Susceptibility of the cysteine-rich N-terminal and C-terminal ends of rat intestinal mucin Muc 2 to proteolytic cleavage

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The present study reveals that partial proteolytic degradation of rat Muc 2 mucin can occur rapidly even in the presence of a battery of proteinase inhibitors. During the initial steps of purification from homogenates of intestinal scrapings, degradation was rapid, causing release of the entire 118 kDa C-terminal glycopeptide and, as shown by N-terminal sequencing, a large (200 kDa) N-terminal glycopeptide fragment. Degradation could be prevented by adding 6 M guanidinium chloride provided that its presence was maintained throughout every step of purification. Even after purification, however, the mucin was still vulnerable to partial proteolysis unless it was stored in guanidinium chloride at −20 °C. These findings imply that a potent proteinase contaminant remains tightly bound to the mucin through every step of purification, or else that the mucin has autocatalytic properties. Because the C- and N-terminal regions of secretory mucins are required for their assembly into linear mucin polymers that form functional gels, our findings emphasize that extreme care is required to purify structurally intact mucin molecules. They also imply that the specific degradation steps described here are likely to occur rapidly after mucins are secreted into the intestinal lumen and come into contact with the products of sloughed cells.

INTRODUCTION

Within the intestinal tract, epithelial mucins are abundant and have important roles in lubrication and host defence [1–5]. For several years we have observed that mucins purified from human and rat intestinal scrapings and then subjected to thiol reducing agents produce a distinctive banding pattern on SDS/polyacrylamide or acrylamide–agarose composite gels. The pattern consists of high-molecular-mass glycoprotein bands near the top of the gel and a major band with a midpoint position corresponding to a molecular mass of 118 kDa. Because the latter contained carbohydrate and was observed only when mucins were reduced, we suggested that the 118 kDa component was a putative 'link' glycopeptide responsible for disulphide linkage of mucin monomers into polymers [6–10]. Another minor band at approx. 200 kDa was also frequently noted in mucin preparations [11,12]. Because it reacted (weakly) with an antibody to the 118 kDa component and had a similar amino acid profile, we postulated that the 200 kDa component might be a dimer of the 118 kDa glycopeptide.

Subsequent studies revealed that the 118 kDa 'link' glycopeptide was not a separate peptide but actually represents the cysteine-rich C-terminus of a large mucin, rat Muc 2 (originally called MLP, mucin-like-peptide [13]). Rat Muc 2 is a homologue of human intestinal MUC2, with known sequence similarity within its N- and C-terminal regions [13–15]. For both mucins, cDNA sequencing indicated that, to release the 118 kDa component, there must be a peptide bond cleaved at a specific Asp-Pro bond located approx. 690 residues from the C-terminus. We assume that, during mucin purification, the 118 kDa fragment remains attached to the rest of the mucin by intramolecular disulphide bonds but can be released by thiol reduction.

The present study addresses the timing of proteolytic cleavage of the 118 kDa fragment from rat Muc 2 and the conditions under which it occurs. Within the published mucin literature there are precedents for both intracellular (post-translational) mucin cleavages [16–20] and cleavages that occur during purification [21]. It is also clear, however, that not all laboratories engaged in the purification of intestinal or other mucins observe a large fragment equivalent to the 118 kDa component [22–29]. A comparison of the purification techniques of Carlstedt et al. with the methods used routinely in our own laboratory reveals potentially significant differences. In their preparations, crude mucus gel is gently dispersed in buffers containing protease inhibitors and 6 M guanidinium chloride (GdmCl). For rat intestinal mucin, the addition of dithiothreitol (DTT) is also recommended, to solubilize otherwise insoluble mucin [30]. In our own mucin purification protocols, we have routinely homogenized tissue scrapings in protease inhibitors but have not included GdmCl or DTT in the protocol [8,9,31]. The lack of prominent fragments in the purified mucins of Carlstedt et al. might therefore be due to less shearing of the molecule and/or the more effective inhibition of proteases. Alternatively it is possible that the mucin is actually cleaved post-translationally within cells and that the subsequent use of GdmCl and DTT during purification effectively separates the cleaved fragment from the rest of the mucin, such that the fragment is removed during subsequent ultracentrifugation steps. If this was so, the cleaved fragment would not appear in the final mucin preparation, and the mucin would be incomplete (i.e. it would lack the C-terminal domain). Neither of these possibilities has been explored.

Because many secretory mucins have now been shown to be structurally similar to rat Muc 2 at their C-terminal ends [3,13,32], and this region of the molecule is assumed to have a pivotal role in disulphide bond-mediated polymerization [4,33], it is important to know the conditions under which this region of the mucin is cleaved. A heightened susceptibility to specific pro-

Abbreviations used: DTT, dithiothreitol; GdmCl, guanidinium chloride.

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teolytic damage might have important implications for the stability of mucin polymeric structures and the function of mucin gels at mucosal surfaces [5,33].

