the radioactivity was not retained. These results indicate that the oligosaccharide side-chains of PHA can be distinguished on the basis of their ability to bind to ConA, as well as by their susceptibility to endo H. The modified side-chain does not bind to ConA and

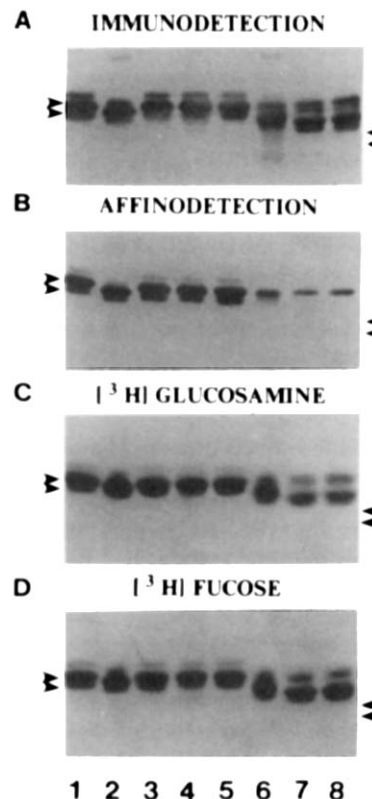


Fig. 1. (A–D) Susceptibility of the oligosaccharide side-chains of mature native and denatured PHA to glycosidases. Native and denatured PHA were treated with α -mannosidase, endo H or endo F, and the polypeptide analyzed by SDS-PAGE and visualization by immunodetection (A), affino detection with ConA/peroxidase (B) or fluorography (C and D). PHA, unlabeled or labeled with [³H]glucosamine or [³H]fucose, was kept in the native configuration or denatured by boiling and then treated for 48 h with one of the three glycosidases. Arrowheads on the left point to the E (largest) and L (smallest) subunits of PHA. Arrowheads on the right point to the positions of unglycosylated PHA subunits (see also Fig. 3). Lanes 1–4, native PHA; lanes 5–8, denatured PHA; lanes 1 and 5, control; lanes 2 and 6, α -mannosidase treated; lanes 3 and 7, endo H treated; lanes 4 and 8, endo F treated

is resistant to endo H, while the high-mannose chain binds to ConA and is susceptible to endo H.

To determine the effect of glycosidases on the oligosaccharides of intact PHA, the lectin was incubated with each of the glycosidases separately and the resulting PHA polypeptides were visualized by immunodetection with antibodies to PHA (Fig. 1A), by affino detection with ConA/peroxidase (Fig. 1B) or by fluorography in the case of [³H]glucosamine-labeled PHA (Fig. 1C) or [³H]fucose-labeled PHA (Fig. 1D). Removal or partial degradation of one or both of the oligosaccharide side-chains is seen as a decrease in the M_r of the glycopolypeptides on the fluorographs or the blots. All of the PHA-L and most of the PHA-E normally have two oligosaccharide chains, and the positions of the twice glycosylated PHA polypeptides are shown by two arrowheads on the left side of Fig. 1. The additional band above the main doublet probably represents PHA-E, which has three oligosaccharide side-chains, as PHA-E is known to have three glycosylation sites [11]. The position of unglycosylated polypeptides of PHA is shown by double arrowheads on the right side of Fig. 1; the electrophoretic mobility values are derived from experiments

in which glycosylation is inhibited by tunicamycin (see also Fig. 3 and [12]). Treatment of native PHA with α -mannosidase produced a small shift in M_r (Fig. 1A–D, lane 2), while a somewhat bigger shift was obtained if the PHA was denatured before enzyme treatment (Fig. 1A, C, D, lane 6). Treatment of denatured PHA with α -mannosidase inhibited the binding of ConA to PHA (Fig. 1B, lane 6), while there was no such effect when native PHA was treated with α -mannosidase (Fig. 1B, lane 2). Since the modified chain of PHA does not bind to ConA, the results indicate that α -mannosidase degrades the high-mannose chain when PHA is denatured, but fails to do so when the PHA is in the native configuration. The small shift in M_r (Fig. 1A, lane 2) does indicate that some mannose residues are lost from native PHA, but not enough to abolish ConA binding.

