Studies on Human Cervical Mucus: The Effect of Proteases on Cervical Mucus Glycoproteins Functions

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(Received 30th April, 1997)

Summary: .The influence of proteolytic enzymes on human cervical mucus glycoproteins has been investigated to assess their influence on cervical function, sperm migration and penetration. The main component of estrogenic cervical mucus, a high molecular weight glycoprotein was treated with Pronase, trypsin and chymotrypsin, and the enzyme-resistant glycoprotein was purified by gel filtration on Bio-Gel P-200 and Sepharose 2-B. A macromolecular component in good yield was recovered containing carbohydrate and protein moieties. Asialoglycoprotein on treatment with Pronase, trypsin and chymotrypsin released more than one glycoproteins. The carbohydrate and amino acid composition of the native and degraded glycoproteins were similar in composition with changes in molecular proportions. Sequential glycosidases treatment, lectin binding and Smith's degradation established main structural features of degraded glycoproteins that showed some differences to that of native glycoprotien. The influence of proteinases on cervical mucus glycoproteins and a possible mechanism of sperm penetration through the cervical mucus and enzyme treated glycoproteins is investigated. It has been shown that in midcycle human and simian cervical mucus glycoproteins there are common epitopes.

Introduction

The mechanism of sperm migration and penetration through the human cervix and cervical mucus is of great significance and importance in reproductive physiology and depends on the intrinsic motility of the sperm and receptivity of the mucus [1, 2]. In addition, the proteolytic activity of the seminal fluid as well as that of spermatozoa has been considered essential for sperm penetration through the cervix [3].

Cervical mucus is a complex mixture produced continually by the endocervical cells. Minor amounts of endometrial, tubal and possibly follicular fluids may contribute to the cervical mucus [4]. The mucus exhibits number of rheological properties such as viscosity, spinnbarkeit, flow elasticity and stickness that are regulated by the ovarian function. The chemical and physical changes in the mucus during the menstrual cycle influence sperm migration and also nutrition and survival of sperm. The cyclic changes in the biophysical properties of the cervical mucus are accompanied by variations in the carbohydrate composition of the cervical glycoproteins [5-7]. The human seminal plasma contains variety of proteinases and the presence of trypsin type enzyme in the sperm has been proposed [8]. Treatment of cervical mucus with commercial pancreatic trypsin or chymotrypsin *in vitro* enhanced sperm migration and decreased the ferning, spinnbarkeit viscosity and gel structure properties [3].

The midcycle mucus possess filamentous structure, and these filaments or channels promote sperm migration. The digestion of the midcycle mucus with Pronase destroyed the filamentous structure of the mucus, suggesting degradation of the protein backbone or of links between the glycoprotien fibrils [8]. Also, it has been proposed that the escape of the spermatozoa from the cervical crypts may arise as a consequence of the breakdown of the glycoprotein filaments by the sperm head protease [8].

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An understanding between the physiological function of the mucus and its macromolecular component, and its clinical implications require, in part, the characterization of the glycoproteins responsible for the biological function and their morphological outlay in the mucus, and for their interaction with proteolytic enzymes.

Fractionation of midcycle mucus by gel exclusion chromatography on Sepharose 2-B provided two high molecular components. The major component was a carbohydrate rich glycoprotein of the mucin type. An antibody raised against the simian periovulatory phase Sepharose 2-B purified glycoprotein reacted positively with the Sepharose 2-B purified human glycoprotein. The simian antibody also reacted with the human cervical mucus that exhibited fibrillar structure. The antigen-antibody complex demonstrated the morphological change in the outlay of the mucus suggesting that the channels in the human mucus are lined by the glycoproteins and thereby showing the physiological role played by the glycoproteins in the mucus. A similar observation has been made for the bonnet monkey mucus [9].

In order to understand the interaction between the cervical mucus and proteolytic enzymes, and their influence on sperm penetration and mucus function, purified glycoprotein and neuraminidase-treated glycoprotein of the ovulatory phase mucus was studied with Pronase, trypsin and chymotrypsin treatment.

