

Human Duodenal Gland (Brunner's Gland) Mucus Glycoprotein Analysis

HENK L. SMITS¹ AND MEBIUS F. KRAMER

*Department of Histology and Cell Biology, State University of Utrecht, School of Medicine,
Nic. Beetsstraat 22, 3511 HG Utrecht, The Netherlands*

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A mucus glycoprotein of the duodenal gland is characterized. The glycoprotein was isolated from a water-soluble homogenate fraction of the submucosal tissue of the most proximal part of the small intestine, containing the duodenal gland, and was purified from contaminating protein by two sequential equilibrium-centrifugation steps in CsCl density gradients. Structural analysis of the purified glycoprotein showed two regions in the protein core: one part characterized by the presence of essentially all of the cysteine residues and another by the presence of most of the serine and threonine. Carbohydrate was found linked to the latter part. Rat (H. L. Smits, P. J. M. van Kerkhof, and M. F. Kramer (1982) *Biochem. J.* 203, 779-785.) and human duodenal gland mucus glycoprotein show homology in chemical composition. Both glycoproteins have a relatively high protein content and contain little sulfate and no neuraminic acid. In man the mucus glycoprotein, however, has a higher content of serine plus threonine, a lower content of *N*-acetylglucosamine, a slightly higher content of fucose, and a lower molar ratio of *N*-acetylgalactosamine relative to serine plus threonine.

Although the existence of the duodenal gland in man has been noted as early as in the seventeenth century (2, 3), and the neural and hormonal control of the secretion of the duodenal gland has been studied in many cases (5), still little is known about the exact nature of the secretory product (6). Recently, we purified a mucus glycoprotein of this gland of the rat (1) and characterized its biochemical properties. The human glycoproteins of the gastrointestinal tract studied originate from the stomach (7, 8) and the small intestine (9).

As is now envisaged, mucus glycoproteins of the gastrointestinal tract share many characteristics (10, 11). They have a relatively low protein content. In the human stomach (8) and the small intestine (9) the protein content of the glycoprotein

only amounts to 17 and 12% (w/w), respectively. The oligosaccharide chains are of the mucin type and are *O*-glycosidically linked via *N*-acetylgalactosamine to serine or threonine in the protein core. They may consist of galactose, fucose, *N*-acetylglucosamine, neuraminic acid, and sulfate. The serine and threonine content of the protein moiety is high and totally amounts to about 30% (w/w). In the native form mucus glycoproteins may possess a multiglycopeptide structure of about equally sized subunits crosslinked by disulfide bridges. The cysteine residues involved in the linking of the subunits are localized almost exclusively in a region of the polypeptide containing few serine and threonine residues. That region can be removed from the polypeptide by proteolytic digestion, resulting in the loss of nearly all cysteine residues and the dissociation of the polypeptide subunits.

These data characterize the mucus glycoprotein of the rat duodenal gland (1) which, however, has a rather high protein

¹ To whom correspondence should be addressed: Laboratory of Pulmonary Function and Toxicology, National Institutes of Health, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, N. C. 27709.

content of about 30% (w/w). This glycoprotein contains only 2% (w/w) sulfate while neuraminic acid is absent. We investigated the mucus glycoprotein of the duodenal gland from patients undergoing surgical resection of parts of the fundic and antral stomach without pathological changes in the most proximal part of the intestine. Like in the rat, histochemistry has shown the presence of neutral mucus substances in the mucous cells of the duodenal gland of man (13).

MATERIAL AND METHODS

Preparation and purification of mucus glycoprotein of the duodenal gland. Tissue containing the duodenal gland was obtained from circular strips, 0.5 to 1.0 cm wide, of the small intestine directly adjacent to the pyloric sphincter. Immediately after resection the tissue was frozen in 0.15 M phosphate-buffered saline (pH 7.2) and stored at -20°C . After thawing, villi and crypts were removed by scraping the tissue with a razor blade. From the remaining tissue, containing the duodenal gland, a water-soluble homogenate fraction was prepared as described previously (1). This fraction contained about 80% of the duodenal gland mucus glycoprotein. The purified glycoprotein derived from this fraction is the nonreduced glycoprotein studied throughout this article. Of the nonsolubilized glycoprotein 40 to 60% could be obtained by reduction with 0.5 M 2-mercaptoethanol at 37°C for 30 min. The still undissolved glycoprotein could be obtained by solubilization with 6 M guanidium chloride. The amount of glycoprotein present in these two fractions was too small to permit a full analysis. The glycoprotein in these fractions showed the same electrophoretic mobility as the purified glycoprotein derived from the water-soluble homogenate fraction also observed for the glycoprotein of the rat (1). The mucus glycoprotein was purified from the pooled water-soluble homogenate fractions of five individuals by repeated equilibrium centrifugation on CsCl density gradients as described previously (1, 12). Glycoprotein was precipitated from gradient fractions by the alcohol-acetic acid method and solubilized by brief sonication at setting 6 for 1 min at 4°C using a Virtis ultrasonic cell disrupter Model 300.

