Characterization of Gastric Mucoproteins Isolated by Equilibrium Density-Gradient Centrifugation in Caesium Chloride

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1. The mucoprotein from pig gastric mucus has been purified by equilibrium centrifugation in a CsCl gradient. 2. This procedure removes the non-covalently bound protein, which is closely associated with the mucoprotein and not easily removed from it by gel filtration. 3. The purified mucoprotein is separable by gel filtration into a high-molecular-weight mucoprotein A (mol.wt. 2.3×10^6) and a low-molecular-weight mucoprotein B/C (mol.wt. 1.15×10^6). 4. These two mucoproteins have the same chemical analysis namely fucose 11.3%, galactose 26%, glucosamine 19.5%, galactosamine 8.3% and protein 13.6%. 5. Mucoprotein A contains 3.1% ester sulphate. 6. These mucoproteins are isolated without enzymic digestion and have a higher protein content than the blood-group-substance mucoproteins from proteolytic digestion of gastric mucus. Detailed amino acid analysis shows that the extra protein in the non-enzymically digested material is composed of amino acids other than serine and threonine. 7. Mucoproteins A and B/C contain respectively 130 and 9 half-cystine residues per molecule of which about 78 and 6 residues are involved in disulphide linkages. 8. Cleavage of these disulphide linkages by mercaptoethanol splits both mucoproteins into four equally sized subunits of mol.wt. 5.2×10^5 for mucoprotein A and 2.8×10^4 for mucoprotein B/C. 9. The sole *N*-terminal amino acid of mucoprotein A is aspartic acid, whereas mucoprotein B/C has several different *N*-terminal amino acid residues.

The viscous and gel-forming properties of pig gastric mucus have been shown to reside in a large-molecular-weight (1.9×10^6) mucoprotein A which, together with a low-molecular-weight (1.1×10^5) mucoprotein B/C, accounts for all the mucosubstance in the mucus (Allen & Snary, 1972). These mucoproteins are isolated without enzymic digestion from the water-soluble mucus and separated by gel filtration on Sepharose 4B (Scheme 1). Mucoprotein B/C, although sedimenting under a single envelope in the ultracentrifuge, is sufficiently polydisperse to be separated by Sephadex G-200 into mucoprotein fractions B and C. The sugar composition of mucoproteins A and B/C is closely similar to that of the gastric blood-group substances prepared by enzymic digestion (Kabat, 1956); however, their protein content is almost one and a half times greater (Snary & Allen, 1971). Mucoproteins A and B/C are closely related, being identical in A and H blood-group activity, chemical analysis and in many biosynthetic and physical properties. For these reasons mucoprotein A is thought to be a high-molecular-weight polymer of mucoprotein B/C. Further, both mucoproteins A and B/C are composed of subunits thought

to be joined by disulphide linkages, and reducing agents such as mercaptoethanol cause a large decrease in the viscosity of the mucus and in the molecular weight of the mucoproteins (Snary *et al.*, 1970).

Before more detailed studies on the chemical structure of the native mucoproteins were possible, it was necessary to ensure the complete removal of non-covalently bound protein, which is closely associated with the mucoprotein and not separable by gel filtration (Snary & Allen, 1971). The present paper describes, first, the separation of the mucoprotein from the non-covalently bound protein by equilibrium density-gradient centrifugation in a CsCl gradient (Creeth & Denborough, 1970) and, secondly, the chemical and physical characterization of the isolated purified mucoprotein. Thirdly, the content and location of the disulphide bridges in the mucoproteins have been investigated.

Methods

The preparation of the water-soluble mucus from the cardiac region of the pig stomach, and the separation by gel filtration on Sepharose 4B of mucoprotein components A and B/C, are described by Snary & Allen (1971, 1972). Neutral sugars were

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Scheme 1. Summary of preparative procedures for the mucoproteins from pig gastric mucus

The data on the mucoproteins which have not been purified by equilibrium density-gradient centrifugation are taken from Snary & Allen (1971). All values of S are the $s_{25,w}^0$ values.

examined chromatographically by the method of Allen & Kent (1968). Physical measurements are described by Snary *et al.* (1970) and Snary & Allen (1971).