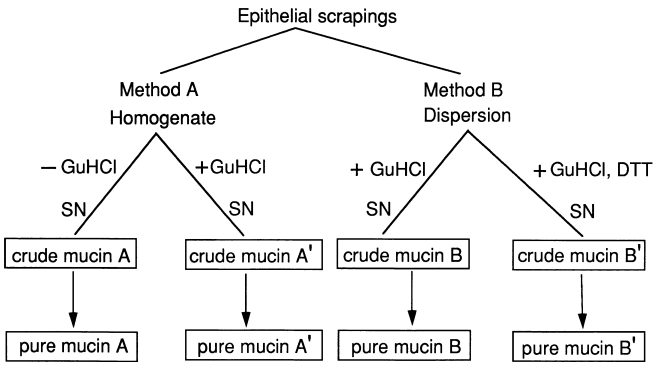
To investigate the cleavage of rat Muc 2 we have (1) compared mucins purified in the absence or the presence of GdmCl, (2) tested the proteolytic activity of crude homogenate solutions containing proteinase inhibitors, (3) examined the effect of storage of purified mucins on their tendency to undergo proteolytic degradation, and (4) determined the origin of the 200 kDa minor band seen after gel electrophoresis of purified mucins.

EXPERIMENTAL

Purification of rat intestinal mucin

Scheme 1 outlines the different mucin preparations used in the present study. Purified mucins A and B differ principally by virtue of the use of homogenization or tissue dispersion at the first step, and by the exclusion or inclusion respectively of GdmCl. Crude mucins A, B, A′ or B′ represent supernatant solutions before they were subjected to density gradient ultracentrifugation. Mucin A was purified essentially as described earlier [31]. In brief, pooled scrapings from the small intestinal epithelium of ten rats (male, Wistar) were added quickly to 75 vol. of 10 mM sodium phosphate, pH 6.5, containing an inhibitor mixture of Na_2EDTA (5 mM), PMSF (1 mM) and *N*-ethylmaleimide (5 mM). The scrapings were homogenized in a Waring blender for 25 s. Insoluble material was removed by centrifugation at 30000 ***g*** for 30 min at 4 °C. Aliquots of the supernatant (crude mucin A) were removed, ultrafiltered to remove components smaller than 10 kDa and either frozen at −20 °C or used immediately for SDS/PAGE. The rest of the supernatant was concentrated to one-third of its original volume and subjected to two sequential CsCl density gradient ultracentrifugations. Carbohydrate-rich (periodic acid/Schiff-positive) [34] mucin fractions from the second gradient (fractions 2–4, ρ 1.40–1.50 g/ml) were pooled, dialysed against distilled water, concentrated 10-fold by freeze-drying and reconstituted in 50 mM Tris/HCl buffer, pH 7.4. This product was called purified mucin A, and the method used to prepare it is referred to as Method A.

In special experiments a slightly different preparation of crude mucin A (called crude mucin A′) was prepared by homogenizing epithelial scrapings for 20 s in the same buffer containing the same proteinase inhibitors plus leupeptin (1 μM) and pepstatin (1 μM), as well as 6 M GdmCl. The homogenate was centrifuged as usual, and 5 ml of the supernatant was mixed with ethanol (95 % final concentration) [35] to precipitate proteins (including mucins) as rapidly as possible. The precipitate was solubilized in Tris/HCl (50 mM, pH 7.4). This was crude mucin A′. An aliquot was subjected immediately to SDS/PAGE. The remainder of the homogenate supernatant was subjected to sequential CsCl–GdmCl density gradient ultracentrifugations (as described below for mucin B). The final product was labelled purified mucin A′. The yields of mucins A and A′ were approximately the same (250–300 μg of mucin protein/g wet weight of mucosal scrapings).



Scheme 1 Purification protocols for rat intestinal mucin

Crude mucins refer to supernatants (SN) of homogenates (left) or dispersions (right) prepared in the presence of (+) or absence (−) of GdmCl (GuHCl). Thereafter purification included three sequential density gradient ultracentrifugation steps in CsCl with (+) or without (−) GdmCl.

For the preparation of mucin B, intestinal mucin was prepared from rat intestinal scrapings by the method of Carlstedt et al. [24], but instead of gentle dispersion by a Dounce homogenizer, the intestinal scrapings were stirred in ice-cooled 6 M GdmCl/Na_2EDTA (5 mM)/*N*-ethylmaleimide (1 mM)/PMSF (1 mM) in sodium phosphate buffer (10 mM, pH 6.5) for approx. 18 h at 4 °C. Insoluble material was removed by centrifugation at 30000 ***g*** for 30 min at 4 °C. The supernatant was designated crude mucin B. One aliquot was stored at −20 °C and the rest was adjusted to a concentration of 4 M GdmCl and CsCl to give a buoyant density of 1.40 g/ml. Density gradient ultracentrifugation was performed at 150000 ***g*** for 48 h at 4 °C. Carbohydrate-rich mucin fractions (fractions 2–5, ρ 1.38–1.48 g/ml) were pooled, and a second, identical, density gradient ultracentrifugation was performed. The mucin-rich fractions (fractions 2–5, ρ 1.44–1.47 g/ml) were dialysed against 10 mM sodium phosphate buffer, pH 6.5, containing 0.2 M GdmCl and 5 mM Na_2EDTA. CsCl was added to give a density of 1.45 g/ml and a third density gradient ultracentrifugation was performed. The final mucin preparation (ρ 1.41–1.48 g/ml) was called purified mucin B. It was dialysed against 4 M GdmCl and stored at −20 °C. Immediately before further study, the mucin was thawed and subjected to ultrafiltration for 30 min at 4 °C (Millipore Ultra PFL filter; 10 kDa cut-off) to remove GdmCl. The mucin was then reconstituted in 20 mM sodium phosphate buffer, pH 7.4. The yield of mucin from method B was only 20 % of that obtained for mucins A and A′ owing to the greater initial insolubility of mucin in dispersions compared with that in homogenates.

