

The Amino Acid Sequences of Glycopeptides Obtained from Taka-Amylase A with Trypsin and Chymotrypsin

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Glycopeptides were obtained from reduced and carboxymethylated Taka-amylase A [EC 3.2.1.1] by digestion with trypsin and chymotrypsin and purified, and their amino acid sequences were determined. The sequence of a glycopeptide obtained with trypsin, T1, was deduced to be Asn-Glu-Trp-Tyr-Asp-Trp-Val-Gly-Ser-Leu-Val-Ser-Asn(CHO)-Tyr-Ser-Ile-Asp-Gly-Leu-Arg. This satisfies the hypothesis that the asparagine linked to the N-acetylglucosamine of the carbohydrate group is followed by residue X and then by a threonine or serine residue.

Taka-amylase A (TAA) [EC 3.2.1.1] is known to contain one carbohydrate group linked to asparagine by a glycosylamine-type linkage (1). Recently, Yamaguchi *et al.* (2-5) determined the complete carbohydrate sequence of a glycopeptide obtained from TAA using the O-methylation method, partial acetolysis and periodate oxidation.

Two hypotheses have been proposed with regard to the components of glycopeptides. One is that the amino acid sequence, Asn-X-Thr/Ser is characteristic of an asparagine-type glycopeptide, where N-acetylglucosamine is linked to an asparagine residue (6). The other is that of Jackson and Hirs (7) that there is some relation between X and the component of the attached carbohydrate unit.

They claimed that if X is hydrophilic, the carbohydrate unit is a heteropolysaccharide containing sialic acid, while if X is hydrophobic, the sugar unit is simpler and contains only mannose and N-acetylglucosamine. Since the composition and the whole carbohydrate sequence of a TAA glycopeptide is known, it seemed of interest to see if these hypotheses were in accord with the present results.

MATERIALS AND METHODS

Materials — Bovine trypsin [EC 3.4.4.4] treated with TPCK, α -chymotrypsin [EC 3.4.4.5], and carboxypeptidases A and B [EC 3.4.2.1 and 3.4.2.2] were purchased from Worthington Biochemical Corp. Carboxypeptidases were used after treatment with diisopropylfluorophosphate. Thermolysin [EC class 3.4.4] was the gift from Dr. Morihara of Shionogi Pharmaceutical Co., Osaka. Thin-layer pre-coated sheets, DC-Fertigplatten Kie-

The abbreviations used in the present paper are: TAA, Taka-amylase A; TPCK, L-(1-tosylamino-2-phenyl)ethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; RCM-, reduced and carboxymethylated; PTH, phenylthiohydantoin.

selgel F-254 were purchased from Merck. The reagents and solvents used for Edman degradation were "sequanal" grade preparations and were exhaustively purified before use. All other chemicals were reagent grade or purer products.

The Taka-amylase A used in the present study was a denatured preparation. During the course of purification, crystalline TAA prepared from Takadiastase by the procedure of Akabori *et al.* (8), was accidentally heated to 40°C in aqueous acetone, so that all the protein must have been denatured before use.

Reduction and S-Carboxymethylation—Reduction and carboxymethylation of TAA were performed by the method of Crestfield *et al.* (9). One gram of TAA, 36.1 g of urea, 3 ml of 5% EDTA, 30 ml of 1.43 M Tris-HCl buffer, pH 8.6, and 1 ml of 2-mercaptoethanol were mixed, and the volume was adjusted to 75 ml with water. Then 8 M urea solution containing 0.2% EDTA was added to a total volume of 120 ml. The solution was kept at room temperature for 4 hr and then 2.68 g of moniodoacetic acid in 10 ml of 1 N sodium hydroxide were added to the reaction mixture. After 15 min reaction in the dark, the reaction mixture was dialyzed and lyophilized.

Amino Acid Analysis—Glycopeptides and peptides were hydrolyzed with 6 N hydrochloric acid in sealed evacuated tubes at 110°C for 18 hr. Analysis was carried out with an automatic amino acid analyzer (Hitachi KLA 3B) by the method of Spackman *et al.* (10). Tryptophan was determined with an automatic amino acid analyzer after hydrolysis with *p*-toluenesulfonic acid for 18 hr according to the method of Liu and Chang (11). A correction was made for destruction of tryptophan during heating in the presence of carbohydrates by measuring the amount remaining in a mixture of tryptophan, mannose, and N-acetylglucosamine in a molar ratio of 1 : 6 : 2 when heated under similar conditions.