Results and Discussions

Sperm migration test

The semen contributed by the donors was normal with respect to volume, sperm count, viability, and structure. The sperm migrated rapidly towards the cervical mucus. Within a few minutes, the spermatozoa penetrated the boundry of the mucus and migrated in an orderly manner. In the case of neuraminidase treated mucus, the sperm migrated randomly.

Immunodiffusion

In immunodiffusion human cervical mucus, and purified glycoprotein Fraction 1 reacted with semian antiglycoprotein antibody.

Purification and fractionation of the mucus glycoproteins

The glycoproteins were eluted as a heterogenous mixture in the void volume of the Bio-Gel P-200 column. Five proteins in addition to glycoproteins were obtained. The agarose and polyacrylamide electrophoresis suggested the presence of periodate-Schiff and Coomassie blue positive components. The glycoproteins had the minimal entry into the gel. The Bio-Gel P-200 purified glycoprotein afforded two main glycoprotein components, Fractions 1 and Fraction 2, on Sepharose 4B chromatography. The main glycoprotein component, Fraction 1, showed in agarose and polyacrylamide electrophoresis only a periodate-Schiff and Coomassie blue staining material and absence of proteins.

Table 1. The carbohydrate and amino acid composition of glycoproteins fractions obtained from the column of sepharose 2B.

Carbohydrate	Fraction 1	Fraction 2
L-Fucose	6.6	5.0
D-Galactose	24.0	20.0
N-Acetylglucosamine	11.8	11.0
N-Acetylgalactosamine	13.0	12.0
N-Acetylneuraminic acid	6.6	4.0
Sulphate	0.50	0.34
Amino acids	Residue per 100 residues	
	Fraction 1	Fraction 2
Asp.	8	5
Thr.	15	12
Ser.	9	7
Glu.	8	11
Pro.	7	8
Gly.	9	10
Ala	9	9
Cys/2	2	3
Val	8	9
Met	1	2
Ileu	5	6
Leu	8	6
Tyr	3	4
Phe	3	2
Lys	2	3
His	1	2
Arg	2	1

Purification and characterization of enzyme-treated glycoproteins

The non diffusible material of the digests obtained by the Pronase, trypsin and chymotrypsin treatment gave a single carbohydrate-protein

containing component on chromatography on a native glycoprotein calibrated Sepharose 4B column (Fig.2). The enzyme-treated glycoproteins were eluted from the Sephare 4B column in the vacinity where the main fraction of the native glycoprotein, Fraction 1, was eluted. The recoveries of the Pronase, trypsin and chymotrypsin degraded glycoproteins after gel chromatography were 65, 60 and 64 respectively. The enzyme-treated glycoproteins in polyacrylamide gel electrophoresis had the minimal entry.

The Pronase-degraded glycoproteins obtained by Sepharose 4B chromatography exhibited a single component in agarose-polyacrylamide gel electrophoresis. The carbohydrate moiety of the degraded glycoprotein contained L-fucose, D-galactose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid residues with an increase in neuraminic acid (Table 2).

Table 2. Carbohydrate composition of Pronase-, trypsin- and chymotrypsin-treated glycoproteins obtained from sepharose 4B columns.

Carbohydrate	Pronase %	Trypsin %	Chymo-trypsin %
L-Fucose	7	6	6
D-Galactose	26	24	26
N-Acetylglucosamine	13	12	12
N-Acetylgalactosamine	14	13	14
N-Acetylneuraminic acid	7	5	6
Total	**67**	**60**	**64**

The main result of the enzyme treatment was the loss of amino acids, as shown by the increase in total carbohydrate content, but the carbohydrate chains lost through enzyme-degradation were not the same for all the enzymes. The most significant difference was decrease in the fucose content after trypsin treatment, and of N-acetylneuraminic acid after trypsin and chymotrypsin treatment. Increase in the quantity of N-acetylglucosamine, N-acetylgalactosamine and galactose was observed (Table 2).