Next we tested the effects of endo H and endo F on native and denatured PHA. Treatment of native PHA with either endo H or endo F did not change the M_r of PHA (Fig. 1A–D, lanes 3 and 4). Endo H cleaves the chitobiose portion of the high-mannose oligosaccharide, but not of the modified side-chain of PHA. Endo F acts in the same manner as endo H on both high-mannose and modified oligosaccharides [22]. Treatment of denatured PHA with either endo H or endo F reduced the M_r of PHA to the same extent (Fig. 1A, lanes 7 and 8). The shift in position of the two major bands of PHA after endo H or endo F treatment indicates that, as expected, only one of the two side-chains has been removed from each polypeptide. Such treatments abolished the binding of ConA (Fig. 1B, lanes 7 and 8) and removed 25% of the radioactivity of [^{3} H]glucosamine-labeled PHA (Fig. 1C, lanes 7 and 8), but none of the radioactivity from [^{3} H]fucose-labeled PHA (Fig. 1D, lanes 7 and 8). The loss of 25% of the radioactivity in [^{3} H]glucosamine was determined on trichloroacetic-acid-precipitable material prior to loading the gels. These results are consistent with the conclusion that endo H and endo F both remove the high-mannose chain of PHA, and can only do so when the PHA has been denatured. Thus, unlike the modified chains of many animal and viral glycoproteins [22] the modified chains of PHA are not sensitive to endo F. Neither does this enzyme appear to act as a peptide *N*-glycosidase F [23] hydrolyzing the asparagine–*N*-acetylglucosamine linkage, since no [^{3} H]fucose was lost from [^{3} H]fucose-labeled PHA, and only 25% of the *N*-[^{3} H]acetylglucosamine from [^{3} H]glucosamine-labeled PHA.

Degradation of oligosaccharide side-chains by α -mannosidase

The results shown in Fig. 1 indicate that the oligosaccharide side-chains of native PHA are somewhat accessible to α -mannosidase, but it is not clear whether the high-mannose chain, the modified chain or both are losing mannose residues. To answer this question we incubated [^{3} H]mannose and [^{3} H]fucose-labeled native PHA with α -mannosidase, then digested the PHA exhaustively with pronase, and sized the resulting glycopeptides on a Bio-Gel P4 column, after separation of the high-mannose and modified glycopeptides on ConA-agarose. Fig. 2A and B show the results in incubating [^{3} H]mannose-labeled native PHA with α -mannosidase. The modified chain which was not bound by ConA, (Fig. 2A) lost about 50% of its radioactivity and moved two fractions to the right indicating a loss of two mannose residues. The high-mannose chain, which was bound by ConA (Fig. 2B), lost 20% of its radioactivity and also moved two fractions to the right. This is consistent with the conversion of a (Man) $_8$ (GlcNAc) $_2$ to a (Man) $_6$ (GlcNAc) $_2$ chain. The extent

