

Structural Analysis of the Carbohydrate Chains of Legume Storage Proteins by 500-MHz $^1\text{H-NMR}$ Spectroscopy

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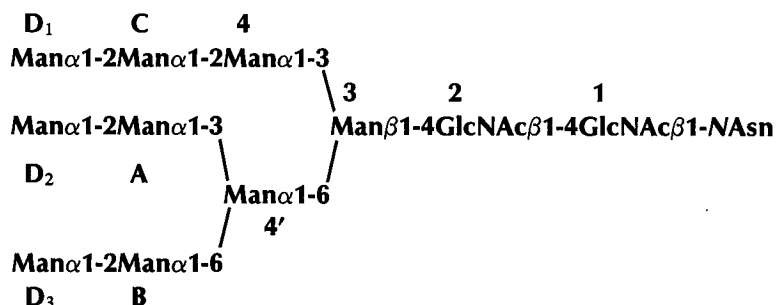
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Kidney bean glycoprotein II and 7S soybean glycoprotein are both legume storage proteins. Sugar analysis of glycopeptides, derived from these glycoproteins, revealed the presence of mannose and *N*-acetylglucosamine as the only sugar constituents, pointing to the presence of oligomannoside-type chains in both legume storage proteins. Glycoasparagines, prepared by extensive Pronase® digestion, were separated on Dowex 50W-X2 and subsequently investigated by 500-MHz $^1\text{H-NMR}$ spectroscopy.

The structures present in kidney bean glycoprotein II ranged from Man_9 - to Man_6 -GlcNAc₂Asn and in 7S soybean glycoprotein from Man_8 - to Man_6 -GlcNAc₂Asn. The Man_9 -structure is the following:



The others are partial structures thereof, lacking one or more D-mannose units.

Abbreviations: endo-H, endo- β -*N*-acetylglucosaminidase-H (EC 3.2.1.96).

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N-Glycosidically-linked oligomannoside-type carbohydrate chains are commonly found in glycoproteins from both animal and vegetable origin [1]. The kidney bean glycoprotein II and the 7S soybean glycoprotein are both legume storage proteins, probably containing oligomannoside-type chains [2, 3]. Our present interest is in the detailed carbohydrate structures since oligomannoside-type chains can occur in several isomeric forms. This knowledge is essential when studying the metabolic route of oligomannoside-type chains in leguminous seeds.

Materials and Methods

Materials

HPTLC pre-coated plates (Silicagel 60) and pronase® E were purchased from Merck (Darmstadt, W. Germany). Endo-H was obtained from Seikagaku Kogyo (Japan) and chicken egg ovalbumin, grade V, from Sigma Chemical Co., St. Louis, MO, USA.

Analytical Methods

Neutral sugars were determined by the phenol-sulfuric acid method [4]. The neutral- and amino-sugar compositions were analysed by capillary GLC on a Carbowax 20M column, after hydrolysis with trifluoroacetic acid and monosaccharide conversion to *O*-methyloxime acetates [5]. HPTLC of oligosaccharides was performed as described [6], but using propanol/nitromethane/water, 5/2/3 by vol, in two consecutive runs. The spots were visualized by dipping the plate [7], then scanned and integrated [8].

Preparation of Glycoproteins, Pronase Digestions and Separation of Glycopeptides

Kidney bean glycoprotein II was isolated from milled white beans [2] and 7S soybean glycoprotein from defatted soy flour [9, 10].

Non-extensive pronase digestion on 1 g of protein, followed by gel filtration (Sephadex G-50, Pharmacia, Sweden), passage through a Dowex 50W-X8 (H⁺-form) column and repeated gel filtration (Sephadex G-25) was performed [9], which gave a mixture of glycopeptides.

Extensive pronase digestion was carried out by simplifying a previously reported method [11]. In both cases 25 g of protein were initially digested by two successive portions of pronase E (500 mg followed by 250 mg). After filtration, passage through a Dowex 50W-X8 (H⁺-form) column and neutralisation, the residue was subjected to three further digestions with pronase E, without any intermediate gel filtration step. The resulting glycoasparagines were purified on Sephadex G-25 eluted with 0.1 M acetic acid and finally separated on a Dowex 50W-X2 (Na⁺-form) column [11, 12]. Each glycoasparagine fraction was desalted on Sephadex G-25, eluted with 0.01 M acetic acid before being subjected to 500-MHz ¹H-NMR spectroscopy. The standard glycopeptide Man₆GlcNAc₂Asn was obtained from ovalbumin [12, 13].