Carbohydrate Analysis—Total neutral sugars were determined by the orcinol-sulfuric acid method: An aliquot of 0.2 ml of sample solution was mixed with 0.5 ml of 1.6% orcinol in 50% (v/v) sulfuric acid and 3.0 ml of 66% (v/v) sulfuric acid were added with vigorous

stirring. The mixture was kept at 80°C for 20 min and then cooled in an ice bath and its absorbance was measured at 540 m μ . Mannose was used as a standard.

Gas liquid chromatography of neutral sugars was carried out using a Shimadzu gas chromatograph (Model GC-4BM), equipped with a flame ionization detector. A 0.3 $\times$ 300 cm glass column was packed with 3% OV-17 on Shimalite W (80–100 mesh), and programmed from 150 to 250°C at the rate of 5°C per min. Neutral sugars in an acid hydrolysate (2 N sulfuric acid for 4 hr at 100°C) were separated from amino acids and peptides by passing them through a column of Dowex 50 after removal of sulfate ion using a Dowex 1 (acetate form) column. Methanolysis of neutral sugars was carried out in 0.5 N methanolic hydrogen chloride in a sealed tube at 65°C for 18 hr. Trimethylsilylation of the methanolysate was performed by the method of Sweely *et al.* (12) and inositol was used as an internal standard. Glucosamine was analyzed with an automatic amino acid analyzer after hydrolysis of glycopeptides by treatment with 4 N hydrochloric acid for 4 hr at 100°C.

Detection of Peptides and Glycopeptides—Peptides eluted from the column were detected by measuring the absorbance at 226 m μ or at 280 m μ in a quartz cuvette of 10 mm light path. Glycopeptides were detected by the phenol-sulfuric acid method: In a typical experiment, 0.5 ml of sample was mixed with 0.5 ml of 5% aqueous phenol. Then 3 ml of concentrated sulfuric acid were added with vigorous stirring. The mixture was cooled and its absorbance was measured at 490 m μ (13).

Sequence Determination—Stepwise degradation of the peptides was carried out using Edman's procedure (14). The PTH derivatives were usually identified by thin layer chromatography, but in some cases subtractive Edman degradation procedure was used. The carboxyl-terminal amino acid was determined by the carboxypeptidase method (15).

RESULTS

Digestion of RCM-TAA with Chymotrypsin and Fractionation of the Resulting Glycopeptides—One gram of RCM-TAA dissolved in 100 ml of 0.1 M Tris-HCl buffer (pH 8.2) was digested with 10 mg of chymotrypsin at 37°C for 5 hr. During the digestion the pH was kept at 7.8 by adding 1 N sodium hydroxide. Then the mixture was adjusted to pH 4.5 with 1 N acetic acid and evaporated to dryness under reduced pressure in presence of octyl alcohol and caprylic acid. The residue was dissolved in 5% acetic acid, and insoluble material was removed by centrifugation. The soluble fraction was chromatographed on a Sephadex G-50 column and the fractions giving a positive phenol-sulfuric acid reaction were collected (Fig. 1).

These fractions were chromatographed on a DEAE-cellulose column, as shown in Fig. 2. Three peaks of material giving a positive phenol-sulfuric acid reaction were obtained. Peak I did not contain glycopeptide since the fraction containing carbohydrate obtained from it by gel-filtration contained no amino acid. Thus, this fraction was not studied further. Peak II was purified on a Bio-Gel P-4 column and separated into two peaks designated as C1 and C2 (Fig. 3). One of these was found to be impure by high voltage paper electrophoresis at pH 3.6. The other (C2) was shown to be homogeneous by paper electrophoresis, and was used for sequence analysis. Peak III could not be purified on Bio-Gel P-4 so it was purified by Dowex 50 column chromatography using the method of Schroeder (16). The glycopeptide fraction (C3) separated appeared to be almost pure judging from its amino acid composition.

