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Isolation and structural analysis of rat gastric mucus glycoprotein suggests a homogeneous protein backbone

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We isolated monomeric gastric mucus glycoprotein from the rat stomach by applying three successive CsCl-density-gradient steps in the continuous presence of guanidinium chloride. The rat gastric mucin was pure as compared with mucin isolated without the chaotropic reagent. In addition, the presence of guanidinium chloride resulted in a better preservation of the protein moiety. The purified mucin was fractionated according to buoyant density and chemically radiolabelled on tyrosine or cysteine residues and digested with specific proteinases. Analysis of mucin fractions of various densities gave identical peptide patterns, suggesting that the fractions contain a common protein backbone. Electron-microscopic images of the individual mucin molecules were recorded using rotary shadowing. They showed large filamentous molecules with a mean length of 208 nm that, after proteolytic digestion, yielded glycopeptides with a mean length of 149 nm. Heterogeneity in buoyant density and electrophoretic mobility is located in this large glycopeptide which remains after proteolytic digestion. Metabolic labelling of the mucin with [^{35}S]sulphate and [^{3}H]galactose, followed by purification and proteolytic digestion, revealed that this glycopeptide accounts for most of the mass and contains relatively little protein, but probably all the oligosaccharides and sulphate. As this protein part is masked by the oligosaccharides, detailed study by the methods described was not possible. The results indicate that rat gastric mucin is homogeneous in a major part of the protein backbone and that the heterogeneity of the molecule originates most likely from differences in sulphate and/or sugar composition.

INTRODUCTION

Gastrointestinal mucus glycoproteins produced in different animal species exhibit a characteristic heterogeneity in molecular size, buoyant density and charge (Allen *et al.*, 1982; Neutra & Forstner, 1987). This feature is largely due to heterogeneity among the large number of oligosaccharides present on these mucins (Allen *et al.*, 1982; Neutra & Forstner, 1987). Whereas the heterogeneity of the oligosaccharides is relatively well established, much less is known about the protein backbone. It has been suggested that human and rat intestinal mucin, as well as pig gastric and colonic mucin, consist of more than one glycoprotein (Gold *et al.*, 1981; Shub *et al.*, 1983; Stanley *et al.*, 1983; Wesley *et al.*, 1985).

Early studies from our laboratory suggested the possible existence of two mucus glycoproteins in mucin preparations from rat stomach (Spee-Brand *et al.*, 1980). It appeared that mucin with a high buoyant density and a corresponding high electrophoretic mobility had an amino acid composition slightly different from that of mucin with both low buoyant density and low electrophoretic mobility. Recently, we have found that these differences in buoyant density and electrophoretic mobility of rat gastric mucin can be explained by differential sulphation (Van Beurden-Lamers *et al.*, 1989). We now present evidence that rat gastric mucin consists of only one glycoprotein. We have purified monomeric mucin by means of three successive density gradients in the continuous presence of guanidinium chloride, a procedure analogous to that used in the purification of human cervical mucin by Carlstedt *et al.* (1983). Guanidinium chloride serves both as an excellent denaturing agent to remove adhering proteins and as an effective proteinase inhibitor. The molecular structure of the highly purified monomeric mucin was investigated by selective proteolytic degradation of radiolabelled mucin.

MATERIALS AND METHODS

Isolation of rat gastric mucus glycoprotein

The mucus glycoprotein from the stomach of the rat was isolated in a manner analogous to that used for the isolation of human cervical mucin by Carlstedt *et al.* (1983). Inbred male Wistar rats (200–250 g body wt.) were fasted for 16 h with free access to water and killed by cervical dislocation. The stomach was removed, turned inside out, rinsed with ice-cold phosphate-buffered saline (0.15 M-NaCl/0.01 M-sodium phosphate, pH 7.2) and stretched over a test tube. The top layer of the fundic mucosa was scraped off, removing the secreted mucus as well as the mucus-producing cells. The following procedures were conducted at 4 °C. The scraped tissue was gently dispersed in 6 M-guanidinium chloride (Grade I; Sigma, St. Louis, MO, U.S.A.)/50 mM-Tris/HCl (pH 7.5)/5 mM-EDTA/1 mM-phenylmethanesulphonyl fluoride in a glass/Teflon Potter–Elvejhem homogenizer. The mucus was solubilized under reducing conditions by

