AMINO ACID SEQUENCE OF GLYCOPEPTIDES OBTAINED FROM CUCUMISIN WITH NAGARSE

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AMINO ACID SEQUENCE OF GLYCOPEPTIDES OBTAINED FROM CUCUMISIN WITH NAGARSE

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Abstract

Cucumisin contains 2.0% carbohydrate as a part of enzyme molecule. Even by gel-filtration on Sephadex G-75 and polyacrylamide disc gel electrophoresis, the carbohydrate moiety could not be separated from the enzyme protein. Glycopeptides were obtained by digestion of cucumisin with nagarse and isolated by a gel-filtration on Bio-Gel P-4 and paper electrophoresis. The three glycopeptides (Ⅰ, Ⅱ, and Ⅲ) were isolated. It was verified that the asparagine residue is the site of attachment of the carbohydrate chain in each peptide. Their amino acid sequences were established: Asn(sugar)-Ala-Ser for peptide Ⅰ and Ⅱ, Asn (sugar)-Arg-Thr for Ⅲ.

Introduction

Among the plant proteases, the carbohydrate chain and its binding site in carbohydrate-containing protease has not been sufficiently studied, except for stem bromelain (*1-3*). Cucumisin [EC 3.4.21.25] is a serine protease isolated from the sarcocarp of melon (*4*). It was proven that the four amino acid sequence, Gly-Thr-Ser-Met, around the reactive serine of cucumisin (*5*) is identical with that of subtilisin which is microbial serine protease (*6-8*). Among proteases of plant derivation, cucumisin is unique because typical plant proteases so far isolated have belong mainly to the thiol protease group. As glucosamine had been detected when amino acid analysis was carried out with acid hydrolysate of cucumisin, it was presumed that cucumisin was a glycoprotein. Further, it became apparent that the purified enzyme preparation contained 2% neutral sugar using D-mannose as a standard.

In this paper we present the isolation of glycopeptides from cucumisin and the determination of their amino acid sequences. In addition, it is shown that asparagine residue is the site of the attachment of carbohydrate chain. A preliminary account of this work has been published (*17*).

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Experimental

Materials-Cucumisin was isolated from Prince melon, *Cucumis melo* L. var. Prince, by the method previously described (*4*). Nagarse, a proteinase from *Bacillus subtilis*, was obtained from Teikoku Chemical Industry. Polybrene was obtained from Aldrich Chemical Co.

Chromatography on Sephadex G-75-Cucumisin was chromatographed on a column (2.5 × 120cm) of Sephadex G-75 equilibrated with 0.1 M acetic acid. Cucumisin in the effluent fractions was detected by measurement of the absorbance at 280nm. Proteolytic activity was assayed against casein at pH 7.8 as previously described (*4*). The carbohydrate content was determined by the phenol-sulfuric acid method of Dubois *et al.* (*9*) with D-mannose as a standard.

Disc Electrophoresis-Electrophoreses in polyacrylamide gel were performed in 7.5% gel with acetate buffer, pH 4.0. Each protein was stained with 1% Amino Black 10B in 7% acetic acid. The carbohydrate moiety was stained with Schiff's reagent after periodic acid oxidation as described by Zacharius *et al.* (*10*).

Paper Electrophoresis-High voltage paper electrophoresis was carried out with Toyo No. 51 paper at pH 3.6 (pyridine-acetic acid-water, 1:10:89) and at pH 6.5 (pyridine-acetic acid-water, 5:0.2:95) both at 2,000 volts for 70～100 min. o-Phthalaldehyde was used for the detection of peptides on paper chromatogram.

Enzymatic Hydrolysis of Cucumisin-Cucumisin (1.2g) was denatured by heating at 100°C for 7 min and then cooled. To the resultant suspension of the coagulated protein were added solid ammonium bicarbonate to 0.1 M and 120 mg of nagarse. The mixture was incubated at 37°C for 25 h and then lyophilized.

Isolation of Glycopeptide-The lyophilized enzymatic hydrolysate was dissolved in 0.1 M acetic acid and centrifuged to remove insoluble materials. The supernatant was applied to a column (2.0 × 135cm) of Bio-Gel P-4 previously equilibrated with 0.1 M acetic acid.

