Separation of the β -Aspartamido-carbohydrate Fractions from Soybean 7S Protein: Protein-Carbohydrate Linkage

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Using a Dowex 50-X2 column and eluting with a 1 mM sodium acetate buffer (pH 2.6), three L- β -aspartamido-carbohydrate (Asn-carbohydrate) fractions have been isolated in 19% yield from the pronase digest of a 7S soybean protein. The Asn-carbohydrates were composed of one asparagine, two glucosamine and seven, eight and nine mannose residues. Further, 1-L- β -aspartamido-2-acetamido-1,2-dideoxy- β -D-glucose (Asn-GlcNAc), which is derived from the protein-carbohydrate linkage, was produced by partial hydrolysis of the Asn-carbohydrates.

Koshiyama has reported that a 7S protein in soybean is a glycoprotein^{1,2)} containing 3.8% mannose and 1.2% glucosamine. Pronase digestion of the 7S protein afforded a glycopeptide fraction that eluted from columns of Sephadex G-25 and Dowex 50-X2 as a single peak. The glycopeptide fraction, composed of 7 amino acid and 39 mannose residues, moved as a single spot on high-voltage electrophoresis and gave aspartic acid as the only amino-terminal residue.³⁾

However, the homogeneity of the carbohydrate and polypeptide chains have not been determined. This paper describes the isolation of three Asn-carbohydrates and Asn-GlcNAc.

MATERIALS AND METHODS

Protein sample. A soybean 7S protein was prepared by the method depending on pH and concentration of the tris-buffers for fractionation and purified on Sepharose 6B according to Thanh *et al.*⁴⁾ The 7S protein showed pure material by the ultracentrifuge analysis.

***Streptomyces griseus* proteinase.** "Pronase P" was obtained from Kaken Chemicals Co., Tokyo, Japan. The enzyme was partially purified according to the procedure described by Murachi *et al.*⁵⁾

Analytical methods. Hexose was analyzed by a phenol-sulfuric acid method.⁶⁾ Glucosamine was

determined with a Hitachi KLA3B amino acid analyzer after hydrolysis with 4 N hydrochloric acid at 105°C for 4 hr. The reducing value was determined by the method of Park and Johnson⁷⁾ with N-acetyl glucosamine as a standard. The ninhydrin reaction was carried out according to Yemm and Cocking.⁸⁾ Analysis for free amino groups was made with trinitrobenzene sulfonic acid (TNBS) using glycine as a standard and the procedure of Satake *et al.*⁹⁾ Amino acid composition was determined with the amino acid analyzer after hydrolysis with 6 N hydrochloric acid at 105°C for 15 hr.

Paper chromatography. Paper chromatography was carried out with the following solvents. Solvent A, *n*-butanol-acetic acid-water (4: 1: 1, by vol.); solvent B, phenol-water (4: 1, by weight); solvent C, *n*-butanol-acetic acid-water (10: 3.2: 10, by vol.).

Avicel thin-layer chromatography. Thin-layer plates were made with Avicel (a microcrystalline cellulose), and developed with solvent D, *n*-butanol-acetic acid-water (3: 1: 1, by vol.) and solvent E, phenol-water (3: 1 by weight).

Paper electrophoresis. High-voltage electrophoresis was carried out with a buffer of formic acid-acetic acid-water (50: 150: 800, by vol., pH 1.5) on a sheet of Toyo No. 51 filter paper (40 cm in length) at 3000 V for 90 min with cooling in *n*-hexane.

Gas-liquid chromatography. Gas-liquid chromatography, according to the procedure of Lamkin and Gehrke,¹⁰⁾ was used for the analysis of amino acids derivatized with *N*-trifluoroacetyl *n*-butyl ester. This method was also found to detect neutral sugars and

hexosamines with prolonged butanolysis. About 1~5 mg of a hydrolyzate of glycopeptide was treated with anhydrous 1.2 N methanolic hydrogen chloride at room temperature for 30 min, followed by anhydrous 1.2 N butanolic hydrogen chloride at 105°C for 4 hr. The resulting butyl esters were trifluoroacetylated at 100°C for 1 hr.

