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Low molecular mass products of depolymerization of purified mucin — attempts at isolation and characterization

Iwona Minkiewicz and Andrzej Gindzieński✉

Department of General and Organic Chemistry, Institute of Chemistry, Medical Academy of Białystok, A. Mickiewicza 2, 15-230 Białystok 8, Poland

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Samples of crude mucin were incubated at room temperature for 48 and 96 h in a sodium azide containing buffer, pH 7.0. Then each sample was purified, reduced and alkylated with iodo[^{14}C]acetamide. Electrophoretic analysis demonstrated that radioactivity was incorporated into the mucin subunits and proteins of 100 and 140 kDa. The results of our experiments suggest that the released proteins can be a part of mucin molecule, cleaved by proteolysis and reduction of disulfide bridges.

Purified mucins isolated from different sources (gastric [1, 2], intestinal [3, 4], tracheobronchial [5] or submaxillary [6]) have of similar structure. They consist of high molecular mass, heterogenous glycosylated monomers (subunits) linked together by disulfide bridges.

On disulfide bond reduction of purified mucin preparation, some authors demonstrated the release of 65–120 kDa (glyco)proteins, structurally related, presumably of different origin [1, 4, 5, 7, 8]. According to others [9], their appearance could be due to accidental proteolytic cleavage of C-terminal mucin fragments, at the early steps of mucin purification. The location of cysteine residues at the C-terminal mucin fragment suggest, that neither proteolysis nor disulfide bond reduction can bring about the release of proteins involved in disulfide linking of mucin "T" domains [9]. However, the other possibility is non covalent trapping of these proteins to the disulfide-maintained three-dimensional mucin network.

The aim of the experiments reported in this paper was to examine whether all the proteins

✉Correspondence to: Andrzej Gindzieński: tel. (48 85) 742 2059 or (48 85) 742 2021, ext. 2246; e-mail zachemog @ amb. ac. bialystok. pl

Abbreviations: SDS, sodium dodecyl sulfate.

released from pig gastric mucin under reductive conditions are disulfide bonded to subunits and whether their concentration in the reduced products depends on the time of exposure to the potential proteolytic activity of mucus at pH 7.0.

MATERIALS AND METHODS

Freshly obtained pig stomachs were washed with water and incubated in 50 mM boric buffer, pH 7.0, containing 0.02% NaN_3 at 4°C for 20 h. The surface epithelium mucus was gently scraped off and the homogenate (divided into three equal pools) was incubated for 48 and 96 h at room temperature. After incubation, the proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA-Na_2, 2 mM *N*-ethylmaleimide, 11 mM iodoacetamide) were added and each sample was stirred for 20 h at 4°C in 6 M urea. Then material insoluble in 6 M urea was removed by centrifugation at 19000 × ***g*** for 30 min. The supernatant was dialysed and the mucin precipitate was resolubilized in 1% sodium dodecyl sulfate (SDS) and purified by chromatography, successively, on two Sepharose Cl 2B columns (2.3 cm × 56 cm and 2.3 cm × 135 cm) and Sephacryl S-500 (2.4 cm × 56 cm) in boric buffer, pH 7.0, containing 10 mM EDTA-Na_2, 0.02% NaN_3 and 1% SDS. Samples were concentrated by dialysis against polyethyleneglycol 35000, and SDS was removed by acetone extraction [10].

Purified mucin (1 mg of each sample) was alkylated with 0.1 M iodoacetamide under nitrogen at 37°C for 4 h in the dark. The excess of iodoacetamide was removed by dialysis against boric buffer, pH 8.0, for 24 h at 4°C. After that, 0.14 μmol of dithiothreitol was added (final concentration 0.14 nM) and the solution was incubated under nitrogen at 37°C for 4 h. Then 0.28 μmol of iodo[^{14}C]acetamide (60 mCi/mmol) was added and incubation was continued under nitrogen at 4°C overnight in darkness. The reduction and alkylation steps were repeated with 10 mM dithiothreitol and 20 mM iodoacetamide, respectively. Samples were dialysed, concentrated and subjected to SDS/polyacrylamide gel electrophoresis (SDS/PAGE). Gels were stained for proteins, dried and submitted to autoradiography by exposing gels in close contact with X-ray film for 2 weeks at −70°C. Mucin from mucosal scrapings incubated for 96 h was also chromatographed on Sephacryl S-500 column with buffer containing 0.02 M 2-mercaptoethanol. Fractions after chromatography were collected into three pools (a, b, c), dialysed, concentrated and submitted to SDS/PAGE and autoradiography.

