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Short Communication

The High Mannose Oligosaccharide of Phytohemagglutinin Is Attached to Asparagine 12 and the Modified Oligosaccharide to Asparagine 60[1]

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ABSTRACT

Phytohemagglutinin, the lectin of the common bean *Phaseolus vulgaris*, has a high mannose and a modified (fucosylated) oligosaccharide on each polypeptide. Fractionation by high performance liquid chromatography of tryptic digests of [^{3}H]fucose or [^{3}H]glucosamine labeled phytohemagglutinin, followed by amino acid sequencing of the isolated glycopeptides, shows that the high mannose oligosaccharide is attached to Asn^{12} and the modified oligosaccharide to Asn^{60} of the protein. In animal glycoproteins, high mannose chains are rarely found at the N-terminal side of complex chains.

Phytohemagglutinin, the lectin of the common bean *Phaseolus vulgaris*, is unique among the plant glycoproteins which have been characterized in that it has one high mannose and one modified oligosaccharide on each polypeptide. The high mannose oligosaccharide has the formula $Man_8(GlcNAc)_2$ whereas the modified oligosaccharide has 3-4 mannose residues as well as xylose and fucose residues (9). Analysis of PHA[3] by SDS-PAGE shows the presence of 2 polypeptides, PHA-E (erythroagglutinating, M_r = 34,000) and PHA-L (leucoagglutinating, M_r = 32,000). Hoffman and Donaldson (4) recently determined the nucleotide sequence of the two genes (dlec1 and dlec2) which encode these polypeptides, and the derived amino acid sequence shows the presence of three glycosylation sites in PHA-E and two glycosylation sites in PHA-L.

The biosynthesis of PHA in developing bean cotyledons affords an excellent opportunity to study the control mechanisms in oligosaccharide modification. Why is one oligosaccharide modified when the protein passes through the Golgi complex (1, 10) whereas the other one remains in the high-mannose configuration? As part of our research to answer this question, we have now identified the glycosylation sites for these two oligosaccharides. The high mannose chain is attached to Asn^{12} and the modified chain to Asn^{60} of the protein.

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[3] Abbreviations: PHA, phytohemagglutinin; TPCK, *N*-tosylphenylalanyl-chloromethylketone; TFA, trifluoroacetic acid; Asn, asparagine.

MATERIALS AND METHODS

Materials. Plants of *Phaseolus vulgaris* L. cv Greensleeves were grown in a greenhouse. Trypsin (TPCK treated) and organic chemicals were purchased from Sigma Chemical Co., acetonitrile (Omnisolv) from EM Science, inorganic chemicals from Mallinckrodt, Inc., and radiochemicals from Amersham Co. ([^{3}H] GlcN) and ICN Biochemicals, Inc. ([^{3}H]Fuc).

Isolation of Radiolabeled PHA. Radioactive labeling of excised cotyledons was done as described by Spencer *et al.* (8) with L-[5,6-^{3}H]fucose (55 Ci/mmol) or D-[6-^{3}H]glucosamine hydrochloride (36 Ci/mmol). Twenty cotyledons were labeled with 6 μCi of precursor each for 24 h, and the radioactive tissue was collected by cutting a thin slice from the cotyledon with a razor blade. The remainder of the cotyledon was discarded.

The radioactive tissue was homogenized in 6 ml of buffer (10 mM Na-phosphate [pH 7.4] with 150 mM NaCl) containing 1% (w/w) Tween 20. The affinity procedure of Felsted *et al.* (2) was used to isolate PHA from the homogenate after it was centrifuged for 10 min at 1000*g*. The PHA eluted from the thyroglobulin affinity column was dialyzed against distilled H_2O, lyophilized, and used for trypsin digestion.

Trypsin Digestion. Trypsin digestion was done as described by Hsieh *et al.* (5). Radiolabeled PHA (1.5 mg) were dissolved in 1 ml of 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 (TPCK-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 of 0.1 M sodium phosphate (pH 2.2), centrifuged to remove particulate matter and filtered through a 0.2 μm Nylon Filter (Western Analytical Prod.).

High Performance Liquid Chromatography. 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). For further purification of the glycopeptides, an Alltech C-8 column (5 μm, 4.6 × 250 mm) was used. In all cases, the peptide separation was carried out at room temperature at a flowrate of 1 ml/min. The gradients are described in detail in the legends of the figures. Fractions of 1 ml were collected. Radioactivity in the fractions was measured by liquid scintillation counting of a 25 μl aliquot.