The analysis was carried out on a Yanagimoto chromatograph, model GC-4APTF fitted with a flame ionization detection. Two columns were used. Column A (a glass tube, 4 mm $\times$ 1.0 m) was packed with 1.5% OV-17 on Chromosorb G (80~100 mesh) and operated at an initial temperature of 80°C, increasing at the rate of 6°C/min to 200°C. Column B (a glass tube, 4 mm $\times$ 1.5 m) was packed with 0.325% ethylene glycol adipate on Chromosorb G (80~100 mesh) and operated at an initial temperature of 80°C, increasing at the rate of 4°C/min to 200°C.

Amino-terminal analyses. The amino-terminal residue was determined by dinitrophenylation (DNP) according to the method of Sanger and Thompson¹¹⁾ and dansylation (DNS) according to the method of Gray and Hartley.¹²⁾ The derivatives resulting from both methods were hydrolyzed with 6 N hydrochloric acid at 105°C in a sealed tube for 13 hr. DNP-amino acids were separated by two-dimensional thin-layer chromatography on a silica gel G plate with solvents of F, toluene-pyridine-ethylene chlorohydrin-0.8 N ammonium hydroxide (100:30:60:60, by vol.) and G, chloroform-benzyl alcohol-acetic acid (70:30:3, by vol.). DNS-amino acids were analyzed by one-dimensional thin-layer chromatography on silica gel G with solvents of H, benzene-pyridine-acetic acid (16:4:1, by vol.) and I, *n*-butanol-acetic acid-water (50:1:50, by vol.).

RESULTS

Preparation of glycopeptide

A purified 7S protein (500 mg, 3.86% of mannose and 1.02% of glucosamine) was digested with the purified pronase. The glycopeptide fragments were separated on a column of Sephadex G-50 followed by Dowex 50-X8 and Sephadex G-25 in a manner similar to the procedure of Koshiyama.³⁾ The resulting glycopeptides were treated twice with pronase, each digestion being followed by fractionation on a column of Sephadex G-25. The yield of the glycopeptide was 19.2 mg and the recovery of carbohydrate was 65.0% of that originally present in the protein.

For a large-scale preparation, the following

method was carried out using the 7S protein. The protein (10.0 g) was dissolved in 1 liter of 0.05 M Tris-hydrochloric acid buffer (pH 8.0) and denatured in a boiling bath for 10 min. The protein suspension was digested initially with 200 mg of pronase P at 40°C, and after 24 hr a further 100 mg of pronase was added. After incubation for a total period of 72 hr, followed by filtration, the digest was evaporated *in vacuo* to about 80 ml, and passed through a column of Dowex-X8 (H⁺ form, 4.5 $\times$ 15 cm). The glycopeptide was eluted with water and the eluate was neutralized with 60 ml of Amberlite IR-4B (CO₃²⁻ form). The glycopeptide solution was evaporated *in vacuo* and the residue was dissolved in the same buffer as described above (1% concentration), and the second pronase digestion (enzyme: substrate = 1:10) was carried out for 24 hr at 40°C. The digestion mixture was passed through a column of Sephadex G-50 (3.8 $\times$ 100 cm) and eluted with 0.1 M acetic acid.

The glycopeptide fraction, detected with phenol-sulfuric acid was further purified of Sephadex G-25 (3.0 $\times$ 120 cm), eluting with 0.1 M acetic acid, was subjected to two further digestions with pronase P (enzyme: substrate = 1:10), followed by filtrations as described above. The glycopeptide fractions were lyophilized to yield average of 180 mg pre-preparation, the recovery of mannose being 47% of that present in the 7S protein.

Separation of Asn-carbohydrates

A column (0.9 $\times$ 150 cm) of Dowex 50-X2 (Na⁺ form), equilibrated with 1 mM sodium acetate buffer at pH 2.6, was prepared according to the method of Huang *et al.*¹³⁾ A 20 mg sample of the glycopeptide was separated with 600 ml of 1 mM sodium-acetate buffer (pH 2.6) and 200 ml of 0.05 M sodium-acetate buffer (pH 6.0) into 6-ml fractions.

The results are shown in Fig. 1. Both glycopeptides prepared by the small and large scale preparations gave practically identical peaks. Solution of each peak were treated with Amberlite IR-4B (CO₃²⁻ form)

FIG. 1. Fractionation of Glycopeptides from the Pronase Digest of Soybean 7S Protein.