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stirring for 24 h in the presence of 100 mM-dithiothreitol under nitrogen. Carboxymethylation was done by the addition of 250 mM-iodoacetamide and stirred for another 24 h in the dark under a nitrogen atmosphere. Insoluble material was removed by centrifugation (30 min; 30000 *g*; Sorvall Superspeed; SS-34 rotor). CsCl (Ultrapure; Boehringer, Mannheim, Germany) was added to a density of 1.40 g/ml and the solution was adjusted to 4 M-guanidinium chloride, 50 mM-Tris/HCl, 5 mM-EDTA and 1 mM-phenylmethanesulphonyl fluoride, pH 7.5. Isopycnic density-gradient centrifugation was performed in a Beckmann ultracentrifuge for 66 h at 150000 *g*. Mucin-containing fractions were re-run on similar gradients. The mucin was finally purified on a third CsCl density gradient in 1 M-guanidinium chloride/50 mM-Tris/HCl/5 mM-EDTA/1 mM-phenylmethanesulphonyl fluoride and CsCl (density 1.50 g/ml; centrifugation conditions as above). The mucin-containing fractions were dialysed against water and stored at −20 °C.

Radiolabelling of the isolated mucin

Carboxymethylated mucin was iodinated with chloramine-T (Hunter & Greenwood, 1962). Typically, 0.5 mCi of $Na^{125}I$ (Amersham International; sp. radioactivity 15 mCi/mmol) was used to iodinate 0.5 mg of mucin. ^{125}I-Mucin was separated from $^{125}I^-$ on a Sephacryl S-200 (Pharmacia) gel-filtration column eluted with 100 mM-NH_4HCO_3, pH 8.0, and stored at −20 °C.

Labelling of cysteine residues was performed on reduced mucin. For this labelling the mucin was isolated without carboxymethylation in the continuous presence of 10 mM-dithiothreitol. The purified reduced mucin was dialysed against water and freeze-dried. The mucin (0.4 mg) was incubated for 60 min at 37 °C under N_2 in 6 M-guanidinium chloride/50 mM-Tris/HCl/5 mM-EDTA/1 mM-dithiothreitol, pH 8.3. Thereafter 3 mM-iodoacetamide (final concn.) was added, together with 50 μCi of iodo[^{14}C]acetamide (Amersham International; sp. radioactivity 53 mCi/mmol). The reaction was allowed to proceed at 37 °C under N_2 in the dark for 60 min. The reaction cycle was repeated with 2 mM-dithiothreitol and 5 mM-unlabelled iodoacetamide to accomplish complete modification of cysteine residues. Unchanged iodo[^{14}C]acetamide was removed by dialysis against 100 mM-NH_4HCO_3. The [^{14}C]mucin was stored at −20 °C.

Metabolic radiolabelling of gastric mucin

The oligosaccharides and sulphate residues on the mucus glycoprotein were labelled by incubating stomach segments for 2 h in the presence of [^{3}H]galactose and [^{35}S]sulphate as described by Van Beurden-Lamers *et al.* (1989). The stomach segments were washed with ice-cold phosphate-buffered saline and the [^{3}H,^{35}S]mucin was homogenized in 6 M-guanidinium chloride-containing buffer, carboxymethylated, and isolated as described above.

Proteolytic digestion of radiolabelled mucin

After dialysis against 100 mM-NH_4HCO_3, pH 8.0, the mucin was digested with one of the following proteinases: trypsin (diphenylcarbamoyl chloride-treated; Sigma), elastase (Merck), thermolysin (type X; Sigma), α-chymotrypsin (Sigma) or proteinase K (Boehringer). The incubations were performed with 1 μg of enzyme/μg of mucin at 37 °C for 16 h. The digestion was monitored by electrophoresis on 4–20 % SDS/polyacrylamide gradient gels.

Analytical methods

Hexose was assayed by the orcinol method of François *et al.* (1962), with galactose and fucose as standards (ratios 2:1). The quantity of hexose was expressed as absorbance at 540 nm. Densities of the CsCl solutions were determined by weighing a known volume using a calibrated pipette. Polyacrylamide-gel electrophoresis was performed, as described by Laemmli (1970), on gels containing 0.1 % SDS. Samples were applied to the gel after boiling for 3 min in sample buffer containing 5 % (v/v) 2-mercaptoethanol and 1 % SDS. The gels were stained with periodic acid/Schiff reagent (Konad *et al.*, 1984), Coomassie Blue or with silver (Silver Stain Kit; Bio-Rad, Richmond, CA, U.S.A.). ^{3}H and ^{35}S radioactivities in SDS/polyacrylamide gels were measured in a liquid-scintillation counter by cutting a dried gel into 0.5 cm segments, which were dissolved in 50 μl of water and 0.5 ml of Solulite (Baker, Phillipsburg, NJ, U.S.A.); the ^{125}I-labelled fragments were counted directly for radioactivity in a γ-radiation counter. Fluorograms were recorded as described by Bonner & Laskey (1974). Quantitative analysis of the monosaccharides was performed by g.l.c. (Kamerling *et al.*, 1975). Determination of amino acid composition after acid hydrolysis of the protein was performed with the use of an automated protein sequencer. Analytical gel filtration was done on a 90 cm × 1.6 cm column of Sepharose CL-2B (Pharmacia) in 4 M-guanidinium chloride/50 mM-Tris/HCl/5 mM-EDTA, pH 7.5. Fractions (2 ml) were collected and analysed for hexose and ^{125}I as described above.