Automatic Protein Sequencing. Samples of glycopeptides from sodium phosphate HPLC gradients were adjusted to neutral pH with 5 M NaOH, lyophilized, and desalted on a small G-15 column prior to amino acid sequencing. Samples from trifluoroacetic acid containing HPLC gradients were lyophilized without further treatments. After lyophilization, the glycopeptides

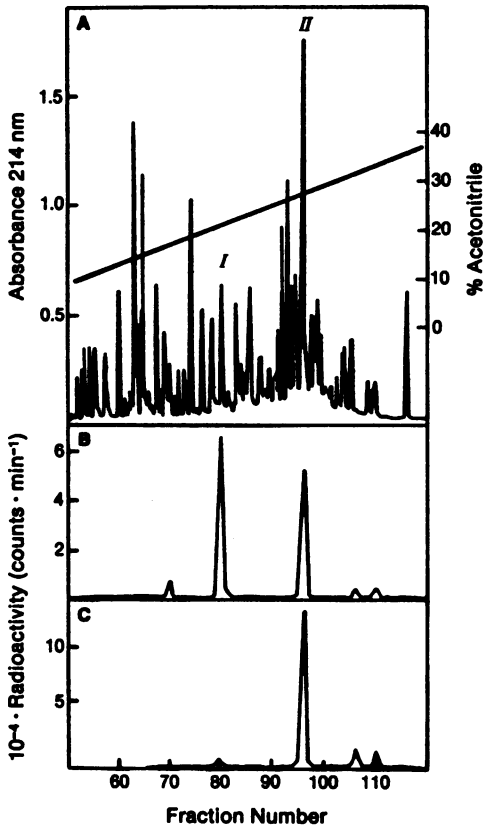

FIG. 1. Reverse phase HPLC separation of tryptic peptides of radiolabeled PHA. Tryptic peptides of PHA were separated on a Vydac C-18 column. Solvent A (100%) (0.1 M sodium phosphate [pH 2.2]) was run for 30 min followed by a linear gradient of 0 to 50% solvent B (acetonitrile) for 120 min. For clarity, the acetonitrile gradient is shown in A only. A, Peptide profile of a peptide separation, obtained by following the *A* at 214 mm; B, radioactivity profile of a peptide separation, PHA labeled with [^{3}H]glucosamine; C, radioactivity profile of a peptide separation, PHA labeled with [^{3}H]Fucose.

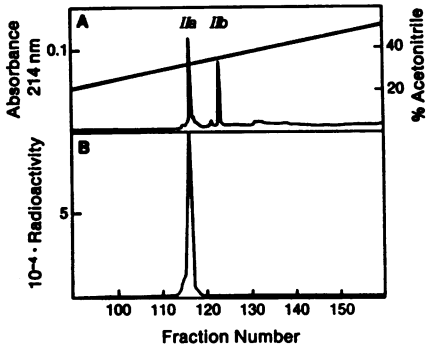

FIG. 2. Reverse phase HPLC purification of the tryptic PHA glycopeptide II. The tryptic PHA glycopeptide II was run over an Alltech C-8 column 100% solvent A (0.1% TFA in water) for 30 min followed by a linear gradient of 0 to 60% solvent B (0.1% TFA in acetonitrile) for 0 min. A, peptide profile of the glycopeptide II purification, obtained by following the absorbance at 214 nm; B, radioactivity profile of the glycopeptide II purification, PHA labeled with [^{3}H]Fucose.

were dissolved in 1% trifluoroacetic acid and subjected to automatic Edman degradations using an Applied Biosystems model 470A Gas-Phase Sequencer employing the standard No Vac program supplied by the manufacturer (3). Phenylthiohydantoin derivatives were identified by HPLC on a Brownke C-18 column using procedures described previously (6). The HPLC system

	10	20	30	40	50
Glycopeptides	F-ETN				
PHA-E	ASQTSFSFQRFNETNLILQRDATVSSKGQLRLTNVNDNGEPTLSSLGRAF				
PHA-L	SNDIYFNFQRFNETNLILQRDASVSSSGQLRLTNLNGNGEPRVGSLGRAF				