The amino acid composition of enzyme-treated glycoproteins was similar to that of native glycoprotein, except for variations in the relative ratios of amino acids (Table 3). A significant increase in threonine and serine, in general, was

Table-3:Amino acid composition of Pronase-, trypsin- and chymotrypsin-treated glycoprotein from sepharose 4B column. Results are expressed as per 100 residues.

Amino acids	Pronase-treated glycoprotein	Trypsin-treated glycoprotein	Chymotrypsin treated glycoprotein
Asp.	12	5	5
Thr.	19	19	18
Ser.	12	13	9
Glu.	6	7	8
Pro.	6	9	10
Gly.	9	9	9
Ala	10	9	8
Cys/2	1	1	1
Val	4	5	3
Met			
Ileu	6	7	8
Leu	9	8	9
Tyr			
Phe	2	2	2
Lys	2	3	4
His	1	2	3
Arg	1	1	3

observed which was concomitant with the increase in the carbohydrate moiety. Also, a decrease in valine occurred suggesting that this amino acid was eliminated.

Preparation and enzyme degradation of asialoglycoprotein : Glycoprotein Fraction 1 contained 64% carbohydrate and nearly three-fourths of the sialic acid was removed to give the asialoglycoprotein. Pronase and trypsin treated asialoglycoprotein afforded two asialoglycoprotein fractions on Sepharose 4B chromatography (Fig.3). In polyacrylamide gel electrophoresis, the enzyme-degraded asialoglycoprotein fractions entered the gel and reacted with priodate-Schiff. The Pronase degraded asialoglycoprotein fractions showed increase in carbohydrates, whereas the trypsin degraded fraction showed decrease in the sugar content (Table 4). Serine and threonine were the two significant amino acids present in all the asialoglycoprotein fractions. The amino acid composition of the asialoglycoprotein fractions were similar to that of the original asialoglycoprotein, but significant variation in the proportions of amino acids in various fractions were observed (Table 5).

The sperm penetration tests were perform on microscopic slide as described by us [10], on asialo-cervical mucus and asialo-Pronase-degraded

Table-4:Carbohydrate composition of Pronase- and trypsin-degraded fractions of asialoglycoproteins from sepharose 4B column.

Carbohydrate	Fraction 1 after treatment with neura-minidase %	Pronase-treated asialoglycoprotein fractions. %			Trypsin-treated asialoglycoprotein fractions. %		
		F1	F2	F3	F1	F2	F3
L-Fucose	7.0	9.0	8.0	7.0	6.0	8.0	9.0
D-Galactose	25.0	26.0	28.0	30.0	22.0	24.0	25.0
N-Acetylglucosamine	12.0	15.0	14.0	11.0	13.0	14.0	9.0
N-Acetylgalactosamine	13.0	16.0	17.0	11.0	12.	11.0	8.0
N-Acetylneuraminic acid	1.4	3.0	1.2	2.0	3.0	4.0	3.0

Table 5: Amino acid composition of Pronase- and trypsin-treated fractions of asialoglycoproteins obtained from Sepharose 4B column. Results are expressed as per 100 residues

Amino acids	Asialogly-coprotein	Pronase-treated asialoglycoprotein fractions. %			Trypsin-treated asialoglycoprotein fractions. %		
		F1	F2	F3	F1	F2	F3
Asp.	7	5	3	6	8	9	6
Thr.	16	20	21	15	19	17	14
Ser.	9	12	12	8	11	10	8
Glu.	8	9	8	12	8	9	6
Pro.	7	8	6	9	9	7	5
Gly.	8	6	8	9	7	4	6
Ala	10	8	5	6	11	8	9
Cys/2	1						
Val	9	6	7	4	7	6	4
Met							
Ileu	4	6	5	9	5	7	11
Leu	8	9	8	5	8	4	10
Tyr	3	2	3	4	2	4	7
Phe	2	3	4	5	2	5	8
Lys	4	4	5	4	3	5	6
His	2	2	3	4		5	
Arg	2		2				

cervical mucus. Both the categories of mucuses did not exhibit linear channels and sperm did not migrate in linearity as exhibited in the case of human midcycle mucus.