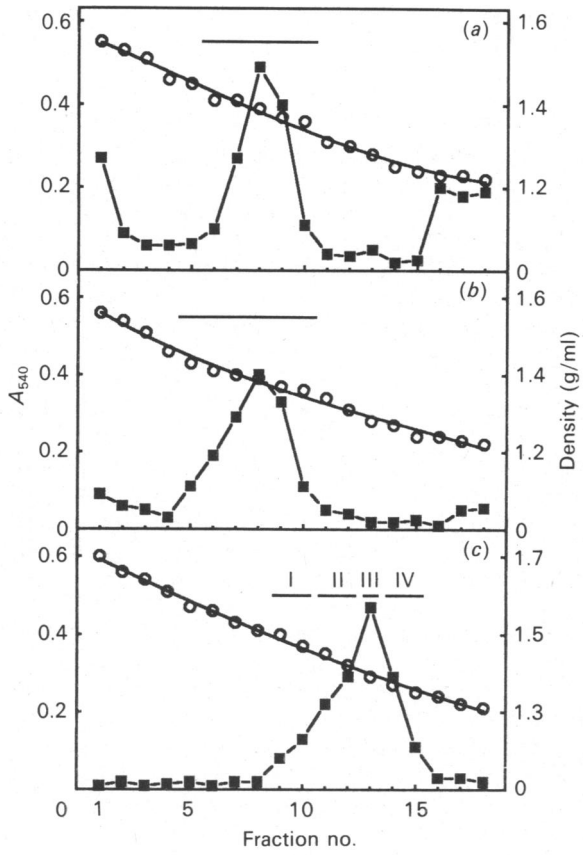
Electron microscopy of isolated gastric mucin

To reveal individual mucin molecules, mucin was sprayed on to mica in the presence of glycerol and rotary-shadowed as described by Fowler & Erickson (1979) for fibrinogen. Briefly, the purified mucus glycoprotein was extensively dialysed against 50 mM-ammonium formate, pH 7.4, and diluted to 100 μg/ml in this buffer containing 40 % (v/v) glycerol (Fluka). Small samples were sprayed on to freshly cleaved mica. The mica sheets were dried *in vacuo* [666 μPa (0.5×10^{-5} Torr)] at room temperature and subjected to rotary shadowing with platinum at an angle between 5 and 10°. The replicas were coated with a supporting film of carbon at a shadowing angle of 90°, floated on water and picked up on copper grids. The specimens were examined in a JEOL 1200 EX electron microscope operating at 80 kV. Micrographs were recorded at magnifications of up to 100000×. Contour lengths were measured by tracing over photographic enlargements on a digital drawing pad connected to an IBM microcomputer.

RESULTS

Isolation and characterization of rat gastric mucin

The extraction of gastric mucin with 6 M-guanidinium chloride was very effective, leaving less than 10 % of hexose-containing material in the 30000 *g* pellet. As Fig. 1(*a*) shows, the distribution of hexose in the first CsCl density gradient showed a peak at a density of 1.40 g/ml,