In special experiments, crude mucin B′ was prepared in a fashion similar to that of crude mucin B, with the exception that 10 mM DTT was also added to the scrapings, as well as pepstatin (1 μM) and leupeptin (1 μM). As expected [30,36], the reducing agent brought about the complete solubilization of otherwise insoluble material. The scrapings were stirred for 4 h at 4 °C, for 4 h at 37 °C, and then alkylated by incubating in the dark with 20 mM iodoacetamide at 4 °C for 17 h. One-tenth of the mixture was subjected to ultrafiltration for 30 min at 4 °C (Millipore Ultra PFL filter; 10 kDa cut-off), to remove GdmCl, and the retained material (crude mucin B′) was applied to SDS/PAGE. The rest of the mixture was subjected to three steps of density gradient ultracentrifugation in GdmCl and CsCl as described for mucin B. The final preparation, purified mucin B′, was dialysed against 4 M GdmCl and stored at −20 °C. The yield of mucin from method B′ was equivalent to that for mucins A and A′ (305 μg of mucin protein/g wet weight of mucosal scrapings).

Reduction and alkylation of purified mucins

Mucins A or B (5–10 mg of protein) were reduced by boiling for 5 min in 0.1 M Tris/HCl, pH 8.5, containing 10 mM DTT and 6 M GdmCl, alkylated for 2 h at 4 °C in the dark with 20 mM

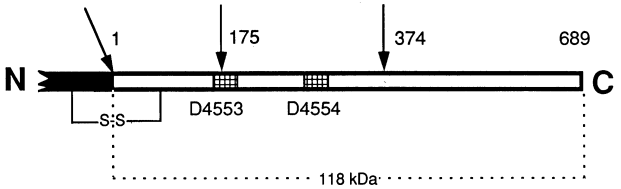


Figure 1 Schematic diagram of the 118 kDa polypeptide core at the C-terminal end of rat Muc 2

Arrows indicate the position of Asp-Pro bonds; upper numbers refer to amino acid residues, with the N-terminal proline residue of the 118 kDa domain set as 1. The solid area represents a zone of high Ser, Thr and Pro content. The hatched boxes indicate the positions of the peptides D4553 and D4554.

iodoacetamide, pH 8.5, and dialysed overnight at 0 °C against 5 mM Tris/HCl, pH 7.5.

Antisera against the C-terminal fragment of rat intestinal mucin

Antiserum against the deglycosylated 118 kDa glycopeptide (previously called 'd-Link') and antisera against synthetic peptides D4553 and D4554 have been described earlier [13,31]. Positions corresponding to D4553 (DEWLVNDPSKPHC) (residues 175–187) and to D4554 (CVGPDNVPREFGE) (residues 325–337) are shown in Figure 1. The first two antisera (diluted 1:1000, v/v) were used in slot-blot immunoassays of mucins, with appropriate preimmune sera as controls. Detection of immunoreactivity was performed with anti-(rabbit IgG) conjugated with alkaline phosphatase, by the Promega protoblot substrate system. Slots were scanned by laser densitometry s(Ultrascan XL Lasex; Pharmacia LKB, Uppsala, Sweden). The three antisera were used in Western blot experiments of mucin samples separated by SDS/PAGE [7.5% (w/v) gel] under reducing conditions [37].

Compositional studies

Fractions from density gradients were identified as carbohydrate-rich by the periodic acid/Schiff assay [34]. Total protein was analysed with the bicinchoninic acid protein assay [38] (Pierce Chemicals, Rockford, IL, U.S.A.). Amino acid analyses of mucin fractions were performed with a Waters Picotag HPLC system (Waters Associates, Mississauga, ON, Canada) after gas-phase hydrolysis of the protein samples in 6 M HCl and 0.05% phenol at 110 °C for 24 h.

Amino acid sequencing of purified mucins

Reduced and alkylated mucin A and B samples (10 μg of protein/200 μl) were coated individually on PVDF (Immobilon) membranes and subjected to amino acid sequencing with a Porton gas-phase microsequencer, model 2090 (Biotechnology Service Centre, The Hospital for Sick Children, Toronto, Ontario, Canada). In some experiments the mucins were first separated by electrophoresis through preparative SDS/PAGE (7.5% gel), transferred to a PVDF membrane and stained with Coomassie Blue, after which a specific band of interest was excised and subjected to amino acid sequencing.