Miscellaneous. For trypsin digestion, the purified glycoprotein was dissolved (2 mg/ml) in 0.1 M NH_4HCO_3 , pH 8.3, containing 10 mM NaN_3 , and digested by diphenylcarbamoyl chloride-treated trypsin from bovine pancreas (type XI; Sigma Chemical Co.) for 5 days at 37°C at an initial concentration of 0.15% (w/w) of glycoprotein. Trypsin was added again after 2 and 4 days (14). Pronase and pepsin digestion were

carried out as described by Mantle *et al.* (15) and Scawen and Allen (14), respectively.

For reduction, glycoprotein was dissolved in 0.5 M Tris-HCl buffer, pH 8.5. After addition of 2-mercaptoethanol to a concentration of 0.5 M, the sample was reduced for 24 h at 4°C . For subsequent carboxymethylation of the reduced glycoprotein, an equivalent volume of 1.5 M Tris-HCl, pH 8.5, containing 1.5 M iodoacetic acid was added and the solution was incubated for 24 h at 25°C in the dark (16). Glycoprotein was collected by precipitation.

For carboxymethylation with iodo[^3H]acetic acid, 500 μCi iodo[^3H]acetic acid (specific activity 61 mCi/mmol, Radiochemical Centre, Amersham, U. K.) in 1.5 M Tris-HCl, pH 8.5, was added in an equal volume to 500 μg reduced glycoprotein (1 mg/ml) in 0.5 M Tris-HCl, pH 8.5, containing 0.1 M dithiothreitol (Calbiochem), and the solution was incubated for 2 h at 37°C in the dark. Then a surplus of unlabeled iodoacetic acid was added and the glycoprotein was precipitated.

Gel filtration on a Sepharose 2B column (0.9×90 cm) and on a Sephadex G 100 column (0.9×90 cm) was performed in 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM NaCl and 1 mM EDTA. If reduced glycoprotein samples were applied, 10 mM dithiothreitol was added to the elution buffer. Fractions of 1.8 ml were collected.

Gel electrophoresis on 2% (w/w) polyacrylamide/0.5% (w/w) agarose gels and on 4% (w/w) polyacrylamide/sodium dodecyl sulfate (SDS)² gels, and analytical procedures were performed as described elsewhere (12).

Carbohydrate was determined by gas chromatography of trimethylsilyl derivatives after hydrolysis with 1 N methanolic HCl for 24 h at 85°C .

Amino acid composition was assayed with a Beckman Multichrom analyzer. Glycoproteins were hydrolyzed in 6 M HCl at 110°C for 24 h. Values of threonine and serine were multiplied by 1.05 and 1.10, respectively, to correct for loss during hydrolysis.

RESULTS

Isolation and purification of duodenal gland mucus glycoprotein. A single equilibrium centrifugation run on a CsCl density gradient of the lyophilized water-soluble homogenate fraction resulted in a suboptimal separation of glycoprotein and protein (Fig. 1A). A second centrifugation of fraction a (Fig. 1A) resulted in an almost complete purification of the glycoprotein (fraction a, Fig. 1B) as judged by the complete absence of protein contaminants

² Abbreviation used: SDS, sodium dodecyl sulfate.