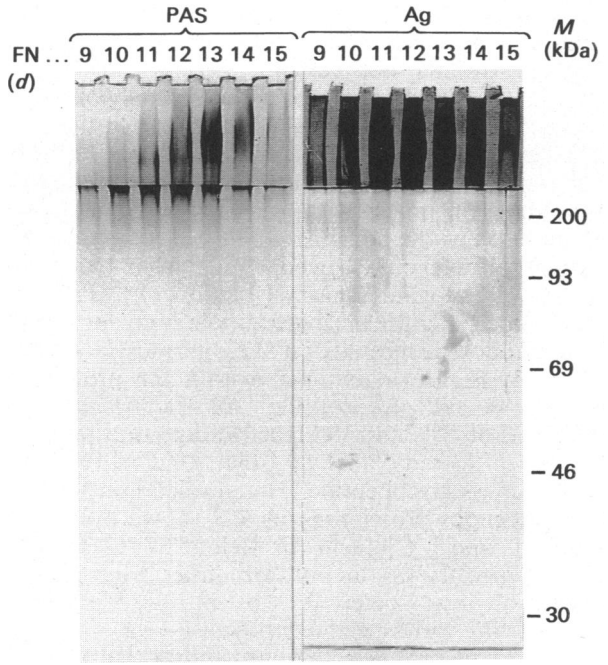


Fig. 1. Isolation of the mucus glycoprotein from rat stomach

The distribution of hexose-containing material after the first CsCl density gradient is shown in (*a*). The gradient had a starting density of 1.40 g/ml and contained 6 M-guanidinium chloride. The mucin-containing fractions were pooled as indicated by the horizontal bar and loaded on an identical gradient represented in (*b*). The mucin fraction indicated by the horizontal bar in (*b*) was purified by a third CsCl density gradient displayed in (*c*). This gradient had a starting density of 1.50 g/ml and contained 1 M-guanidinium chloride. The mucin-containing peak was subdivided into four fractions as indicated by the bars, designated I–IV (○, density; ■, hexose). The purity of the mucin after the final CsCl density gradient was assessed by analysis of aliquots on SDS/10%-polyacrylamide gels run with a 3.5% stacking gel (*d*). The fraction numbers (FN) refer to those of the third CsCl-density gradient (*c*); 'PAS' indicates periodic acid/Schiff, and 'Ag' indicates silver staining. Molecular masses (*M*) are shown on the right.

Table 1. Amino acid composition of purified rat gastric mucin

Amino acid	Composition (residues/100 residues)
Asx	7.4
Thr	15.7
Ser	14.8
Glx	10.5
Pro	8.9
Gly	10.2
Ala	7.3
Cys	1.2
Val	5.6
Met	0.3
Ile	1.7
Leu	3.6
Tyr	0.3
Phe	1.4
His	3.2
Lys	3.8
Arg	0.9

which is characteristic for mucins. The major contaminants in this fraction were small amounts of proteins, mainly present in the top of the gradient, and nucleic acids. These nucleic acids were found in the bottom fractions of the first and second gradient (fraction 1–8) and fractions 4–8 of the third gradient and these were characterized by a 2-fold higher absorption at 260 nm than at 280 nm. The contaminating proteins were removed by the second CsCl density gradient, and removal of nucleic acids was accomplished by the third gradient. The mucin preparation was free from contaminating substances as shown by SDS/polyacrylamide-gel electrophoretic analysis of 25 μg of mucin and subsequent silver staining (Fig. 1*d*).

The resulting mucin preparation contained more than 90% of the initial amount of mucin, estimated by the recovery of high-molecular-mass hexose-containing material from the initial homogenate. Omission of the carboxymethylation step before the actual purification did not influence this recovery (three independent experiments).

Table 1 shows the amino acid composition of the isolated mucin. Serine, threonine, proline and glycine are present in high amounts. Attempts to determine the *N*-terminal amino acid residue failed. This indicates that the mucin preparation was free from contaminating

Table 2. Monosaccharide composition of the four mucin fractions

The data are expressed as molar ratios relative to the amount of *N*-acetylgalactosamine.

Monosaccharide	Composition (molar ratio) (Fraction no.)... I	II	III	IV
Fucose	1.40	1.50	1.40	1.10
Mannose	0.06	0.05	0.06	0.09
Galactose	2.20	2.50	2.50	2.20
N-Acetylgalactosamine	1.00	1.00	1.00	1.00
N-Acetylglucosamine	3.00	3.00	3.10	3.00
Sialic acid	0.10	0.05	0.05	0.08

proteins. Moreover, it suggests that the gastric mucin has a blocked *N*-terminal amino acid residue.

To elucidate the heterogeneity, as reflected in the broad range in buoyant densities (1.33–1.52 g/ml), we divided the hexose peak from the third CsCl density gradient into four fractions designated I–IV. The monosaccharide composition of the four fractions was determined after hydrolysis (Table 2). The results, expressed as molar quantities relative to *N*-acetylgalactosamine, reveal a nearly identical monosaccharide composition for all four fractions. The composition shows high amounts of fucose, galactose, *N*-acetylgalactosamine and *N*-acetylglucosamine and trace amounts of mannose and sialic acid. This composition is indicative of a high abundance of *O*-linked oligosaccharides; the presence of mannose indicates the possible occurrence of *N*-linked oligosaccharides.