Incubation of purified mucins with crude mucin A

Purified mucins A (10–13 μg of protein) or B (7 μg of protein) were incubated with a dilute solution of homogenate supernatant (crude mucin A) (0.7–1.3 μg of protein). In some cases the homogenate was boiled for 10 min before use. Incubations were performed in 10 mM sodium phosphate buffer, pH 7.4, for 1, 2 or 16 h at 37 °C in a final volume of 500 μl. Control incubations contained homogenate alone (no added mucin) or purified mucin alone (no added homogenate). After incubation, samples were boiled in reduced SDS/PAGE sample buffer and subjected to SDS/PAGE; protein bands were stained with silver [39]. Equivalent non-stained samples were transferred to PVDF (Immobilon) membranes and subjected to Western blot analyses.

Storage of mucins

Purified mucins A and B were dialysed at 4 °C for 16 h against six changes of 10 mM sodium phosphate buffer, pH 7.4. Purified mucins A′ and B′ were dialysed against 4 M GdmCl. All mucin samples were then stored at −20 °C for 18 months. At 6–8 week intervals the mucins were thawed to room temperature and then returned to the freezer. After 18 months the mucins were subjected to ultrafiltration, SDS/PAGE and Western blotting with the antiserum to the deglycosylated 118 kDa domain of rat Muc 2 (1:3000, v/v).

RESULTS

Characteristics of mucin A and mucin B

Purified mucins A and B were harvested from density gradients as carbohydrate-rich soluble glycoproteins with a buoyant density range of 1.40–1.47 g/ml. Both mucins were similarly enriched in hydroxyamino acids (results not shown), which is typical of mucin molecules. As expected from many earlier studies in our own and other laboratories, the mucins barely entered 7.5% gels during SDS/PAGE, even under reducing conditions (Figure 2). Unlike mucin B, reduced mucin A produced a prominent silver-stained band at approx. 120 kDa, and a less prominent band at approx. 200 kDa, both of which reacted with the antiserum to the deglycosylated 118 kDa fragment (d-Link). Occasionally preparations of mucin A also showed poorly stained lower-molecular-mass bands in the region of 70 kDa. No bands were

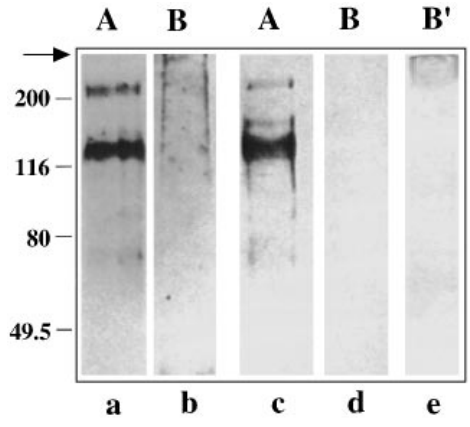


Figure 2 SDS/PAGE of purified mucins A, B and B′

Gels were processed for silver staining (lanes a, b and e) or Western blotting (lanes c and d) with the antiserum against the deglycosylated 118 kDa domain of rat Muc 2. Total protein loading was 4, 4 and 6 μg respectively for lanes a, b and e. The arrow marks the junction of the stacking (4%) and separating (7.5%) gels. The positions of molecular mass standards (in kDa) are indicated at the left.

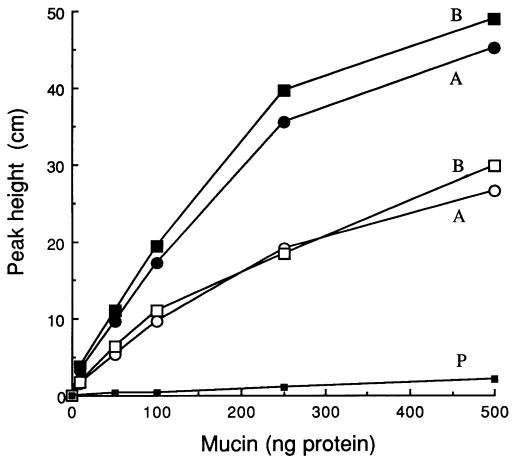


Figure 3 Immunoreactivity of purified mucins A and B

Increasing concentrations of reduced mucin A or mucin B were incubated with the antiserum against the deglycosylated 118 kDa domain (upper filled symbols) or with the antiserum against peptide D4553 (open symbols) (1:1000 dilution, v/v) in slot-blot immunoassays. The slots were scanned by densitometry and the peak heights were measured. The line marked P represents the results of preimmune sera.

Table 1 N-terminal amino acid sequence of purified mucin A

Cycle number	Retention time (min)	Major peak (pmol)	Minor peak (pmol)
1	18.125	Pro (30.8)	–
	24.003	–	Leu (5.8)
2	10.890	His (24.2)	–
	9.691	–	Glu (6.6)
3	22.677	Phe (40.8)	–
	23.990	–	Leu (8.1)
4	19.058	Val (48.2)	–
	8.540	–	Gln (5.9)
5	8.759	Thr (27.3)	–
	23.618	–	Lys (4.8)
6	22.678	Phe (32.4)	–
	9.676	–	Glu (5.8)
7	6.730	Asp (28.6)	–
	12.203	–	Ala (4.9)
8	9.317	Gly (30.2)	–
	–	–	–
9	24.013	Leu (32.1)	–
	–	–	–
10	14.913	Tyr (25.4)	–

Major sequence: Pro-His-Phe-Val-Thr-Phe-Asp-Gly-Leu-Tyr
Minor sequence: Leu-Glu-Leu-Gln-Lys-Glu-Ala

seen in preparations of mucin B, which seems to be much more resistant to degradation.