Digestion of RCM-TAA with Trypsin and Fractionation of the Resulting Glycopeptides—One gram of RCM-TAA was dissolved in 100 ml of water. The solution was adjusted to pH 8 with 1 N sodium hydroxide, and 13.5 mg of TPCK-treated trypsin were added. The mixture was incubated at 37°C for 4.5 hr, keeping the solution at pH 8.0 using a pH stat. Then the reaction mixture was applied

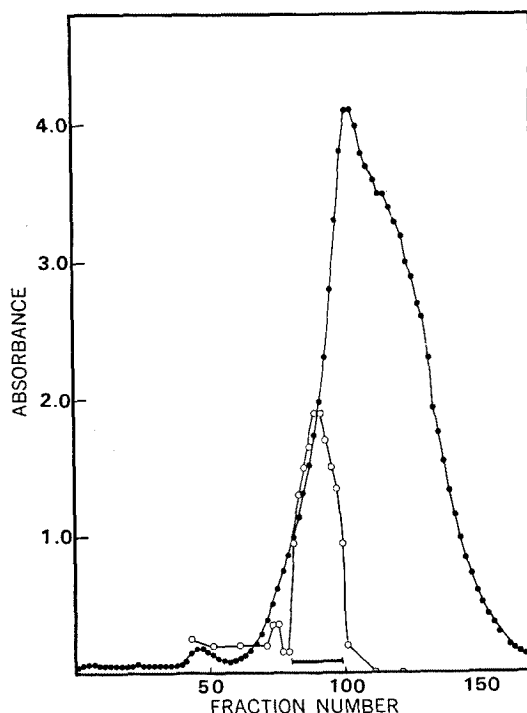


Fig. 1. Gel filtration of glycopeptides of RCM-TAA obtained with chymotrypsin. A digest (1 g) of RCM-TAA with chymotrypsin was chromatographed on a Sephadex G-50 column (3×100 cm) equilibrated with 5% acetic acid. Fractions of 5 ml were collected. Solid points show absorbance at 280 mμ and open circles that at 490 mμ (phenol-sulfuric acid reaction). Fractions indicated by the bar were combined.

on a DEAE cellulose column equilibrated with 0.05 M ammonium acetate buffer, pH 8. The column was eluted with a linear gradient of 0.05 to 2 M acetate, and two peaks containing carbohydrate were obtained, as shown in Fig. 4. Peak A was separated into two peaks on a Sephadex G-50 column (2×100 cm) equilibrated with 0.05 M pyridine. However, pure glycopeptides could not be obtained from either of these peaks. Peak B in Fig. 4 was purified by successive gel filtration on columns of Sephadex G-50, Sephadex G-25 and finally Bio-Gel P-4, as shown in Fig. 5. The purified glycopeptide (T1) seemed to be almost pure as judged by amino acid analysis.

Digestion of Glycopeptide T1 with Chymotrypsin and Fractionation of the Peptides—