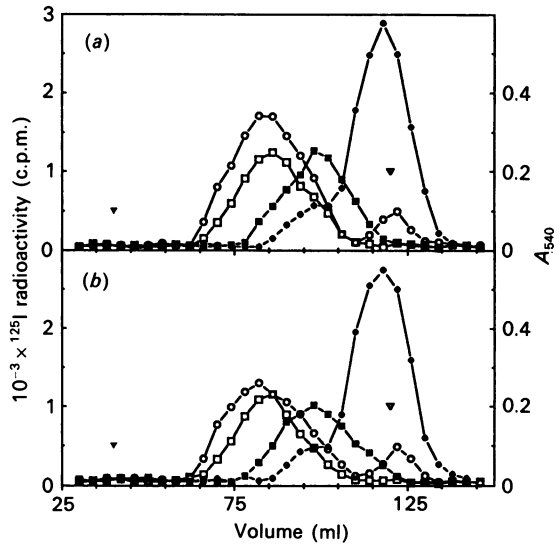


Fig. 2. Analysis of ^{125}I-mucin on a Sepharose CL-2B gel-filtration column (1.6 cm × 90 cm)

(*a*) Analysis of mucin fraction I; (*b*) analysis of mucin fraction IV. Fraction numbers are indicated as in Fig. 1(*c*). □, ■ Hexose profiles before (□) and after (■) digestion; ○, ●, ^{125}I-labelled material before (○) and after (●) digestion. The closed (▼) and open (▽) arrowhead indicate the void (V_0) and total (V_t) volumes of the column respectively.

Specific proteolytic digestion of rat gastric mucin

The heterogeneity of the gastric mucin was further investigated by specific proteolytic digestion of radiolabelled mucin. To increase the sensitivity of the assays the protein moiety of the mucin was first labelled either with ^{125}I or iodo[^{14}C]acetamide. The iodination of the mucin yielded specific radioactivities between 4.3 and 5.4×10^8 c.p.m./mg. Carboxymethylation using iodo-[^{14}C]acetamide yielded specific radioactivities of 7800–9200 d.p.m./mg; the recovery after dialysis was more than 90% for both labelling methods. We compared the behaviour of the ^{125}I-mucin fractions I and IV on Sepharose CL-2B gel filtration. The mucins from both fractions show identical elution profiles. The hexose peaks co-migrated with the ^{125}I radioactivity; the small peak at V_t originated from free $^{125}I^-$ (Fig. 2). Analysis of trypsin-digested ^{125}I-mucin on a Sepharose CL-2B column showed a glycopeptide which contains all the hexose, but only a small amount of the ^{125}I, whereas most of the ^{125}I-peptides were eluted at the V_t of the column (Fig. 2). These elution profiles are identical for all isolated mucin fractions. Fig. 3 shows a comparison of the four mucin fractions on SDS/4–20% (w/v)-polyacrylamide gradient gels. The control fractions show higher electrophoretic mobilities with increasing densities. This increased mobility was detected in periodic acid/Schiff staining as well as in the distribution of ^{125}I- and [^{14}C]mucins (Fig. 3). To gain an insight into the primary structure of the mucin, the four mucin fractions, both ^{125}I- and ^{14}C-labelled, were digested with specific proteinases. The digestion conditions employed in this experiment resulted in complete digestion of the protein part of the mucin (results not shown). Periodic acid/Schiff staining of the digestion products showed only one large glycopeptide, with a mobility slightly higher than the original mucin, irrespective of the proteinase used (Fig. 3*a*). It was also noted that the resulting glycopeptide originating from mucin with a higher buoyant density (fraction I) keeps the highest mobility as compared with the high-molecular-mass glycopeptide from the mucin with a lower buoyant density (fraction IV). Thus the features underlying the heterogeneity in buoyant density and electrophoretic mobility on SDS/polyacrylamide-gel electrophoresis, are maintained within the proteinase-resistant remnant glycopeptide. Autoradiograms and fluorograms of ^{125}I- and ^{14}C-labelled digestion products show very little radiolabel of either kind in the high-molecular-mass glycopeptide. The purified mucin is free from endogenous proteinases, as was shown by incubation of ^{125}I- and [^{14}C]mucin for 16 h at 37 °C (Figs. 3*b* and 3*c*; control lanes), as no degradation of the radiolabelled protein was detected. Most of the radioactivity after digestion with specific proteinases was found in numerous small peptides. Fig. 3(*b*) shows that the ^{125}I-peptides after digestion with trypsin, α-chymotrypsin, elastase and thermolysin were typically smaller than 14 kDa. Digestion of [^{14}C]mucin with trypsin also produced small peptides. However, the peptides resulting from digestion of [^{14}C]mucin with a α-chymotrypsin and elastase yielded larger peptides up to 70 kDa (Fig. 3*c*). Therefore we conclude that there are several cysteine-containing peptides in the proteinase-sensitive part of