In the current study, the mucus was collected from donors at the time of estrogen surge. The mucus contained a heterogeneous mixture of glycoproteins and several proteins. On purification of the mucus by gel chromatography on Sepharose 4B two glycoproteins were obtained with similar carbohydrate and amino acid composition. The chromatographic behavior of the enzyme-degraded glycoproteins indicate that these represent high molecular weight material. There has been minor changes in the molecular sizes of Fraction 1 glycoprotein suggesting resistance to degradation. The recovery of glycoproteins after protease treatment (60-65%) indicates that all enzymes degraded the original glycoprotein to a similar extent, indicating that major portion of the glycoprotein is well protected by the carbohydrate chains. Furthermore, the size of the macromolecule in gel filtration on Sepharose 4B as well as the carbohydrate analysis suggest some differences in the degraded glycoproteins. Minimum change in Fraction 1 glycoprotein was observed with the Pronase, whereas trypsin and chymotrypsin had more pronounced effect in agreement with the previous observation [11]. The carbohydrate moiety of all the degraded glycoproteins contained all the sugars present in the native glycoprotein and represented nearly 80% of the sugars in the starting material. This suggested that only a small proportion of the sugar residues were linked to peptides cleaved by proteases. Proteolytic degradation of human cervical mucus has been investigated after various chemical modifications [8]. The Pronase degradation generated glycoproteins that apparently differed mainly in the relative proportion of carbohydrate residues, particularly for the sialic acid and N-acetylglucosamine content. This observation may be explained by the presence of carbohydrate rich and carbohydrate deficient domains in the glycoprotein which could influence their susceptibility to the proteolysis. The recoveries after protease treatments indicate that all enzymes degraded the original glycoprotein to a similar limited extent, indicating that some areas in the molecules are relatively easily available to

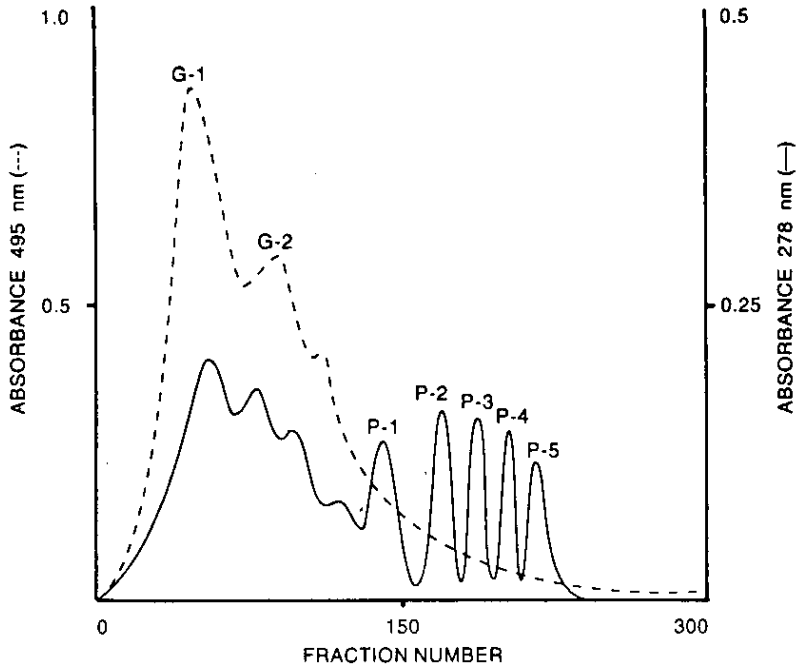

Fig. 1. Purification of crude human cervical mucus on a column (60 x 2.8 cm) of Bio-Gel P-200. The column was washed with phosphate buffer, pH 7.0. Fractions of 1 ml were collected and the carbohydrate (495 nm) and protein (278 nm) containing fractions were combined, dialyzed and lyophilized.