Glycopeptide samples containing 150 μg mannose were incubated with 20 mU endo-H for 24 h at 37°C in 500 μl 3.3 mM citric acid/11.7 mM Na_2HPO_4 buffer, pH 6, to which 25 μl toluene was added. The reaction was stopped by boiling for 3 min. The mixtures were subsequently treated with Amberlite MB-3 and lyophilized before being subjected to HPTLC analysis.

500-MHz $^1\text{H-NMR}$ Spectroscopy

Deuterium exchanged glycoasparagines were obtained by three-fold lyophilization of their $^2\text{H}_2\text{O}$ solutions. Finally the samples were dissolved in 0.4 ml $^2\text{H}_2\text{O}$ (99.96 atom % ^2H , Aldrich, Milwaukee, WI, USA). 500-MHz $^1\text{H-NMR}$ spectra were recorded on a Bruker WM 500 instrument (SON hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University, The Netherlands) operating in the pulsed Fourier-transform mode at a probe temperature of 27°C. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.002 ppm [14].

Results

Sugar analysis of kidney bean glycoprotein II revealed the presence of mannose, *N*-acetylglucosamine and xylose as main sugar constituents, together with traces of galactose and arabinose. Non-extensive pronase digestion of kidney bean glycoprotein II yielded a glycopeptide fraction containing mannose and *N*-acetylglucosamine in the molar ratio 8:2. Other sugar components of the starting material eluted with the void volume of the Sephadex G-50 column, indicating high molecular weight carbohydrate-rich material not digested by pronase E. The 7S soybean glycoprotein contained mannose and *N*-acetylglucosamine only. Analysis of the glycopeptides derived from this glycoprotein showed these sugar constituents to be present in the molar ratio 7:2.

When subjected to endo-H, glycopeptides from both glycoproteins were completely digested, releasing the corresponding oligosaccharide mixtures. These mixtures could be resolved by HPTLC using authentic $\text{Man}_6\text{-GlcNAc}$ as standard compound (Fig. 1). According to the integrations of HPTLC scans, $\text{Man}_6\text{-}$, $\text{Man}_7\text{-}$, $\text{Man}_8\text{-}$ and $\text{Man}_9\text{-GlcNAc}$ were released from kidney bean glycoprotein II glycopeptides in a molar ratio 14:31:16:39. The molar ratio of $\text{Man}_6\text{-}$, $\text{Man}_7\text{-}$ and $\text{Man}_8\text{-GlcNAc}$ released from 7S soybean glycopeptides was 22:27:51.

Extensive pronase digestion of 25 g glycoprotein resulted in glycoasparagines which were separated on Dowex 50W-X2 (Na^+ -form). Samples from each of these fractions were then subjected to endo-H digestion. HPTLC of the resulting oligosaccharides showed that all the fractions were homogeneous with respect to the number of mannose residues present (see Fig. 1).

