Structure, Position, and Biosynthesis of the High Mannose and the Complex Oligosaccharide Side Chains of the Bean Storage Protein Phaseolin*

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Phaseolin, the major storage protein of the common bean (Phaseolus vulgaris), is a glycoprotein which is synthesized during seed development and accumulates in protein storage vacuoles or protein bodies. The protein has three different N-linked oligosaccharide side chains: Man₆(GlcNAc)₂, Man₇(GlcNAc)₂, and Xyl-Man₃(GlcNAc)₂ (where Xyl represents xylose). The structures of these glycans were determined by ¹H NMR spectroscopy. The Man₆(GlcNAc)₂ glycan has the typical structure found in plant and animal glycoproteins. The structures of the two other glycans are shown below.

Phaseolin was separated by electrophoresis on denaturing gels into four size classes of polypeptides. The two abundant ones have two oligosaccharides each, whereas the less abundant ones have only one oligosaccharide each. Polypeptides with two glycans have Man₇(GlcNAc)₂ attached to Asn25₂ and Man₆(GlcNAc)₂ attached to Asn3₄₁. Polypeptides with only one glycan have Xyl-Man₃(GlcNAc)₂ attached to Asn25₂. Both these asparagine residues are in canonical glycosylation sites; the numbering starts with the N-terminal methionine of the signal peptide of phaseolin. The presence of the 1Man₃(GlcNAc)₂ and of Xyl-Man₃(GlcNAc)₂ at the same asparagine residue (position 252) of different polypeptides seems to be controlled by the glycosylation status of Asn3₄₁. When Asp3₄₁ is unoccupied, the glycan at Asn25₂ is complex. When Asn3₄₁ is occupied, the glycan at Asn25₂ is only modified to the extent that 2 mannosyl residues are removed.

The processing of the glycans, after the removal of the glucose residues, involves enzymes in the Golgi apparatus as well as in the protein bodies. Formation of the Xyl-Man₃(GlcNAc)₂ glycan is a multistep process that involves the Golgi apparatus-mediated removal of 6 mannose residues and the addition of 2 N-acetylglucosamine residues and 1 xylose. The terminal N-acetylglucosamine residues are later removed in the protein bodies. The conversion of Man₆(GlcNAc)₂ to Man₇(GlcNAc)₂ is a late processing event which occurs in the protein bodies. Experiments in which [³H]glucosamine-labeled phaseolin obtained from the endoplasmic reticulum (i.e. precursor phaseolin) is incubated with jack bean α-mannosidase show that the high mannose glycan on Asn25₂, but not the one on Asn3₄₁, is susceptible to enzyme degradation. Incubation of [³H]

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The asparagine-linked oligosaccharides found on plant glycoproteins, like those of other eukaryotes, fall into two general categories: high mannose and complex oligosaccharides. The high mannose oligosaccharides generally have the formula \( \text{Man}_n(\text{GlcNAc})_m \), with a branching pattern of mannose residues similar to that of the high mannose oligosaccharides of animal and yeast cells (1, 2). The complex oligosaccharides have a \( \text{Man}_n(\text{GlcNAc})_m \) core to which one or more of the following sugars may be attached: xylose, fucose, N-acetylglucosamine, and/or galactose. The fucose is attached to the proximal N-acetylglucosamine of the chitobiose core, as in lima bean agglutinin (3) and laccase of \( \text{Acer pseudoplatanus} \) (4); whereas the xylose is attached to the \( \beta \)-linked mannose residue, as in stem bromelain (5), the protease inhibitor of \( \text{Caesalpinia pulcherrima} \) (6), and laccase (4). The structures of only a few N-linked glycans of plant glycoproteins have been determined. Because of our interest in the biosynthesis and transport of storage proteins and lectins in developing legume seeds (7), we have made a detailed study of the structure and biosynthesis of the oligosaccharide side chains of phaseolin, the major storage protein of the common bean. This protein accumulates during seed development in special protein storage vacuoles (protein bodies).

When phaseolin isolated from cotyledons of beans (\( \text{Phaseolus vulgaris} \) cv. Greensleeves) is subjected to SDS-PAGE, it can be separated into four polypeptides, more accurately called size classes (8). These four glycosylated polypeptide classes, referred to as A, B, C, and D, range in M, from 52,000 to 45,000. Each polypeptide band can be further resolved by two-dimensional electrophoresis into two or more polypeptides with different isoelectric points near pH 5 (9, 10).

Previous reports from our and other laboratories detailed some important events during the biosynthesis and processing of phaseolin (11). When cotyledon mRNA is translated in vitro, two phaseolin polypeptide size classes of \( M, 48,000 \) (a) and 45,000 (b) are made (12). In vitro, phaseolin is synthesized on polysomes bound to the rough ER (13). The polypeptides are cotranslationally glycosylated with either one or two oligosaccharide chains, converting the \( \alpha \)-polypeptides into polypeptides A and B, with A having two oligosaccharide chains and B having one (11). Analogously, the \( \beta \)-polypeptides become the glycosylated polypeptides C and D, with C having two oligosaccharide chains and D having one. Transport of these glycosylated polypeptides through the Golgi complex (14) and their ultimate deposition in the protein bodies (15) are accompanied by further processing steps.

The N-linked oligosaccharides of plant glycoproteins are synthesized initially as \( \text{Glc}_{3}\text{Man}_{0}(\text{GlcNAc})_3 \) groups (16, 17) which are cotranslationally transferred from dolichol pyrophosphate to nascent polypeptide chains. Processing of the oligosaccharides starts in the ER, continues in the Golgi apparatus, and involves numerous glycosidases and glycosyltransferases. There is a considerable amount of information concerning the processing of glycoproteins in animal cells (18, 19), but very little is known about these events in plant cells. The processing of N-linked glycans in plant cells also begins with the loss of 3 glucose residues, followed by the removal of up to 7 mannose residues and the possible addition of fucose, xylose, N-acetylglucosamine, and galactose residues. The result of these processing events is a variety of glycans that range in size up to Hex5,6-Man3,GlcNAc2 (where Hex represents hexose) when assayed on Bio-Gel P-4 columns (20). Developing cotyledons of leguminous seeds represent an excellent system to study the processing of N-linked glycans (7, 22).