degradation, whereas the others are protected by the carbohydrate residues. Also, the size of the macromolecule in gel chromatography as well as the carbohydrate composition suggest some differences in the degraded glycoproteins. Proteolysis of the asialoglycoprotein unlike that of the native glycoprotein, generated more than one glycoprotein fragments with lower recoveries. The influence of sialic acid residues on the integrity of the glycoprotein towards proteases could not be assessed completely as in the native glycoprotein a portion of the sialic acid was resistant to the neuraminidase. It can be safely assumed that lack of sialic acid in mucus and glycoproteins enhance its susceptibility towards proteases. The incomplete removal of sialic acid and also the presence of sulphate groups on the sugar residues make it complicated to assess the role of sialic acid towards the proteases. The cleavage of asialoglycoproteins into several fragments, however, suggests that the lack of sialic acid residues in the glycoproteins increases the susceptibility to proteolytic cleavage. Several glycoprotiens, serum as well as secretory, are known to lose their resistance after treatment with neuraminidase [12-14]. It appears that peptide chains that are in the vicinity of oligosaccharides devoid of sialic acid residues, are those that are eliminated.

The studies, Smith degradation and lectin binding, on Pronase-treated glycoprotein indicate that alhough the main structural features of the native glycoprotein remaind unchanged, distinct changes occured, namely, (i) Fraction 2 glycoprotein was degraded, (ii) an increase in sialic acid content in Fraction 1 glycoprotein, (iii) likely that oligosaccharide chains of pronase-resistant glycoprotein fragment contained sialic acid.

The midcycle human cervical glycoprotein showed similarities to the simian midcycle cervical glycoprotein in basic chemical structure [15] as well as in the peptide backbone, as both the glycoproteins have common epitopes. From the studies on the asialomucuses and asialoglycoproteins, it appears that for proper

sperm migration neuraminic acid residues are significant and their removal from the glycoprotein increases the glycoprotein suceptibility to proteases.

Experimental

Collection of cervical mucus

The cervical mucus was collected by aspiration with a mild suction pump at the time of midcycle estrogen surge. The secretion was promptly frozen and maintained in the frozen state until used.

Sperm migration

The sperm penetration was performed on the midcycle mucus and neuraminidase treated mucus according to the method of Moghissi [9] Semen was collected from sexually mature donors.

Analytical methods

The hexose content was estimated by the phenol-sulphuric acid method [16], the protein content by measuring absorbance at 278 *nm* or by using the procedure of Lowry *et al.* [17], and the sialic acid content by thiobarbituric acid method of Warren [18] after acid hydrolysis with 50 mM sulphuric acid or by gas chromatography [19]. All solutions were dialyzed in hydrogen carbonate-treated cellulose tubing at 4 °C.

Gas-liquid chromatography (Glc)

Glc determination of carbohydrate of the glycoproteins was carried out according to the procedure of Reinhold [19].

Column chromatography

Bio-Gel P-200 (Bio-Rad Laboratories) and Sepharose 4B (Pharmacia Fine Chemical Ltd.) columns were run in 50 mM sodium phosphate (pH 7.0).

Gel electrophoresis

Agarose gel electrophoresis was performed in 50 mM barbital buffer (PH 8.2) on slides containing 1% agarose gel. Polyacrylamide-gel electrophoresis was performed according to the procedure of Holden *et al.* [20,21]. The polyacryl-amide gel and agarose slides were stained with Coomassie blue, and with the periodate-Schiff reagent. For agarose electrophoresis 0.25 mg per ml of each substance was used, and each well had nearly 12-15 µl of the solution.

Purification of the mucus glycoprotein

The crude human cervical mucus was solublized in 50mM sodium phosphate (pH 7.0) containing 0.02% sodium azide by studying for 16 hours at 4 °C. The insoluble materials, cellular debris and other suspended materials, were removed by centrifugation at 2500 rpm. The supernatant was dialyzed and the non-diffussible materials lyophilized to give the crude mucus glycoproteins. The solution was applied to a column (60 x 2.8 cm) of Bio-Gel P-200. The column washed with phosphate buffer (pH 7.0, 300 ml) and fractions of 1 ml were collected. The carbohydrate and protein containing fractions as well as protein containing fractions were pooled. The pH was adjusted to 5.5 with 4 M acetic acid and the solution was extensively dialyzed and lyophilized to give the purified mucus and protein fractions (Fig.1).