The following analytical methods were used: fucose (Gibbons, 1955); sialic acids (Aminoff, 1961) with *N*-acetylneuraminic acid as standard; phosphate (Chen *et al.*, 1956). Carbohydrate material eluted from columns was determined by the anthrone method of Seifter *et al.* (1950). Quantitative values of hexose were determined by the method of Weimer & Moshin (1953) with galactose as standard. Values obtained for the total hexose by the orcinol method were corrected for interference by fucose, which had 66% of the absorbance of galactose at the same molarity. Glucosamine and galactosamine were analysed as described by Minnikin & Allen (1973).

Sulphate was determined by the method of Clarke & Denborough (1971). Samples (5mg) were hydro-

lysed with 1.5 ml of 1 M-HCl at 105°C for 5 h in a sealed tube. After hydrolysis the hydrolysate was centrifuged (1000g for 10min) and sulphate in the supernatant was measured. The sulphate content was determined by mixing with 1.5 ml of 3.8% (w/v) trichloroacetic acid, followed by the addition of 0.5 ml of BaCl₂-gelatin reagent (Dodgson, 1961). The mixture was left for 20min at room temperature and then the extinction at 360 and 500nm was read. Correction was made for the small u.v. absorption of the material by estimation of a blank containing everything but BaCl₂.

Amino acid analyses were performed by the method of Mahowald *et al.* (1962) with a Locarte amino acid analyser. Samples were hydrolysed for 10, 18, 30, 40 and 70h and the correction factors were determined for the decay of serine and threonine and for the slow appearance of leucine. Cysteine and cystine were measured by the method of Press *et al.* (1966).

Duplicate 10mg samples were dissolved in 10ml of a solution of 6M-guanidinium chloride in 0.5M-Tris-HCl buffer, pH8.5, for the determination. In some experiments, before the *S*-carboxymethylation, the disulphide bonds of the cystine residues were completely reduced by incubation at 37°C for 5h with 0.5M-2-mercaptoethanol. The *S*-carboxymethylcysteine was analysed in the standard amino acid analysis.

The *N*-terminal amino acids were identified as dansyl derivatives by chromatography on polyamide sheets by the method of Hartley (1970).

Polyacrylamide-gel electrophoresis was performed in 5mM-Tris buffer, containing 0.1% sodium dodecyl sulphate, and adjusted to pH8.3 with 0.1M-glycine. A 7.5% (w/v) gel was used and samples were applied in a solution of 0.1M-2-mercaptoethanol containing 1% sodium dodecyl sulphate. The gel was run at 5mA for 2h.

The method used for separating the mucoprotein from the protein by equilibrium density-gradient centrifugation in a CsCl gradient was based on that of Creeth & Denborough (1970). The mucoprotein fractions were dissolved in a 38% (w/w) CsCl solution to give a mucoprotein concentration of about 1.2mg/ml. The solution was centrifuged at $1.5 \times 10^5 g$ at 5°C for 48h, after which time density-gradient formation was assumed to be complete (Creeth & Denborough, 1970). Each centrifuge tube was fractionated into six equal fractions. The density of each fraction, determined by weighing a 2ml sample, varied linearly from 1.38g/ml (for the top fraction) to 1.56g/ml (for the bottom fraction) (Fig. 1).