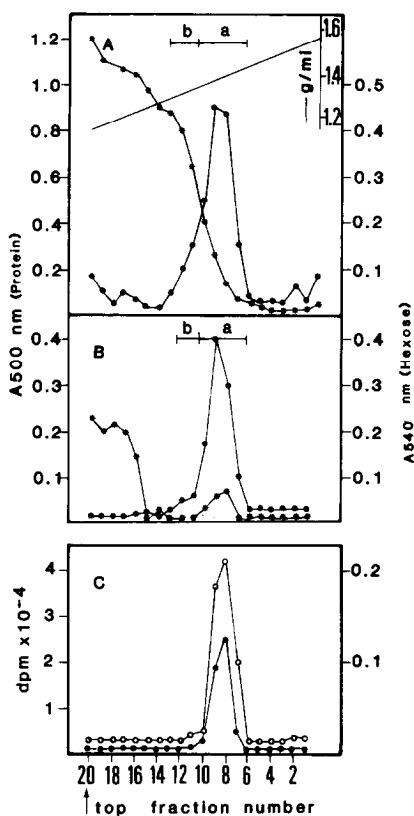


FIG. 1. Centrifugation of mucus glycoprotein of the duodenal gland on CsCl density gradient. (A) First centrifugation of water-soluble homogenate fraction. (B) Recentrifugation of glycoprotein fraction a (A). (C) Centrifugation of [carboxy- ^3H]methylated mucus glycoprotein (fraction a, B). (●, hexose; ×, protein; ○, radioactivity).

when analyzed by electrophoresis on a 4% polyacrylamide gel loaded with 200 μg of hexose. The twice-purified glycoprotein had a buoyant density of 1.40 g/ml on the CsCl density gradient and the reduced glycoprotein hardly entered a 2%-polyacrylamide/0.5%-agarose gel containing 0.1% SDS. No stacking gel was used on these gels as the electrophoretic mobility was that low that the reduced glycoprotein was completely retarded by the stacking gel. About the same buoyant density (1.37 g/ml on CsCl) and electrophoretic immobility was observed for the mucus glycoprotein of the rat duodenal gland (1). Like in the case of the rat duodenal gland mucus glycoprotein (1), the electrophoretic behavior

of the glycoprotein was not altered if 6 M guanidinium chloride, 8 M urea, 0.1% dithiothreitol, 0.05% 2-mercaptoethanol, or 0.01% Nonidet P40 was added to the gel. Glycoprotein present in fraction b (Fig. 1A and 1B) showed the same electrophoretic behavior as the main glycoprotein fraction, and therefore is assumed not to differ from the latter. As fraction b contained a high amount of protein contaminants, it was omitted from further study. About 1.25 mg (hexose) of purified glycoprotein was obtained per individual. It should be noted however, that the glycoprotein studied only represents the glycoprotein derived from the most proximal (0.5 to 1.0 cm) part of the duodenal gland.

Glycoprotein analysis. The chemical composition of the purified glycoprotein is shown in Table I. Like in the rat (1) the protein content is relatively high (33%) and the only sugars present are galactose, fucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine, while on a weight percentage basis little sulfate and no neuraminic acid is present. Apart from a higher molar proportion of *N*-acetylglucosamine and a somewhat lower proportion of fucose in rat, the molar proportion of sugars is similar

TABLE I

CHEMICAL COMPOSITION OF DUODENAL GLAND MUCUS GLYCOPROTEIN

	Composition (% by wt)	
	Native glycoprotein	Digested glycoprotein
Galactose	18.4	17.6
Fucose	11.9	13.4
<i>N</i> -Acetylglucosamine	9.2	9.7
<i>N</i> -Acetylgalactosamine	18.4	20.0
Total sugar	58.0	60.7
Protein	33.0	25.0
Sulfate	3.0	1.5
Total recovery	94.0	87.2

Note. The protein value was calculated by adding up the amounts of individual amino acids. The results are expressed as percentages by weight of freeze-dried material. Molar proportions in native glycoprotein: Gal:Fuc:GlcNAc:GalNAc = 1.0:0.65:0.5:1.0; GalNAc:(Ser + Thr) = 0.80:1.0. Molar proportions in digested glycoprotein: Gal:Fuc:GlcNAc:GalNAc = 1.0:0.75:0.55:1.15; GalNAc:(Ser + Thr) = 1.02:1.0.

in the two species. The total recovery (Table I) is only 94%. This may well be due to the presence of water or low amounts of salts. The amino acid composition (Table II) is characteristic for mucus glycoproteins, showing a high content of serine plus threonine (34.6%). The molar ratio of *N*-acetylgalactosamine to serine plus threonine (0.80) is lower than that observed for the glycoprotein of the rat and indicates that only part of the serine and threonine residues is glycosylated. The high content of serine and threonine together with the absence of contaminating sugars like mannose, ribose, deoxyribose, and glucose is indicative for the purity of the glycoprotein.