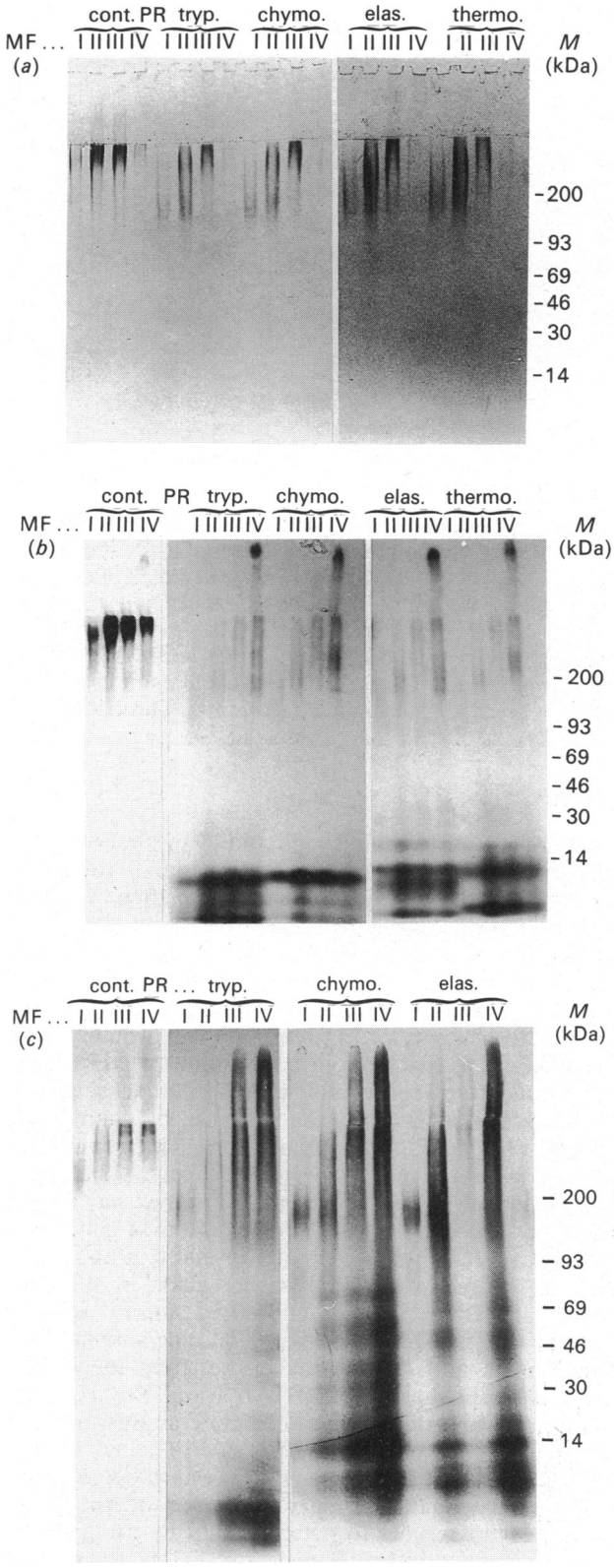


Fig. 3. Analysis of proteolytic-digestion products of [^{14}C]- and ^{125}I-mucin on SDS/4–20% polyacrylamide gels (3.0% stacking gel)

Control incubations were performed as described in the text, but without addition of proteinase. (*a*) Periodic acid/Schiff staining; (*b*) autoradiogram of ^{125}I-labelled digestion products; (*c*) fluorogram of ^{14}C-labelled digestion products. The Roman numbers I–IV indicate the mucin fractions as defined in Fig. 1(*c*). Abbreviations: Cont., control incubation; *M*, molecular mass; PR, proteinase; tryp., trypsin; elas., elastase; chymo., α-chymotrypsin; thermo., thermolysin; MF, mucin fractions. [The digestion of [^{14}C]mucin with thermolysin in (*c*) is not shown.]

Table 3. Distribution of hexose and radioactive sulphate, galactose and amino acid residues in the proteolytic products of digested mucin

The hexose distribution is based on Sepharose CL-2B gel-filtration patterns. The distribution of radioactivity is based on analysis of SDS/polyacrylamide gel-electrophoretic results. The SDS/polyacrylamide gels are dried directly to minimize the loss of small peptides from the gels and subsequently cut into fragments for determination of radioactivity. Abbreviation: nd, not detectable.

	Distribution (%)	
	Glycopeptide	Peptides
Hexose	100	nd
[^{3}H]Galactose	98	2
[^{35}S]Sulphate	99	1
[^{14}C]Cysteine	11	89
^{125}I-tyrosine	9	91

the protein that do not contain tyrosine and which have molecular mass up to 70 kDa.

The mucin from the different fractions labelled with the same radioisotope and digested with the same proteinase show identical peptide patterns. Our conclusion is that the portion of the peptide backbone which is degraded by the proteolytic enzymes is homogeneous within the whole range of buoyant densities.

