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THE INTERACTION OF α_2 MACROGLOBULIN WITH TRYPSIN RELEASES A SOLUBLE GLYCOPEPTIDE

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SUMMARY: When human α_2 macroglobulin (α_2M) or its asialo-[^{3}H]galactose derivative reacts with trypsin, a glycopeptide of molecular weight 3500-4000 is released from the α_2M. The glycopeptide was purified on Biogel P-4 columns and its amino acid and carbohydrate composition were determined. The oligosaccharide contains sialic acid, galactose, mannose and GlcNAc in a ratio of 1.0:0.73:3.85:2.85 and is apparently attached to protein in a GlcNAc→asparagine linkage.

INTRODUCTION

Human $\alpha_2$macroglobulin (α_2M) is a plasma glycoprotein that can bind active forms of endopeptidases to give complexes that retain proteolytic activity toward low molecular weight substrates but can no longer hydrolyze high molecular weight substrates (1-4). Studies by Swenson and Howard (4) have shown that trypsin cleaves the α_2M near the middle of the polypeptide chain to give two 85,000 molecular weight fragments from each of the four subunits. In this paper, we show that trypsin also release a small glycopeptide from the α_2M. The amino acid analysis of this glycopeptide indicates that it does not arise from the amino terminus but may come from a second cleavage near the middle of the molecule.

METHODS

Purification and Assay of α_2M. Human α_2M was isolated and purified from outdated blood essentially by the method of Wickerhauser and Hao (5). The α_2M was homogeneous by the criterion of pore gradient gel electrophoresis on commercially prepared polyacrylamide gel slabs (Pharmacia). Assays for α_2M were prepared by adding trypsin to samples of α_2M and measuring either the soybean trypsin inhibitor-resistant hydrolysis of α-N-benzoyl-D-L-arginine-p-nitroanieide HCl (BAPNA) (6), or the inhibition of hydrolysis of the high molecular weight substrate, Hide Powder Azure (Calbiochem).

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Isolation and Purification of Glycopeptides. Purified α_2M (or asialo-α_2M) was incubated with an equimolar amount of trypsin for 1 hour at room temperature and then trichloroacetic acid was added to a final concentration of 5%. The precipitate was removed by centrifugation and the supernatant liquid was extracted 5-6 times with diethyl ether to remove the trichloroacetic acid. The supernatant liquid was lyophilized and glycopeptides were isolated by chromatography on a calibrated 2 x 100cm column of Biogel P-4 in 0.02 N acetic acid. Glycopeptides were detected either by radioactivity or by hexose determination. Glycopeptides were treated with Pronase as previously described (7).

Radioactive Labeling and Analysis of Glycopeptides. Glycopeptides were treated with neuraminidase or with 0.1 N HCl at 80° for 1 hour to remove sialic acid and exposed galactose residues were oxidized with galactose oxidase and reduced with NaB^3H_4 (8). Glycopeptides were purified by paper electrophoresis and gel filtration. Glycopeptides were subjected to mild (0.1 N NaOH, 0.1 M NaB^3H_4, 24 hours, 37°) and strong (1 M NaOH, 1 M NaB^3H_4, 8 hrs, 100°) alkaline digestion to determine the linkage of carbohydrate to protein. Glycopeptides were also subjected to acetolysis (9) to cleave 1→6 glycosidic bonds and products were isolated by gel filtration on Biogel P-2 and P-4.

Paper chromatography was done on Whatman 3 MM paper in solvent 1:n-butanol:pyridine:0.1 N HCl (5:3:2); solvent 2:n-butanol:pyridine:H_2O (40:30:40) and solvent 3; ethyl acetate:acetic acid:formic acid:H_2O (18:3:1:4). Sialic acid was determined by the thiobarbituric acid method (10); galactose with galactose oxidase, hexose by the anthrone procedure (11) and hexosamine by the method of Blix (12). Neutral sugars were identified by paper chromatography and gas liquid chromatography of the alditol acetates. Amino acids were identified on a Durrum Amino Acid Analyzer.

RESULTS AND DISCUSSION

Both native α_2M and asialo-α_2M were equally active in their ability to inhibit trypsin activity towards high molecular weight substrates, but in both cases activity towards low molecular weight substrates remained intact. In order to study the effect of trypsin on the α_2M molecule, both native α_2M and asialo-$[^3H]\alpha_2M$ were reacted for 1 hour with trypsin; after which time protein was precipitated with trichloroacetic acid. Both the supernatant liquid and the precipitate were tested for hexose and for radioactivity as shown in Table 1. The results showed that a large amount of radioactivity or hexose was released into the supernatant liquid (about 25% of the total) when either native α_2M or asialo-α_2M were treated with trypsin. Controls, in which trichloroacetic acid was added before trypsin also showed some radioactivity or hexose in the supernatant liquid but this was much less than in the incubated samples. These experiments suggested that a glyco-