Slot-blot immunoassays of mucins A and B

Although the 118 kDa fragment was not released by the reduction of mucin B, it was not clear whether mucin B (most of which does not enter 7.5% gels) actually contained this segment in a non-cleaved form. This was resolved by performing quantitative slot-blot immunoassays over a range of mucin concentrations (0–500 ng/100 μl), with two antisera specific for the 118 kDa fragment. Figure 3 shows that both mucins A and B reacted with each antiserum in a dose-dependent fashion. Mucin B therefore contains the 118 kDa domain in a non-cleaved form. Assuming that the antisera have the same avidity for the cleaved and non-cleaved forms of the mucin, the content of the 118 kDa component per unit mass of total protein is about the same in each mucin.

Detection of C-terminal cleavage by amino acid sequencing

The Asp-Pro cleavage that releases the 118 kDa fragment from mucin A, and the lack of the same cleavage in mucin B, were confirmed by subjecting both purified mucin samples to N-terminal amino acid sequencing. In mucin A the sequence obtained was Pro-His-Phe-Val-Thr-Phe-Asp-Gly-Leu-Tyr, which corresponds to the known sequence of residues 1–10 at the N-terminus of the 118 kDa glycopeptide fragment [13]. Each of the first seven cycles also produced a second (minor) amino acid peak, as summarized in Table 1, giving rise to the sequence Leu-Glu-Leu-Gln-Lys-Glu-Ala. Mucin B gave no N-terminal data. These findings suggest that in mucin B the N-terminus was blocked and there was no internal cleavage to release the 118 kDa component. In mucin A there was one (possibly two) cleavage(s), the major one involving the Asp-Pro cleavage site (labelled 1 in Figure 1) of the 118 kDa C-terminal fragment. The second (minor) N-terminal sequence obtained from mucin A is discussed later.

Cleavage of mucin A occurs at an early stage of purification by method A

Crude mucin A (intestinal homogenate supernatant containing three proteinase inhibitors) and crude mucin B (the soluble supernatant of a dispersion of intestinal scrapings in proteinase inhibitors plus GdmCl) represent the starting points for the two mucin purification protocols. Aliquots of each crude mucin were subjected to SDS/PAGE (under reducing conditions) and then Western blotted with the antibody to the 118 kDa peptide (Figure 4). A dominant immunoreactive 118 kDa band was noted in crude mucin A, but not in crude mucin B, suggesting that cleavage occurred during homogenization (method A), even in the presence of the three proteinase inhibitors. The results were the same in the presence of two additional proteinase inhibitors (results not shown). To prove that homogenization itself was not problematic if GdmCl was present in the homogenizing solution, crude mucin A was prepared again in the presence of five proteinase inhibitors, but this time the homogenizing buffer contained 6 M GdmCl. This preparation is called crude mucin A′. The amount of released 118 kDa fragment was decreased drastically (Figure 4, lane A′). Thus cleavage of the mucin in method A seems to be due to an agent present in initial tissue homogenates, active despite the addition of proteinase inhibitors, but sensitive to GdmCl.

Effect of reduction and alkylation on mucin preparations

Because method B solubilizes only approx. 20% of the mucin present in intestinal scrapings, additional experiments were performed to determine whether subjecting tissue dispersions to thiol reduction and alkylation reactions would enhance the yield of mucin and preserve its structure in an intact (undegraded) state. Accordingly, crude mucin B was prepared as before from tissue dispersions in GdmCl, but 10 mM DTT was added to ensure that the mucin would be solubilized [30] and the mixture was then alkylated by incubating it for 17 h with iodoacetamide (see the Experimental section for details). After ultrafiltration

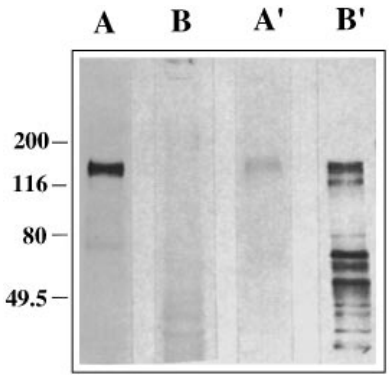


Figure 4 SDS/PAGE and Western blotting of crude mucins A, B, A′ and B′

Total protein loading on the gels was 20, 17, 15 and 10 μg, from left to right. Blotting was performed with the antiserum against the deglycosylated 118 kDa domain of rat Muc 2 (1:3000 dilution, v/v). The positions of molecular mass standards (in kDa) are indicated at the left.

