The Action of Proteolytic Enzymes on the Glycoprotein from Pig Gastric Mucus

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(Received 5 October 1976)

A glycoprotein of mol.wt. 2×10^6 was isolated in homogeneous form from pig gastric mucus by isopycnic centrifugation in CsCl but without enzymic digestion or reductive cleavage of disulphide bonds. Digestion of the purified glycoprotein with trypsin, pepsin or Pronase resulted in the formation of glycoprotein subunits, of mol.wt. 5.2×10^5 – 5.8×10^5 , one-quarter that of the undigested glycoprotein. The glycoprotein subunits were isolated by gel filtration and shown to contain all the carbohydrate present in the undigested glycoprotein, but 18.6–25.6% of the total amino acids originally present were lost on digestion. The relative amount of threonine, serine and proline had increased from 41% (w/w) in the undigested glycoprotein to 61–67% of the total amino acids in the glycoprotein subunits after digestion. The results support the previously proposed structure for the glycoprotein, namely that of four subunits joined by disulphide bridges. These results show the presence of two distinct regions in the glycoprotein molecule, one rich in threonine, serine and proline, which is glycosylated and resistant to proteolysis, whereas the other, with an amino acid composition more characteristic of a globular protein, is not glycosylated and is susceptible to proteolysis. In addition, the region that is susceptible to proteolysis contains the disulphide bridges which join the glycoprotein subunits together to form the gastric glycoprotein.

A high-molecular-weight glycoprotein (mol.wt. 2×10^6) possessing A and H blood-group activity has been shown to be the component that is responsible for the viscous and gelling properties of pig gastric mucus (Allen & Snary, 1972; Allen, 1977). This glycoprotein can be solubilized by homogenization of the mucous gel and isolated free of contaminating protein by equilibrium-density-gradient centrifugation in CsCl (Starkey *et al.*, 1974; Robson *et al.*, 1975). The purified glycoprotein has a protein core, 13% by weight of the glycoprotein, with carbohydrate side chains attached. The carbohydrate composition is 24.9% galactose, 14.3% fucose, 23.3% glucosamine, 8.7% galactosamine and 3.1% ester sulphate residues expressed as percentage by weight of the freeze-dried glycoprotein. When the glycoprotein from gastric mucus is treated with 2-mercaptoethanol it loses its characteristic viscous and gelling properties and dissociates into four equal-sized glycoprotein subunits of one-quarter the molecular weight of the starting material (Snary *et al.*, 1970; Starkey *et al.*, 1974).

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Digestion with pepsin and other proteolytic enzymes has been used to solubilize the gastric-mucus gel (Kabat, 1956; Kristiansen & Porath, 1968; Slomiany & Meyer, 1972; Snary & Allen, 1972), but such treatment has a considerable effect on the physical properties of the material when compared with the material isolated without previous enzymic digestion. For example, digestion of both the ovarian-cyst blood-group-substance glycoprotein and the glycoprotein from pig gastric mucus with proteolytic enzymes caused a large fall in the viscosity (Pusztai & Morgan, 1961; Allen & Starkey, 1974). The digestion products of the ovarian-cyst blood-group substance contained less protein but still contained all of the carbohydrate present in the starting material (Morgan & Pusztai, 1961). Such studies have led to the suggestion that the blood-group-substance glycoproteins consist of two distinct regions, one of which is glycosylated and one which is not glycosylated and is susceptible to proteolytic attack (Dunstone & Morgan, 1965; Kristiansen & Porath, 1968; Donald, 1973; Scawen & Allen, 1975).

The present paper is concerned with the effect that digestion with proteolytic enzymes has on the structure and composition of the purified pig gastric glycoprotein. These studies are of interest because (1) they clearly demonstrate the effect of proteolytic

digestion on the purified glycoprotein (mol.wt. 2×10^6), splitting the molecule into subunits (mol.wt. 5×10^5); (2) they provide information about the structure of the non-glycosylated region of the glycoprotein, which is accessible to proteolytic enzymes and contains the disulphide bridges that join the glycoprotein subunits together to form the native glycoprotein of the gastric mucus; (3) these results also explain at the molecular level the decrease by proteolytic enzymes in the viscous and gel-forming properties of the gastric mucus.