TABLE 1

RELEASE OF GLYCOPEPTIDE FROM α_2MACROGLOBULIN BY TRYPSIN

Treatment	Fraction Tested	Radioactivity (CPM x 10^6)	Hexose (Anthrone O.D.)
Trypsin, then TCA	Supernatant	7.7	1.06
Trypsin, then TCA	Pellet	21.4	2.56
TCA, then Trypsin	Supernatant	2.5	0.30
TCA, then Trypsin	Pellet	24.8	> 3.0

Native α_2M or asialo-^{3}H-α_2M was reacted with trypsin and the protein was precipitated with trichloroacetic acid. Both the supernatant liquid and the pellet were analyzed for their content of ^{3}H or for their hexose content. Controls were prepared in which α_2M was treated with trichloroacetic acid before the addition of trypsin.

peptide was released as a result of trypsin action. Also the removal of sialic acid from α_2M did not prevent its interaction with trypsin.

In order to characterize the soluble product released by trypsin, a large amount of α_2M was incubated with trypsin and the protein was removed by precipitation with trichloroacetic acid. The supernatant liquid was extracted with ether, lyophilized and chromatographed on a Biogel P-4 column as shown in Figure 1. Both the radioactivity and the

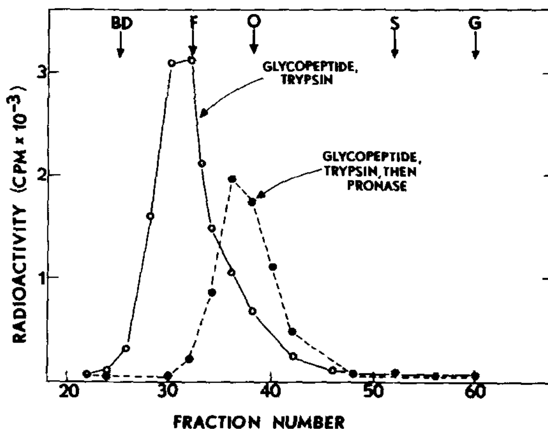


Figure 1. Gel Filtration of Glycopeptides on Biogel P-4. Asialo-[^{3}H]glycopeptide was placed on a 2 x 100 cm column of Biogel P-4 and the column was eluted with 0.1% acetic acid. Three ml fractions were collected and an aliquot of each tube was analyzed for its content of radioactivity. The trypsin released glycopeptide was digested with pronase and the digest was rerun on the Biogel P-4 column. Markers are blue dextran (BD), Fetuin (F), ovalbumin (o) stachyose (S), mannose (M).

TABLE 2

AMINO ACID AND CARBOHYDRATE COMPOSITION
OF α_2MACROGLOBULIN GLYCOPEPTIDE

Amino Acid[a]	g/100 g Glycopeptide	Carbohydrate Ratio)
Aspartic Acid	15.0	Sialic acid 1.0
Threonine	7.4	Galactose 0.73
Serine	4.9	Mannose 3.85
Glutamic Acid	13.7	GlcNAc 2.85
Proline	3.8	
Cysteine	---	
Glycine	3.4	
Alanine	4.0	
Valine	8.3	
Methionine	---	
Isoleucine	3.9	
Leucine	13.6	
Tyrosine	4.6	
Phenylalanine	5.1	
Histidine	5.3	
Lysine	5.2	
Arginine	1.8	
Tryptophan	---	

[a]Determined for 24 hour hydrolysis in 6NHCl at 110°C.

anthrone positive material emerged in an area indicating a molecular weight of 3500-4000. Also as seen in Figure 1 when this peak was further digested with the proteolytic enzyme, pronase, its molecular weight was shifted to 1800-2000 indicating the loss of a number of amino acids.

Analysis of the native glycopeptide from the P-4 column showed that it contained sialic acid, galactose, mannose and GlcNAc in a ratio of 1.0:0.73:3.85:2.85. The identification of mannose and galactose, and their ratio, was confirmed by paper chromatography and by gas-liquid chromatography. A preliminary amino acid analysis of the peptide is presented in Table 2. This data is from a 24 hour hydrolysate and has not been corrected for destruction of amino acids, nor has any possible tryptophan been determined. However it does show the presence of about 20 amino acids with aspartic acid and serine (thrèonine) being prominent. This amino acid composition is incompatible with the N-terminal sequence previously determined (7), suggesting that the glycopeptide may arise from a cleavage near the middle of the α_2M.