Column; Dowex 50 W-X2. Elution; initially with sodium acetate buffer, pH 2.6, 1 mM in Na^+ and sodium acetate, pH 6.0, 0.05 M in Na^+ at the elution volume of 600 ml.

batchwise, and then passed through Sephadex G-25. The carbohydrate-positive fractions were lyophilized. The total recovery of mannose from the fractionation procedure was 94%, in which the ratio based on the mannose contents of I, IIa, IIb, IIc and III were 7.6, 7.6, 5.2, 6.2 and 74.3%, respectively.

Detection of components by thin-layer chromatography

For the detection of amino acids, about 1 mg of each peak was subjected to hydrolysis with 6N hydrochloric acid at 105°C for 15 hr. After evaporation of the hydrochloric acid, two dimensional thin-layer chromatography on an Avicel plate was carried out with solvents D and E. Ninhydrin was used for detection of the amino acids and hexosamine. Peak I showed only a glucosamine, and Peak II showed both glucosamine and aspartic acid. Peak III gave glycine, threonine, alanine, valine and leucine (trace), together with aspartic acid and glucosamine.

For detection of the sugars, about 1 mg of each peak was hydrolyzed with 1 N hydro-

chloric acid at 100°C for 3 hr. After neutralization with Amberlite IR-4B (CO_3^{2-} form), two dimensional thin-layer chromatography on an Avicel plate was performed using the same solvents as for the amino acids. Detection with aniline hydrogen phthalate showed mannose and glucosamine in all peaks.

Therefore, the peaks IIa, IIb and IIc contained Asn-carbohydrates.

Detection of components in the Asn-carbohydrates by gas-liquid chromatography

Gas-liquid chromatography of peak IIa are shown in Fig. 2. Peak IIb and IIc gave

FIG. 2. Gas-liquid Chromatogram of the Standard Mixture (a) and the Component Sugars of Peak IIa (b), as Trifluoroacetyl *n*-Butyl Ester Derivatives.

Column; 1.5% OV-17 on chromosorb G; linear temperature programming (6°C/min) from 80° to 200°C.

practically the same chromatograms which included aspartic acid, glucosamine and mannose. These results agreed with those from thin-layer chromatograms. Chromatography on column B clearly confirmed glucosamine (relative retention time to ornithine, 0.89) as the hexosamine component, being distinguished from a galactosamine (relative retention time, 0.96).

Paper chromatography and electrophoresis of the Asn-carbohydrates

The peak II and III were subjected to paper chromatography by double ascents. The components were detected with alkaline silver nitrate as shown in Fig. 3. Peaks IIa, b and

FIG. 3. Paper Chromatogram of Glycopeptides from Soybean 7S protein.

Solvent; *n*-butanol-acetic acid-water (10:3.2:10, by vol.), double ascent technique; detection by alkaline-silver nitrate. (1), isomalto-oligosaccharides from glucose (top) to isomalto-octasaccharide; (2) IIa; (3), IIb; (4), IIc; (5), III.

c showed a single spot and the different migration from peak III.

Figure 4 shows a high-voltage electrophoretogram. Alkaline silver nitrate was used again for detection. Peak IIa, b and c gave a single spot.

Quantitative analysis of the isolated fractions

Aspartic acid and glucosamine were analyzed with an amino acid analyzer after hydrolysis. The molar ratio of glucosamine was determined with reference to one mole of aspartic acid. The molar ratio of glucosamine to aspartic acid was nearly two to one.

The molar ratios of mannose, free amino group and reducing equivalent were calculated with reference to one mole of aspartic acid using the methods described above. These results are shown in Table I.

Each glycopeptide contained one mole equivalent of aspartic acid. The values from

FIG. 4. Paper Electrophoretogram of the Glycopeptides from Soybean 7S Protein.

With buffer of formic acid-acetic acid-water (50:150:800, by vol. pH 1.5) at 75 V per cm for 90 min. (1), IIa; (2), IIb; (3), IIc; (4), III and (5), Asn-GlcNAc.