Phaseolin is encoded by a small multigene family (23). Gene copy number analysis indicates that there are approximately seven phaseolin genes/haploid genome (24). The phaseolin gene family can be divided into two main gene types, \( \alpha \) and \( \beta \), which encode the two polypeptide size classes \( \alpha \) and \( \beta \) detectable after translation of mRNA in vitro. The nine longest of the published nucleotide sequences of phaseolin cDNA clones show 98% homology between the \( \alpha \) and \( \beta \) type genes (24). All amino acid sequences derived from these cDNA sequences have two canonical glycosylation sites. We refer to these sites as \( \text{Asn}^\beta \) and \( \text{Asn}^\alpha \), counting amino acid residues from the initiating methionine of the signal sequence. \( \text{Asn}^\beta \) is located in a sequence Gly-Asn-Leu-Thr-Glu in a hydrophilic protein domain, and \( \text{Asn}^\alpha \) is in a Val-Asn-Phe-Thr-Gly sequence in a hydrophobic protein domain.

In this paper, we report the structures of the three different phaseolin oligosaccharides as determined by \(^1H\) NMR spectroscopy. We show their distribution among the various phaseolin polypeptides and the specific glycosylation sites (asparagine residues). In addition, we provide evidence for specific processing steps in the Golgi complex and the protein bodies.

**MATERIALS AND METHODS AND RESULTS**

The structures of the three principal glycans present in Pronase digests of purified phaseolin were determined by \(^1H\) NMR and are shown in the Miniprint (Figs. 1-5). The principal polypeptides A and C each have \( \text{Man}_n(\text{GlcNAc})_m \) and \( \text{Man}_n(\text{GlcNAc})_m \) in equal proportions. Polypeptide D has mainly a small complex glycan: Xyl-Man-3(\text{GlcNAc})_m. We have no analytical data on polypeptide B because we were unable to purify sufficient amounts. However, indirect evidence indicates that its glycan resembles the one from polypeptide D. Both polypeptides B and D have a single glycan (11), the glycopeptides stain poorly with the Schiff stain for glycoproteins (42) (data not shown), and the glycan is endo-\( \beta \)-N-acetylglucosaminidase H-resistant (Fig. 2).

**Identification of the Glycosylation Site for the Three Different Phaseolin Oligosaccharides**—The amino acid sequences of...
the phaseolin polypeptides derived from the nucleotide sequences of nine different cDNAs show two possible glycosylation sites/polyepitope: Asn\(^{232}\) and Asn\(^{341}\) (16, 46). To find out which oligosaccharide is attached to which asparagine residue, affinity-purified phaseolin from cotyledons labeled for 24 h with \([^{3}H]\)glucosamine was digested with trypsin. The resulting mixture of peptides and glycopeptides was fractionated by HPLC on a C\(_{18}\) column (Fig. 6A). Only one major radioactive peak (fraction 81) was observed (Fig. 6B). An aliquot of the peak fraction was digested with Pronase, and gel filtration of the resulting glycopeptides resolved three peaks corresponding in size to Man\(_{6}(\text{GlcNAc})_{2}\)Asn, Man\(_{9}(\text{GlcNAc})_{2}(\text{Gly})\)Asn, and Xyl-Man\(_{3}(\text{GlcNAc})_{2}(\text{Gly})\)Asn (data not shown). Next, the tryptic glycopeptides from HPLC fraction 81 were fractionated by ConA chromatography. When the radioactive material which did not bind to ConA was rerun on the reverse-phase C\(_{18}\) column, a major peak at fraction 81 appeared again (Fig. 6C). Amino acid sequencing of the first five amino acids of this tryptic glycopeptide (peak I) revealed the sequence Gin-Asp-Asn-Thr-Ile (QDNTI), which is identical with the amino acid sequence of the predicted tryptic glycopeptide that includes Asn\(^{232}\) (Fig. 7). Sizing of the glycan on Bio-Gel P-4 after exhaustive Pronase digestion showed it to be the complex phaseolin glycan Ph\(_{1}\). When the fraction 81 glycopeptides that bound ConA were fractionated by HPLC on a C\(_{18}\) column, we observed two major radioactive peaks (Fig. 6D, peaks II and III). These glycopeptides were subjected to amino acid sequencing, and the oligosaccharides were analyzed by gel filtration after exhaustive digestion with Pronase. Analysis of peak II identified it as Man\(_{9}(\text{GlcNAc})_{2}\) attached to a peptide starting with the sequence Ala-Thr-Ser-Asn-Val (ATSNV). Similarly, peak III was identified as Man\(_{7}(\text{GlcNAc})_{2}\) attached to a peptide starting with the sequence Gin-Asp-Asn-Thr-Ile (QDNTI). Comparison of these amino acid sequences with the two predicted tryptic glycopeptides indicates that Man\(_{6}(\text{GlcNAc})_{2}\) is attached to Asn\(^{232}\) and Man\(_{9}(\text{GlcNAc})_{2}\) is attached to Asn\(^{341}\) (Fig. 7).

Biochemical Characterization of the Phaseolin Oligosaccharide Intermediates.—To study the biosynthesis and processing of the phaseolin oligosaccharides, we labeled cotyledons for 3 and 24 h with \([^{3}H]\)glucosamine, isolated phaseolin, and separated the polypeptides by preparative SDS-PAGE. Polypeptides A, C, and D were obtained by electrophoresis and digested with Pronase, and the glycopeptides were fractionated on ConA (Fig. 8). The polypeptide size class B, which was not clearly separated from polypeptides A and C on the gels, remained as a contaminant in the size classes A and C. The glycopeptide prepared from polypeptides A and C after a 3-h labeling period all bind tightly to ConA-agarose. After 24-h labeling, 88% of this glycopeptide fraction still binds to ConA. In contrast, polypeptides of the phaseolin size class D isolated after 3-h labeling contained a mixture of glycan which either did not bind to the ConA column or were slightly retarded (Fig. 8, lower left panel, peaks A and B, respectively) or bound tightly and were eluted with \(\alpha\)-methylmannoside. After 24-h labeling, the proportion of the complex glycan was about 70% of the total and now eluted as one major peak from the ConA column (Fig. 8, lower right panel).