Molecular mass of purified mucin was estimated by chromatography on Sephacryl S-1000 column according to Shogren *et al.* [11]. The proteolytic activity was determined according to Habeeb [12] and neutral sugars by the phenol sulfuric acid method [13]. Polyacrylamide gel electrophoresis was performed as described by Laemmli [14]. The proteins in gels were stained by the silver method [15].

RESULTS AND DISCUSSION

In the experiments performed in this work pig gastric mucin was purified by gel exclusion chromatography in buffers with the use of proteinase inhibitors and SDS. In these conditions, we have not found any substantial increase in the content of free $-NH_2$ groups in crude mucin (not shown). Chromatography of the purified mucin on a Sephacryl S-1000 column revealed that the isolated material is a mixture of mucin glycoforms of molecular mass ranging from 2.2 to 5.9 × 10^6 Da (Table 1).

In order to examine the suggested proteolytic origin of the released proteins we have tested whether their content in the purified preparations is dependent on duration of the preparation procedure. Crude mucin scrapings were submitted to incubation last-

Table 1. Apparent molecular mass of purified mucin.

After SDS removal, the Sephacryl S-500 column V_o peak of pure mucin was dissolved in boric buffer, pH 7.0, containing 5 M guanidine chloride. Sample (1.5 ml) was applied to Sephacryl S-1000 column (1 cm × 110 cm) and eluted with the same buffer. Coliphage T4 DNA and adenosine 5′-monophosphate were used as markers for the column void volume V_o and the total volume V_t determination, respectively. After chromatography, the eluate was assayed for absorbance at 280 nm and for carbohydrates according to Dubois *et al.* [13]. Distribution coefficients $K_D = [(V_e - V_o)/(V_t - V_o)]$ were determined and molecular mass was estimated according to Shogren *et al.* [11].

Sephacryl S-500 column fraction number	K_D	Average molecular mass (kDa)
25	0.07	5900
27	0.15	4700
30	0.26	3400
32	0.33	2800
34	0.41	2200

ing 48 and 96 h at room temperature in a buffer of pH 7.0 containing sodium azide. Then, after purification in the presence of proteinase inhibitors and SDS, samples of the incubated mucin were reduced and alkylated with iodo[^{14}C]acetamide according to a special procedure. First, free -SH groups were blocked with an excess of cold iodoacetamide. Then, partial reduction and alkylation of disulfide bonds was performed with dithiothreitol and iodo[^{14}C]acetamide, respectively. Full dissociation of subunits was achieved after the second cycle of reduction and alkylation with cold iodoacetamide. A part of both mucin scraping and mucin's sample was subjected to SDS/PAGE and autoradiography and the rest was chromatographed on Sephacryl S-500 column un-

Figure 1. Electrophoresis (A) and autoradiography (B) of purified mucin reduced and alkylated with iodo[^{14}C]acetamide.

Mucus scrapings were incubated for 48 and 96 h at room temperature, then the samples were purified, reduced and alkylated with iodo[^{14}C]acetamide (see Methods). Lanes a, b, c, samples after 0, 48 and 96 h incubation, respectively. Lane d, molecular mass markers.

der reducing conditions [16]. After electrophoresis (Fig. 1A), two diffuse bands of stained protein were observed of approximately 80 and 120 kDa; their intensity increased with the growing time of incubation. The radioactivity was incorporated into the subunit fractions non-penetrating the gel and into two diffuse bands of approx. 100 and 140 kDa (Fig. 1B). Interestingly, the two bands stained for proteins were located on the gel symmetrically, below those which were detected by autoradiography. This could mean, that the labeled and stained proteins are not of the same class and that proteins bound to mucin by the disulfide bonds occur in the purified mucin preparations in a low amount.

In order to determine the elution position in gel exclusion chromatography of the released proteins, a reduced and alkylated mucin sample (from mucus scrapings incubated for 96 h) was chromatographed on Sephacryl S-500 column. As we can see from Fig. 2, most of the radioactivity was eluted as a heterogeneous peak (fractions 20–49) and in a small peak eluted just a few fractions later (fractions 50–57). Fractions 20–57 were divided into three regions – a, b, c (Fig. 2) and submitted to SDS/PAGE and autoradiography. The radiolabeled 100 and 140 kDa bands and those stained for protein of 80 and 120 kDa, were eluted in fractions 35–49 (Fig. 3).

The results obtained in our experiments show that no 70–75 kDa radioactive proteins [1] were released on disulfide bond reduction either from mucin scrapings or mucin preparation. The molecular mass of the released proteins was 100 and 140 kDa. As the amount of the incorporated radioactivity was dependent on the reduction time we presume that these proteins were proteolytically cleaved off from mucin. However, according to some authors [9, 17, 18] these proteins could be linked to mucin subunits by disulfide bonds and become released upon thiol reduction.