TABLE I. COMPOSITION OF THE ISOLATED FRACTIONS

Values (*) are expressed as molar ratios relative to two glucosamine residues. The other values are expressed as molar ratios with respect to aspartic acid.

	I	IIa	IIb	IIc	III
Aspartic acid	—	1.0	1.0	1.0	1.0
Glucosamine	2.0*	1.7	1.7	1.7	2.1
Mannose	7.7*	8.7	7.8	6.7	8.5
Free amino group	0.2*	1.1	1.1	1.1	0.8
Reducing equivalent	0.9*	0.0	0.0	0.0	0.1

the amino acid analyzer and the TNBS procedure were in agreement. Peak I had little free amino group, but approximately one reducing group per mole. No reducing group was detected in peaks IIa, b and c. Compared to one mole of aspartic acid, the mannose ratio was 8.7, 7.8 and 6.7 mole in peaks IIa, IIb and IIc.

Separation of Asn-GlcNAc

Twenty mg of the Asn-carbohydrate (a mixture of Peak IIa, b and c) was hydrolyzed partially with 2 N hydrochloric acid for 20 min

at 100°C,¹⁴⁾ followed by removal of hydrochloric acid with Amberlite IR-4B (CO₃²⁻ form). The hydrolyzate was passed through a column (1.0×60 cm) of Dowex 50-X4,¹⁴⁾ 200~400 mesh, which had been equilibrated with 0.15 M pyridine, adjusted to pH 5.0 with acetic acid and elution was carried out with the same buffer. The first peak, eluted from the column and checked by ninhydrin and phenol-sulfuric acid methods, was further purified on paper chromatography with solvents A and B. Elution from the paper gave a 450 µg of a single spot, calculated by analysis of the glucosamine.

Identification of Asn-GlcNAc

The isolated Asn-GlcNAc was identified as follows. 1) After hydrolysis with 1 N hydrochloric acid at 105°C for 3 hr, paper chromatography of the hydrolyzate showed aspartic acid and glucodamine, but no mannose. Hydrolysis with 4 N hydrochloric acid at 105°C for 4 hr gave a molar ratio of aspartic acid: glucosamine, 1.0:0.9 by the amino acid analyzer. 2) Paper chromatography of the Asn-GlcNAc with solvent A and B showed the same mobility as that of the authentic sample, kindly supplied by Dr. Yamashina. The spot showed a characteristic color of bluish purple, identical to the authentic one on reaction with ninhydrin.¹⁴⁾ 3) Thin-layer chromatography of DNS-Asn-GlcNAc using solvent F and G showed the same mobility as that of the authentic sample which was detected by ultraviolet light. 4) Paper electrophoresis at pH 1.5 gave the same migration towards the cathode as that of the authentic compound (Fig. 4).

DISCUSSION

The glycopeptides, isolated by the same method as Koshiyama, were further separated to Asn-carbohydrates (peaks IIa, b and c) and glycopeptides (peak III) (see Fig. 1). The higher concentration of buffer, such as in 0.2 M on a column of Dowex 50, gave one peak of a mixture of glycopeptides. However, Asn-carbohydrates have been separated at the

low concentration (1 mM) of buffer. The content of Asn-carbohydrates in the original pronase digest was about 20%.

There were at least three kinds of Asn-carbohydrate (see Fig. 1) that may have been due to paucidisparity of the carbohydrate group.¹³⁾ They have two moles of glucosamine and seven, eight and nine moles of mannose in each residues in the Asn-carbohydrate. When the mole of mannose is taken to be seven to nine in combination with two moles of N-acetylglucosamine, the molecular weight of the carbohydrates are 1,558 to 1,882, respectively. Since the molecular weight of 7S protein has been reported 180,000~210,000¹⁵⁾ and the carbohydrate is 5%, it may be calculated five or six carbohydrate groups are present in one mole of 7S protein.

Isolation of the Asn-glucosamine from partial hydrolyzate demonstrated the protein-carbohydrate linkage is typical of those in many glycoproteins. In soybean, a hemagglutinin has been shown to be a glycoprotein¹⁷⁾ and have the same protein-carbohydrate linkage¹⁸⁾ as that of the soybean 7S protein. Several other glycoproteins have been isolated from plant sources. However, these chemical structures remain to be studied in detail.

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