The glycopeptides resolved by ConA chromatography were analyzed by sizing on a long column (1 x 100 cm) of Bio-gel P-4 before and after treatment with various glycosidases. The results of these experiments are presented in Figs. 9-11 and can be summarized as follows. Initially (3-h labeling period), both polypeptides A and C have two Man\(_{6}(\text{GlcNAc})_{2}\) glycans. One glycan remains as Man\(_{6}(\text{GlcNAc})_{2}\), and the other is slowly processed to Man\(_{6}(\text{GlcNAc})_{2}\). Polypeptide D has mainly a complex glycan which, in short-time labeling experiments, has terminal \(N\)-acetylglucosamine residues. After 24-h labeling, these terminal \(N\)-acetylglucosamine residues are no longer present.

Localization of the Phaseolin Oligosaccharide Processing Events.—To determine whether the different oligosaccharide
processing events take place in the cell, cotyledons were labeled for 1 h with \[^{3}H\]glucosamine, and the organelles were fractionated on sucrose gradients. The homogenization procedure disrupts the large protein bodies, and their proteins become admixed with cytosolic proteins (28). Rough ER and the Golgi apparatus were collected according to their sucrose densities of 1.13 and 1.18 g cm\(^{-3}\), respectively. Radiolabeled phaseolin, isolated from the membrane preparations with anti-phaseolin-Sepharose in the presence of Tween 20 and from the soluble fraction by the affinity method of Stockman et al. (27), was digested with Pronase; and the glycopeptides obtained were submitted to ConA affinity chromatography.

The glycopeptides of total phaseolin isolated from the ER carry high mannose-type oligosaccharides exclusively as they all bind to ConA-agarose (Fig. 12, upper panel). On the other hand, about 25% of the glycopeptides of phaseolin isolated from the Golgi apparatus and the soluble fractions do not bind to ConA (Fig. 12, middle and lower panels). This is consistent with the known role of the Golgi apparatus in the conversion of high mannose to complex side chains on glycoproteins (see Ref. 19).

The various glycopeptides obtained by ConA affinity chromatography were further analyzed by gel filtration (Fig. 13). In addition to the glycopeptides described above, those obtained from soluble phaseolin labeled for 3 and 24 h with N-\[^{3}H\]acetylglucosamine were analyzed. Gel filtration of the ConA-binding (ConA+) glycopeptides from the ER-localized phaseolin shows that they co-migrated with Man\(_{9}(GlcNAc)_3\)Asn. They are not distinguishable from the ConA+ glycopeptides found in the Golgi apparatus or the soluble fraction after 1-h labeling. However, the ConA+ glycopeptides of soluble phaseolin labeled for 3 h can be resolved into a major peak corresponding to Man\(_{9}(GlcNAc)_3\)Asn and a minor peak that co-migrated with Man\(_{9}(GlcNAc)_3(Gly)\)Asn. After a 24-h labeling period, the Man\(_{9}(GlcNAc)_3\)Asn and Man\(_{9}(GlcNAc)_3(Gly)\)Asn peaks are present in a ratio of 1:1, as found for the mature protein. Thus, the processing from Man\(_{9}(GlcNAc)_3\) to Man\(_{9}(GlcNAc)_3\) is only apparent after a long labeling period and is therefore presumed to take place after the transport of phaseolin to the protein bodies.

Gel filtration of the Golgi apparatus-derived phaseolin glycopeptides that did not bind ConA (ConA–) yields two peaks that eluted in positions corresponding to the complex glycopeptides obtained from 3-h-labeled polypeptide D (compare the Go\(\tilde{g}\)i panel in Fig. 13 to the CONTROL panel in Fig. 10). Treatment of these Golgi apparatus-derived glycopeptides with \(\beta\)-N-acetylglucosaminidase generated a product which

**Fig. 8.** Analysis of the glycopeptides from phaseolin size classes A, C, and D by ConA affinity chromatography. Each of 20 cotyledons was labeled with 6 \(\mu\)Ci of \[^{3}H\]glucosamine for 3 and 24 h, and phaseolin was isolated by immunoaffinity chromatography. Purified phaseolin was subjected to preparative SDS-PAGE, and the individual polypeptides were recovered by electroelution (polypeptide B remained as a contaminant in polypeptides A and C). Glycopeptides were obtained by exhaustive digestion with Pronase, followed by gel filtration on a small column (2 \(\times\) 18 cm) of Bio-Gel P-4 to remove small peptides and amino acids. The purified glycopeptides were then submitted to ConA affinity chromatography. The arrows indicate the starting point of elution with 200 mM \(\alpha\)-methylmannoside.