Fractionation of the purified mucus glycoprotein

The purified mucus glycoprotein (54mg) in 8 ml of phosphate buffer was applied to a column (65 x 1.4 cm) of Sepharose 4B. The glycoproteins were eluted with 50 mM phosphate buffer (pH 7.0) and fractions of 1 ml were collected. Fractions containing carbohydrate and proteins were combined, dialyzed and lyophilized. Two fractions, a major fraction, Fraction 1, and another fraction, Fraction 2, were obtained (Fig.2) with carbohydrate and amino acid composition given in Table-1.

Enzyme degradation

Pronase treatment of the purified glycoprotein Fraction 1 (15 mg) was performed in 50 mM phosphate buffer (10 ml, pH 8.0) containing 0.1% sodium azide with insolublized Pronase (10 mg, Enzite Protease, Miles Laboratories Inc.). The mixture was shaken at 22 °C until the glycoprotein was dissolved (5h), and then incubated with stirring for 6 days at 37 °C. A further amount of prewashed enzyme (2mg) was added to the reaction mixture and the solution was incubated for another 3 days. The insoluble enzyme

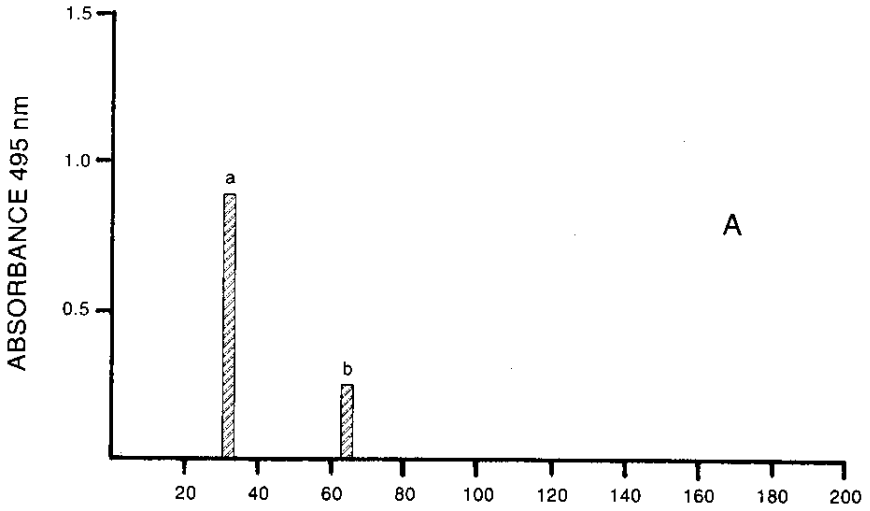

Fig. 2A: The purified mucus glycoprotein was fractionated on a column (65 x 1.4 cm) of Sepharose 4B. The glycoproteins were eluted with 50 mM phosphate buffer. Two glycoprotein fractions, Fraction 1 and Fraction 2, were obtained.