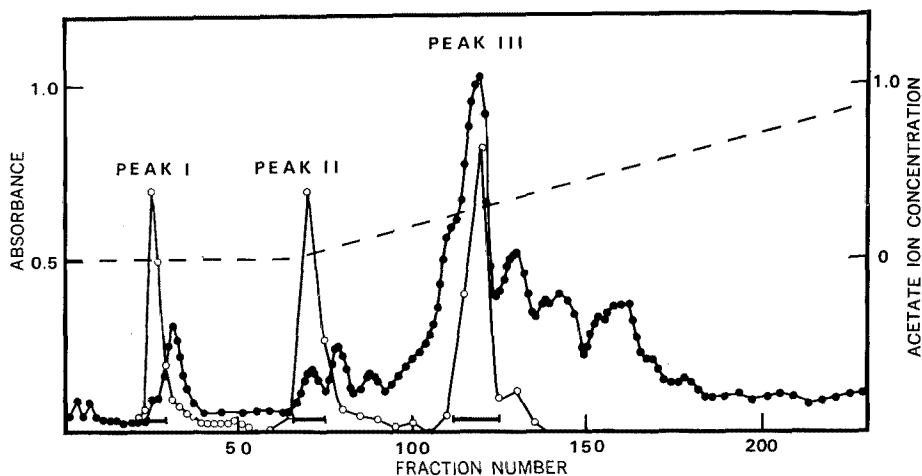


Fig. 2. Chromatogram of glycopeptides obtained with chymotrypsin on a DEAE-cellulose column. The glycopeptide fraction (Fig. 1) was applied to a DEAE-cellulose column (2.4×40 cm) equilibrated with 0.001 M ammonium acetate buffer (pH 5). The column was washed with 280 ml of the starting buffer and then eluted with a linear gradient obtained with 1,000 ml of 0.001 M and of 1 M ammonium acetate buffer (pH 5.0). The first twenty fractions were 10 ml each and the rest were 5 ml each. Solid points show absorbance at $280\text{ m}\mu$ and open circles that at $490\text{ m}\mu$.

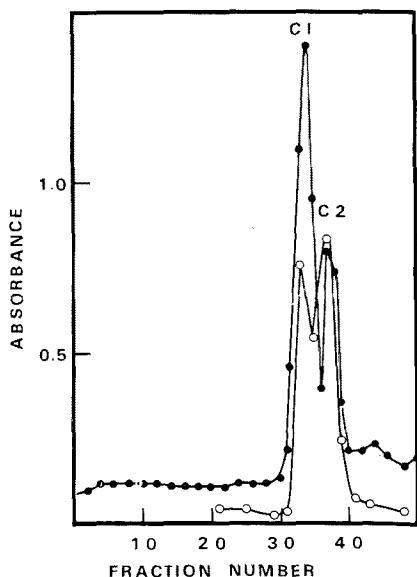


Fig. 3. Gel filtration of Peak II on a Bio-Gel P-4 column. Peak II in Fig. 2 was chromatographed on a Bio-Gel P-4 column (1.2×100 cm) equilibrated with 0.1 M acetic acid. Fractions of 2.5 ml were collected. Solid points show absorbance at $226\text{ m}\mu$ and open circles that at $490\text{ m}\mu$.

Glycopeptide T1 (1.8 mg) was digested with 0.28 mg of chymotrypsin in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.1) for 18 hr at 37°C . Then the mixture was applied on a column of Bio-Gel P-2 (Fig. 6). The first peak containing a glycopeptide (T1-C2) was purified further by gel filtration on a Bio-Gel P-4 column (Fig. 7).

Digestion of Glycopeptide T1 with Thermolysin and Fractionation of the Peptides—Glycopeptide T1 (1.8 mg) was digested with 0.25 mg of thermolysin in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.6) for 4 hr at 37°C . The reaction mixture was chromatographed on a Bio-Gel P-4 column as shown in Fig. 8. Fractions 41–46 in Fig. 8 were collected and chromatographed on a Dowex 1 column (1.3×10 cm) which had been equilibrated with 0.1 M ammonium acetate buffer (pH 8.0). Elution with the same buffer gave a peptide, T1-Th7, and subsequent gradient elution with 50 ml each of this buffer and 2 M acetic acid, gave a peptide, T1-Th1. Fractions 48–53 in Fig. 8 were also purified in a similar way on a Dowex 1 column. A peptide, T1-Th5, was obtained by gradient elution.

