A STUDY OF THE 11S GLOBULIN FROM COTTON SEEDS

IX. STRUCTURE OF THE GLYCOPEPTIDE

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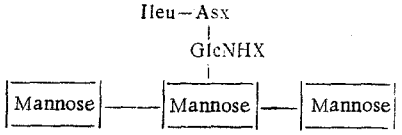
We have shown previously that the 11S globulin is a complex protein consisting of three types of subunits [1]. Subunits I', I", and II (A, B, and C, respectively) were investigated for the presence of carbohydrates [2]. Subunit C proved to be a glycoprotein. After the acid hydrolysis of the glycoprotein obtained, mannose was obtained, being identified by TLC and GLC (in the latter case, in the form of the TMS derivative of mannose). In order to study the structure of the carbohydrate moiety of the glycoprotien we performed the digestion of subunit C with pronase. The reaction was continued for about three days, its course being followed by TLC on cellulose in system 1. The residue after the freeze-drying of the reaction mixture was dissolved in 50% aqueous pyridine. The mixture was separated on a column of Sephadex G-25 equilibrated with the same solution. The results of chromatography are presented in Fig. 1.

Two fractions containing glycopeptides were obtained. Both fractions were rechromatographed on a column of Sephadex under the same conditions as in first case. In this way we obtained peptide (I) in the pure form. Fraction (II), after rechromatography did not consist of a pure peptide and therefore it was purified further by paper chromatography in system 1. Since the yield of glycopeptide (II) was considerably smaller than that of (I), we investigated the latter. Glycopeptide (I) was found to contain isoleucine and aspartic acid, the N-terminal amino acid being isoleucine with a small amount of aspartic acid. The amount of mannose, as determined after acid hydrolysis by GLC, was about 50%, which corresponds to 2.5 moles of mannose per mole of peptide. Furthermore, by the Elson–Morgan method we detected about 12% of glucosamine, which was also determined on the amino-acid analyzer, the residence time for glucosamine on the column of the Swedish amino-acid analyzer, type LKB 4101, being the same as for tyrosine. The amino group in the glucosamine was blocked, as was shown by the products of the dansylation reaction before and after complete acid hydrolysis.

To determine the sequence of carbohydrate residues in the chain of the glycoprotein we performed partial acid hydrolysis. This gave a mixture of glycopeptides and mannose which was separated by paper chromatography in system 2 (Fig. 2).

On analyzing the compositions of the peptides obtained, we found mannose in all the fractions apart from III, which contained Ileu, Asp, and $GlcNH_2$. Fractions I and II contained Asp and $GlcNH_2$, IV contained Ileu, Asp, and $GlcNH_2$, and VI pure mannose. Consequently, the partial acid hydrolysis led to the incomplete splitting off of the mannose. No splitting off of $GlcNH_2$ or GlcNHX was observed. It may be concluded from this that the $GlcNH_2$, the amount of which is 0.5 mole per mole of peptide, is the connecting link between the carbohydrate and peptide moieties. The glycopeptide was methylated by Hakomori's method [3]. The completeness of the methylation was checked by TLC. The methylated glycopeptide was hydrolyzed, and the hydrolyzate was investigated by TLC in system 3. Two products, M_1 and M_2 with R_{fM_1} 0.25 and R_{fM_2} 0.6 were obtained. Product M_1 gave no reaction for an α-diol grouping and was identified with an authentic sample as dimethylmannose. Substance M_2 was identified as tetramethylmannose. The periodate oxidation of the glycopeptide led to the complete loss of the mannose, which shows the absence of a substituent in the hydroxyl in position 3. Consequently, the oligosaccharides of the glycoprotein have branching, probably in the 4,6 position:



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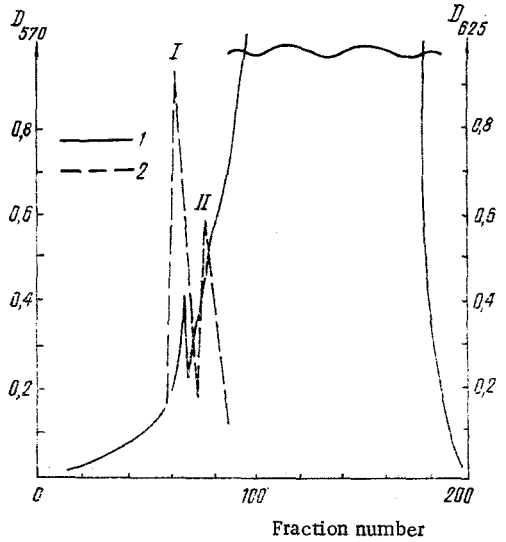

Fig. 1

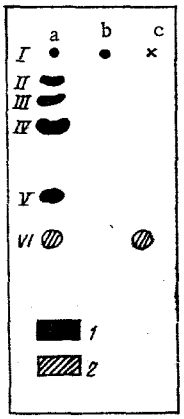

Fig. 2

Fig. 1. Chromatography of pronase-digested subunit C on Sephadex G-25: 1) absorption at 570 nm; 2) anthrone reaction (625 nm) (rate of elution 15 ml/h; fraction volume 5 ml).

Fig. 2. Paper chromatography of the glycopeptide after partial acid hydrolysis: 1) ninhydrin staining; 2) aniline phthalate staining; a) glycopeptide after partial acid hydrolysis; b) native glycopeptide; c) mannose.