To locate the oligosaccharides and sulphate within the molecule, we incubated rat stomach segments in the presence of both [^{3}H]galactose and [^{35}S]sulphate and subsequently isolated the double-labelled mucin. This purified mucin was then digested with trypsin and analysed on SDS/4–20%-polyacrylamide gels. All ^{35}S and ^{3}H radioactivity was present on the glycopeptide, and no radioactivity was found in the small peptides (results not shown).

Table 3 summarizes the distribution of hexose and the various radioactive labels in tryptic digests of purified mucin. It shows that probably all saccharides and sulphate groups are localized within the proteinase-resistant glycopeptide, which comprises most of the molecular mass. The periphery of the molecule is susceptible to proteolysis and apparently contains all of the labelled cysteine and tyrosine residues, but hardly any galactose or sulphate.

Electron-microscopic image of the isolated mucin

To get an indication of the shape of the mucus glycoprotein we used rotary shadowing to measure the contour length of the individual molecules. The purified mucin appeared as long, filamentous, flexible molecules, as shown in Fig. 4, with an average length of 208 nm (Table 4).

In the previous section we showed that proteolytic digestion of the mucin results in a small decrease in

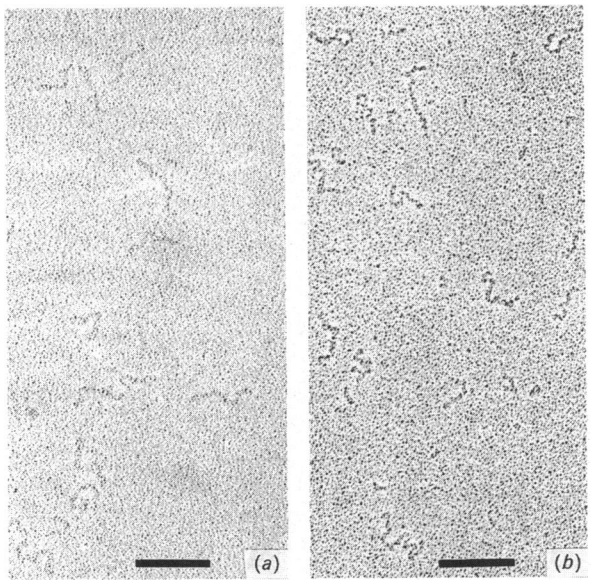


Fig. 4. Electron-microscopic images of isolated mucin molecules revealed by rotary shadowing

Rat gastric mucin isolated in the presence of guanidinium chloride before (*a*) and after (*b*) proteinase K treatment is shown. The bars indicate 100 nm.

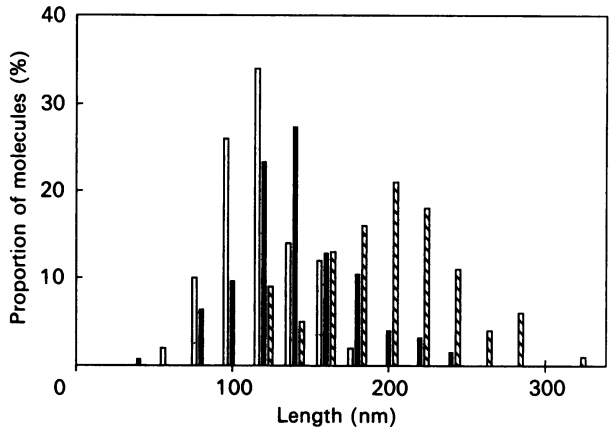


Fig. 5. Effect of proteolytic digestion on the length distribution of mucin molecules

The molecules were made visible by rotary shadowing. The length of the molecules was measured for magnifications of × 100000. Only clearly separated molecules, free from kinks, were measured. Actual values and averages for the observations are shown in Table 4. □, Mucin purified by the method of Spee-Brand *et al.* (1980); ▨, mucin purified in the presence of guanidinium chloride; ■, this described mucin after digestion with proteinase K.

molecular mass and, at the same time, a loss of 90% of tyrosine and cysteine residues. To illustrate this phenomenon we treated purified mucin with proteinase K before the rotary-shadowing procedure. Proteinase K digestion decreased the mean length of the molecules significantly from 208 nm to 149 nm ($P < 0.01$; Table 4). The electron-microscopic image of the rat gastric mucin molecules shows no differences in diameter between the glycosylated and the proteinase-sensitive part of the molecule (Figs. 4*a* and 4*b*).

The electron-microscopic observations led to the conclusion that the proteinase-resistant glycopeptide of the gastric mucin contains 72% of the molecule's length (Table 4). This value could well explain the small difference in apparent molecular mass between the mucin molecule and the glycopeptide observed in gel filtration and on SDS/polyacrylamide-gel electrophoresis.