(approx. 30 min duration), the retained material (crude mucin B′) was subjected to SDS/PAGE. Surprisingly, there were multiple immunoreactive bands noted at 118 kDa and smaller (Figure 4, lane B′), indicating extensive fragmentation of the mucin. Three additional samples of crude mucin B′ were prepared independently and all exhibited the same evidence of degradation (results not shown). However, degradation at this stage was virtually eliminated if the alkylated crude mucin mixture was not filtered to remove GdmCl but instead was carried through the entire density gradient ultracentrifugation process to yield purified mucin B′ (Figure 2). Western blots did not reveal any degradation fragments. Thus the exposure of mucin to reducing agents accelerated mucin degradation by homogenate factors (crude mucin B′), but only if GdmCl was removed.

Effect of crude mucin A (supernatant of intestinal homogenate) on purified mucins

Because pure mucin A exhibited definite (although limited) signs of proteolytic damage, we expected that the homogenate (i.e. crude mucin A) should be able to cleave purified (undegraded) mucin B at the appropriate Asp-Pro cleavage site to release the 118 kDa fragment. Accordingly, purified mucin B (7 μg of protein) was incubated with a dilute solution of crude mucin A (0.7 μg of total protein) for 2 h at 37 °C, after which the reaction was terminated by boiling and the mixture was subjected to SDS/PAGE. As shown by silver staining in Figure 5 (lanes a–c), degradation of mucin B occurred, but the major degradation product was 85 kDa rather than 118 kDa. This product reacted strongly with the antiserum to the 118 kDa fragment (d-Link) (Figure 5, lane e). A similar-sized immunoreactive product was also released from purified mucin A after it had been subjected to the same 2 h incubation conditions (Figure 5, lane g), with a concomitant decrease in intensity of the 118 kDa band. With longer (16 h) incubations (Figure 5, lane h), the 118 kDa band decreased even more markedly and the 85 kDa band increased. These findings, as well as the amino acid composition of the 85 kDa fragment (Table 2), support the interpretation that the 85 kDa fragment is derived from the 118 kDa component. Thus intestinal homogenates, even those containing major classes of protease inhibitors, remain a potent catalyst of mucin degradation involving the C-terminal domain. The minor band at 200 kDa (in mucin A) did not seem to change in intensity during these incubations (Figure 5, top of gel in lanes g and h) and was investigated separately (see below).

Table 2 Composition of rat intestinal mucin components

Purified mucin B (10 μg) (reduced and alkylated) was incubated with crude mucin A (1 μg) for 2 h at 37 °C; the products were separated by preparative SDS/PAGE and transferred to a PVDF membrane. The 85 kDa band was excised and subjected to amino acid analysis. Values are the means of two separate analyses. Abbreviations: n.d., not determined; CMC, carboxymethyl cysteine. Values for the 118 kDa glycopeptide are taken from [13].

	Composition (mol %)	
Amino acid	118 kDa glycopeptide	85 kDa fragment
Asp	11.2	11.5
Glu	15.3	16.6
Ser	6.9	9.1
Gly	8.1	7.8
His	2.2	1.8
Arg	3.3	3.2
Thr	7.2	6.3
Ala	6.5	6.5
Pro	8.7	8.0
Tyr	2.7	2.6
Val	6.3	6.2
Met	0.6	0.4
CMC	4.8	4.0
Ile	3.6	3.9
Leu	6.0	5.6
Phe	4.1	3.1
Lys	1.1	3.4
Trp	n.d.	n.d.

Incubation of purified mucin A (13 μg of protein) with crude mucin A (1.3 μg of protein) was repeated, but in this case the crude mucin A was boiled for 10 min before incubation. The amount of released 85 kDa protein was decreased markedly (Figure 5, lanes i and j). Thus the homogenate factor (enzyme?) responsible for cleavage within the 118 kDa domain of mucin A and B is sensitive to high temperature.

The 85 kDa fragment was reactive with the anti-(d-Link) antibody but did not react with the antibody against the D4553 peptide (Figure 5, lanes k and l). Because this peptide corresponds to a short sequence within the 118 kDa component (Figure 1) [40] and contains an Asp-Pro bond, cleavage might have occurred within or near this site and destroyed the immunoreactive epitope. If so, the 85 kDa degradation product would still be expected to react with anti-D4554, which represents a more C-terminal position of the mucin (Figure 1). As shown in Figure 5 (lane m), the anti-D4554 antibody did indeed detect bands at 118 and 85 kDa as well as two more minor bands at approx. 70 and 60 kDa. Thus more than one cleavage of the mucin C-terminal region might have taken place during the 2 h incubation period.