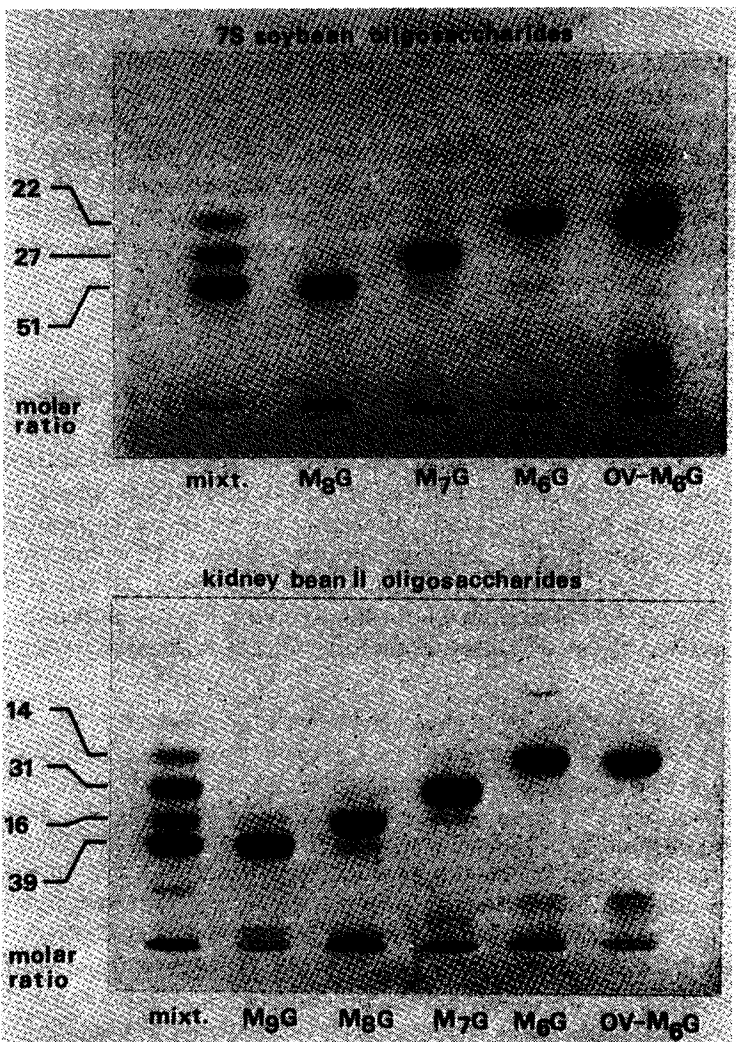


Figure 1. HPTLC patterns of oligosaccharides released from legume storage glycopeptides by endo-H. Mixt = oligosaccharides from the glycopeptide mixture; M_6 to M_9 = $\text{Man}_6\text{GlcNAc}$ to $\text{Man}_9\text{GlcNAc}$, oligosaccharides from the pure glycoasparagine fractions; OVM_6G = $\text{Man}_6\text{GlcNAc}$ standard obtained from ovalbumin (see [12, 13]). Note that the reported molar ratios obtained by scan integrations under conditions for linear response [8] are not faithfully reflected by the above picture.

After desalting on Sephadex G-25, the purified glycoasparagines were subjected to 500-MHz $^1\text{H-NMR}$ spectroscopy. The chemical shifts of the H-1 and NAc-signals of the analysed glycoasparagines are compiled in Table 1. The α -anomeric regions of the $^1\text{H-NMR}$ spectra of the kidney bean glycoprotein II are given in Fig. 2.

For kidney bean glycoprotein II the largest glycoasparagine, $\text{Man}_9\text{GlcNAc}_2\text{Asn}$ (for spectrum see Fig. 2a), was found to be identical to the Man_9 -structure isolated from soybean agglutinin [15] (cf. compound 72 in [14], and Fig. 3). The other structures isolated lack one or more of the D-mannoses. This can be inferred from the chemical shifts of the H-1 signals of Man-A, Man-B and Man-C [14] as outlined in Table 2.

Table 1. ^1H Chemical shift reference data of H-1 atoms of pertinent mannose residues in oligomannoside structures [14].

$\text{D}_2\text{-A-R}^{\text{a}}$		$\text{D}_3\text{-B-R}$		$\text{D}_1\text{-C-R}$	
Residue	δ^{b}	Residue	δ	Residue	δ
Man-A _{term}	5.40	Man-B _{term}	4.90	Man-C _{term}	5.30
Man-A _{subst}	5.09	Man-B _{subst}	5.15	Man-C _{subst}	5.05
Man-D ₂	5.04	Man-D ₃	5.04	Man-D ₁	5.06

^a R is the rest of the oligomannoside-type glycopeptide. For coding of the residues see Fig. 3.

^b Chemical shifts are expressed in ppm downfield from internal DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate).

Man₈-GlcNAc₂Asn from kidney bean glycoprotein II was found to be heterogeneous. In the $^1\text{H-NMR}$ spectrum (Fig. 2b) two signals are present for H-1 of Man-A and H-1 of Man-B. The intensities of the signals show that Man-D₂ and Man-D₃ occur in the ratio 1:3. The chemical shift of H-1 of Man-C indicates that this residue is completely substituted with Man-D₁ (compare Tables 1 and 2). The structure of Man₈-GlcNAc₂Asn is presented in Fig. 3.