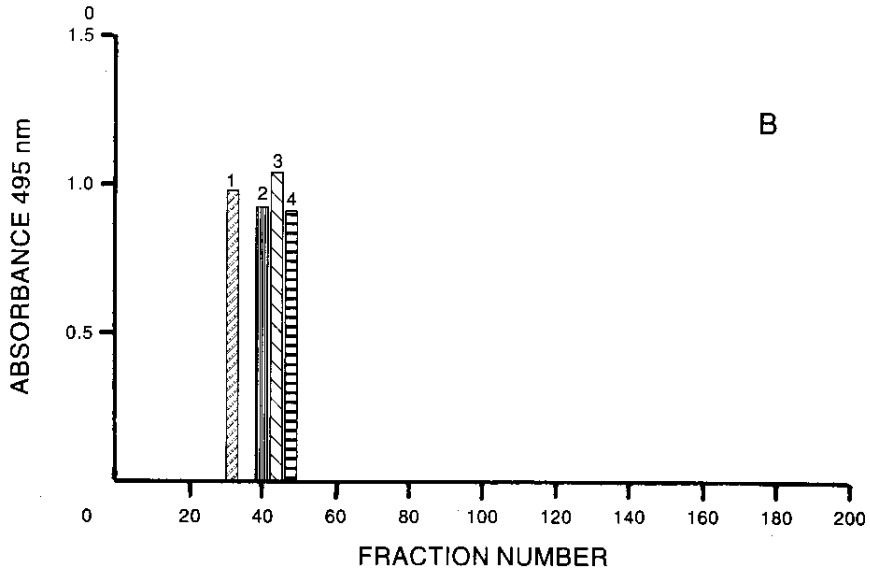

Fig. 2B: Fractionation of Pronase- (2), trypsin- (3) and chymotrypsin-treated Fraction 1 glycoproteins on calliberated column of Sepharose 4B.
The column was washed with phosphate buffer pH 7.0.

was removed at the centrifuge and the residue was washed with buffer. The pH of the supernatant and the washings was adjusted to pH 5.0 with acetic acid and the solution dialyzed against distilled water at 4 °C. The non-dialyzable material was lyophilized and a portion of the residue (8 mg) was applied to a calibrated (0.8 x 12 cm) column of Sepharose 4B. Fractions containing carbohydrate and protein were combined and dialyzed, and the retentate was lyophilized to give the Pronase-resistant (degraded) glycoproteins.

Trypsin treatment

The Fraction 1 glycoprotein (15 mg) was treated with trypsin, 0.8mg, in 5 ml of 40 mM Tris-HCl buffer, pH 8.2, for 20 hours at 37 °C. After

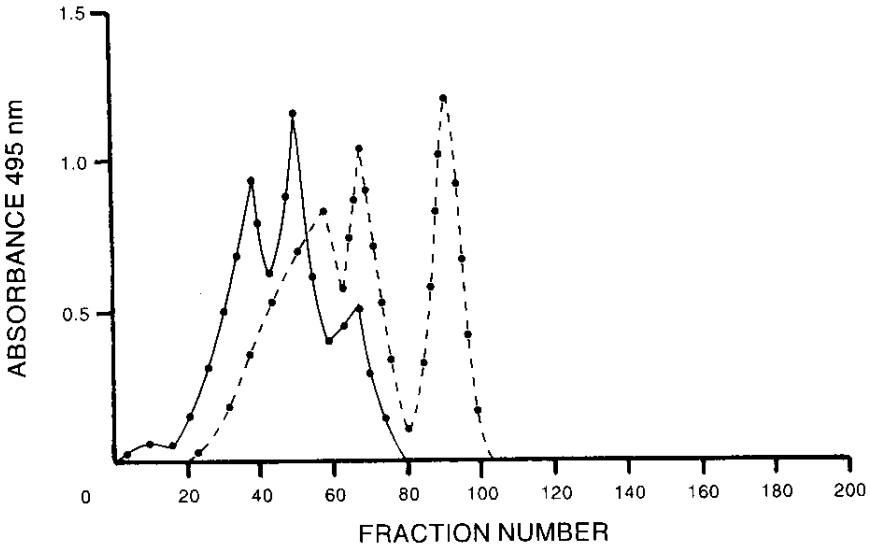

Fig. 3: Fractionation of Pronase- and trypsin-treated (Fraction 1) asialoglycoproteins on a calliberated Sepharose 4B column. The column was washed with 50 mM phosphate buffer, pH 7.0.

incubation for 20 hours at 37 °C another portion of trypsin, 100 μg, in 250 μl of Tris-HCl buffer was added, and the mixture was further incubated for 10 hours. Trypsin was inactivated by the addition of 8 fold excess N-α-p-tosyl-L-lysine chloromethyl ketone-HCl (TLCK, Sigma), the solution was acidified to pH 4.5 with 1 M HCl, and dialyzed. The non-diffusible material was lyophilized (8 mg) and the residue was applied to the calibrated Sepharose 4B column (0.8 x 12 cm). The carbohydrate-protein containing fractions were combined, dialyzed and lyophilized to give trypsin resistant glycoproteins.