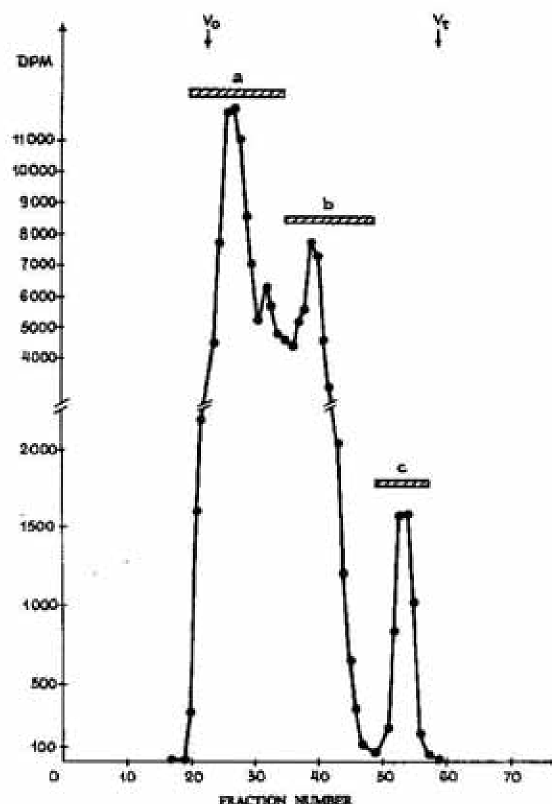

Figure 2. Column chromatography of purified mucin, reduced and alkylated with iodo-[^{14}C]acetamide.

Mucin from mucus scrapings incubated for 96 h at room temperature was purified, reduced and alkylated with iodo[^{14}C]acetamide (see Methods) and applied to the Sephacryl S-500 column. The column was eluted with boric buffer containing 0.2 M 2-mercaptoethanol. Fractions were assayed by scintillation counting. The fractions from the peaks indicated by the bars were pooled, concentrated and submitted to further analysis.

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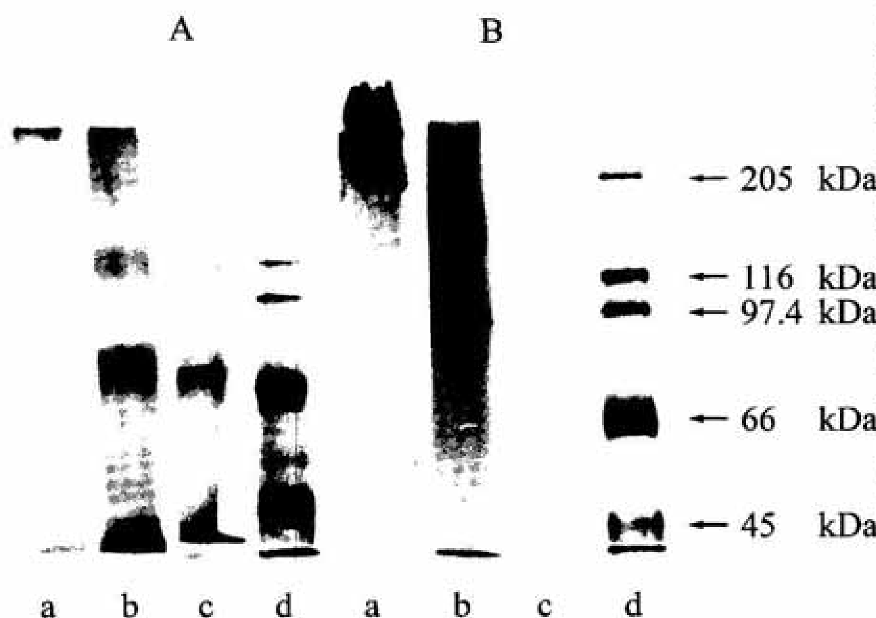

Figure 3. Electrophoresis (A) and autoradiography (B) of the eluate from Sephacryl S-500 column.

The mucin sample isolated from mucus scrapings after 96 h of incubation was chromatographed as shown in Fig. 2. Fractions under the peaks indicated by bars were pooled and submitted to electrophoresis, protein staining and to autoradiography. Lane a, fractions 20–34 (peak a from Fig. 2), lane b, fractions 35–49 (peak b), lane c, fractions 50–57 (peak c), lane d, molecular mass markers.

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