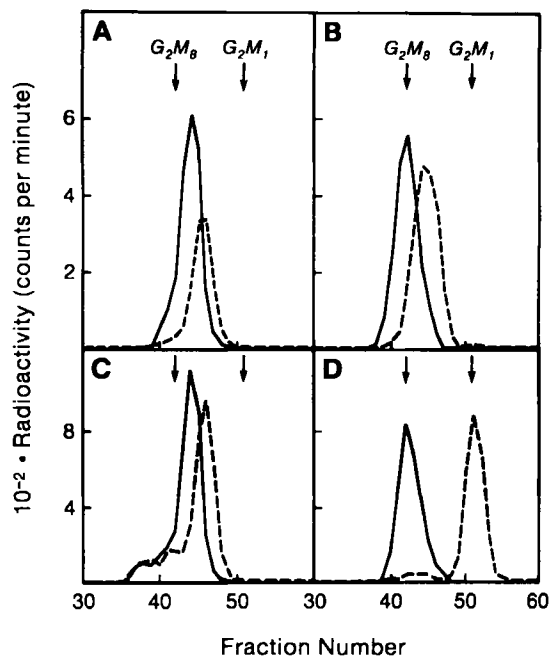


Fig. 2. (A–D) Susceptibility of oligosaccharide side-chains and of glycopeptides of mature PHA to degradation by α -mannosidase. Radioactive mature PHA (A and B) or glycopeptides derived from PHA (C and D) were treated with α -mannosidase; the size-distribution of the glycopeptides was analyzed by gel filtration on Bio-Gel P4. (A, B) [^{3} H]Mannose-labeled PHA was treated with α -mannosidase and then subjected to proteolysis with pronase. The glycopeptides were separated by ConA-agarose into a fraction which does not bind to ConA (A) and binds to ConA (B). (C) [^{3} H]Fucose-labeled PHA was digested with pronase, the glycopeptide purified and then digested with α -mannosidase. (D) [^{3} H]Glucosamine-labeled PHA was digested with pronase and the digest fractionated on ConA-agarose. The glycopeptide which bound to ConA was digested with α -mannosidase. Control (no α -mannosidase) (—); digestion with α -mannosidase (---). Standards are indicated by vertical arrows: G_2M_8 , Asn-(GlcNAc) $_2$ (Man) $_8$; G_2M_1 , Asn-(GlcNAc) $_2$ Man

of the degradation by α -mannosidase on the two types of chain when they are not attached to protein is shown in Fig. 2C and D. In this case labeled PHA was first incubated with pronase, the glycopeptides were separated by affinity chromatography on ConA, and then incubated with α -mannosidase. [^{3} H]Fucose was used to label the modified oligosaccharide chain, and its position shifts two fractions to the right after α -mannosidase digestion (Fig. 2C). [^{3} H]Glucosamine was used to label the high-mannose chain and its position shifts by nine fractions so that it now coelutes with (Man) $_{1-2}$ (GlcNAc) $_2$ indicating the removal of seven mannose residues by α -mannosidase. Together these results indicate that the modified chain can be degraded equally either in glycopeptide form, or attached to a native, globular protein. The high-mannose chain, on the other hand, is much less degraded when attached to the native globular protein than when it exists as a glycopeptide.

Effect of endo H on newly synthesized PHA from the endoplasmic reticulum

Precursors of mature PHA are present in the ER and Golgi complex [18, 28], and the ER contains newly synthesized PHA in which all the chains are in the high-mannose configuration. To obtain such precursors we isolated radioactively labeled

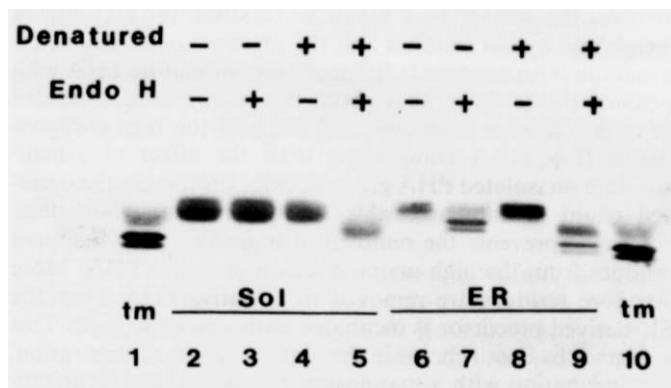


Fig. 3. Analysis of the accessibility of high-mannose chains of mature PHA and its ER-derived precursor to endo H. Fluorograph of [^3H]leucine-labeled PHA obtained from the soluble fraction (Sol) or membranous organelles (ER), and treated with (+) or without (-) endo H, with (+) or without (-) denaturation by heating. The position of unglycosylated polypeptides of PHA is shown in the tunicamycin (tm) samples (lanes 1 and 10)