Materials and Methods

Enzymes

Trypsin (bovine, twice recrystallized), pepsin (pig, twice recrystallized) and Pronase (type VI) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

Purification and characterization of pig gastric glycoprotein

The scrapings of pig gastric mucosa were collected and homogenized to solubilize the gel (Robson *et al.*, 1975). The glycoprotein was isolated from the soluble gel by equilibrium-density-gradient centrifugation in CsCl (initial density 1.42g/ml), followed by gel filtration on Sepharose 4B (Starkey *et al.*, 1974). The analytical methods used for the colorimetric determination of total hexose, galactose, fucose, amino sugars and amino acids were those given by Starkey *et al.* (1974).

Enzymic digestion

These were carried out in the following buffers: trypsin, 0.2M-NH₄HCO₃ adjusted to pH8.5 with 5M-NH₃; Pronase, 0.2M-ammonium acetate adjusted to pH6.5 with 6M-HCl; pepsin, 0.2M-citric acid adjusted to pH2.2 with 5M-NaOH. All buffers contained 0.02% NaN₃. Digestion was carried out on 50mg samples of the gastric glycoprotein at a concentration of 5mg/ml for 72h at 37°C and an initial enzyme/substrate ratio of 0.15 (w/w). Additions of enzyme equal to the first were made after 24 and 36h. After digestion the glycoprotein product was separated from low-molecular-weight peptide material and added enzyme by gel filtration on a column (60cm $\times$ 9.5cm) of Sepharose 4B eluted with 0.2M-NaCl. The excluded fraction was exhaustively dialysed against water and freeze-dried. This material was used for all subsequent physical and chemical studies.

Sedimentation and diffusion coefficients

Sedimentation runs were performed at 25°C, in a Beckman model E analytical ultracentrifuge. Linear relations were obtained when values of $1/s$ were plotted against the mean plateau-region concentrations obtained from the schlieren peak areas.

Height-area diffusion coefficients were calculated from the spreading of boundaries formed between solution and solvent in a double-sector synthetic-boundary cell in the analytical ultracentrifuge. Runs were carried out at 25°C and at 2600 rev./min and the diffusion coefficients were corrected for solvent viscosity. Values of $1/D$ were plotted against $C_0/2$ to give $D_{25,w}^0$.

Values of $s_{25,w}^0$ and $D_{25,w}^0$ were combined to give average molecular weights by making use of the Svedberg equation and a value for the partial specific volume, $\bar{v} = 0.64$ ml/g obtained from a comparison of published values of a range of glycoproteins. The frictional coefficients were calculated from the diffusion coefficients.

Sedimentation and diffusion runs were made in the following buffers: 0.18M-KCl, 0.02M-potassium acetate adjusted to pH5.5 with acetic acid, 0.02% NaN₃ ($\eta_{r,25} = 1.017$; $\rho_{25} = 1.011$ g/ml); 0.18M-KCl, 0.02M-sodium veronal adjusted to pH8.5 with HCl, 0.02% NaN₃, 0.2M-2-mercaptoethanol ($\eta_{r,25} = 1.037$; $\rho_{25} = 1.013$ g/ml).

N-Terminal analyses

These were carried out by using the dansyl* chloride procedure (Gray, 1972). Dansyl-amino acids were identified by chromatography on polyamide thin layers (Woods & Wang, 1967) by using the solvent system described by Ramshaw *et al.* (1970).