On gel filtration on Sepharose 2B the native and the reduced and carboxymethylated glycoprotein preparations both eluted in and just behind the void volume of the column (Figs. 2A and B). The native

TABLE II
AMINO ACID COMPOSITION OF DUODENAL GLAND
MUCUS GLYCOPROTEIN

	Amino acid composition (residues/100 residues)		
	Non-reduced	Reduced and carboxymethylated	Trypsin digested
Asp	1.1	1.0	1.0
Thr	21.9	19.1	22.8
Ser	12.7	13.2	16.4
Glu	5.1	5.3	5.2
Pro	16.0	16.3	11.6
Gly	5.0	5.1	6.0
Ala	5.9	5.9	6.6
Val	3.7	4.0	4.1
Met	0.3	0.4	0.3
Ile	7.0	7.0	6.9
Leu	5.0	5.9	5.5
Tyr	1.1	1.2	0.9
Phe	2.6	2.6	2.0
Lys	2.8	2.8	2.3
His	7.0	6.7	5.2
Arg	1.9	1.8	1.5
Cys	1.5	—	—
CmCys	—	1.2	—

Note. CmCys, carboxymethylcysteine.

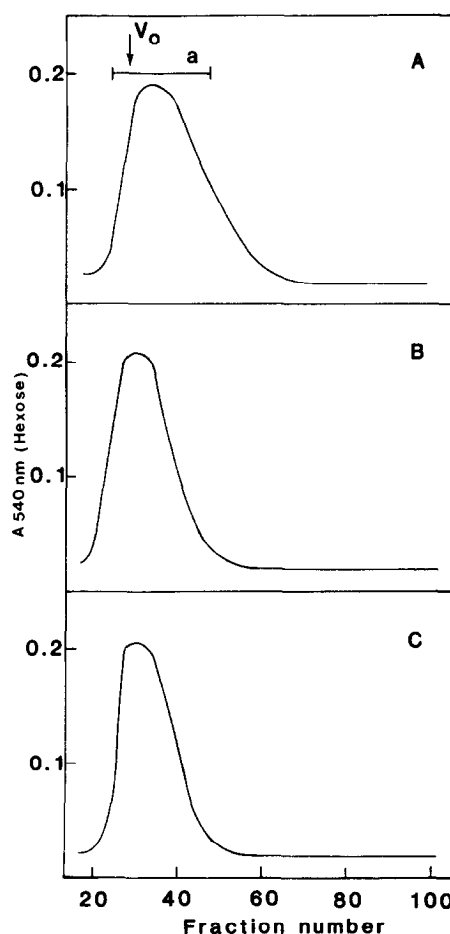


FIG. 2. Gel filtration of purified mucus glycoprotein of the duodenal gland on Sepharose 2B. (A) nonreduced glycoprotein; (B) reduced, carboxymethylated glycoprotein; (C) trypsin digest of reduced, carboxymethylated glycoprotein. Fractions were analyzed for total hexose.

glycoprotein formed a trailing edge, indicating the presence of some degraded material. Whether this material is formed during the isolation procedure or whether it was already present in the native mucus is not known. The same phenomenon was observed for rat duodenal gland mucus glycoprotein (1) as well as for many other mucus glycoproteins.

Upon digestion of the reduced and carboxymethylated glycoprotein (fraction a, Fig. 2B) with trypsin, the digested glycoprotein still eluted in and just behind the void volume of the column (Fig. 2C). Gel

electrophoresis of that trypsin-digested glycoprotein (fraction a, Fig. 2C) showed that the electrophoretic mobility of the glycoprotein is not altered by digestion (not shown). Like the reduced glycoprotein, the digested glycoprotein hardly entered the gel and remained just below the origin. Also, digestion with Pronase and pepsin did not increase the mobility on gels.