Results and Discussion

Purification of gastric mucoprotein by using CsCl-density-gradient centrifugation

Equilibrium centrifugation in a CsCl density gradient (Creeth & Denborough, 1970) clearly separated the non-covalently bound protein from the mucoprotein. There were two peaks of absorption at 280nm with maxima in fraction 1 (top of tube; protein) and fraction 6 (bottom of tube; mucoprotein), whereas there was only one peak for fucose and hexose with a maximum in fraction 6 (Fig. 1). There was 90% recovery by weight of the freeze-dried water-soluble mucus after density-gradient centrifugation in CsCl and 45% of this was the purified mucoprotein in fractions 5 and 6. Some of the 10% by weight of non-recoverable material was observed as precipitated protein adhering to the walls at the tops of the centrifuge tubes. Mucoprotein from fractions 5 and 6 was pooled and re-run for 48h in another CsCl density gradient containing 4M-guanidine hydrochloride. After this re-run there was only one 280nm-absorbing peak with a maximum in fraction 6 (mucoprotein),

showing that no further protein had separated from the mucoprotein. Therefore it is reasonable to assume that all the non-covalently bound protein associated with the mucoprotein is removed by the initial separation in the CsCl gradient and that the remaining protein is covalently bound to the carbohydrate.

The mucoprotein from equilibrium centrifugation of the water-soluble mucus was fractionated into mucoproteins A_{CsCl} and B/C_{CsCl} by gel filtration on Sepharose 4B (Scheme 1). Elution of the column with 0.2M-NaCl produced two orcinol-positive and 280nm-absorbing peaks, one in the excluded volume consisting of the higher-molecular-weight mucoprotein A_{CsCl} and the other in the included volume consisting of the lower-molecular-weight mucoprotein B/C_{CsCl}. The peak containing the lower-molecular-weight mucoprotein B/C_{CsCl} was dialysed against water, freeze-dried and rechromatographed on a Sephadex G-100 column eluted with 0.2M-NaCl. Only one peak containing all the orcinol-positive and 280nm-absorbing material was eluted from the Sephadex G-100 column and this peak was in the excluded fraction. Both mucoproteins A_{CsCl} and B/C_{CsCl} on sedimentation analysis gave respectively single apparently homogeneous peaks with $s_{25,w}^{0,0}$ 33S and 5.75S, and from a combination with the $D_{25,w}^0$ values, molecular weights of 2.3×10^6 and 1.15×10^5

Fig. 1. Fractionation of water-soluble mucus by density-gradient centrifugation in a CsCl gradient

The starting density of the CsCl was 1.42g/ml. Density after centrifugation (----) was 1.38g/ml for fraction 1 and 1.56g/ml for fraction 6. ■, Fucose; ▲, total hexose by the orcinol method; ○, protein by E_{280} . Each tube was fractionated into six 4ml fractions.

Table 1. *Physical properties of mucoproteins A_{CsCl} and B/C_{CsCl} before and after reduction with 0.2M-2-mercaptoethanol*

The analytical techniques used are described in the Methods section. $M_{s,D}$ is the molecular weight determined by the combination of sedimentation and diffusion coefficients.

Mucoprotein	Buffer	$s_{25,w}^0$	Molecular weight ($M_{s,D}$)
A _{CsCl}	0.2M-KCl, pH 5.5	33	2.3×10^6
	0.2M-KCl-0.2M-mercaptoethanol, pH 8.5	14.6	5.21×10^5
B/C _{CsCl}	0.2M-KCl, pH 5.5	5.7	1.15×10^5
	0.2M-KCl-0.2M-mercaptoethanol, pH 8.5	3.5	—

Table 2. *Chemical composition of mucoproteins A_{CsCl} and B/C_{CsCl}*

The analytical techniques used are described in the Methods section. The protein value was from a summation of the amino acid analyses (Mahowald *et al.*, 1962). The values are not corrected for moisture and ash content in the material. The results are average values from at least four experiments. Paper chromatography confirmed the presence of galactose and fucose and the absence of ribose and deoxyribose.