Amino Acid and Carbohydrate Composi-

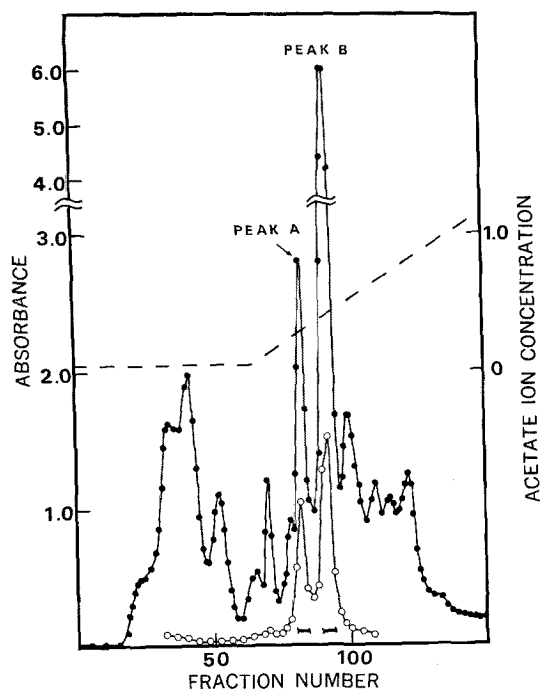


Fig. 4. Chromatogram of a digest obtained with trypsin on a DEAE-cellulose column. A digest (1 g) of RCM-TAA with trypsin was chromatographed on a column of DEAE-cellulose (3×35 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 8.0). The column was washed with 350 ml of the starting buffer and then eluted with a linear gradient obtained with 1,000 ml of 0.05 M and of 2 M acetate buffer (pH 8.0). Fractions of 9 ml were collected. Solid points show absorbance at 280 m μ and open circles that at 490 m μ .

tions of Purified Peptides—The amino acid and carbohydrate compositions of the two glycopeptides, C3 and T1, and the amino acid compositions of glycopeptide C2, and peptides of glycopeptide T1 obtained with chymotrypsin and thermolysin are summarized in Table I. Mannose was identified as the only neutral sugar of glycopeptides C3 and T1 by gas liquid chromatography.

Sequence Determination of Peptides—*Glycopeptide C2*: PTH-valine was detected at the first step of Edman degradation, but, the second step of the degradation was not successful. Digestion of peptide C2 for 2 hr with carboxypeptidase resulted in release of 40% of the tyrosine in the original peptide. These results showed that the sequence of this

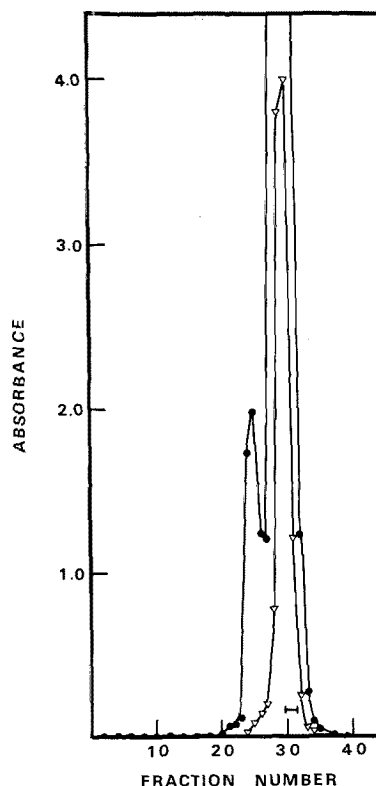


Fig. 5. Gel filtration of Peak B from DEAE-cellulose on a Bio-Gel P-4 column. Peak B in Fig. 4 was chromatographed on a Bio-Gel P-4 column (1×130 cm) equilibrated with 0.1 M ammonium acetate buffer (pH 8.6), and fractions of 2.5 ml were collected. Solid points show absorbance at 226 m μ and triangles that at 280 m μ .

peptide is Val-(Ser, Asn)-Tyr.

Glycopeptide C3: Seven steps of Edman degradation revealed the amino-terminal sequence of glycopeptide C3 as Asp-Trp-Val-Gly-(Ser)-Leu-Val-. At the fifth step of degradation, no clear spot of PTH-serine was found on a thin layer chromatogram, although a typical absorption curve of PTH-amino acid was obtained. These results suggest that serine may be the fifth amino acid from the amino terminus of the peptide. Taking into consideration the amino acid composition, the specificity of chymotrypsin, and the amino acid sequence of glycopeptide C2, the sequence of glycopeptide C3 is concluded to be Asp-Trp-Val-Gly-(Ser)-Leu-Val-(Ser, Asn)-Tyr.