**Fig. 12.** ConA chromatography of glycopeptides from phaseolin isolated from rough ER, Golgi, and soluble fractions. Each of 20 cotyledons was labeled with 6 \(\mu\)Ci of \[^{3}H\]glucosamine for 1 h. The radioactive tissue was homogenized in buffered sucrose containing 2 mM MgCl\(_2\) and then separated into an organelle and soluble fraction on a column of Sepharose 4B. The organelles were fractionated on linear gradients of 16–54% (w/w) sucrose, and the rough ER (RER) and Golgi apparatus were identified by marker enzymes as described (14). Fractions containing the rough ER and Golgi apparatus were diluted with an equal volume of phosphate-buffered saline containing 1% Tween, and the phaseolin was isolated using anti-phaseolin-Sepharose. Phaseolin from the soluble fraction was isolated by immunoaffinity chromatography. Each fraction of phaseolin was digested by Pronase, purified by passage through the short Bio-Gel P-4 column, and then fractionated on ConA-agarose columns as described for Fig. 1. The arrows indicate the starting point of elution with 200 mM \(\alpha\)-methylmannoside.
of undenatured ER-derived phaseolin with jack bean α-mannosidase converts approximately half of the Man₉(GlcNAc)₂ groups into an oligosaccharide shortened by 4 mannose residues (data not shown). To determine if this accessibility to α-mannosidase in vitro related to the attachment site of the oligosaccharide (Asn³⁻ or Asn⁴⁻), we analyzed tryptic glycopeptides of the α-mannosidase-treated phaseolin.

The mixture of tryptic peptides was separated by C₃ reverse-phase HPLC (see Fig. 6D). The glycopeptides were identified by measuring the radioactivity in each fraction, and the N termini of the glycopeptides were determined by amino acid sequence analysis. The oligosaccharides attached to these glycopeptides were analyzed by sizing them on a calibrated column of Bio-Gel P-4 after exhaustive digestion with Pronase (Fig. 14, middle panel). Two controls are included in Fig. 14. Tryptic peptides, obtained from phaseolin which was not incubated with α-mannosidase, were separated on the C₃ column. One-half of each glycopeptide fraction was digested with Pronase, and the other half with α-mannosidase followed by Pronase. The reaction products were analyzed by sizing them on a calibrated column of Bio-Gel P-4 (Fig. 14, upper and lower panels, respectively). The results can be summarized as follows. The N-terminal region of the glycopeptide in HPLC fraction 118 (Fig. 6, HPLC peak II) is H₂N-Ala-Thr-Ser-Asn-Val (ATSNV) and carries Man₉(GlcNAc)₂. Comparison with the amino acid sequences of the two predicted glycopeptides (Fig. 7) shows that this high mannose oligosaccharide is attached to Asn⁴⁺ in the hydrophobic protein domain. When present in the undenatured ER-derived glycoprotein, this oligosaccharide is not accessible to jack bean.

FIG. 14. Gel filtration of glycopeptides from rough ER-de- derived phaseolin. The procedures for labeling and isolating phaseolin from rough ER were as described for Fig. 12 with the exception that the cotyledons were labeled for 2 h. An aliquot of phaseolin (25,000 cpm) was treated for 24 h with α-mannosidase. Both the treated sample and an untreated sample were then digested with trypsin, and the tryptic peptides were separated on a C₃ HPLC column as described for Fig. 6. The tryptic glycopeptides so obtained were then digested with Pronase, and the reaction products were analyzed by gel filtration on the long Bio-Gel P-4 column (upper and middle panels). In addition, samples of phaseolin not treated with α-mannosidase were subjected to trypsin digestion, HPLC fractionation, and finally α-mannosidase treatment before Pronase digestion and gel filtration (lower panel). Arrows M₀ and M₁ indicate the elution positions of Man₉(GlcNAc)₂Asn and Man₉(GlcNAc)₃Asn, respectively.

FIG. 13. Gel filtration of glycopeptides from phaseolin iso- lated from rough ER, Golgi, and soluble fractions. Phaseolin glycopeptides obtained by ConA affinity chromatography as described for Fig. 12 were analyzed by gel filtration as described for Fig. 9. Glycopeptides of phaseolin from the soluble fraction labeled for 3 and 24 h with [³H]glucosamine were included. Glycopeptides which were not retained by ConA-agarose columns are labeled as ConA−, and those which required α-methylmannoside elution are designed ConA+. Arrows M₀ and M₁ indicate the elution positions of Man₉(GlcNAc)₂Asn and Man₉(GlcNAc)₃Asn, respectively.

co-migrated with Xyl-Man₉(GlcNAc)₂(Gly)Asn, and simultaneous treatment with β-N-acetylglucosaminidase and α-mannosidase resulted in a product that co-migrated with Xyl-Man₉(GlcNAc)₂(Gly)Asn (data not shown). The complex (ConA−) glycopeptides obtained from the soluble phaseolin fraction after 1-h labeling showed the same size distribution as was found for the same in the Golgi apparatus. After a 3-h labeling period, the glycopeptide corresponding to the peak with less terminal N-acetylglucosamine residues increased in level. After 24-h labeling, all the ConA− glycopeptides co-migrated with Xyl-Man₉(GlcNAc)₂(Gly)Asn. Thus, the processing of a high mannose glycan to a complex glycan occurs in the Golgi complex where the complex glycan obtains terminal N-acetylglucosamine residues. This is followed by the slow removal of terminal N-acetylglucosamine residues after the protein arrives in the protein bodies.

Accessibility of Phaseolin High Mannose Oligosaccharides to α-Mannosidase Digestion in Vitro—Phaseolin isolated from the rough ER carries only high mannose-type oligosaccharides with 9 mannose residues (Fig. 13, upper left panel). Digestion
α-mannosidase. It is readily degraded by α-mannosidase in the corresponding tryptic glycopeptide; however, the main digestion product co-migrates on the Bio-Gel P-4 column with Man₆(GlcNAc)₂Asn (fraction 48), although a significant proportion is still somehow protected by the attached peptide and only shortened by 2–4 mannose residues.