	% by weight of freeze-dried material	
	Mucoprotein A _{CsCl}	Mucoprotein B/C _{CsCl}
Galactose	26.0 (±0.4)	26.3 (±1.0)
Fucose	11.3 (±0.9)	11.2 (±0.6)
Glucosamine	19.5 (±1.3)	18.1 (±1.5)
Galactosamine	8.3 (±0.5)	7.0 (±0.6)
Sialic acid	0.2	0.2
Protein	13.6 (±0.7)	14.3 (±1.1)
Phosphate	0.2	0.2
Sulphate	3.1	

(Table 1). The change in the sedimentation coefficients, but not molecular weights, of the mucoproteins A_{CsCl} and B/C_{CsCl} after the density-gradient centrifugation is due to a CsCl-induced irreversible contraction in the tertiary structure of these mucoproteins (Snary *et al.*, 1974). Associated with this CsCl effect is a large decrease in the intermolecular interactions between the mucoproteins, which may account for why, after gel filtration, mucoprotein A, without CsCl treatment, contains mucoprotein B/C (Snary & Allen, 1971) but not after CsCl treatment.

The alternative method used for preparing mucoproteins A_{CsCl} and B/C_{CsCl} was to separate mucoprotein fractions A and B/C from the water-soluble mucus by gel filtration (Scheme 1, Snary & Allen, 1971) and then to use equilibrium centrifugation of the individual mucoprotein fractions in the CsCl gradient. With both mucoprotein fractions A and B/C non-covalently bound protein, which was eluted with the mucoproteins on gel filtration, separated out from the mucoprotein in the CsCl gradient. The resulting mucoproteins prepared by this method had

identical physical properties with the mucoproteins A_{CsCl} and B/C_{CsCl} prepared by the other method above.

The protein content of mucoprotein A_{CsCl} of 13.6% (Table 2) compares with a protein content of mucoprotein A of 16.1% when it was isolated by gel filtration before fractionation in a CsCl gradient. It should be noted that the analytical values published previously (Snary & Allen, 1971) were approximate only. This reflects 15.7% of the protein being non-covalently bound in the mucoprotein A isolated without CsCl-density-gradient centrifugation. The nature of this non-covalently bound protein was further investigated. Electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel was performed on the protein in fractions 1 and 2 from equilibrium centrifugation of mucoprotein A isolated by gel filtration of the water-soluble mucus. The gel was stained with Coomassie Blue and showed that at least four distinct protein bands were separated by electrophoresis. The molecular size of these proteins on electrophoresis was such that normally they would have been expected to separate on Sepharose 4B from the excluded mucoprotein A. This suggests that there is not one specific non-covalently bound protein associated with the mucoprotein, and that small amounts of different proteins may be loosely entangled with the complex tertiary structure of the mucoprotein preventing their removal by gel filtration. Neuraminidase activity, which is associated with mucoproteins A and B/C isolated by gel filtration, is absent from the mucoproteins isolated by a CsCl density gradient (Allen & Starkey, 1974a).

Analysis of the CsCl-purified mucoprotein A_{CsCl}

The native mucoprotein A_{CsCl}, free from non-covalently bound protein, contains 40% more amino acids than the blood-group substance prepared by pepsin digestion (10.9% protein, Table 3). The proportion of threonine and serine, the amino acids involved in the peptide-carbohydrate linkage (Marshall, 1972; Slomiany & Meyer, 1972), although still constituting 31% of the protein (Table 3), is lower than the 37% found for the proteolytically digested

Table 3. *Amino acid composition of mucoproteins A_{CsCl} and B/C_{CsCl}*

The samples were S-carboxymethylated in 6M-guanidine hydrochloride by the method of Press *et al.* (1966). Where stated reduction was with 0.5M-2-mercaptoethanol. The results are average values from at least three experiments.

	(μmol/mg of freeze-dried material)		* Gastric blood-group-substance preparation
	Mucoprotein A _{CsCl}	Mucoprotein B/C _{CsCl}	
Asp	0.096	0.098	0.044
Thr	0.198	0.205	0.225
Ser	0.188	0.176	0.173
Glu	0.105	0.115	0.062
Pro	0.140	0.132	0.182
Gly	0.095	0.098	0.067
Ala	0.090	0.086	0.082
Val	0.076	0.074	0.073
Met	0.007	0.005	0.005
Ile	0.048	0.049	0.023
Leu	0.076	0.086	0.046
Tyr	0.025	0.029	0.007
Phe	0.030	0.036	0.013
His	0.023	0.026	0.023
Lys	0.032	0.042	0.021
Arg	0.039	0.037	0.037
Reduction, carboxymethylcysteine (+ carboxymethyl-half-cystine)	0.056	0.060	
No reduction, carboxymethylcysteine	0.023	0.020	