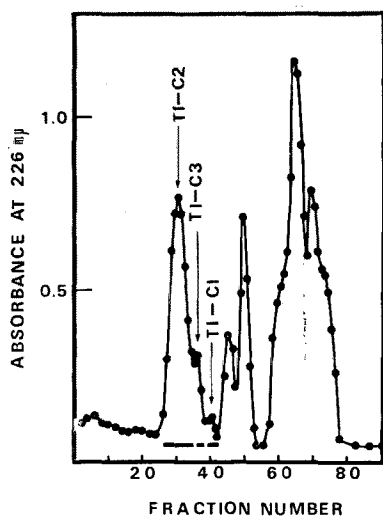


Fig. 6. Gel filtration of a digest of T1 with chymotrypsin. A digest of T1 with chymotrypsin was chromatographed on a column of Bio-Gel P-2 (1.2×100 cm) equilibrated with 0.1M ammonium acetate buffer (pH 8.0), and fractions of 1.6 ml were collected.

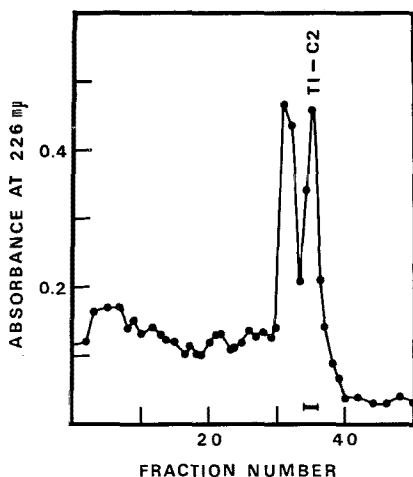


Fig. 7. Gel filtration of T1-C2 in Fig. 6 on a Bio-Gel P-4 column. T1-C2 in Fig. 6 was purified on a column of Bio-Gel P-4 (1×130 cm) equilibrated with 0.1M ammonium acetate buffer (pH 8.6), and fractions of 2.4 ml were collected.

Glycopeptide T1: Results of sequence studies on glycopeptide T1 are summarized in Table II. Six steps of Edman degradation revealed the amino terminal sequence of this peptide as Asn-Glu-Trp-Tyr-Asp-Trp-. At the

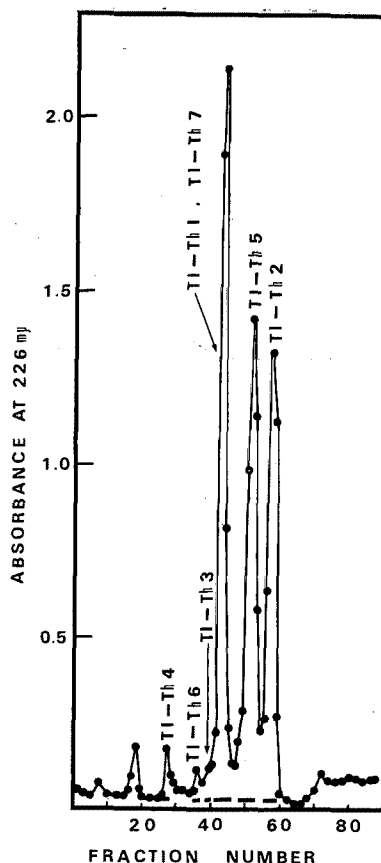


Fig. 8. Gel filtration of a digest of T1 with thermolysin. A digest of T1 with thermolysin was chromatographed on a column of Bio-Gel P-4 (1×100 cm) equilibrated with 0.1M ammonium acetate buffer (pH 8.0), and fractions of 2.5 ml were collected.

second step of degradation, a spot of PTH-aspartic acid was also observed besides the main spot of PTH-glutamic acid on the plate. Therefore, the possibility of variation in the amino acid at this position cannot be excluded completely. The presence of tryptophan at the sixth position from the amino terminus is uncertain because of the low yield of its PTH derivative. No PTH-amino acid was detected at the seventh step of degradation. After the seventh step of Edman degradation, the residual peptide was digested with carboxypeptidase B, and release of arginine was observed. This indicates that arginine is the carboxyl terminal of the parent peptide T1.

TABLE I. Amino acid and carbohydrate compositions of glycopeptides obtained from RCM-TAA and chymotrypsin and thermolysin peptides of glycopeptide T1. Values are molar ratios to the amino acid marked with an asterisk (*). Numbers in parentheses are the nearest integers or numbers determined after sequence analysis.