The N-terminal region of the glycopeptide in HPLC fraction 128 (Fig. 6, HPLC peak III) is H₂N-Gln-Asp-Asn-Thr-Ile (QDNTI) and also carries Man₆(GlcNAc)₂. Comparison with the two predicted glycopeptides (Fig. 7) indicates that this oligosaccharide is attached to Asn° in the hydrophilic protein domain. When ER-derived phaseolin (undenatured) is treated with jack bean α-mannosidase, this high mannose oligosaccharide is shortened by 2–4 mannose residues. Most of the corresponding tryptic glycopeptide is totally accessible to α-mannosidase, which becomes trimmed to Man₆(GlcNAc)₂(Gly)Asn (fraction 51). However, as seen for the HPLC peak II tryptic glycopeptide, some of the material is partially resistant to α-mannosidase and only shortened by 2–4 mannose residues. It is not entirely clear why glycopeptides obtained by trypsin digestion are only partially degradable by α-mannosidase, as the corresponding glycopeptides from Pronase digests are completely degraded by α-mannosidase to Man₆(GlcNAc)₂(Gly)Asn (data not shown). We favor the explanation that the peptide portion of the glycopeptide causes steric hindrance of the enzyme. The experiments discussed here show that the oligosaccharides of ER-derived phaseolin, which are accessible to jack bean α-mannosidase in vitro, are the same ones which are normally modified: Man₀(GlcNAc)₂ to Man₆(GlcNAc)₂ in the phaseolin polypeptides A and C in the protein bodies and Man₀(GlcNAc)₂ to Xyl-Man₀(GlcNAc)₂ in glycoprotein D in the Golgi apparatus.

Accessibility of the Terminal N-Acetylgalactosaminidase Residues of the Complex Phaseolin Oligosaccharide to β-1,4-Acetylgalactosaminidase Digestion in Vitro—Phaseolin isolated from the Golgi fraction of cotyledons labeled for 1 h with [3H]glucosamine was digested with Pronase, and the resulting glycopeptides were separated by ConA chromatography. When the glycopeptides that did not bind ConA were treated with β-N-acetylgalactosaminidase, more than 90% of the label appeared as free N-acetylgalactosamine (data not shown). Thus, more than 90% of the N-[3H]acetylgalactosamine incorporated into the complex side chain of 1-h-labeled phaseolin resides in terminal β-N-acetylgalactosaminide-accessible (residues of the Complex Phaseolin Oligosaccharide to β-1,4-Acetylgalactosaminidase Digestion in Vitro) glycopeptides.

The sensitivity of the terminal N-acetylgalactosaminidase residues of the complex oligosaccharides in native phaseolin to β-1,4-acetylgalactosaminidase digestion in vitro was studied by treating N-[3H]acetylgalactosamine-labeled phaseolin with the enzyme. The phaseolin was obtained from cotyledons which had been labeled for 1 h with [3H]glucosamine. After this short labeling time, most of the N-[3H]acetylgalactosamine is in terminal N-acetylgalactosamine residues rather than in the chitobiose core. After inactivation of β-1,4-acetylgalactosaminidase, phaseolin was digested with Pronase, and the glycopeptides obtained were separated by ConA affinity chromatography. Analysis of the nonretarded glycopeptide fraction of the enzyme-treated and control phaseolin showed that the terminal N-acetylgalactosamine residues can be removed by treatment of native phaseolin with β-1,4-acetylgalactosaminidase. The amount of radioactivity in N-[3H]acetylgalactosamine in in vitro treated phaseolin was less than 10% compared to the controls. Gel filtration analysis of the same glycopeptides from in vitro treated phaseolin showed that the glycopeptides co-migrated with the mature glycopeptides obtained from mature phaseolin in the protein bodies (data not shown).

Discussion

The results reported in this paper confirm and extend the limited amount of information available on the structure of N-linked glycans of plant glycoproteins in general and seed storage glycoproteins in particular. Plant glycoproteins have been shown to contain both high mannose and complex N-linked glycans, and it is known that the latter derive from the former (1–7, 14, 23, 47, 48). We isolated three different abundant glycans from affinity-purified phaseolin and determined their structures. Two are typical high mannose oligosaccharides, and one is complex. The two high mannose oligosaccharides have 9 and 7 mannose residues and share the unique branching pattern of mannosyl residues reported for high mannose glycans of yeast and animal cells (1, 2). In addition to these, phaseolin contains small amounts of Man₆ and Man₇ glycans (48). By collecting the peak fractions of the glycopeptides separated on Bio-Gel P-4, we obtained samples for 1H NMR analysis which were uncontaminated by these minor glycan species. The complex glycan of phaseolin with 3 mannose residues and 1 xylose residue linked β1–2 to the β1–linked mannose seems to be a common component of complex N-linked plant oligosaccharides (4–6). A β1–2-linked xylose residue has been found in only one animal glycan (44). In addition to this core, most complex glycans of plant glycoproteins have an α1–3–linked fucose residue on the proximal N-acetylglucosamine, and some have N-acetylgalactosamine, galactose, and fucose residues emanating from the core.

By sequencing tryptic phaseolin glycopeptides which were separated by reverse-phase HPLC and identified by their specific oligosaccharide, we have shown that Man₆(GlcNAc)₂ is always attached to Asn°, whereas Man₆(GlcNAc)₂ and Xyl-Man₆(GlcNAc)₂ are always attached to Asn°. Thus, the Man₆(GlcNAc)₂ glycan is located in a hydrophilic protein domain, and the glycans which undergo processing are both in a hydrophilic part of the protein (24).

Polypeptides A and C have only high mannose glycans with Man₆(GlcNAc)₂ attached to Asn° and Man₆(GlcNAc)₂ attached to Asn°. Polypeptide D has only one glycan: mostly Xyl-Man₆(GlcNAc)₂ and a smaller amount of Man₆(GlcNAc)₂. It is likely that polypeptide D is a mixture of two slightly different glycoproteins: one with Man₆(GlcNAc)₂ and one with Xyl-Man₆(GlcNAc)₂. Xyl-Man₆(GlcNAc)₂ is always attached to Asn°, and Man₆(GlcNAc)₂ also occupies this site on the related polypeptide, as it does on polypeptides A and C. The presence of a glycine residue at the N-terminal side of asparagine clearly identifies this glycosylation site when glycopeptides are analyzed. Whether polypeptide D lacks a second glycosylation site or has an unoccupied glycosylation site is not clear. All nine cDNAs which have been sequenced give rise to polypeptides with two slightly different polypeptides: one with Man₆(GlcNAc)₂ and one with Xyl-Man₆(GlcNAc)₂. Xyl-Man₆(GlcNAc)₂ is always attached to Asn°, whereas Man₆(GlcNAc)₂ also occupies this site on the related polypeptide, as it does on polypeptides A and C.