* Prepared by pepsin digestion and phenol extraction of gastric mucus. Data from Snary & Allen (1971).

gastric blood-group substances. There is, in particular, a higher content of aspartic acid, glutamic acid, leucine and isoleucine residues in mucoprotein A_{CsCl} than in the pepsin-digested blood-group substance. These residues, which are more characteristic of an average globular protein, therefore constitute a significant part of the enzymically accessible protein in the mucoprotein molecule. The higher protein content of mucoprotein A_{CsCl} compared with the pepsin-digested blood-group substance (Table 3) is evidence that the isolation from the water-soluble mucus of mucoproteins which are not enzymically digested has been at least partly, or possibly completely, successful. Further, on treatment of mucoprotein A with enzymes such as pepsin, trypsin and Pronase, there is a marked decrease in the viscosity (Allen & Starkey, 1974b). This again indicates the absence of proteolytic digestion in mucoprotein A and that the covalently bound protein digested by proteolytic enzymes is essential in maintaining the structure that confers on the molecule the viscous properties.

The composition of the constituent sugars of the mucoprotein A_{CsCl} (Table 2) is very similar to that of mucoprotein A before fractionation in a CsCl gradient (Snary & Allen, 1971), as well as to the blood-group substances isolated from pig stomach by enzymic digestion (Kabat, 1956; Slomiany & Meyer, 1972). Therefore with respect to the carbohydrate chains the above mucoprotein preparations are likely

to be the same. There is 3.1% of ester sulphate in the large-molecular-weight mucoprotein A_{CsCl}. Sulphated mucoproteins have been isolated from a number of gastro-intestinal sources, including sheep colonic mucosa (Kent & Marsden, 1963), dog gastric mucosa (Pamer *et al.*, 1968), as well as pig gastric mucosa (Slomiany & Meyer, 1972). The very low values of sulphate reported previously for these gastric mucoproteins (Snary & Allen, 1971) is attributed to the use here of a simpler method of determination and the previous estimation being performed only in the impure water-soluble mucus. The characteristically low sialic acid content of these gastric mucoproteins (Table 2) may, in part, be due to neuraminidase, probably of bacterial origin, that is closely associated with the mucoprotein (Allen & Starkey, 1974a).

Subunit structure of the mucoprotein A_{CsCl}

Mucoprotein A has been shown to consist of subunits one-quarter of the molecular weight of the parent molecule (Snary *et al.*, 1970). The subunits are produced by reduction of the mucoprotein with mercaptoethanol suggesting that disulphide linkages may be involved in their polymerization. Clear evidence for this is provided by the finding that mucoprotein A_{CsCl} contains about 130 half-cystine residues per molecule, of which 78 (59%) are involved in disulphide bridges that are split on reduction with

mercaptoethanol (Table 3). The splitting by mercaptoethanol of the purified mucoprotein A_{CsCl} into subunits one-quarter of the molecular weight (Table 1) shows that the cleavage is a function of the protein portion of the mucoprotein and not a result of changes in some non-covalently attached protein. Only one peak was present on ultracentrifugation of mucoprotein A_{CsCl} in mercaptoethanol. Further, the *S*-carboxymethylated mucoproteins had, within the experimental limits, the same amino acid analysis as the non-*S*-carboxymethylated mucoproteins. Since after *S*-carboxymethylation the mucoproteins are dialysed before acid hydrolysis, there is no production of appreciable amounts of dialysable peptides on treatment with mercaptoethanol. Therefore each mucoprotein A_{CsCl} molecule is broken down into four subunits of about the same molecular weight. A closely similar chemical structure for these subunits is suggested by the finding by the dansyl method (Hartley, 1970) that aspartic acid is the only *N*-terminal amino acid of the mucoprotein. There were negligible traces of glycine, alanine, leucine and isoleucine.