Man₇-GlcNAc₂Asn from kidney bean glycoprotein II (for spectrum see Fig. 2c) was also found to be heterogeneous. All three Man-D residues are present to some extent (compare Tables 1 and 2). Taking into account the intensities of the signals, the amounts of the Man₇-GlcNAc₂Asn isomers from kidney bean glycoprotein II can be calculated as indicated in Fig. 3.

The $^1\text{H-NMR}$ spectrum of Man₆-GlcNAc₂Asn from kidney bean glycoprotein II (Fig. 2d) shows a single compound. Man-A, Man-B and Man-C are present in the terminal position (Fig. 3).

The structures of the glycoasparagines derived from 7S soybean glycoprotein can be derived by analogy. The structures are presented in Fig. 3.

Discussion

Our present results provide a new example of the occurrence of oligomannoside-type chains in plant glycoproteins. The xylose present in the kidney bean glycoprotein II preparation was not a constituent of the glycopeptide fraction derived from this glycoprotein, but was present in the high molecular weight fraction that was not digested by pronase E. Our detailed characterisation of the 7S soybean oligosaccharides (Man₆, Man₇, Man₈- and Man₉-GlcNAc₂) correct the previously reported structures from this glycoprotein (Man₇, Man₈- and Man₉-GlcNAc₂ [3, 11]) and show that only one type of branching is involved. Finally, all structures established here are identical with oligomannoside-type chains found in many other glycoproteins from various origins, thus confirming the general occurrence of such a unique pattern of branching in a wide variety of organisms, from yeasts to higher animals [14, 15].