Amino Acid Analysis and Amino Acid Sequence Determination-Amino acid composition of glycopeptide was analyzed with an amino acid analyzer, model JLC-5AH (JAPAN ELECTRON OPTICS Ltd.), after hydrolysis in constant boiling HCl at 110°C for 24 h. Sequence analysis of glycopeptide was performed by the manual Edman method (*11*) with the exception that Polybrene was added to the reaction tube. For confirmation, the amino acids released were identified by the subtractive method (*12*).

Analysis for Carbohydrate-The glycopeptide was hydrolyzed in 1 N H_2SO_4 at 100°C for 6 h. To remove the sulfate ion Dowex 1-X2 carbonate form was added to the hydrolysate. The resulting solution was lyophilized. Quantitative analysis of neutral sugars was performed using a column of Dowex 1-X4 by the method of Walborg, Jr. and Christensson (*13,14*). After acid hydrolysis (4 N HCl, 100°C, 6～8 h) of glycopeptide, hexosamine was estimated with the amino acid analyzer mentioned above.

Alkali Treatment of Glycopeptide-It is well known that o-glycosidic linkages to serine or threonine are alkalisensitive (*15*). Treatment with mild alkali causes cleavage of the

glycosidic bond by β-elimination and simultaneously, conversion of threonine to α-aminocrotonic acid and serine to dehydroalanine. The glycopeptides were treated with a solution of 0.16 N NaOH, contained 0.3 M $NaBH_4$, at 25°C for 24 h.

Results and Discussion

Evidence for the Presence of a Carbohydrate Moiety in Cucumisin-The sample of cucumisin proved to contain carbohydrate amounting to 2.0% using mannose as a standard, when the content was analyzed directly by the phenol-sulfuric acid method. The ratio of the carbohydrate to protein in cucumisin did not decrease upon repeated gel filtration on Sephadex G-75. The elution profile of protein was completely coincident with that of the carbohydrate as shown in Fig. 1. Furthermore, after polyacrylamide disc gel electrophoresis, the band corresponding to cucumisin was stained by Schiff's reagent as shown in Fig. 2. These results indicate that cucumisin contains a carbohydrate moiety covalently bound to the protein.

Isolation and Homogeneity of the Glycopeptides-The heat-denatured cucumisin was digested completely with nagarse. The resulting lyophilized hydrolysate was dissolved in