The splitting of mucoprotein A into subunits by mercaptoethanol is accompanied by a large decrease in the viscosity of the water-soluble mucus. This explains at the molecular level the success of the use of mucolytic agents like *N*-acetylcysteine in the treatment of bronchial conditions characterized by exceptionally viscous mucus secretions (Sheffner, 1963; Snary *et al.*, 1970). It is interesting that other mucoproteins from a variety of mucous secretions, including those from canine tracheal, canine gastric and pig submaxillary mucus, but not ovine and bovine submaxillary mucus (Holden *et al.*, 1971), have been shown to be split into subunits by dithioerythritol. Such reducing agents have also been shown to decrease the viscosity (Sheffner, 1963; Galzigna & Reggiani, 1965) and increase the solubility (Dunstone, 1969; Kim & Horowitz, 1971) of other mucous secretions. All this suggests that mucoprotein subunits joined by interchain disulphide bridges found in gastric mucoproteins may be a feature of the structure of mucoproteins from several other mucous secretions.

The lower-molecular-weight mucoprotein B/C from the water-soluble mucus has also been shown to be cleaved by mercaptoethanol into subunits of one-quarter the molecular weight of the parent molecule ($s_{25,w}^0$ 3.5S and mol.wt. 27700; Snary *et al.*, 1970). Mucoprotein B/C_{CsCl} fractionated by equilibrium centrifugation is presumed to undergo a similar cleavage because in mercaptoethanol it has the same $s_{25,w}^0$ value of 3.5S (Table 1). There are nine half-cysteine residues per mucoprotein B/C molecule of which six are involved in disulphide bridges which are split on mercaptoethanol reduction (Table 3). The three disulphide bridges are the minimum number necessary to join the four mucoprotein B/C subunits

together. As with mucoprotein A_{CsCl} there is no loss of material on mercaptoethanol reduction of mucoprotein B/C. A calculation from the protein content of mucoprotein B/C (14.3%, Table 2) shows that there are in the region of 40 amino acid residues in the peptide of each mucoprotein subunit.

There is a very close similarity between the higher-molecular-weight mucoprotein A and the lower-molecular-weight mucoprotein B/C. Thus within the limits of the determinations mucoprotein B/C_{CsCl} has the same sugar and amino acid analysis as mucoprotein A_{CsCl} (Tables 2 and 3). Previous work has shown that mucoproteins A and B/C are the same in many biosynthetic, immunological and physical parameters (Allen & Snary, 1972). It has therefore been suggested that the higher-molecular-weight mucoprotein A is a polymer of lower-molecular-weight mucoprotein B/C units (Snary & Allen, 1971). The two mucoproteins are not interconvertible, although the ratio of the two mucoproteins in the water-soluble mucus preparations varies considerably. In this series of experiments the amount of the low-molecular-weight mucoprotein B/C in the preparations has been particularly low. It is unlikely that non-covalent-type linkages only are involved in construction of mucoprotein A from mucoprotein B/C units since 2.5M-KCl, 3.5M-CsCl, or 6M-guanidine hydrochloride do not affect the proportion of the two mucoproteins present (Snary *et al.*, 1971, 1974). Further, splitting of the interchain disulphide bonds still preserves the difference between the high-molecular-weight subunit $A_{\text{mercaptoethanol}}$ (mol.wt. 5.21×10^5) and the low-molecular-weight subunit B/C_{mercaptoethanol} (mol.wt. 27700). The only clear distinction (apart from the molecular weight) between mucoproteins A and B/C is in the *N*-terminal amino acid residue. In mucoprotein A_{CsCl} the only *N*-terminal amino acid is aspartic acid, whereas in mucoprotein B/C several different *N*-terminal amino acid residues are present, namely glycine, aspartic acid, valine, alanine, leucine and isoleucine.

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