EXPERIMENTAL

For chromatography we used systems: 1) pyridine–water–butan-1-ol–acetic acid (10:12:15:3), 2) pyridine–water–butanol-1-ol (4:3:6), and 3) benzene–acetone (2:1).

Cleavage of Subunit C with Pronase [4]. Pronase (Merck) was added to a solution of 3.5 g of carboxymethylated subunit C in 60 ml of 0.15 M Tris acetate buffer, pH 7.8, containing 1.5 mM potassium acetate to give an enzyme: substrate ratio of 1:100. After 20 h, a new portion of enzyme (1:100) was added, and the pH was brought with dry Tris to 7.8. After 48 h, a third portion of pronase (1:50) was added and after 16 h a fourth portion of the enzyme (1:50). The reaction mixture was centrifuged at 6000 rpm and the supernatant liquid was freeze-dried.

Gel Filtration of the Products of the Cleavage of Subunit C by Pronase. The hydrolyzate obtained was dissolved in 18 ml of 50% aqueous pyridine and deposited on a column (4.0 × 70 cm) of Sephadex G-25 (fine) equilibrated with 50% aqueous pyridine. The fractions were analyzed by the ninhydrin reaction after alkaline hydrolysis (2.5 N NaOH, 90°C, 2.5 h), and their absorptions were measured at 570 nm. The glycopeptides were detected by the reaction with anthrone (100°C, 16 min), the absorption being measured at 625 nm.

Quantitative Determination of Glucosamine [5]. The glycopeptide (1 mg) was hydrolyzed with 2 N HCl at 100°C for 16 h. The hydrolyzate was freeze-dried and the dry residue was dissolved in 2 ml of distilled water and 5.5 ml of acetylacetone. The reaction mixture was kept in the boiling water bath for 20 min and cooled with ice, and 2 ml of the solution was distilled into a flask containing 8 ml of the Ehrlich reagent. The absorption was measured at 548 nm; $D_{548} = 0.265$. A calibration curve was plotted with standard glucosamine.

Determination of the Neutral Sugars. A weighed sample of the substance was hydrolyzed with 3 N HCl at 100°C for 4 h. The hydrolyzate was diluted with water, treated with Dowex 50 × 4 resin (H^+ form, 400 mesh) and filtered. The resin was washed several times with water, and the filtrate was freeze-dried. The TMS derivatives were obtained as described by Gorovits [6]. GLC was performed on a Tsvet-4 instrument with a flame-ionization detector on a column (2 m long, 4 mm in internal diameter) filled with 5% of SE-30 on Chromaton N-AW (0.2-0.25 nm) at a column temperature 170°C with helium as the carrier gas (50 ml/min).

Partial Acid Hydrolysis of the Glycopeptide. The glycopeptide (5 mg) was hydrolyzed with 0.5 ml of 3 N HCl at 100°C for 20 min. The mixture was evaporated to dryness, the residue was dissolved in water, and

the solution was deposited on FN-17 chromatographic paper (Filtrak). Chromatography was carried out in system 2. The fractions obtained were eluted with 0.005% ammonia, the eluates were evaporated in a rotary apparatus, and the N-terminal amino acids and compositions were determined.

The glycopeptide was methylated by Hakomori's method [3]. The completeness of methylation was checked by TLC, for which purpose aliquots were taken and were analyzed after acid hydrolysis (3 N HCl, 100°C, 5 h) on plates (6 × 9 cm) with a fixed layer of type KSK silica gel in system 3. The revealing agent used was a 3% solution of anisidine in n-butanol and benzidine–sodium metaperiodate [7].

Periodate Oxidation of the Glycopeptide. A suspension of 2 mg of the substance in 2 ml of 0.005 M KIO_4 solution was left overnight at 4°C. The mixture was freeze-dried and the sugars were determined as described above.

SUMMARY

1. The oligosaccharide of the glycopeptide (I) from subunit C of the 11S globulin of cotton seeds consists of one molecule of glucosamine and three molecules of mannose.

2. The oligosaccharide is attached to the protein through the glucosamine and has a branched structure.

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THE GLOBULINS OF COTTON SEEDS

X. STRUCTURES OF TRYPTIC PEPTIDES OF THE 7S GLOBULIN

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In preceding communications we have considered the isolation, purification, and characteristics of the amino-acid compositions of peptides from the tryptic hydrolysis of the 7S globulin [1, 2]. In the present paper we described the determination of the amino-acid sequences of the tryptic peptides.

Judging from the amount of basic amino acids in the polypeptide chain of the protein (12 arginine and five lysine residues) and from peptide maps [3], tryptic hydrolysis should give about 20 peptides. We have obtained such results by performing the hydrolysis of subunit I and the 7S globulin with TPCK-trypsin [1]. For preparative purposes we used unmodified trypsin (Spofa, Czechoslovakia) [2].

In all the peptides obtained, the sequences of the amino acids were determined by the method of Edman degradation with direct identification of the PTH derivatives of the amino acids, and also of the DNS derivatives. The structures of the long peptides were determined by comparing their N-terminal sequences while taking into account the structures of their fragments and the structures of overlapping chymotryptic peptides.

The amino-acid sequences of the tryptic peptides are as follows (x in the case of subunit I denotes an amide and in the case of subunit II an acid; the numbering of the peptides is given in the order of their sequence in the chain from the N-end):

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