To investigate the existence of nonglycosylated region(s) rich in cysteine residues in mucus glycoprotein of human duodenal gland, we labeled cysteine residues present in the glycoprotein by carboxymethylation with iodo³H]acetic acid after reduction. Under the conditions used all cysteine residues have been carboxymethylated, as is shown by the amino acid analysis (Table II). Labeling by carboxymethylation was observed only after reduction. This demonstrates the specificity of the labeling reaction and the involvement of all cysteine residues in disulfide bridges in the native glycoprotein. Density gradient centrifugation of the thus labeled glycoprotein (Fig. 1C) showed that the labeled glycoprotein had the same buoyant density as the native glycoprotein. As all radioactivity present in fractions 1-6 and 11-19 (Fig. 1C) coeluted with free radioactivity on a Sephadex G 100 column, no high-molecular-weight material with other density than the mucus glycoprotein had been labeled. Elution of the labeled glycoprotein on a Sephadex G 100 column showed radioactivity in material of high molecular weight in the void volume of the column only, as did 95% of the hexose. Some radioactivity eluted in the same included fractions of the column as did free [*carboxy*-³H]methylcysteine. This material presumably represented some noncovalently bound iodoacetic acid, as it coeluted with NaCl on a small Bio-gel P₂ column. Upon trypsin digestion of the radioactively labeled glycoprotein, radioactivity was almost completely digested and eluted as a polydispersed fraction of low molecular weight, containing little if any hexose (Fig. 3B), which almost all still eluted in the void volume of the column. The loss of radioactivity from the high-molecular-weight material upon digestion with tryp-

sin is in agreement with the nearly complete loss of cysteine residues as observed by amino acid analysis (Table II), while serine and threonine are retained. This proves that cysteine is present in discrete regions of the polypeptide sensitive to proteolytic digestion. The digestion of a cysteine-rich fragment in the peptide chain is consistent with an increase of the molar proportion of serine plus threonine from 33.6 to 39.2% of the total amount of amino acids and with an increase of the molar ratio of sugar to protein from 1.02 to 1.38 on proteolytic digestion. The resistance to digestion by trypsin of the region containing serine and threonine may well be explained by its protection by the carbohydrate moiety.

We were unable to measure accurate sedimentation-coefficient values of both native and reduced glycoprotein. In both cases sedimentation was observed at low speed and over a wide range.

DISCUSSION

It has been proposed that the alkaline, viscous, secretion fluid of the duodenal gland might contribute to the protection of the small intestinal mucosa in the proximal part of the duodenum against the erosive effect of the gastric juice (5). The protection given by the mucus to the mucosa depends at least in part on its viscous and gel-forming properties which in turn largely depend on the polymeric nature of the native mucus glycoprotein (17, 18), which forms the major macromolecular constituent of the mucus besides protein (19). Structural studies of gastric (20) and small intestinal mucus glycoproteins (15, 21) from the pig have presented a molecular basis for these properties. The non-degraded mucus glycoprotein consists of multiglycopeptide built up from about equal-sized subunits linked by disulfide bridges present in regions of the protein core essentially devoid of carbohydrate. The integrity of this polymeric structure seems to be the primary requirement for the optimal physiological function of the glycoprotein. The properties of the mucus glycoproteins of the human stomach (7)