Chymotrypsin treatment

The Fraction 1 glycoprotein (15 mg) was treated with 0.5 mg α-chymotrypsin (Worthington Biochemical) in 0.1 M sodium phosphate, pH 7.0, (4 ml) at 37 °C for 24 hours. The pH of the solution was adjusted to 4.5 with 1 M HCl, and the mixture was dialyzed. The non-diffusible solution was lyophilized, and a portion of the residue (8 mg) was applied to the calibrated Sepharose 4B column. The carbohydrate and protein containing fractions were combined, dialyzed and lyophilized to give chymotrypsin resistant glycoproteins.

Polyclonal antibodies

Antibodies to the simian glycoproteins were raised as previously described by us [10]. Cordis 11 plates (Cordis, Miami, Fla.) or 1% agarose microscope slights were employed for immunodiffusion. Human cervical mucus and Sepharose 2B Fraction 1 were reacted in immunodiffusion with antisemian antibody against purified semian glycoprotein. The plates were developed for 96 to 120 hours.

Removal of sialic acid

Sialic acid was removed from crude mucus (3ml), after keeping the mucus in a boiling water bath for a minute, by treatment with neuraminidase (1ml, 500 units, *Vibro cholerae*). The mucus was treated with neuraminidase twice, kept in a boiling bath for a minute and sperm penetration test performed.

Sialic acid was removed from Fraction 1 glycoprotein (30 mg) by treatment with neuraminidase (*Vibrio cholerae*, 0.5 ml: 250 units, Behring diagnostics) in 0.1 M sodium acetate (20 ml) containing 0.1% calcium chloride, pH 5.5, for 24 hours at 37 °C. After a further addition of

neuraminidase (100 µl), the solution was incubated for another 10 hours. The reaction was terminated by immersing the mixture in a boiling water bath for 3 minutes. The mixture was dialyzed and the nondialyzable material was lyophilized. The residue in 0.1 M sodium acetate buffer (pH 5.0, 15 ml) was incubated at 37 °C for 32 hours with neuraminidase (2 mg, *Clostridium perfringens*, Sigma chemical, Type VI). The mixture was dialyzed and the rententate lyophilized to give asialoglycoprotein. Degradation of asialoglycoprotein with Pronase and trypsin was carried out in a manner similar to that described for Fraction 1 glycoprotein.

Sialic acid was removed from human mucus (3 ml), after keeping the mucus in a boiling water bath for a minute, by treatment with neuraminidase (1 ml). The mucus was treated with neuraminidase twice, kept in a boiling water bath for a minute and then treated with insolublized Pronase (3 mg). The Pronase was removed and sperm penetration test performed.

Smith-degradation

Periodate oxidation-sodium borohydride reduction of the Pronase-degraded glycoprotein. The Pronase degraded glycoprotein (25 mg) was oxidized with 0.1 M sodium metaperiodate (5 ml) for 16 hours at room temperature in the dark. The excess of periodate was destroyed with ethyleneglycol and the solution was dialyzed against distilled water and the retentate was lyophilized. The residue in water (1.5 ml) was treated with sodium borohydride (10 mg) for 4 hours at 4 °C. A fresh portion of sodium borohydride (4 mg) was added and the solution was allowed to stand at room temperature for 2 hours. The excess borohydride was removed with 4 M acetic acid at 4°C, the solution was dialyzed and the retentate lyophilized to give periodate-resistant glycoprotein.

Second periodate oxidation

The periodate resistant glycoprotein (9mg) was treated with 0.1 M sodium metaperiodate (2.5 ml), the solution was kept for 22 hours at 24°C in the dark. The periodate treated material was processed as described above to give the periodate resistant material (4.2 mg).

Lectin-binding studies

The native-, Pronase-degraded and Smith degraded glycoproteins were treated with wheat germ and *Ricinum communis* lectins as previously described [22].

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