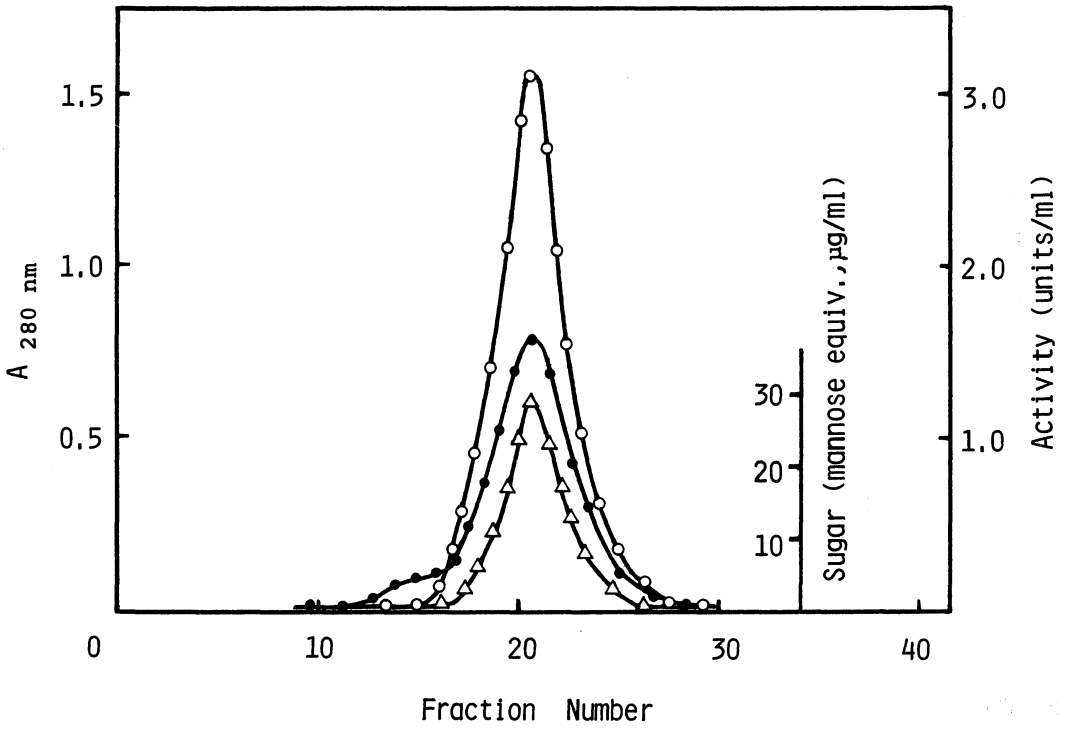

Fig. 1. Gel filtration of cucumisin on Sephadex G-75.
A column (2.5 × 120cm) was equilibrated and eluted with 0.1 M acetic acid. Absorbance at 280 nm (●); cucumisin activity (○) was assayed against casein; carbohydrate content (△) was measured by the phenol-sulfuric acid method (*9*).

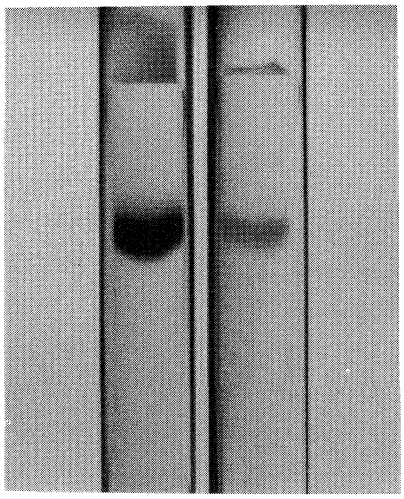

Fig. 2. Polyacrylamide disc gel electrophoresis of cucumisin.
About 100 μg of enzyme was applied to 7.5% polyacrylamide gel containing acetate buffer (pH 4.0) and electrophoresed at 18°C for 2.0 h at 3 mA per tube. Separate gels run in the same conditions were stained for protein with 1% Amino Black 10B in 7% acetic acid, and for carbohydrate with Schiff's reagent after periodic acid oxidation. PAS, periodic acid-Schiff's reaction.