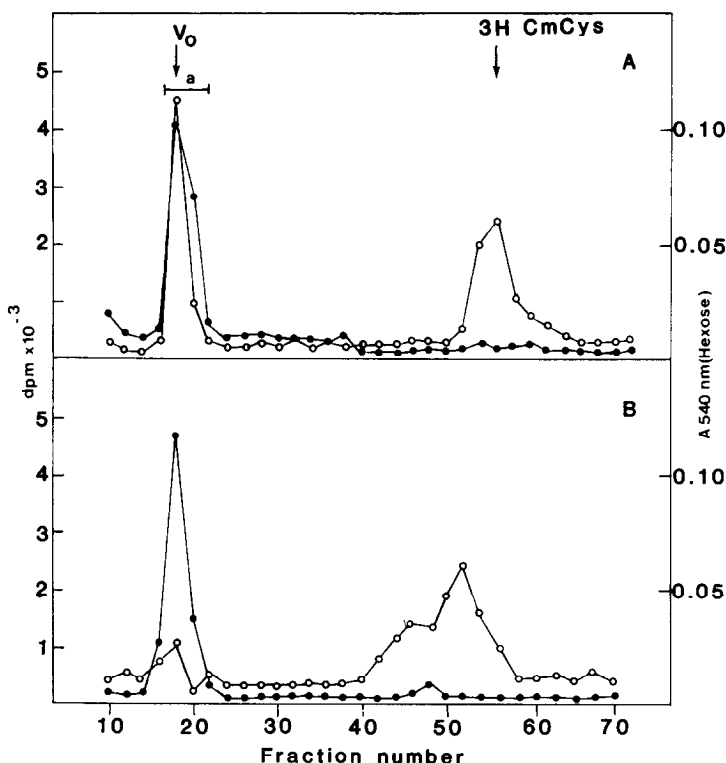


FIG. 3. Gel filtration of [*carboxy*- ^3H]methylated mucus glycoprotein of duodenal gland on Sephadex G 100. (A) Reduced glycoprotein, labeled by carboxymethylation with iodo[^3H]acetic acid. (B) Trypsin digest of the [*carboxy*- ^3H]methylated glycoprotein. (O, radioactivity; ●, hexose.) [^3H]CmCys, [*carboxy*- ^3H]methylcysteine; V_0 , void volume.

and the duodenal gland of the rat (1) fit this concept of a multiglycopeptide structure. Also, in the protein core of the mucus glycoprotein of the duodenal gland of man, cysteine residues are present mainly in discrete regions containing little serine and threonine residues, and carbohydrate, as could be shown by proteolytic digestion of the glycoprotein (Tables I and II, Fig. 3). However, column chromatography and sedimentation-velocity analysis of native and reduced glycoprotein did not show the existence of glycoprotein subunits linked by disulfide bridges, as glycoprotein eluted in and just behind the void volume of a Sepharose 2B column both before and after reduction (Figs. 2A and B) and sedimentation coefficients even of the reduced glycoprotein could not be determined. This was presumably due to the formation of aggregates by both the native and the reduced glycoprotein. Our results therefore

neither show nor exclude the existence of subunits in this particular glycoprotein. The electrophoretic immobility of the reduced glycoprotein under various conditions also suggests an abnormal behavior of the reduced glycoprotein due to the formation of aggregates. Such an abnormal behavior may well be due to special viscoelastic properties of the glycoprotein. However, gastric (20) and small intestinal mucus glycoproteins (21) of the pig lose their gel-forming and viscous properties on reduction. Incomplete reduction of the glycoprotein, however, can be ruled out, as chemical analysis of the glycoprotein has shown that upon reduction and carboxymethylation all cysteine residues are transformed into carboxymethylcysteine (Table II).

The mucus glycoproteins of rat (1) and human duodenal glands both have a relatively high protein content and contain

little sulfate and no neuraminic acid. Beside these similarities, some differences in chemical composition are found. In man the mucus glycoprotein has a higher content of serine plus threonine combined with a lower ratio of *N*-acetylgalactosamine to serine plus threonine, and a higher molar content of fucose combined with a lower molar content of *N*-acetylglucosamine.

Recently, we studied the primary structure of the oligosaccharides of the mucus glycoproteins of the duodenal gland of rat and pig by ^1H NMR spectroscopy (22). The primary structure of the main oligosaccharides of the mucus glycoprotein of the rat could be determined. The carbohydrate moiety of this glycoprotein consists of a mixture of branched tetra-, penta-, and hexasaccharides containing terminal *N*-acetylglucosamine $\alpha(1 \rightarrow 4)$ -linked to galactose as a characteristic constituent. The oligosaccharides of the mucus glycoprotein of the pig are more complex, but show the same characteristics. As the carbohydrate composition of the mucus glycoprotein of the duodenal gland of rat, pig, and man differ only slightly, the characteristics of the latter may well be similar, too.

It has been suggested that in gastric (23, 24) and small intestinal mucus (15) of the pig a nonglycosylated protein with a molecular weight of 70,000 and 90,000, respectively, is associated with the native glycoprotein and might be involved in crosslinking the glycoprotein subunits by disulfide bridges. As after labeling of the reduced glycoprotein with iodo[^3H]acetic acid no radioactively labeled high-molecular-weight material could be detected other than the glycoprotein component (Fig. 1C), such a protein is unlikely to be part of the duodenal gland mucus glycoprotein.

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