PHA from the ER fraction of a homogenate of pulse-labeled cotyledons (2–3 h labeling). When the cotyledons are labeled with [^3H]leucine, [^3H]mannose or [^3H]glucosamine most of the PHA recovered from the total organelle fraction is from the ER because the ER is a large compartment relative to the Golgi. Mature PHA, which has been transported to the protein bodies, was obtained from the soluble fraction of a homogenate after removal of the organelles by centrifugation at $150000 \times g$ for 90 min. Most of the protein bodies break during homogenization and their contents are released as soluble protein. The accessibility of the oligosaccharide side-chains to glycosidases was compared for these two fractions. The results shown in Fig. 3 illustrate the effect of endo H on [^3H]leucine-labeled PHA obtained from the organelles and the soluble fraction. The PHA either remained in the native globular condition or was boiled to denature it prior to endo H treatment. The position of unglycosylated polypeptides synthesized in the presence of tunicamycin is shown in lanes 1 and 10. Undenatured mature PHA is not affected by endo H (lane 3) but denaturation followed by endo H digestion causes a decrease in size (lane 5) corresponding to the removal of one oligosaccharide chain. ER PHA is susceptible to endo H both in its native form and when denatured. The shift in mobility after treatment indicates that one chain is removed from native ER PHA (lane 7) while both chains can be removed after denaturation (lane 9). These results indicate that ER PHA has a high-mannose chain, which is accessible to endo H even when the PHA has not been denatured. Denaturing by boiling uncovers a second high-mannose chain, which is also present in mature PHA.

The oligosaccharide on ER-derived PHA, which is accessible in vitro is the same one which is normally modified in the Golgi

We recently determined that the high-mannose chain of PHA is attached to Asn¹² while the modified chain is attached to Asn⁶⁰ [14]. This information was obtained by determining the amino acid sequence of glycopeptides purified from a tryptic digest of [^3H]fucose and [^3H]glucosamine-labeled PHA. We wished to find out if the oligosaccharide which is accessible to glycosidases in ER-derived PHA is the same one

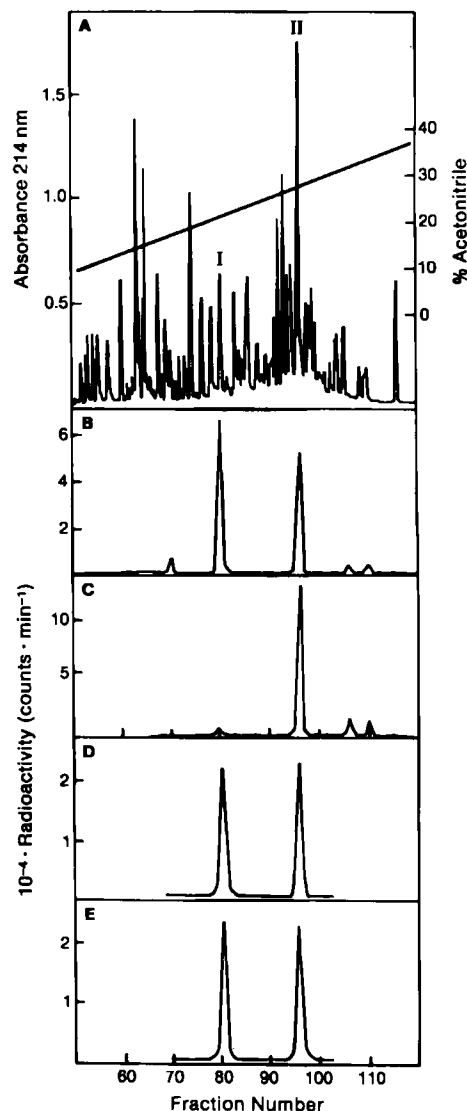


Fig. 4. Reverse-phase HPLC separation of tryptic peptides of radio-labeled PHA. Tryptic peptides of PHA were separated on a Vydac C-18 column. 100% solvent A (0.1 M sodium phosphate, pH 2.2) was run for 30 min followed by a linear gradient of 0–50% solvent B (acetonitrile) for 120 min. For clarity, the acetonitrile gradient is shown in (A) only. (A) Peptide profile of a peptide separation, obtained by following the absorbance at 214 nm; (B) radioactivity profile of a peptide separation, PHA labeled with [^3H]glucosamine for 24 h; (C) radioactivity profile of a peptide separation, PHA labeled with [^3H]fucose for 24 h; (D) radioactive profile of a peptide separation, PHA isolated from the rough ER of cotyledons labeled with [^3H]glucosamine for 3 h; (E) radioactive profile of a peptide separation, PHA isolated from the rough ER of cotyledons labeled with [^3H]glucosamine for 3 h and treated *in vitro* with α -mannosidase for 24 h at 37°C