Table 4. Length of individual mucin molecules as assessed by measuring of electron-microscopic images produced by rotary shadowing

For details, see the legend to Fig. 5. The mucin in the preparation method without guanidinium chloride is not significantly longer than the proteinase K-treated mucin ($P < 0.01$). The undigested mucin prepared as described in the Materials and methods section was significantly longer than both the digested mucin ($P < 0.01$) and the mucin prepared as described by Spee-Brand *et al.* (1980) ($P < 0.01$).

Mucin treatment or preparation	No. of molecules	Length (nm)	S.D.
Spee-Brand *et al.* (1980)	50	128	26
With guanidinium chloride	104	208	42
Proteinase K-treated	124	149	36

DISCUSSION

In a previously published study we described the purification and partial characterization of rat gastric mucin (Spee-Brand *et al.*, 1980). Since then, Carlstedt *et al.* (1983) developed a method for mucin isolation in high yields and with maximum protection against proteolytic degradation. We have employed this refined purification method on rat gastric mucin and we have found it to be superior in preserving the protein part of the molecule, as was most clearly demonstrated by length measurements of individual molecules. The lengths of the molecules isolated in the presence of guanidinium chloride were significantly greater than those of molecules isolated without the chaotropic reagent. The mucin preparation described in the present paper was pure as was demonstrated by SDS/polyacrylamide-gel-electrophoretic analysis followed by silver staining and by the absence of free *N*-terminal amino acids in the preparation. Blockage of the *N*-terminal amino acid was also demonstrated for ovine and porcine submaxillary-gland mucin and canine tracheobronchial mucin (Hill *et al.*, 1977; Eckhardt *et al.*, 1987; Ringler *et al.*, 1987). The monosaccharide and amino acid compositions were characteristic for mucins (Neutra & Forstner, 1987).

On the assumption that the mucin was intact and pure, we investigated the primary structure of the mucin molecule with respect to heterogeneity by using specific proteolytic degradation. Specific proteolytic digestion of mucin radiolabelled on specific amino acid residues showed that all mucin fractions, independent of their buoyant density, yielded identical peptide patterns. We conclude that the protein part of the molecule, which is susceptible to proteolysis, is likely to be homogeneous. At present, no amino acid sequence data for the oligosaccharide-containing part of the molecule are available. Therefore, detailed comparison of the complete protein moieties of the different fractions were not possible using

the methods described. Swallow *et al.* (1987) have shown that PUM (**P**eanut-lectin-binding **U**rinary **M**ucin), a human tumour-associated mucin, is coded by a hypervariable gene locus. They suggest that the heterogeneity of this protein is due to unequal crossing-over events between tandem repeats in the gene. These tandem repeats are probably located within the glycosylated part of the protein. If the gene coding for rat gastric mucin contains repeated structures comparable with the *PUM* locus, this kind of heterogeneity would not be detected by our assay.

The heterogeneity of the mucin in SDS/polyacrylamide-gel-electrophoretic mobility was maintained within the large glycopeptide comprising the major proteolytical degradation product. This glycopeptide, which contains all the oligosaccharides and sulphate groups of the molecule, showed a monosaccharide composition and an apparent molecular mass on gel filtration independent of the buoyant density of the mucin. We have shown previously that the major cause for this heterogeneity of the mucin in respect of buoyant density and in electrophoretic behaviour is a differential degree of sulphation of the molecules (Spee-Brand *et al.*, 1980; Van Beurden-Lamers *et al.*, 1989). Our present observation that all sulphate is located within the glycopeptide supports this conclusion.

These observations taken together, we can describe the rat gastric mucin as a filamentous (which is a common feature for this group) molecule (Rose *et al.*, 1984; Sheehan *et al.*, 1986). If we assume that the radioactive labellings of the tyrosine and cysteine residues were uniform for the whole molecule, we conclude that probably all cysteine and tyrosine residues are located in the proteinase-sensitive part of the molecule. The presence of a large proteinase-resistant part of the molecule, containing most of the oligosaccharides, and a proteinase-sensitive part, which contains hydrophobic amino acid residues and cysteine residues, was also found for pig small-intestinal mucin (Mantle *et al.*, 1981; Mantle & Allen, 1981), rat small intestine mucin (Smits *et al.*, 1982) and canine tracheobronchial mucin (Ringler *et al.*, 1987).

From our present data we are unable to say whether the non-glycosylated part of the protein backbone, which comprises 28% of the molecule's length, protrudes at one end, or both ends, of the molecule. We have shown that efficient proteinase inhibition is essential for the isolation of gastric mucin. This means that monomeric mucin is not intrinsically resistant against proteolysis. Further studies need to address the question as to how the proteinase-sensitive part of the molecule is protected *in vivo* against proteolysis by pepsin.

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