	C2	C3	T1	T1-C1	T1-C2	T1-C3	T1-Th1
Aspartic acid	1.00* (1)	2.00* (2)	4.00* (4)	0.44	1.32 (1)	0.93 (1)	1.00 (1)
Threonine ¹⁾	0.29		0.19	0.23	0.17		
Serine ¹⁾	0.82 (1)	1.59 (2)	3.07 (3)	1.07 (1)	0.99 (1)	0.84 (1)	
Glutamic acid			1.09 (1)	0.16	0.15		0.96 (1)
Glycine	0.18	1.10 (1)	2.13 (2)	0.85 (1)	0.22	1.00 (1)	
Alanine	0.18		0.16	0.24	0.17		
Valine	0.76 (1)	1.89 (2)	1.79 (2)	0.96 (1)	1.00 (1)		
Isoleucine			0.82 (1)	0.20		1.05 (1)	
Leucine		1.10 (1)	1.90 (2)	1.00 (1)		1.18 (1)	
Tyrosine ²⁾	0.35 (1)	0.64 (1)	1.67 (2)		0.50 (1)		
Lysine				0.30			
Arginine			1.13 (1)			0.73 (1)	
Tryptophan ²⁾		0.43 (1)	1.85 (2)				+ (1)
Total	4	10	20	4	4	6	3
Glucosamine	+	2.03	1.86		+		
Neutral hexose		5.95	5.44				
Molar yield (%) ³⁾	2.5	6.3	9.7 (42.0)				
	T1-Th2	T1-Th3	T1-Th4	T1-Th5	T1-Th6	T1-Th7	
Aspartic acid	1.00 (1)	0.19	1.00 (1)		1.08 (1)		
Threonine ¹⁾							
Serine ¹⁾		1.15 (1)	0.92 (1)	1.00 (1)			
Glutamic acid							
Glycine		1.00 (1)			1.00 (1)		
Alanine		0.12					
Valine		1.12 (1)	0.97 (1)				
Isoleucine					0.93 (1)		
Leucine		0.27	0.81 (1)			1.05 (1)	
Tyrosine ²⁾	0.70 (1)			0.81 (1)			
Lysine							
Arginine						1.00 (1)	
Tryptophan ²⁾	+	(1)					
Total	3	3	4	2	3	2	
Glucosamine			+				
Neutral hexose							
Molar yield (%) ³⁾							

¹⁾ Values of threonine and serine were not corrected. ²⁾ The low values of tyrosine and tryptophan might be caused by oxidation or halogenation during purification or hydrolysis of peptides. ³⁾ Peptides were estimated quantitatively by amino acid analysis, and the molecular weight of TAA was taken as 50,000 (20). The value in parenthesis is that corrected for losses during the purification process and amino acid and sugar analyses.

T1	Asn-Glu-Trp-Tyr-Asp-Trp-Val-Gly-Ser-Leu-Val(Ser, Asn) [*] Tyr-Ser-Ile-Asp-Gly-Leu-Arg
T1-C1	Val-Gly-Ser-Leu
T1-C2	Val(Ser, Asn) [*] Tyr
T1-C3	Ser- or -Asp-Gly(Ile or, Arg) Leu Ile
T1-Th1	Asn-Glx-Trp
T1-Th2	Tyr-Asx-Trp
T1-Th3	(Val, Gly, Ser)
T1-Th4	(Leu, Val, Ser, Asx) [*]
T1-Th5	Tyr-Ser
T1-Th6	Ile-Asx-Gly
T1-Th7	Leu-Arg

Peptide T1-C3 was concluded to be at the carboxyl terminus of the parent peptide T1 because it contained carboxyl terminal arginine. Peptide T1-Th5 provided the overlap between glycopeptide T1-C2 and peptide T1-C3, and glycopeptide T1-Th4 showed that peptide T1-

C1 was followed by glycopeptide T1-C2. The sum of the amino acid compositions of the amino terminal hexapeptide, peptides T1-C1, T1-C2, and T1-C3 accounts for the amino acid composition of the original peptide T1. So the alignment of the chymotrypsin and thermolysin peptides of glycopeptide T1 was concluded to be as shown in Table II. The sequence of chymotrypsin peptide C3 is in agreement with the sequence of the part between aspartic acid, the fifth amino acid from the amino terminus, and tyrosine, the seventh amino acid from the carboxyl terminus of peptide T1.