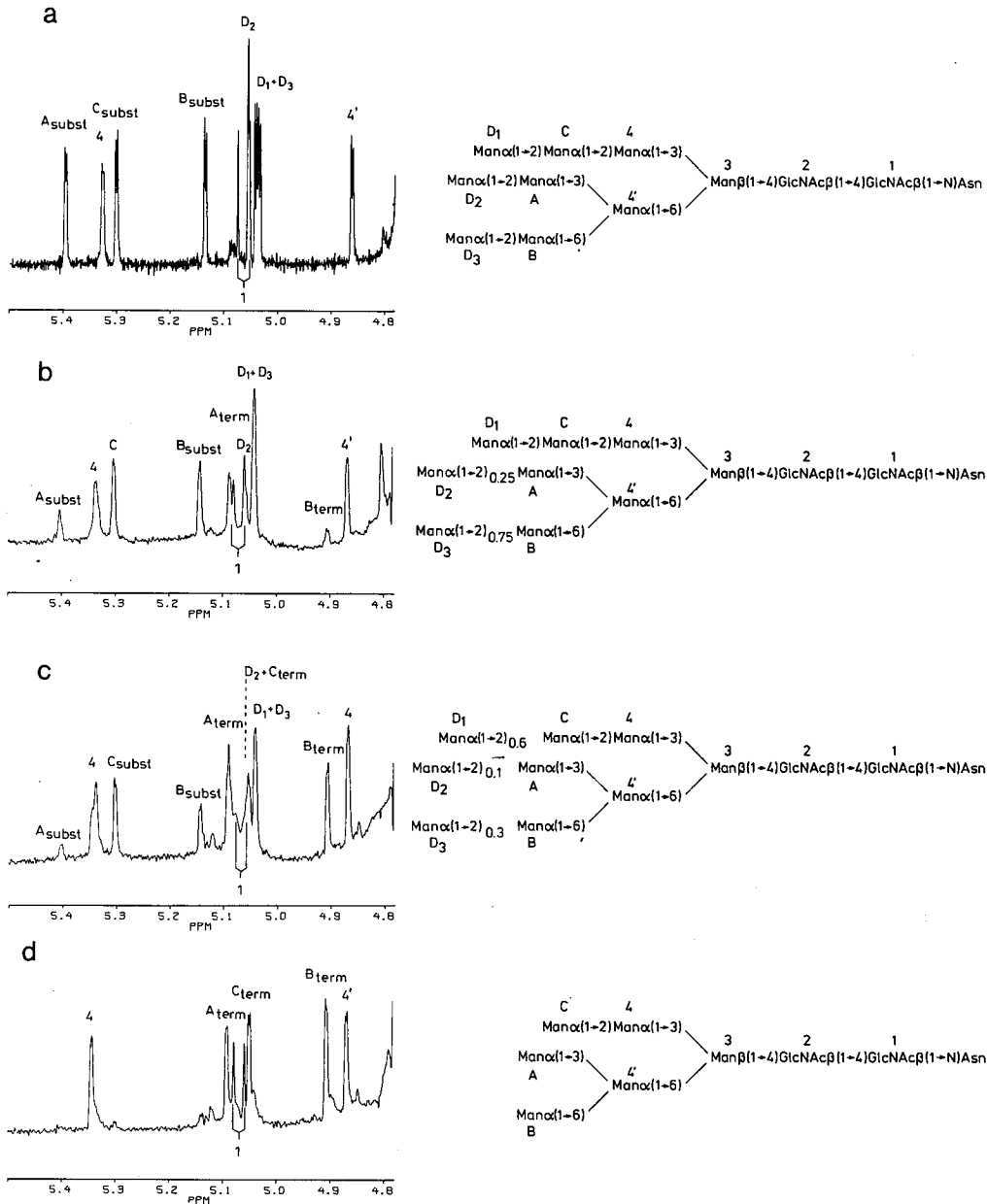


























































































































Figure 2. α -anomeric regions of the 500-MHz ^1H -NMR spectra of glycoasparagines obtained from kidney bean glycoprotein II. (a) $\text{Man}_9\text{GlcNAc}_2\text{Asn}$; (b) $\text{Man}_8\text{GlcNAc}_2\text{Asn}$; (c) $\text{Man}_7\text{GlcNAc}_2\text{Asn}$; (d) $\text{Man}_6\text{GlcNAc}_2\text{Asn}$.

Table 2. ¹H Chemical shifts of structural-reporter-group protons of the constituent monosaccharides for glycoasparagines derived from kidneybean glycoprotein II and 7S soybean glycoprotein.

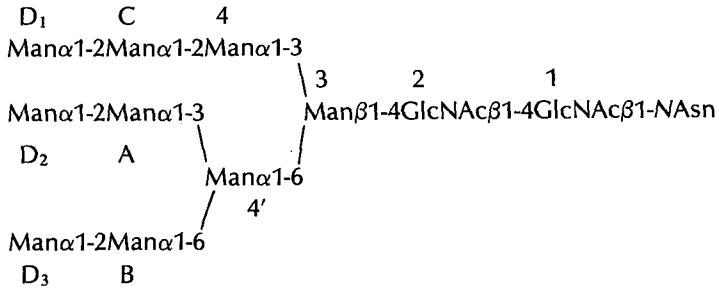
Reporter group	Residue	Chemical shift ^a in ^b																				
		Kidney bean					Soybean															
		Man9	Man8	Man7	Man6	Man8	Man7	Man6	Man7	Man6	Man6											
H-1	1											5.065	5.069	5.069	5.069	5.069	5.069	5.069	5.069	5.069	5.069	
	2											4.605	4.601	4.602	4.602	4.602	4.602	4.602	4.602	4.602	4.602	4.602
	4											5.329	5.336	5.344	5.344	5.344	5.344	5.344	5.344	5.344	5.344	5.344
	4'											4.865	4.866	4.867	4.867	4.867	4.867	4.867	4.867	4.867	4.867	4.867
	A											5.398	5.403	5.088	5.400	5.090	5.090	5.090	5.090	5.090	5.090	5.090
	B											5.138	4.903	5.142	4.905	5.142	4.905	5.142	4.905	5.142	4.905	5.142
	C											5.302	5.302	5.302	5.302	5.052	5.052	5.052	5.303	5.303	5.050	5.302
	D ₁											5.038	5.040	5.040	—	—	—	—	5.040	—	—	—
	D ₂											5.057	5.055	—	5.052	—	—	—	—	—	—	—
	D ₃											5.038	—	5.040	—	5.040	—	—	—	—	5.041	—
NAC	1											2.012	2.010	2.009	2.009	2.009	2.009	2.011	2.011	2.011	2.011	2.011
	2											2.063	2.062	2.062	2.062	2.062	2.062	2.062	2.062	2.061	2.061	2.061

^a Chemical shifts are given at 27°C, in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate in ²H₂O.