Effect of storage of mucins on proteolytic degradation

The tendency of intestinal mucin to undergo limited peptide cleavage even in the presence of a cocktail of enzyme inhibitors suggests that a potent enzyme in homogenates remains attached to mucin during purification and is inaccessible to specific enzyme inhibitors. Alternatively, one might question whether the mucin itself (especially thiol-reduced mucin) is autocatalytic once it is separated from GdmCl. In either of these models we would predict that even highly purified mucins would undergo some degradation if they were stored in the absence of GdmCl. Mucins

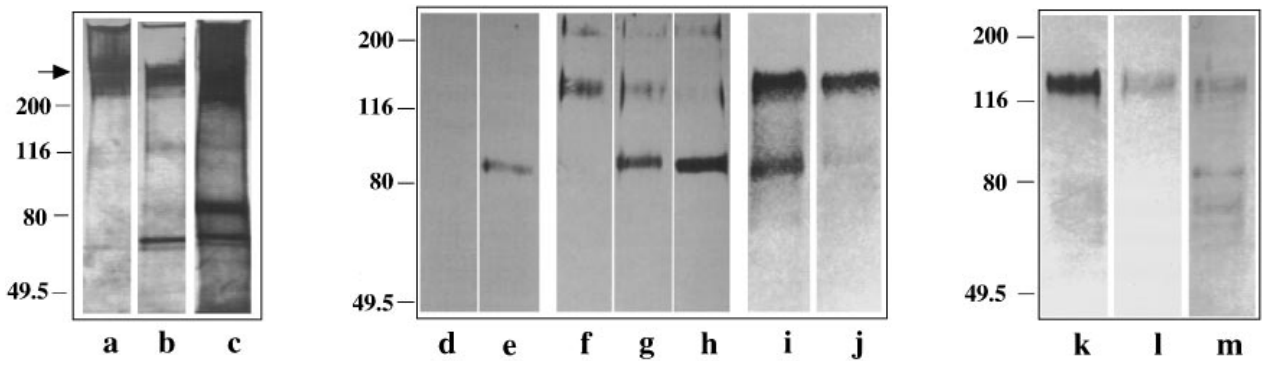


Figure 5 SDS/PAGE and Western blotting of purified mucins incubated with crude mucin A (intestinal homogenate supernatant)

Lanes a–c, silver staining of mucin B (10 μg of protein) after incubation for 2 h at 37 °C without (lane a) or with (lane c) crude mucin A (1 μg). Lane b, crude mucin A alone (1 μg). An arrow marks the junction of the stacking (3.5%) and separating (7.5%) gels. Lanes d–j, Western blotting with antiserum against the deglycosylated 118 kDa domain (d-Link) (1:1000 dilution, v/v). Purified mucin B (7 μg of protein) was incubated for 2 h at 37 °C without (lane d) or with (lane e) crude mucin A (0.7 μg). Lanes f and g represent incubations comparable to those in lanes d and e, but with purified mucin A (10 μg of protein) instead of mucin B. Lane h, as in lane g but the incubation time was 16 h. Lanes i and j, purified mucin A (13 μg) incubated for 1 h at 37 °C with crude mucin A (1.3 μg) (lane i) or with crude mucin A that had been heated previously for 10 min at 100 °C (lane j). Lanes k–m, Western blotting with antisera against peptides D4553 and D4554 (1:1000 dilution, v/v). Purified mucin A (10 μg of protein) was incubated at 37 °C for 2 h in the absence (lane k) or presence (lane l) of crude mucin A (1 μg), and blots were probed with anti-D4553. Lane m, as in lane l except that anti-D4554 was used on Western blots. The positions of molecular mass standards (in kDa) are indicated at the left of each group of lanes.

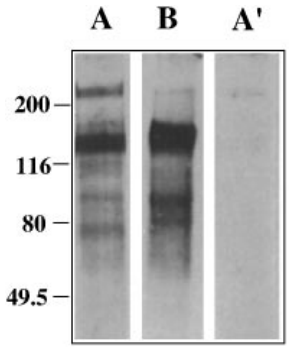


Figure 6 Degradation of purified mucins during storage

Purified mucins A and B were stored at −20 °C in PBS, pH 7.0, and purified mucin A′ in GdmCl (4 M). Every 6–8 weeks the samples were removed from the freezer, thawed (1–2 h at room temperature), and then restored to −20 °C. After 18 months, samples were filtered rapidly and subjected to SDS/PAGE and immunoblotting with the antiserum against the deglycosylated 118 kDa domain of rat Muc 2. The positions of molecular mass standards (in kDa) are indicated at the left.

A and B were therefore stored in PBS for 18 months at −20 °C, with intermittent thawing and restorage. At the end of this period, samples were ultrafiltered and subjected to SDS/PAGE and Western blotting. The results (Figure 6) indicate that purified mucins A and B underwent partial degradation, with the most prominent immunoreactive bands at approx. 200, 118 and 70–90 kDa. Degradation was largely absent after storage of purified mucin A′ (Figure 6, lane A′) or B′ (results not shown) in the presence of 4 M GdmCl. These findings indicate that despite their purity, mucins remain vulnerable to degradation if not stored in GdmCl.