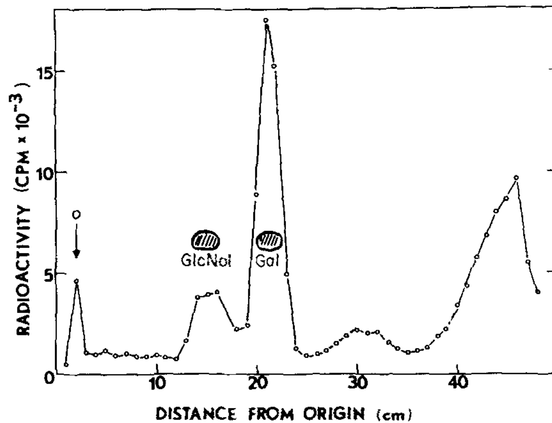


Figure 2. Paper chromatography of ^{3}H-sugars released by strong acid hydrolysis. The ^{3}H-glycopeptide was treated with strong alkali in the presence of NaB^3H_4 to label the reducing end of the oligosaccharide. The oligosaccharide was hydrolyzed in 4 N HCl and the products were chromatographed on Whatman 3 MM paper in n-butanol:pyridine:0.1N HCl (5:3:2). Papers were cut into 1 cm sections and their content of ^{3}H was measured in the liquid scintillation counter.

The [^{3}H]galactose-labeled α_2M was treated with mild and strong alkali in the presence of NaB^3H_4 to reduce the oligosaccharide and the digestion mixture was subjected to paper electrophoresis in formate buffer, pH 1.5. In mild alkali, a small peak of radioactivity remained at the origin but this was greatly enhanced by strong alkaline digestion. Similar results were observed with ovalbumin. The radioactivity at the origin was eluted, hydrolyzed in strong acid and chromatographed on paper as seen in Figure 2. Two radioactive peaks were detected which corresponded to galactose and glucosaminitol. The rapidly migrating peak and that at the origin are apparently due to contaminants in the NaB^3H_4. This data suggests that the oligosaccharide is attached in a GlcNAc→asparagine bond.

The oligosaccharide obtained by strong alkaline digestion was treated with β-galactosidase and the digestion mixture was chromatographed on Biogel P-4 (Figure 3). A large peak of radioactivity was eluted in the monosaccharide area and was identified as galactose by paper chromatography (Figure 3B). A rather broad peak of radioactivity remained in approximately the same area as the original oligosaccharide (Figure 3A) and is probably

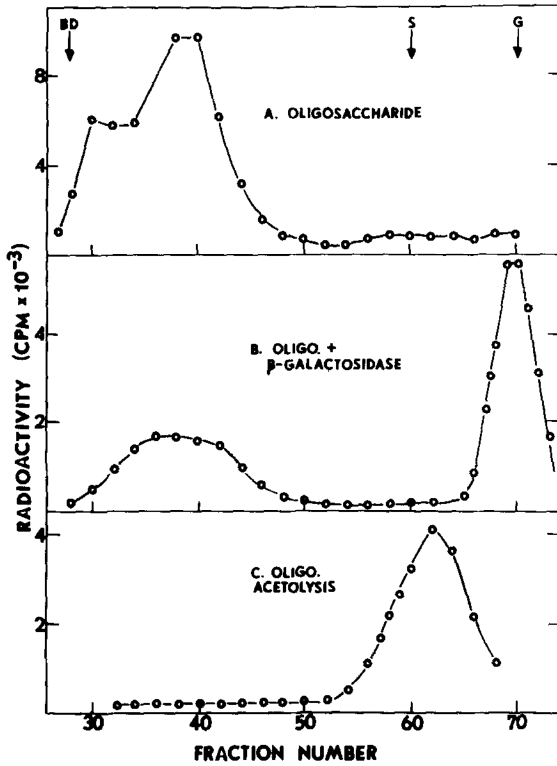
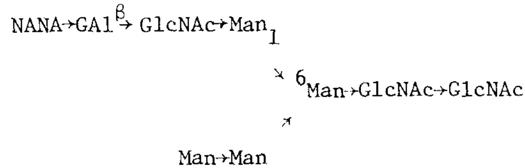


Figure 3. Biogel P-4 Chromatography of ^{3}H-Oligosaccharide and Its Degradation Products. The ^{3}H-oligosaccharide was obtained by alkaline digestion of glycopeptide and was purified on the Biogel column (upper scan). The oligosaccharide was incubated with 1 unit of β-galactosidase overnight in 0.001 M acetate buffer (middle scan). The [^{3}H]-oligosaccharide was subjected to acetolysis and the degradation products were separated on the Biogel P-4 column. A shoulder of radioactivity in the trisaccharide area was pooled and rerun on the same column (lower scan).

the agalacto-oligosaccharide labeled at the reducing end. The asialo-[^{3}H]glycopeptide was subjected to acetolysis and the mixture was chromatographed on the P-4 column (Figure 3C). A radioactive peak emerged in the trisaccharide area of the column. This peak was reduced with NaB^3H_4 and hydrolyzed to give rise to [^{3}H]galactose and [^{3}H]mannitol by paper chromatography. Thus the structure of this trisaccharide is probably Gal→GlcNAc→Man, and it is attached to the main chain in a 1→6 linkage.

The data presented here indicate that when α_2M reacts with trypsin, a glycopeptide of molecular weight 3500–4000 is released from the α_2M. The oligosaccharide is apparently of the complex type and is linked to the

protein in a GlcNAc→asparagine bond. Although the oligosaccharide has not been completely characterized, a possible structure that is compatible with the data is



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