Results and Discussion

Purity and composition of the glycoprotein

Before any extensive chemical analyses could be carried out, it was necessary to establish that the glycoprotein, purified by equilibrium centrifugation in a CsCl gradient and then by gel filtration, was homogeneous and free of non-covalently bound protein. The schlieren pattern of the glycoprotein sedimented in the analytical ultracentrifuge was that of a single unimodal polydisperse peak. This shows that the glycoprotein is one species with respect to size (Gibbons *et al.*, 1970), although it possesses microheterogeneity, presumably owing to the number and size of the carbohydrate chains per molecule. A second preparative-density-gradient centrifugation of the purified glycoprotein in another CsCl gradient containing 6M-guanidinium chloride (starting density 1.40g/ml) (Starkey *et al.*, 1974) showed the presence of only one component in the gradient (as measured by hexose and A_{280}) at an equilibrium density of 1.46g/ml, with no evidence for non-covalently bound protein separating at the lower densities (<1.40g/ml). A more sensitive method of

* Abbreviation: dansyl or Dns, 5-dimethylaminonaphthalene-1-sulphonyl.

detecting contaminating protein in glycoprotein preparations is by the presence of material remaining at the meniscus in analytical-density-gradient centrifugation in Cs_2SO_4 (Creeth & Denborough, 1970; Bhaskar & Creeth, 1974). When this method was applied to the glycoprotein from gastric mucus, no evidence was found for contaminating protein in the glycoprotein that had been purified by equilibrium centrifugation in a CsCl gradient. Also, the distribution of the material in Cs_2SO_4 about the gradient was symmetrical in each case. From the above evidence the glycoprotein was considered free from non-covalently bound protein. Different preparations of the glycoprotein purified from the gastric mucus had a consistent amino acid and carbohydrate composition. The mol.wt. was also consistent: $2.09 \times 10^6 \pm 0.19 \times 10^6$ for six entirely different glycoprotein preparations. Previous work has shown that the mol.wt. was not decreased by treatment with 2.5M-KCl or 6M-guanidinium chloride (Allen *et al.*, 1974). Further, when the glycoprotein was boiled in 2% (v/v) sodium dodecyl sulphate there was no significant change in the *s* value measured at one glycoprotein concentration before (26S) and after (24S) boiling. From this result it would also seem likely there has been no change in molecular weight under these conditions.

N-Terminal analysis, by the dansyl method, of four entirely different preparations of the purified glycoprotein showed consistently the same three *N*-terminal amino acids, phenylalanine, valine and isoleucine. The apparent intensity of the Dns-Ile spot on the chromatogram was always about twice that of the Dns-Phe and Dns-Val spots. A fourth intensely fluorescing spot, initially thought to be Dns-Asp (Starkey *et al.*, 1974), proved to be *O*-dansyl-tyrosine. The presence of three *N*-termini in the purified glycoprotein cannot be due to the presence of

non-covalently bound protein impurities for the reasons given above. In addition, the unpurified homogenized mucus always contained a wide range of *N*-terminal amino acids, in contrast with the three always found in the glycoprotein after purification in one or two CsCl density gradients, even when the second gradient contained 6M-guanidinium chloride. The three *N*-terminal amino acids found could result from partial proteolytic digestion of the glycoprotein, either *in vivo* or during the isolation procedure, but this is unlikely in view of the consistent results obtained from four independent preparations of the glycoprotein, and in any case proteolysis could not have been that extensive. Alternatively, the three *N*-terminal amino acids could be due to the presence of three different polypeptide chains in the subunits of the glycoprotein, or the subunits might have a single species of polypeptide chain which exhibits some polymorphism. Quantitative *N*-terminal analysis has so far proved impracticable, principally owing to the large amount of the high-molecular-weight glycoprotein required.

Results of enzyme digestion

The purified glycoprotein was digested with trypsin, pepsin or Pronase for 72h. The time of incubation used was twice that required to minimize the viscosity of the glycoprotein (Allen & Starkey, 1974). The digested glycoprotein when chromatographed on Sepharose 4B was almost completely excluded and therefore readily separable from lower-molecular-weight peptide material and the added enzyme. Analysis of the purified digestion products is shown in Table 1; although enzymic digestion of the glycoprotein caused a decrease in the protein content ranging from 18.6 to 25.6%, within the limit of analyses the high-molecular-weight digestion products contained all of the carbohydrate present in the

Table 1. Carbohydrate composition of the glycoprotein from pig gastric mucus before and after digestion with proteolytic enzymes

Protein content is calculated from the amino acid composition (Table 3). n.d., Not determined. Sugars were analysed colorimetrically. The other constituents of the glycoprotein not analysed below were *N*-acetylneuraminic acid (2.4%), ester sulphate (3.1%) and moisture content (about 10%) (Allen, 1977).