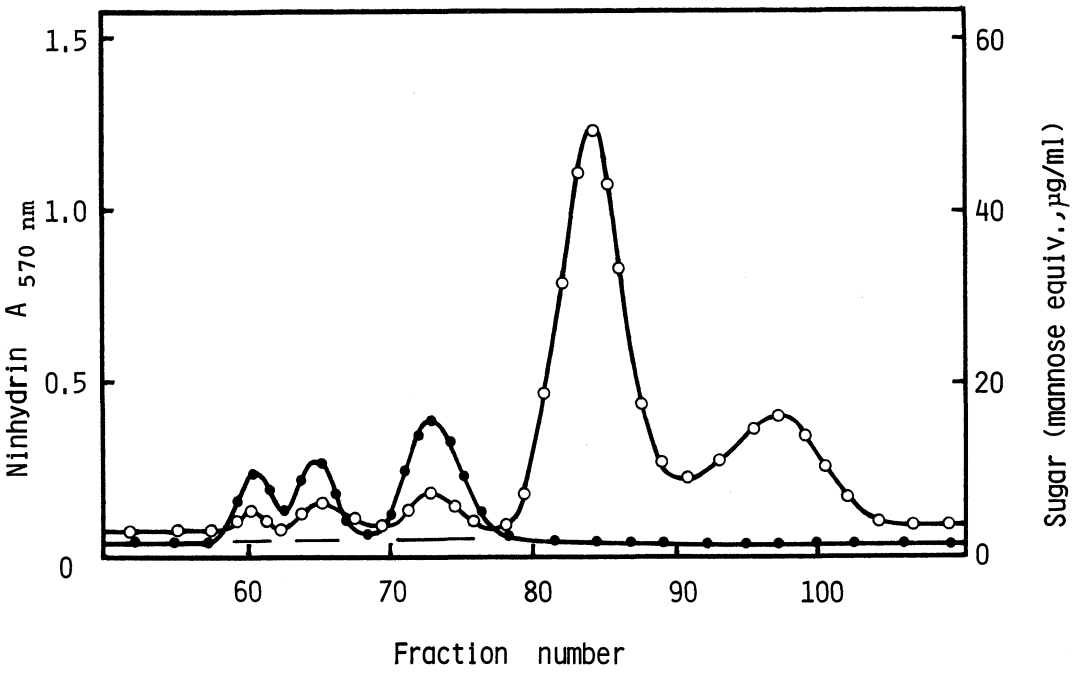

Fig. 3. Gel filtration of an enzymatic digest of cucumisin on Bio-Gel P-4.
A column (2.0 × 135cm) was equilibrated and eluted with 0.1 M acetic acid. The fraction size was 5.0 ml. Carbohydrate content (●); ninhydrin reaction after alkaline hydrolysis (○). Fractions were pooled as indicated the solid bars.

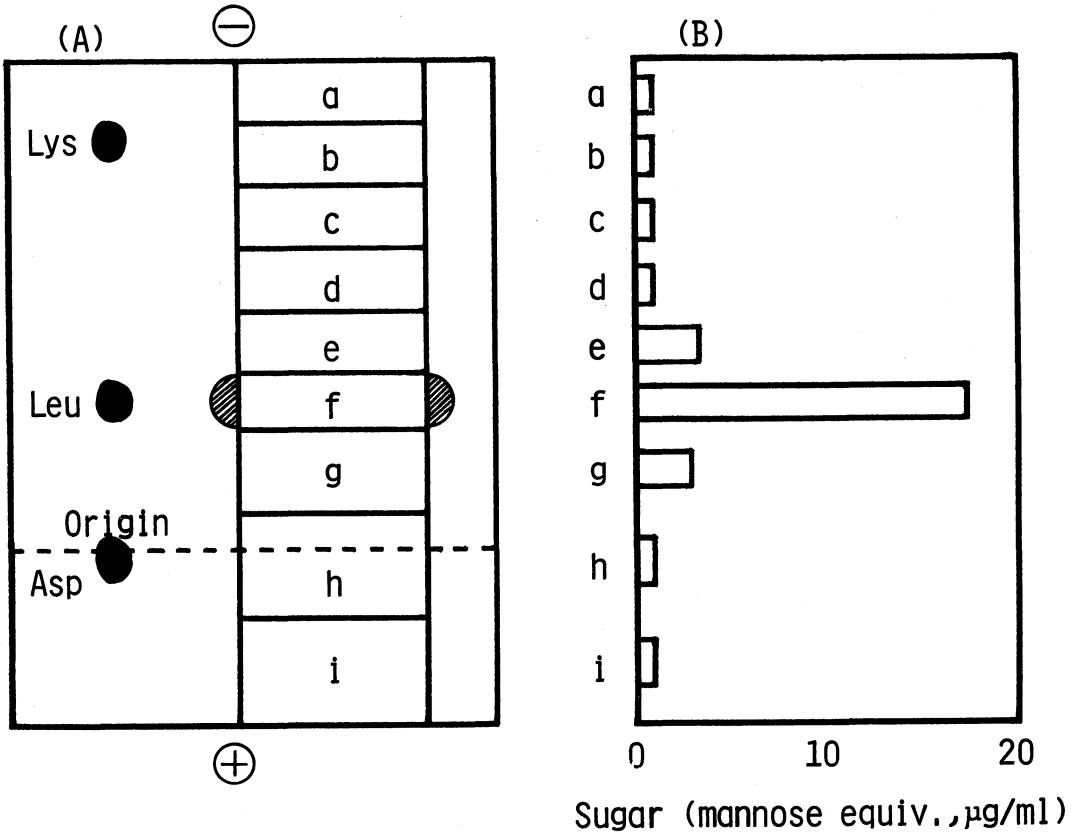

Fig. 4. Tracing of electrophoretograms of the Fraction I (A) and carbohydrate analysis of each strip (B). (A) Electrophoresis was carried out at pH 3.6, at 2,000 volts for 80 min. The positive staining of o-phthalaldehyde is indicated with hatched areas. (B) Extracts from each strip were used for determination of carbohydrate contents by the phenol-sulfuric acid method. A similar result was also obtained for fraction II.