Biochemical Characterization and Localization of the Phaseolin Oligosaccharide Intermediates—By labeling cotyledons for different intervals with [3H]glucosamine, we were able to
identify early and late glycan processing events, and subcellular fractionation experiments permitted us to place the early processing events in the Golgi apparatus and late events in the protein bodies. The various processing steps are summarized in Fig. 15. In the ER, we find only the Man9(GlcNAc)2 form of glycans, indicating that the removal of terminal glucose residues occurs very quickly. In the Golgi apparatus, we find unprocessed high mannose glycans and fully processed complex glycans with terminal N-acetylglucosamine residues. This indicates that the intermediate processing steps occur rapidly in the Golgi apparatus. Transport of the glycoproteins to the protein bodies occurs with terminal N-acetylglucosamine residues attached to the complex glycans. Our findings confirm that in plants the transformation of high mannose oligosaccharides into complex glycans occurs in the Golgi complex (14). Plant glycan processing enzymes such as mannosidases I (49) and II(5) have been recently characterized but not yet localized, whereas some of the glycosyltransferases such as fucosyltransferase (50) and N-acetylglucosamine transferase (51) have been identified and localized in the Golgi apparatus. Our finding that processing (removing of N-acetylglucosamine and mannose) occurs in the protein bodies is consistent with the observation that protein bodies contain α-mannosidase and β-N-acetylglucosaminidase (52).

Accessibility of Glycans to Modifying Enzymes—Experiments with glycoproteins from plants (63), yeast (64), and virus envelopes (55) show that the control of glycan modification is, in large part, determined by the accessibility of glycans to the processing enzymes in the Golgi complex. This hypothesis derives from studies in which native glycoproteins are incubated in the absence or presence of glycosidases (endo-β-N-acetylglucosaminidase H, α-mannosidase) and then run on SDS gels. Any change in M, caused by glycosidase treatment can yield an estimate of the extent ofican's accessibility to modifying enzymes. The results obtained here are in agreement with the hypothesis stated above. We observed that the Man9(GlcNAc)2 on Asn30 of ER-derived phaseolin is partially susceptible to α-mannosidase (2–4 mannose residues are removed), whereas the Man9(GlcNAc)2 on Asn341 is not altered by exposure to the enzyme. Thus, only the glycan which can be modified in the Golgi apparatus is accessible to the glycosidase in vitro. As discussed above, the glycan on Asn262 only becomes complex when the second glycosylation site is unoccupied. In addition, we found that the terminal N-acetylglucosamine residue(s) on the complex glycan of Golgi apparatus-derived phaseolin can be removed by incubation of the native protein with β-N-acetylglucosaminidase. It is of particular interest in this regard that the glycan which is accessible to glycosidases (and presumably to glycosyltransferases as well) is in a hydrophilic region of the polypeptide (Asn262), whereas the glycan which is not accessible (Asn341) is in a hydrophobic region of the polypeptide (24). It may well be that high mannose chains in hydrophobic pockets are sequenced and thus remain unmodified. However, the converse is not necessarily true. When analyzing the glycans of phytohemagglutinin, we found an unmodified chain, which is not accessible to α-mannosidase in vitro, attached to an asparagine residue in a hydrophilic region (56).

Of particular interest with respect to the processing of glycans is our finding that the presence of a glycan on Asn341 may determine whether the glycan at Asn262 is processed or not. We interpret our results as showing that the absence of a glycan from Asn341 results in the more extensive processing of the glycan on Asn262. If glycans are not accessible, they remain unmodified. The absence of a glycan on Asn341 may result in greater accessibility to the Golgi enzymes of the glycan attached to Asn262.

Role of Terminal N-Acetylglucosamine Residues—Our results show that the complex side chain of phaseolin polypeptide D acquires terminal N-acetylglucosamine residues in the Golgi apparatus, which are later removed in the protein bodies. Earlier (57), we made a similar observation for the complex side chain of phytohemagglutinin, which, like phaseolin, moves via the ER-Golgi apparatus-protein body transport pathway in beans. Since protein bodies are similar to lysosomes (both contain a full complement of acid hydrolases (see Ref. 7), it is tempting to speculate that these terminal N-
acetylglycosamine residues may play a role in targeting in the same manner that terminal phosphate residues target lysosomal hydrolases to lysosomes (58). Such a role for the terminal N-acetylglycosamine residue seems unlikely in view of the finding that tunicamycin does not inhibit the transport of unglycosylated phytohemagglutinin to the protein bodies (59). Furthermore, many protein body proteins, such as soybean glycinin and pea legumin, are not glycoproteins.

Our laboratory has recently investigated (60) the structural requirements of oligosaccharides that can accept xylose and/or fucose residues during the formation of complex glycans in the plant Golgi apparatus. Only glycans containing at least 1 terminal N-acetylglycosamine residue can serve as acceptors of xylose and fucose. As both phytohemagglutinin and phaseolin contain glycans with transient N-acetylglycosamine residues and these same glycans contain xylose and/or fucose, then the terminal N-acetylglycosamine residues may simply constitute recognition markers for later Golgi apparatus-mediated processing events.

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Structure of Phaseolin Glycans

** vindenin of Supplemental Material:**
Structure and position of the high mannose and complex oligosaccharides on the N- and O-linked glycoproteins of phaseolin

** MATERIALS AND METHODS**

** Plant Material:**
Phaseolin (Vigna unguiculata) was grown in a greenhouse as described (1). Seeds were harvested for the isolation of mature phaseolin seeds (pod) and stored at -20°C. The outer layer (seed coat) was removed, and phaseolin was extracted with 4°C water and high-speed centrifugation.