	Composition (% by weight of freeze-dried glycoprotein)			
	Undigested glycoprotein	Digested glycoprotein		
		Trypsin	Pepsin	Pronase
Galactose	26.0	27.5	n.d.	27.8
Fucose	11.3	11.5	n.d.	11.7
Glucosamine	19.5	20.7	22.3	20.1
Galactosamine	8.3	8.0	8.2	9.5
Protein	12.9	10.5	9.7	9.6
Fall in protein (% of undigested protein)	—	18.6	24.8	25.6

starting material. The amount of material actually lost on digestion is only small (about 3%, w/w) and so has very little effect on the analytical results for carbohydrate. Further, analyses for total hexose (anthrone) of the enzyme digests fractionated by gel filtration on Sepharose 4B showed that only the excluded peak (i.e. digested glycoprotein) was carbohydrate-positive and that the included peak of peptides and added enzyme was carbohydrate-negative. Thus no significant amounts of low-molecular-weight carbohydrate-containing material are released on enzymic digestion.

Only a single unimodal peak was obtained on sedimentation analysis of all preparations of proteolytically digested glycoprotein and the molecular weight of the glycoprotein after digestion with trypsin, Pronase or pepsin was decreased to about one-quarter that of the starting material (Table 2). The values of molecular weight for the glycoproteins from the different enzyme digests are, within the limits of the experiment, the same, since the poly-disperse nature of these glycoproteins (Gibbons, 1972) means that values of the molecular weight may vary by 10%. Since this digested glycoprotein excluded on Sepharose 4B accounts for 96% of the original native glycoprotein (mol.wt. 2×10^6), it can be concluded that there are an average of four glycoprotein subunits (mol.wt. 5×10^5) produced for each glycoprotein molecule. This splitting by proteolytic enzymes of the glycoprotein of pig gastric mucus into four subunits of equal molecular weight is similar to that obtained when the glycoprotein is reduced in 0.2M-2-mercaptoethanol (Table 2, and Snary *et al.*, 1970). When the product of trypsin digestion was treated with 0.2M-2-mercaptoethanol, there was no change in the sedimentation coefficient at the one glycoprotein concentration used ($s_{25,w} = 12.3S$ before and 12.8S after reduction); from this it was assumed that no further change in molecular weight had occurred, although the removal of a small peptide moiety cannot be ruled out.

The decrease in frictional ratio that occurs on

digestion (Table 2) indicates that either the molecule has become less asymmetric or that it has assumed a less-expanded structure. It is more likely to be the latter in this case, in view of the approximately spherical shape of the glycoprotein before enzymic digestion (Allen *et al.*, 1974).

The amino acid compositions of the various purified enzymically digested glycoproteins are shown in Table 3. Each of the enzymes used caused a decrease in the amino acid content of the glycoprotein which, although representing only about 3% by weight of the total glycoprotein, is between 18 and 25% by weight of the protein core. In general, the magnitude of the decrease caused by the enzymes is in the order trypsin < pepsin < Pronase. With all three enzymes the loss of serine, threonine and proline, which are the amino acids characteristic of the glycosylated region in these glycoproteins (Slomiany & Meyer, 1972; Donald, 1973), is much less than the losses observed for all the other amino acids. Thus in the starting material, serine, threonine and proline together accounted for 41% (w/w) of the total amino acids, whereas after digestion they accounted for 61% (trypsin), 66% (pepsin) and 67% (Pronase) (w/w) of the total amino acids. The amino acids lost are those more characteristic of an average globular protein and include most of the hydrophobic and basic amino acids.