	60	70	80	90	100
Glycopeptides	YSAPIQIWD-TT				
PHA-E	YSAPIQIWDNTTGAVAASPTSFTFNIDVPNNSGPADGLAFVLLPVGSQPK				
PHA-L	YSAPIQIWDNTTGTVASFATSFTFNIQVPNNAGPADGLAFALVPVGSQPK				

FIG. 3. A comparison of the amino acid sequences of the glycopeptides and the amino acid sequence of PHA-E and PHA-L as derived by Hoffman and Donaldson (4) from the nucleotide sequences of the genes.

used with this column consisted of a Perkin Elmer Series 4 liquid chromatograph, an LC-85B spectrophotometric detector equipped with a 1.4-μl flow cell, an ISS-100 automatic sample injector, an LCI 100 computing integrator, and a model 7500 computer employing Chrom III software.

RESULTS AND DISCUSSION

The amino acid sequence of phytohemagglutinin derived from the nucleotide sequence of the cDNA shows that the E polypeptide has three possible glycosylation sites at Asn^{12}, Asn^{60}, and Asn^{80} and the L polypeptide has two possible glycosylation sites at Asn^{12} and Asn^{60} (4). We have shown previously (9) that both E and L subunits have one high mannose and one modified (fucosylated) oligosaccharide. To find out which oligosaccharide is attached to which Asn residue, cotyledons were labeled with [^{3}H]GlcN or [^{3}H]Fuc for 24 h, and the PHA isolated by affinity chromatography. The PHA was digested with trypsin and the resulting mixture of peptides and glycopeptides fractionated by HPLC on a C-18 column (Fig. 1A). Radioactivity in each fraction was determined, and we observed two major peaks for [^{3}H]GlcN (fractions 80 and 96) and one major peak for [^{3}H]Fuc (fraction 96) (Fig. 1, B and C). These results are in agreement with our previous finding that PHA contains approximately equal amounts of high mannose and modified oligosaccharides. The fucose-labeled material in fraction 96 was recovered and further resolved by HPLC on a C-8 column into two peptides (Fig. 2A). The peak at fraction 116 was labeled with [^{3}H]Fuc, and the peak at fraction 123 had no radioactivity (Fig. 2B). The fractions of interest (80 from the C-18 column and 116 from the C-8 column) were subjected to amino acid sequencing. The results indicate that the high mannose oligosaccharide is attached to a peptide with the sequence Phe-X-Glu-Thr-Asn at the N-terminus, and the modified oligosaccharide to a peptide with Tyr-Ser-Ala-Pro-Ile-Gln-Ile-Trp-Asp-X-Thr-Thr at the N terminal end. In each case, X denotes an unidentified amino acid which presumably represents the Asn residue to which the oligosaccharides are attached. A comparison with the published sequence of PHA (Fig. 3) shows that these unidentified amino acids correspond to Asn^{12} (high mannose oligosaccharide) and Asn^{60} (modified oligosaccharide) of the protein. It appears that the TPCK-treated trypsin hydrolyzed the peptide bond between Arg^{10} and Phe^{11} as expected from the specificity of trypsin; unexpectedly, it also hydrolyzed the bond between Phe^{50} and Tyr^{51} to generate the glycopeptide with the modified oligosaccharide. The possibility that the modified oligosaccharide is attached to Asn^{80} in the E polypeptide can be eliminated by the observation that no Asn was recovered in position 60. A recovery of 50% as much Asn as was found for other amino acids was to be expected if on the E polypeptide, all the modified chains were attached to Asn^{80}; a recovery of 25% would be expected if there was an equal distribution of the chains between Asn^{60} and Asn^{80} on the E polypeptide.

The assignment of the high-mannose oligosaccharide to Asn^{12} and of the modified oligosaccharide to Asn^{60} holds true for most

of the PHA in this cultivar (Greensleeves). A small proportion of the PHA-E is glycosylated three times, presumably with an oligosaccharide at Asn^{80}, and this oligosaccharide is always modified (A Vitale, R Bollini, personal communication). A small proportion of the PHA-L is totally resistant to digestion by endoglycosidase H, indicating that both oligosaccharides are modified (L Faye, MJ Chrispeels, unpublished observation).

A survey of 50 animal glycoproteins whose amino acid sequence, glycosylation site, and type of glycosylation at a particular site are known, showed that high mannose chains are rarely found at the N-terminal side of a complex oligosaccharide (7). PHA, therefore, belongs to a class of glycoproteins which, based on this criterion, has only a few members.

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