** Glucosylation and Structure of Phaseolin Glycans**

** Isolation of Exogenous Palatins:**
Endogenous labeling of exogenous phaseolin was done as described (1), and the glycosylation of phaseolin was analyzed as described (2). The purified phaseolin was digested with glycosidases and the glycosylation products were separated on a 12.5% SDS-PAGE gel and stained with Coomassie blue. The glycosylation of phaseolin was analyzed on a 12.5% SDS-PAGE gel and stained with Coomassie blue. The glycosylation of phaseolin was analyzed on a 12.5% SDS-PAGE gel and stained with Coomassie blue. The glycosylation of phaseolin was analyzed on a 12.5% SDS-PAGE gel and stained with Coomassie blue. The glycosylation of phaseolin was analyzed on a 12.5% SDS-PAGE gel and stained with Coomassie blue.
which is in agreement with the presence of Glycogen (41). The chemical shifts of the other signals of Ph 1 are essentially identical with those of E. Therefore, the structure of the glycans of Ph 1 is:  

\[ \text{Mann}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-\text{Glc}(1^\text{b})^{-}} \]

The 1H-NMR data of Ph 1 are essentially the same as those for Man(1^\text{a})\text{Glc}(1^\text{b})\text{Man} (61) from hordei lectin, and the following has the structure:

\[ \text{Mann}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Man}(1^\text{a})^{-\text{Glc}(1^\text{b})^{-}} \]

**Characterization of the Glucose Receiver:** The two high mannose glycoproteins from polyepitides A and C were extracted with cold 80 % ethanol and the products were analyzed by 1H-NMR spectroscopy. The spectra of these two glycoproteins were essentially identical to those of Glycogen (41). Therefore, the structure of the glycoprotein of Ph 1 is:  

\[ \text{Glc}(1^\text{a})^{-}\text{Glc}(1^\text{b})^{-}\text{Glc}(1^\text{c})^{-}\text{Glc}(1^\text{d})^{-}\text{Glc}(1^\text{e})^{-}\text{Glc}(1^\text{f})^{-}\text{Glc}(1^\text{g})^{-}\text{Glc}(1^\text{h})^{-}\text{Glc}(1^\text{i})^{-}\text{Glc}(1^\text{j})^{-}\text{Glc}(1^\text{k})^{-}\text{Glc}(1^\text{l})^{-}\text{Glc}(1^\text{m})^{-}\text{Glc}(1^\text{n})^{-}\text{Glc}(1^\text{o})^{-}\text{Glc}(1^\text{p})^{-}\text{Glc}(1^\text{q})^{-}\text{Glc}(1^\text{r})^{-}\text{Glc}(1^\text{s})^{-}\text{Glc}(1^\text{t})^{-}\text{Glc}(1^\text{u})^{-}\text{Glc}(1^\text{v})^{-}\text{Glc}(1^\text{w})^{-}\text{Glc}(1^\text{x})^{-}\text{Glc}(1^\text{y})^{-}\text{Glc}(1^\text{z})^{-}\text{Glc}(1^\text{aa})^{-}\text{Glc}(1^\text{ab})^{-}\text{Glc}(1^\text{ac})^{-}\text{Glc}(1^\text{ad})^{-}\text{Glc}(1^\text{ae})^{-}\text{Glc}(1^\text{af})^{-}\text{Glc}(1^\text{ag})^{-}\text{Glc}(1^\text{ah})^{-}\text{Glc}(1^\text{ai})^{-}\text{Glc}(1^\text{aj})^{-}\text{Glc}(1^\text{ak})^{-}\text{Glc}(1^\text{al})^{-}\text{Glc}(1^\text{am})^{-}\text{Glc}(1^\text{an})^{-}\text{Glc}(1^\text{ao})^{-}\text{Glc}(1^\text{ap})^{-}\text{Glc}(1^\text{aq})^{-}\text{Glc}(1^\text{ar})^{-}\text{Glc}(1^\text{as})^{-}\text{Glc}(1^\text{at})^{-}\text{Glc}(1^\text{au})^{-}\text{Glc}(1^\text{av})^{-}\text{Glc}(1^\text{aw})^{-}\text{Glc}(1^\text{ax})^{-}\text{Glc}(1^\text{ay})^{-}\text{Glc}(1^\text{az})^{-}\text{Glc}(1^\text{aa})^{-}\text{Glc}(1^\text{ab})^{-}\text{Glc}(1^\text{ac})^{-}\text{Glc}(1^\text{ad})^{-}\text{Glc}(1^\text{ae})^{-}\text{Glc}(1^\text{af})^{-}\text{Glc}(1^\text{ag})^{-}\text{Glc}(1^\text{ah})^{-}\text{Glc}(1^\text{ai})^{-}\text{Glc}(1^\text{aj})^{-}\text{Glc}(1^\text{ak})^{-}\text{Glc}(1^\text{al})^{-}\text{Glc}(1^\text{am})^{-}\text{Glc}(1^\text{an})^{-}\text{Glc}(1^\text{ao})^{-}\text{Glc}(1^\text{ap})^{-}\text{Glc}(1^\text{aq})^{-}\text{Glc}(1^\text{ar})^{-}\text{Glc}(1^\text{as})^{-}\text{Glc}(1^\text{at})^{-}\text{Glc}(1^\text{au})^{-}\text{Glc}(1^\text{av})^{-}\text{Glc}(1^\text{aw})^{-}\text{Glc}(1^\text{ax})^{-}\text{Glc}(1^\text{ay})^{-}\text{Glc}(1^\text{az})^{-}\text{Glc}(1^\text{aa})^{-}\text{Glc}(1^\text{ab})^{-}\text{Glc}(1^\text{ac})^{-}\text{Glc}(1^\text{ad})^{-}\text{Glc}(1^\text{ae})^{-}\text{Glc}(1^\text{af})^{-}\text{Glc}(1^\text{ag})^{-}\text{Glc}(1^\text{ah})^{-}\text{Glc}(1^\text{ai})^{-}\text{Glc}(1^\text{aj})^{-}\text{Glc}(1^\text{ak})^{-}\text{Glc}(1^\text{al})^{-}\text{Glc}(1^\text{am})^{-}\text{Glc}(1^\text{an})^{-}\text{Glc}(1^\text{ao})^{-}\text{Glc}(1^\text{ap})^{-}\text{Glc}(1^\text{aq})^{-}\text{Glc}(1^\text{ar})^{-}\text{Glc}(1^\text{as})^{-}\text{Glc}(1^\text{at})^{-}\text{Glc}(1^\text{au})^{-}\text{Glc}(1^\text{av})^{-}\text{Glc}(1^\text{aw})^{-}\text{Glc}(1^\text{ax})^{-}\text{Glc}(1^\text{ay})^{-}\text{Glc}(1^\text{az})^{-}\text{Glc}(1^\text{aa})^{-}\text{Glc}(1^\text{ab})^{-}\text{Glc}(1^\text{ac})^{-}\text{Glc}(1^\text{ad})^{-}\text{Glc}(1^\text{ae})^{-}\text{Glc}(1^\text{af})^{-}\text{Glc}(1^\text{ag})^{-}\text{Glc}(1^\text{ah})^{-}\text{Glc}(1^\text{ai})^{-}\text{Glc}(1^\text{aj})^{-}\text{Glc}(1^\text{ak})^{-}\text{Glc}(1^\text{al})^{-}\text{Glc}(1^\text{am})^{-}\text{Glc}(1^\text{an})^{-}\text{Glc}(1^\text{ao})^{-}\text{Glc}(1^\text{ap})^{-\text{Glc}(1^\text{aq})^{-}} \]