The cysteine content (arising from cystine and cysteine) of the enzymically digested glycoproteins was very low, being not greater than 8% of the amount present in the undigested glycoprotein (Table 3). This value is consistent with the observed lack of effect of 2-mercaptoethanol on the trypsin-digested glycoprotein. This shows that the cysteine is present mainly in the non-glycosylated region of the molecule, the region which is not rich in serine, threonine and proline. Although these results are quite convincing, the values for cysteine may be low because it was not determined as *S*-carboxymethylcysteine. This explains why the amount of cysteine (as cystine and cysteine) found in this preparation of the glycoprotein

Table 2. *Physical properties of glycoprotein from pig gastric mucus before and after digestion with proteolytic enzymes*
The molecular weights are calculated from the sedimentation and diffusion coefficients, and are the averages of two or three separate determinations. Frictional coefficients were calculated from the diffusion coefficients.

	Undigested glycoprotein	Digested glycoprotein			Glycoprotein reduced with 2-mercaptoethanol*
		Trypsin	Pepsin	Pronase	
$s_{25,w}$ (S)	28.7	18.9	18.0	21.1	14.6
$D_{25,w}$ ($\times 10^7$ cm ² /s)	0.94	2.48	2.30	2.49	1.92
Mol.wt.	2.09×10^6	5.21×10^5	5.38×10^5	5.79×10^5	5.21×10^5
f/f_0	3.2	1.9	2.0	1.9	2.1

* Data from Starkey *et al.* (1974).

Table 3. Amino acid composition of the glycoprotein from pig gastric mucus before and after digestion with proteolytic enzymes
Compositions are expressed as $\mu\text{mol}/\text{mg}$ of freeze-dried material.

Composition	Undigested glycoprotein ($\mu\text{mol}/\text{mg}$)	Digested glycoprotein					
		Trypsin		Pepsin		Pronase	
		($\mu\text{mol}/\text{mg}$)	(% loss)	($\mu\text{mol}/\text{mg}$)	(% loss)	($\mu\text{mol}/\text{mg}$)	(% loss)
Asp	0.059	0.026	55.9	0.018	69.5	0.014	76.3
Thr	0.223	0.191	14.3	0.190	14.8	0.184	17.5
Ser	0.195	0.172	11.8	0.150	11.8	0.168	13.8
Glu	0.095	0.056	41.0	0.041	56.8	0.035	63.2
Pro	0.189	0.160	15.3	0.148	21.7	0.130	31.2
Gly	0.072	0.041	43.1	0.031	56.9	0.030	58.3
Ala	0.058	0.035	39.7	0.029	50.0	0.028	51.7
Cys	0.038	0.003	92.1	0.003	92.1	0.003	92.1
Val	0.070	0.043	38.6	0.035	50.0	0.031	55.7
Ile	0.041	0.022	46.3	0.020	51.2	0.017	58.5
Leu	0.044	0.020	54.5	0.014	68.2	0.018	59.1
Tyr	0.023	0.009	60.9	0.006	73.9	0.008	65.2
Phe	0.021	0.010	52.4	0.010	52.4	0.007	66.7
His	0.019	0.013	31.6	0.010	47.4	0.007	63.2
Lys	0.048	0.012	75.0	0.007	85.4	0.013	72.9
Arg	0.040	0.017	59.5	0.007	82.5	0.006	85.0

(0.038 $\mu\text{mol}/\text{mg}$) is somewhat lower than that by *S*-carboxymethylcysteine analysis of the reduced glycoprotein (0.056 $\mu\text{mol}/\text{mg}$; Starkey *et al.*, 1974).