Origin of the 200 kDa component of mucin

The 200 kDa band observed in purified mucin A was not prominent in the other mucin preparations, indicating that its appearance was prevented by adding GdmCl at the earliest steps (homogenization) of purification. The band reacted weakly with the antibody against d-Link (Figure 2), but did not react with the antibody against D4553 or D4554 (results not shown), and did not change in mobility with thiol-reducing agents. It also did not seem to be a source of the 85 kDa degradation fragment of mucin. Thus its behaviour was not appropriate for a disulphide-bonded dimer of the 118 kDa glycopeptide. When the 200 kDa component was subjected to N-terminal analyses, the sequence obtained was Leu-Glu-Leu-Gln-Lys-Glu-Ala-Arg-Ser-Arg-Asn-His-Val-Xaa-Ser-Xaa. For those amino acids that could be identified, this sequence is identical with the published N-terminal sequence of mature rat Muc 2 as deduced from cDNA cloning by Ohmori et al. [15]. We note also that the first seven residues of this sequence match exactly the minor sequence obtained from purified mucin A (Table 1). Therefore in addition to a C-terminal cleavage to produce the 118 kDa component, another cleavage near the N-terminal region of mucin A must have occurred during its purification. The site of the cleavage is unknown, but the presence of GdmCl in homogenates and throughout the purification of mucin prevented cleavage.

DISCUSSION

The present study reveals that the N- and C-terminal regions of rat Muc 2 are very susceptible to proteolytic degradation during tissue disruption (gentle homogenization), even in the presence of several proteinase inhibitors and even after the mucin is purified. Findings with crude mucin B′ reveal that prior thiol reduction leads to an even more extensive degradation if GdmCl is removed before the density gradient stage of purification. Boiling of homogenates offered partial protection. To achieve full protection, however, required the presence of GdmCl throughout all steps of the purification process and even during low-temperature storage of the final purified mucin product.

As judged from the large degradation fragments identified by SDS/PAGE and Western blotting (200, 118 and 85 kDa), the cleavages seemed to be restricted to a selected number of sites in the mucin peptide core. One site, which liberates the 118 kDa

C-terminus, is an Asp-Pro bond located 689 residues from the C-terminal end of Muc 2. This was deduced from results of N-terminal amino acid sequencing. The second major cleavage site was C-terminal to the first and released an 85 kDa fragment. Unlike the 118 kDa glycopeptide itself, the 85 kDa product failed to react with the antibody against peptide D4553, which corresponds to a short sequence containing an internal Asp-Pro bond (Figure 1) [13]. An antibody against peptide D4554, corresponding to a more C-terminal position, was reactive, however, indicating that the 85 kDa fragment derives from the 118 kDa glycopeptide. There is a third Asp-Pro bond C-terminal to D4554 (at residue 374; Figure 1). It is not yet known whether cleavage at this site is responsible for the release of the 60 kDa band recognized by anti-D4554.

If the degradation products all arise from cleavage of Asp-Pro bonds, as implied, a common causative agent can be considered. One possibility is a chemical rupture, although the acidic conditions required for acid hydrolysis of proteins [41] were not used during any of the purification steps. Recently recognized [42] is the likelihood of an acid shift in pH during boiling of proteins in the standard Laemmli sample buffer (0.0625 M Tris buffer, pH 6.8), and consequent Asp-Pro cleavage reactions. This is unlikely to explain our results, however, because the SDS/PAGE buffer we used was pH 7.4 and, despite identical treatment of all mucin samples during electrophoresis, only those purified without continuous exposure to GdmCl exhibited degradation. A second possibility is an unidentified neutral Asp-Pro endopeptidase that is active in intestinal homogenates. There are two known proline-specific neutral peptidases (pH optima 8.0–8.4) that are exopeptidases [43,44] but both have a requirement that proline be located at the penultimate position of an N-terminus. Two other potential enzymes that cleave at aspartic residues are neprilysin, a brush border zinc metalloenzyme related to the thermolysin family and active at neutral pH [45], and ICE-LAP3, an intestinal cysteine protease related to the family of interleukin-1β-converting enzymes [46]. Further experiments are needed to explore these suggestions.

The third major Muc 2 cleavage reaction generated a 200 kDa fragment, which we identified by amino acid sequencing as the large N-terminal end of the mucin. The actual peptide bond cleavage site is not yet known.

A surprising feature in the present study was the susceptibility of purified mucins to degradation during storage at -20 °C with intermittent thawing steps. Once again, GdmCl prevented degradation. The degradative agent must be present in purified mucin samples as a contaminant or else it is an integral part of the mucin structure and causes autocatalysis. The agent has not yet been identified. For practical purposes we conclude that to keep mucins intact they are best prepared and stored in solutions containing 4 M (or higher) GdmCl.

The heightened sensitivity of each end of the mucin molecule to proteolysis might be important not only for purification protocols but also in consideration of normal mucin physiology. Under conditions *in vivo* of continuous secretion of mucin from goblet cells, rapid peptide cleavages ('nicking') of mucin molecules would be expected to occur within the intestinal lumen, catalysed by enzymes liberated from sloughed epithelial cells. The result would be disruption of the linear (end-to-end joined) polymers of mucin [2,26] and destabilization of mucin gels. This might represent an important step in the gradual fluid washout and removal of the adhesive gel from mucosal surfaces. In conditions of epithelial cell damage and/or excessive sloughing due to inflammation, infection or malignancy, the rate and extent of mucin degradation might increase significantly, thereby weakening the barrier function of the surface mucus layer.

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