**Structure of Phaseolin Glycans**

1. **Structure of Phaseolin Glycans**

   - **Fig. 1.** Effect of ads 0.5 treatment on the mobility of phaseolin glycoproteins in classes A, C, and B. Each of 20 conductivity vials was labeled with 3H-glucosamine for 24 h. Phaseolin was isolated and purified by affinity chromatography. The 1H-13C NMR of phaseolin was then sequenced with 1H and 13C labeling in 250 mM buffer with N-acetylglucosamine, 80% ethanol, and 20% ethanol.

   - **Fig. 2.** Effect of ads 0.5 treatment on the mobility of phaseolin glycoproteins in classes A, C, and B. Each of 20 conductivity vials was labeled with 3H-glucosamine for 24 h. Phaseolin was isolated and purified by affinity chromatography. The 1H-13C NMR of phaseolin was then sequenced with 1H and 13C labeling in 250 mM buffer with N-acetylglucosamine, 80% ethanol, and 20% ethanol.
Structure of Phaseolin Glycans

Fig. 9. Gel filtration of 1-3h and 2-4h labeled phaseolin glycopeptides before and after treatment with ami-no-glycosidase F and endo H. An aliquot of the glycopeptides which were treated with aminoglycosidase F were applied to the long (0.7 x 60 cm) Biogel P-4 column. The arrows in the upper panels indicate the elution positions of M_2, M_1, M_0, M_{-2}, and M_{-4}, respectively. The elutes in the lower panels indicate the elution positions of M_{-6}, M_{-8}, M_{-10}, and free glycans, respectively.

Fig. 10. Gel filtration of 1-3h labeled phaseolin glycopeptides before and after treatment with β-N-acetylglucosaminidase, aminoglycosidase F and endo H. An aliquot of the glycopeptides that were not treated by aminoglycosidase F (Fig. 1, lower left panel) was further treated by gel filtration on a calibrated 200 cm column of Biogel P-4. See Fig. 9 for explanation of arrows.

Fig. 11. Gel filtration of 1-3h labeled phaseolin glycopeptides before and after treatment with β-N-acetylglucosaminidase, aminoglycosidase F and endo H. An aliquot of the glycopeptides that were not treated by aminoglycosidase F (Fig. 1, lower left panel) was further treated by gel filtration on a calibrated 200 cm column of Biogel P-4. See Fig. 9 for explanation of arrows.
### Structure of Phaseolin Glycans

Table 1: 1H-chemical shifts of structural reporter group proton of the constituent monosaccharides for the glycopolypeptides derived from phaseolin (Ph 1-5), together with those for reference compound R (44).

|       | Ph 1 | Ph 2 | Ph 3 |
|-------|------|------|------|
| NAc   |      |      |      |
| 1     | 2.053| 2.010| 2.009| 2.012| 2.064|
| 2     | 2.073| 2.070| 2.060| 2.064|       |
| 10-1  |      |      |      |
| 1     | 4.034| 4.085| 4.096| 4.064| 4.086|
| 2     | 4.041| 4.056| 4.070| 4.076| 4.084|
| 3     | 3.122| 3.121| 3.117| 3.112| 3.113|
| 4     | 4.913| 4.887| 4.875| 4.871| 4.866|
| 5     | 5.048| 5.045| 5.042| 5.042| 5.038|

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|        | 1     | 2     | 3     | 4     | 5     |
|--------|-------|-------|-------|-------|-------|
|        | 4.238| 4.085| 4.238| 4.238|       |
| 2      | 4.239| 4.239| 4.239| 4.239|       |
| 3      | 3.985| 3.981| 3.981| 3.981|       |
| 4      | 4.083| 4.083| 4.083| 4.083|       |
| 5      | 4.904| 4.904| 4.904| 4.904|       |

10-5 of 1

|        | 1     | 2     | 3     | 4     | 5     |
|--------|-------|-------|-------|-------|-------|
|        | 3.577| 3.577| 3.577| 3.577|       |

10-6 of 1

|        | 1     | 2     | 3     | 4     | 5     |
|--------|-------|-------|-------|-------|-------|
|        | 3.250| 3.248| 3.248| 3.248|       |

*Assignments may have to be interchanged.