which is normally modified in the Golgi. For this experiment we incubated [^3H]glucosamine-labeled PHA with α -mannosidase, then subjected it to tryptic digestion and separated the peptides and glycopeptides by HPLC. The controls for this experiment are shown in Fig. 4A–D. Fig. 4A shows the distribution of peptides along the acetonitrile gradient. When [^3H]glucosamine-labeled PHA from the protein bodies was used we obtained two radioactive peaks in fractions 80 and 96 (labeled I and II respectively), and when [^3H]fucose-labeled PHA from the protein bodies was used we obtained a single

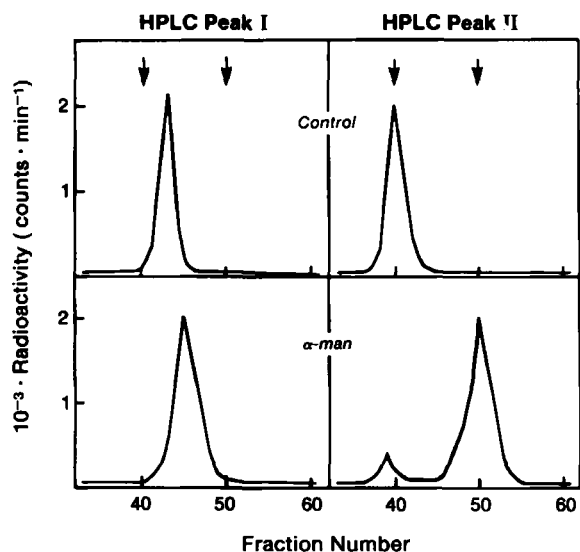


Fig. 5. Analysis of PHA oligosaccharides by gel filtration on a Bio-Gel P4 column. [^3H]Glucosamine-labeled PHA from the rough ER was treated *in vitro* for 24 h with α -mannosidase. An untreated control sample and the α -mannosidase-treated protein were digested with trypsin and the tryptic peptides separated on a C-18 HPLC column. The glycopeptides obtained (Fig. 4D and E, HPLC peaks I and II) were further digested with pronase. The oligosaccharides were then fractionated on a 100-cm-long Bio-Gel P4 column

radioactive peak in fraction 96 (Fig. 4C). This indicates that the complex oligosaccharide is attached to a tryptic peptide, which elutes in fraction 96. When [^3H]glucosamine-labeled PHA from the ER was used we obtained the same two radioactive peaks in fractions 80 and 96 both for the control (Fig. 4D) and for the PHA, which had been incubated with α -mannosidase (Fig. 4E). This result shows that treatment of ER-derived PHA with α -mannosidase did not alter the elution position of the tryptic glycopeptides derived from the PHA.

The radioactive peaks shown in Fig. 4D and E were collected, subjected to pronase degradation and the size of the oligosaccharide side-chains determined on Bio-Gel P4 (Fig. 5). The results show that ER-derived PHA has high-mannose oligosaccharide chains corresponding to $(\text{Man})_9(\text{GlcNAc})_2$ and $(\text{Man})_8(\text{GlcNAc})_2$. After incubation of native PHA with α -mannosidase the oligosaccharides are smaller and correspond to $(\text{Man})_{1-2}(\text{GlcNAc})_2$ and $(\text{Man})_6(\text{GlcNAc})_2$. Most important is the observation that the oligosaccharide chain which was attached to the tryptic peptide in fraction 80 lost only 1–2 mannose residues, while the oligosaccharide chain which was attached to the tryptic peptide in fraction 96 lost 7–8 mannose residues. Thus, it is the oligosaccharide chain which normally becomes fucosylated *in situ* which is also most accessible to degradation when ER PHA is incubated with α -mannosidase *in vitro*.