0.1 M acetic acid and then centrifuged to remove insoluble materials. The supernatant was applied to a gel filtration on a Bio-Gel P-4 column with 0.1 M acetic acid as eluent. As shown in Fig. 3, three carbohydrate-containing fractions were detected in front of large peaks and pooled respectively. The recovery of carbohyrate as determined by the phenol-sulfuric acid method was 66.5% of the original protein: fraction I (12.4%), II (13.9%), and III (40.2%).

The isolated glycopeptides were examined for homogeneity on high voltage paper electrophoresis. The pooled carbohydrate-containing fractions were applied linearly on an origin line of a paper sheet, and then electrophoreses were carried out at pH 3.6, at 2,000 volts for 80 min. After electrophoresis, guide strips were cut out on both edges and stained with o-phthalaldehyde. The peptides were located on the unstained paper sheet by the aid of guide strips (Fig. 4A), and the unstained paper sheet was cut out parallel to the origin line at appropriate intervals as shown in Fig. 4A. The migrated materials were extracted from

each strip with 0.01 M acetic acid. Aliquots of each extract were examined for carbohydrate content by the phenol-sulfuric acid method. The carbohydrate content of each extracts was shown in Fig. 4B. Fraction I and II gave a single band with the same mobility as detected with the o-phthalaldehyde and phenol-sulfuric acid method as shown in Fig. 4A and 4B.

Therefore, glycopeptide I and II were found to be already single with gel filtration on Bio-Gel P-4. But an electrophoretogram of the fraction III indicated three peptide bands (data not shown). Only one band contained carbohydrate among them. Glycopeptide III was obtained after the second electrophoresis of this carbohydrate-containing band. The second electrophoresis carried out pH 6.5, at 2,000 volts for 75 min.

Compositions of Glycopeptides-The isolated glycopeptides were dissolved in a respectively appropriate, small volumes of water. An exactly measured aliquot of each solution was hydrolyzed according to the respective method to analyze the three components: amino acid, hexosamine, and neutral sugar. Amino acid and hexosamine contents were estimated with the amino acid analyzer, and neutral sugars were performed by ion-exchange column chromatography using D-mannose and D-fucose as a standard. The rates of recovery on hydrolysis were calculated by the data of the following materials which were hydrolyzed in the same conditions. The individual recoveries were as follows: hen egg white lysozyme (95%) for amino acid, N-acetylglucosamine (82%) for hexosamine, and D-mannose (90%) for neutral sugars. The compositions of these three glycopeptides are shown in Table I. The correlated values of the three components in Table I were calculated on the basis of

Table I. Composition of glycopeptides

Residues	Number of residues per molecule of glycopeptide[a]		
	Glycopeptide		
	I	II	III
Asp	1.00 (1)	1.00 (1)	1.00 (1)
Thr			0.91 (1)
Ser	0.81 (1)	0.84 (1)	
Ala	0.95 (1)	0.90 (1)	
Arg			1.10 (1)
Glucosamine	2.87 (3)	2.18 (2)	2.05 (2)
Mannose	1.76 (2)	1.75 (2)	1.65 (2)
Fucose	0.71 (1)	0.78 (1)	0.69 (1)

[a]The number of residues was calculated assuming the number of aspartic acid to be 1.00 per molecule of glycopeptide.

the used volumes of the glycopeptide solutions for the respective analyses. These glycopeptides (Ⅰ, Ⅱ, and Ⅲ) were all presumed to be composed of tripeptide because after one cycle of Edman degradation these peptides became their respective dipeptides. The details of the Edman degradation are described later. The compositions of glycopeptide Ⅰ and Ⅱ are the same except for a difference in glucosamine content. However, it is not certain whether the difference was significant or was due to experimental error. Glycopeptide Ⅲ is distinct from Ⅰ and Ⅱ. A remarkable difference is observed in the peptide moiety. Aspartic acid residue is the only component these three glycopeptides have in common.