The above results show that the glycoprotein from pig gastric mucus consists of two distinct regions. One of these is glycosylated and is not susceptible to attack by proteolytic enzymes. It is also rich in the amino acids threonine, serine and proline, the site of attachment of the polysaccharide side chains by serine and threonine ether links (Slomiany & Meyer, 1972; Donald, 1973). The second region is not glycosylated and is susceptible to attack by proteolytic enzymes. It is richer in those amino acids more characteristic of a globular protein. This region also contains the cysteine residues and is therefore that part of the molecule responsible for the joining of the four glycoprotein subunits by disulphide bridges to form the gastric glycoprotein (mol.wt. 2×10^6). Thus the effect of proteolytic digestion is to remove this peptide portion complete with disulphide bridges. This causes the molecule to dissociate into four subunits similar in size to those obtained by reductive cleavage in 2-mercaptoethanol (Snary *et al.*, 1970). Both the viscous (Allen & Starkey, 1974) and gelatinous properties (Robson *et al.*, 1975) of the gastric glycoprotein are lost on either proteolytic digestion or reductive cleavage in 2-mercaptoethanol, showing that the four-subunit polymeric structure of the glycoprotein is essential for the rheological properties of both it and the gastric mucus.

The trypsin-digested protein, after removal of the

low-molecular-weight material, had the same *N*-terminal analysis as the undigested glycoprotein, namely phenylalanine, valine and isoleucine. This suggests that the part of the glycoprotein that is susceptible to proteolytic digestion, i.e. the non-glycosylated region, is the C-terminal part of the polypeptide backbone.

Preliminary experiments on the effects of proteolytic enzymes on the purified glycoprotein (Allen & Starkey, 1974) had shown a difference in the nature of the products formed after digestion with trypsin or Pronase. Although both enzymes caused a decrease in the viscosity of the impure water-soluble mucus, the products resulting from digestion of the purified glycoprotein had different mol.wts. (9.6×10^5 for trypsin and 1.5×10^6 for Pronase-digested material). Further, reduction of both these materials in 2-mercaptoethanol caused a further decrease in mol.wt. to about 5×10^5 , the subunit size always found in all the studies described in this paper. Since the preparations of glycoprotein and enzymes used were the same as in the present paper, it seems that under certain, as yet undefined, conditions, the conformation of the glycoprotein can change in a manner which renders it less susceptible to attack by proteolytic enzymes.

Pepsin digestion was also performed on the impure water-soluble mucus and gave a single high-molecular-weight component, as shown by examination in the analytical ultracentrifuge. After chromatography on Sepharose 4B, sedimentation and diffusion

measurements gave a mol.wt. of 5.30×10^5 for the high-molecular-weight digestion product. This value is again about one-quarter of the mol.wt. of the gastric glycoprotein before centrifugation in a CsCl density gradient (1.9×10^6 ; Allen & Snary, 1972). This shows that there has been no major change in the susceptibility of the glycoprotein molecule to proteolytic digestion after purification by density-gradient centrifugation in CsCl. The digestion by pepsin of the glycoprotein from gastric mucus is interesting physiologically, although the conditions prevailing in the stomach may be different from those *in vitro*. If pepsin is a factor in the continual erosion of mucus, presumably this is outweighed by the rate of mucus secretion.

Pusztai & Morgan (1961) found that the plant proteinases papain and ficin decreased the viscosity and serological activity of the blood-group-substance glycoprotein, although chymotrypsin, trypsin and pepsin were reported to have no effect on the serological activity, in contrast with the ready digestion of the glycoprotein of pig gastric mucus by two of these enzymes. The non-diffusible digestion products of the blood-group-substance glycoprotein contained all of the carbohydrate present in the original glycoprotein and were enriched in the amino acids threonine, serine and proline, although the protein content was lower (Morgan & Pusztai, 1961). The presence of a non-glycosylated region in the peptide core of ovarian-cyst blood-group-substance glycoproteins has been shown by Pronase digestion, which gave a product containing all of the carbohydrate, and in which threonine, serine and proline accounted for about two-thirds of the total amino acids (Donald, 1973). The work described in the present paper on the glycoprotein from gastric mucus clearly demonstrates the presence of the non-glycosylated region and its role in determining the structure of the native glycoprotein. The use of proteolytic enzymes to solubilize glycoproteins from other mucous secretions suggests that non-glycosylated regions may be present in their peptide cores and play an important part in determining the rheological properties (Kent & Marsden, 1963; Palmer *et al.*, 1968; Hough & Jones, 1972; Gottschalk, 1972).

We thank the Medical Research Council for financial support.

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