DISCUSSION

Phytohemagglutinin is unique among the plant glycoproteins that have been studied in that it has both a high-mannose chain, attached to Asn¹², and a complex chain, attached to Asn⁶⁰, on each polypeptide [7, 14]. Newly synthesized PHA in the ER has two high-mannose chains and one of these is modified in the Golgi complex as the protein is transported to the protein bodies [13, 28]. This modification

involves the loss of 5–6 mannose residues, the addition of fucose and xylose residues and the presence of transient terminal GlcNAc residues [13]. Incubation of mature PHA with α -mannosidase shows that about two mannose residues can be removed from both the modified and the high-mannose chains (Fig. 2). A comparison with the effect of α -mannosidase on isolated PHA glycopeptides shows that the modified chain is fully accessible to the enzyme, while steric hindrance prevents the removal of more than two mannose residues from the high mannose chain of native PHA. More mannose residues are removed from native PHA when the ER-derived precursor is incubated with α -mannosidase. This precursor has both chains in the high-mannose configuration, and incubation with α -mannosidase results in the formation of a $(\text{Man})_6(\text{GlcNAc})_2$ and a $(\text{Man})_{1-2}(\text{GlcNAc})_2$ chain (Fig. 5). The appearance of the $(\text{Man})_{1-2}(\text{GlcNAc})_2$ chain results from the presence on the PHA precursor of a highly accessible high-mannose chain. That this is the same chain which is normally modified *in vivo* is shown by the HPLC separation of the tryptic digests of radioactive PHA (Fig. 4). The peptide which carries the modified chain (eluting at fraction 96) in mature PHA, also carries the high-mannose chain, which is readily accessible to α -mannosidase when ER-derived PHA is incubated with the enzyme (Fig. 5).

Denaturation of mature or organelle PHA exposes the high-mannose chain to degradation by α -mannosidase so that the α -mannosidase-digested protein is now smaller and cannot be visualized by affinity detection with ConA (Fig. 1B). The results obtained with α -mannosidase are confirmed by the susceptibility of the high-mannose chains to endo H. Native mature PHA is not susceptible to endo H, while native ER-derived precursor, incubated with this enzyme, can lose one of its chains. Denaturation of either mature PHA or precursor results in the loss of one additional high-mannose chain (Fig. 3). Native PHA precursor has one high-mannose chain which is accessible to enzymes all the way down to the chitobiose core, and another chain, also present on mature PHA, which cannot be degraded further than $(\text{Man})_6(\text{GlcNAc})_2$.

Thus, *in vivo* modification of the oligosaccharide chains does not affect the high-mannose chain, shown here to be almost completely protected from glycosidase digestion *in vitro*. Reciprocally the high-mannose oligosaccharide chain precursor of the modified chain of mature PHA is highly accessible to glycosidases *in vitro* without protein denaturation. In conclusion, there is a strong correlation between the oligosaccharide accessibility observed *in vitro* on the native glycoprotein and the extent of processing modifications occurring *in vivo*.

The finding that $(\text{Man})_5(\text{GlcNAc})_2$ is an obligatory intermediate in the modification of oligosaccharides in the Golgi apparatus of plant cells (Johnson and Chrispeels, unpublished) strengthens the conclusion that the high-mannose chains that cannot be trimmed *in vitro* further than $(\text{Man})_6(\text{GlcNAc})_2$ are not sufficiently exposed *in vivo* to the α -mannosidase which carries out the first processing step from $(\text{Man})_9(\text{GlcNAc})_2$ to $(\text{Man})_5(\text{GlcNAc})_2$. These findings confirm and extend results obtained with viral glycoproteins [15] and yeast glycoproteins [16] where the *in vitro* accessibility of oligosaccharide side-chains to endo H correlates with their *in vivo* fate in the Golgi complex. Accessible chains become modified, whereas inaccessible chains remain in the high-mannose form. The physical accessibility of chains to modifying enzymes appears to be the major determinant in whether they are modified or not.

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