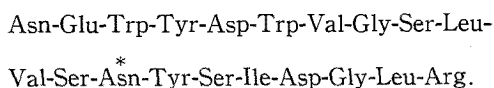
Edman degradation of peptide T1-C3 did not establish whether the second amino acid from the amino terminus of the peptide is isoleucine or leucine. However, the presence of isoleucine in this position was confirmed by the compositions of peptides T1-Th6 and T1-Th7.

DISCUSSION

Digests of RCM-TAA with trypsin were sparingly soluble in acidic media, and were also difficult to dissolve in the small amount of alkaline buffer used for gel filtration. So the digestion mixture was applied directly to a DEAE cellulose column of pH 8.0. The glycopeptides separated were all purified further using alkaline buffer because of their insolubility in neutral or acidic media. Purified T1, after lyophilization, was initially soluble in water but gradually formed a gel-like precipitate. To keep T1 in solution, the solution was raised to pH 10 by addition of triethylamine. This unusually low solubility of the glycopeptide in water might be because it contains many hydrophobic amino acids, two residues each of tryptophan, tyrosine, leucine, and valine, and one isoleucine residue.

From their amino acid sequences and the carbohydrate compositions, all the glycopeptides described here seem to be derived from the same part of the parent protein. Yamaguchi *et al.* reported the homogeneity (3) and complete sequence (5) of a glycopeptide obtained from TAA. It seems likely that the glycopeptides isolated in the present study are

similar to theirs, but have longer peptide chains. The structure of the glycopeptide containing serine obtained from TAA has been established as -Ser-Asn-^{*} (1), so the undetermined part (Ser, Asn^{*}) of the sequence of our glycopeptides around the carbohydrate side chain must be -Ser-Asn^{*}-. Thus the sequence of glycopeptide T1 can be written as follows;



Two other fractions containing carbohydrate in digests with chymotrypsin, peak I in Fig. 2 and fraction C1 in Fig. 3 were not studied. Peak I seemed to be polysaccharide contaminating TAA which could not be removed during the purification process, because it contained a large amount of glucose, in addition to glucosamine and mannose but no amino acid. Fraction C1 could not be purified, but it probably contained the glyco-

peptide, Val-Gly-Ser-Leu-Val-Ser-Asn^{*}-Tyr, judging from the results of amino acid analysis and subtractive Edman degradation of impure material. Two fractions containing carbohydrate in digests with trypsin derived from peak A in Fig. 4 might contain glycopeptides having longer peptide chains than T1, as judged by amino acid analysis. The contaminating polysaccharide mentioned above might be eluted in peak A or dispersed in front of this peak.

The sequence -Asn-Tyr-Ser-^{*} in TAA conforms with the hypothesis by Neuberger and Marshall (17) of the occurrence of the particular amino acid sequence, -Asn-X-Thr/Ser- in glycopeptides. Another hypothesis on the composition of glycopeptide proposed by Jackson and Hirs (7) is that when the residue next to the asparagine linked to carbohydrate is hydrophilic the carbohydrate side chain is of a complex type. However, when this residue is hydrophobic, a simpler, mannose-N-acetylglucosamine side chain is formed.

The carbohydrate side chain of TAA is of the simpler type (2) and the residue next to asparagine is tyrosine which is hydrophobic. Thus results conform with the hypothesis of Jackson and Hirs.

Shepherd *et al.* reported the existence of a labile amide group in porcine adrenocorticotrophic hormone (18). In their experiment, exposure of the hormone to 0.1M sodium bicarbonate (pH 9) at 25°C for 18–22 hr resulted in deamination of an amide group. On reinvestigation of the amino acid sequences of porcine and human adrenocorticotrophic hormones Rinker *et al.* (19) ascribed this instability to the -Asn-Gly- sequence. Glycopeptide T1 has the sequence -Asp-Gly-. Glycopeptide T1 was kept in alkaline solution (pH 10) throughout our experiments to keep it in solution, so the sequence might be -Asn-Gly- instead of -Asp-Gly-.

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