Characterization of the Threonine and Serine Residues in the Glycopeptides- These glycopeptides contained two kinds of amino acid attachable to carbohydrate. Glycopeptide Ⅰ and Ⅱ had asparagine and serine residues, and Ⅲ had asparagine and threonine. The amino acid analyses of glycopeptides showed that the values of serine in Ⅰ and Ⅱ and of threonine in Ⅲ did not vary before and after the mild alkali treatment. Therefore, it was confirmed that the o-glycosidic linkages in these glycopeptides were not present.

Sequence Analysis- Amino acid sequence of the glycopeptide I was established by the Edman method. After each Edman degradation step, the amino acid composition of the residual peptide was established. In order to obtain the residual peptide from the first step, after trifluoroacetic acid treatment the reaction mixture which contained a released PTH-amino acid and the residual peptide was applied to paper electrophoresis according to the method shown in Fig. 4A and 4B. The residual peptide which contained a new, free α-amino group was detected by o-phthalaldehyde and recovered from the unstained strip by the method mentioned above. The residual peptide after the first step was composed of equimolecular amounts of serine and alanine. Therfore, it was estimated that aspartic acid was removed by the first step of Edman degradation. The aspartic acid was deduced to be N-terminal amino acid of the glycopeptide Ⅰ. As shown in the second step of Table Ⅱ, the phenol-sulfuric acid reaction was negative when the aspartic acid was released from the

Table II. The amino acid sequence of glycopeptide I. Residues per mol calculated on the basis of one serine. Carbohydrate was detected by the phenol-sulfuric acid method.

	1	2	3		
	Asn(sugar)-	Ala	———	Ser	
Amino acid composition :	1.2	1.1	1.0		
Edman degradation :				Carbohydrate	PTH derivative identified by TLC
0 cycle	1.2	1.1	1.0	+	
1st cycle	0	1.0	1.0	—	n.d.
2nd cycle	0	0.1	1.0		Ala

n.d. , not determined.

Table III. The amino acid sequence of glycopeptide II. Residues per mol calculated on the basis of one serine. Carbohydrate was detected by the phenol-sulfuric acid method.

	1	2	3		
	Asn(sugar)-	Ala	———	Ser	
Amino acid composition :	1.0	1.1		1.0	
Edman degradation :				Carbohydrate	PTH derivative identified by TLC
0 cycle	1.0	1.1		1.0	+
1st cycle	0	1.2		1.0	— n.d.
2nd cycle	0	0.1		1.0	Ala

n.d., not determined.

Table IV. The amino acid sequence of glycopeptide III. Residues per mol calculated on the basis of one threonine. Carbohydrate was detected by the phenol-sulfuric acid method.

	1	2	3		
	Asn(sugar)-	Arg	———	Thr	
Amino acid composition :	1.2	1.1		1.0	
				Carbohydrate	PTH derivative
0 cycle	1.2	1.1		1.0	+
1st cycle	0	1.1		1.0	— n.d.[a]
2nd cycle	0	0.1		1.0	Arg[b]

[a] Not determined. [b] Identified as ornithine by amino acid analyzer after hydrolysis with 0.1 N NaOH.

glycopeptide I. Consequently, this aspartic acid, actually asparagine in nature, was identified as the site of carbohydrate attachment. The amino acid sequence of glycopeptide I was established as Asn(sugar)-Ala-Ser. As shown in Table III, that of glycopeptide II was also established in the same manner as Asn(sugar)-Ala-Ser which is cleary identical to I. The residual peptide of glycopeptide III at the first step migrated nearly to the standard lysine(data not shown). This peptide was composed of equimolecular amounts of threonine and arginine. The carbohydrate of glycopeptide III was also removed by the first Edman degradation. The amino acid sequence of glycopeptide III was established as Asn(sugar)-Arg-Thr as shown in Table IV. Consequently, two kinds of amino acid sequence were identified. Glycopeptide I and II seem to be derived from the same part of the parent protein. The difference between I and II may be due to the carbohydrate chain.

The sequence-Asn(sugar)-Ala-Ser- and -Asn(sugar)-Arg-Thr- in cucumisin conform with the hypothesis by Neuberger and Marshall*(16)* of the occurrence of the paticular amino acid sequence, -Asn(sugar)-X-Thr/Ser in glycopeptides.

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