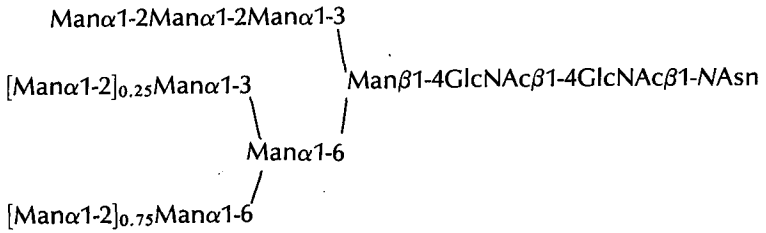
^b For complete structures see Fig. 3.

Kidney bean glycoprotein II glycoasparagines

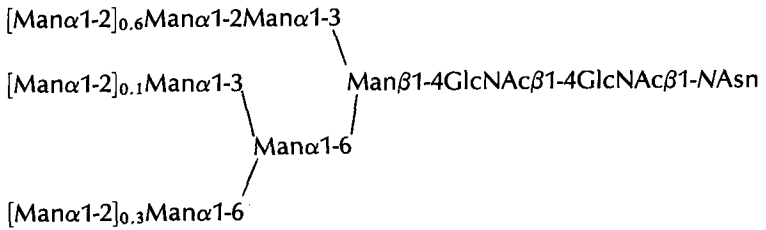
Man₉GlcNAc₂Asn



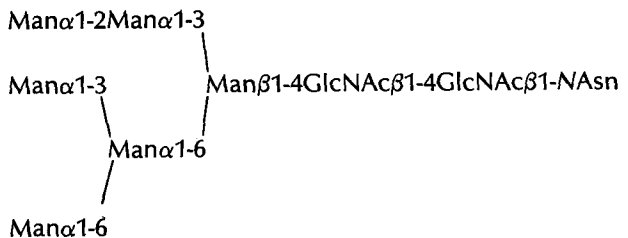
Man₈GlcNAc₂Asn



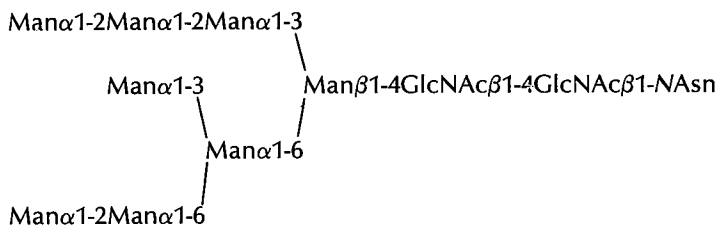
Man₇GlcNAc₂Asn



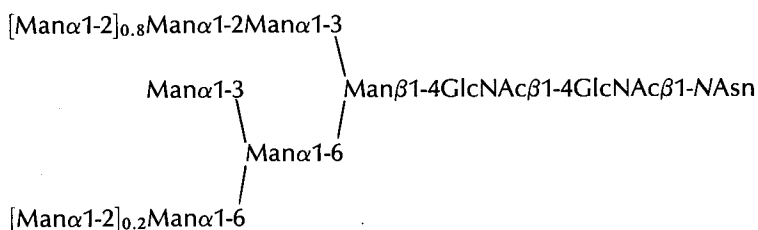
Man₆GlcNAc₂Asn



Man₈GlcNAc₂Asn



Man₇GlcNAc₂Asn



Man₆GlcNAc₂Asn

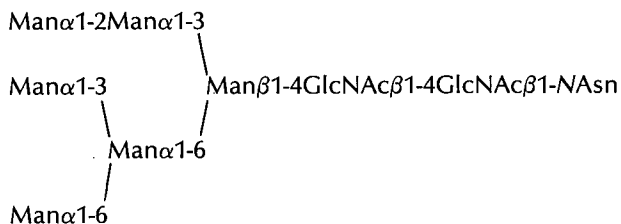


Figure 3. Structures of the glycoasparagines isolated from kidney bean glycoprotein II and 7S soybean glycoprotein.

Acknowledgements

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