A glycosulphatase that removes sulphate from mucus glycoprotein

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A novel glycosulphatase has been purified from a mucus glycopeptide-degrading *Prevotella* from the colon. The purified enzyme removed inorganic [^{35}S]sulphate from ^{35}S-labelled native rat gastric mucus glycoprotein. Desulphation of mucus glycoprotein was initially rapid (19% complete after 10 min) but then plateaued, reaching only 33% after 3 h. Crude periplasmic extracts could remove 79% of the radioactivity as inorganic sulphate. These results suggest that steric hindrance may limit the access of the purified glycosulphatase to the mucus glycoprotein oligosaccharide chains in the absence of glycosidases, and/or that the enzyme may have the wrong specificity for some of the remaining sulphated sugars in the chains. The apparent molecular mass of the enzyme was 111 kDa as judged from gel exclusion chromatography, and it appeared to be composed of two identical subunits. The enzyme was localized in the periplasm of the bacterium, and using pig gastric mucus glycopeptide as a growth substrate markedly increased enzyme levels. Enzymic activity increased at the end of the growth phase. The substrate specificity of the enzyme was tested against low-molecular-mass sulphated molecules. The monosaccharides glucose 6-sulphate and *N*-acetylglucosamine 6-sulphate were rapidly desulphated, the latter being the major sulphated sugar in some mucus glycoproteins. Lactose 6-sulphate, galactose 6-sulphate, sulphated steroids and unsaturated disaccharide sulphate breakdown products from chondroitin sulphate were not desulphated. Glycosulphatases which can remove sulphate from mucus glycoproteins may play an important role in the degradation of highly sulphated mucus glycoproteins in the digestive tract, and could modify the effectiveness of mucus glycoproteins in mucosal protection.

INTRODUCTION

The digestive tract is covered with a layer of mucus throughout its length, and this acts as the first line of defence against many aggressive luminal factors. The main structural components of this mucus layer are the mucus glycoproteins. The mucus glycoproteins secreted in different regions of the digestive tract differ in many respects, including size, composition and antigenicity. Thus the predominant type of mucus glycoprotein differs from one region to another (Filipe, 1979). Neutral, sialo- and sulpho-mucus glycoproteins are secreted by different mucus-secreting cells. Moreover, the type of mucus glycoprotein secreted by a mucus-secreting cell can change as the cell matures. In certain diseases, changes in the sulphate content of mucus glycoproteins can be detected histologically. Such alterations are useful as a diagnostic tool, even though the actual structures yielding the staining properties have not been well established (Allen and Hoskins, 1988).

There is some evidence that high levels of sulphate esterification of macromolecules can affect their properties, making them more resistant to degradation. Mian et al. (1979) showed that chemically sulphated glycopeptides inhibited neuraminidase activity against sialoglycopeptides and submaxillary mucus glycoprotein. Houdret et al. (1989) found that the proportion of the major neutral glycopeptide in respiratory mucus is decreased in cystic fibrosis patients with Pseudomonas infection, and suggested this was a reflection of the ability of bacteria to degrade neutral mucus glycoproteins relative to sulpho-mucus glycoproteins. Stanley et al. (1986) showed that bacterial degradation of mucus glycopeptides was less complete against more sulphated preparations.

There has been little work on glycosulphatases in colonic bacteria which may act on sulphated mucus glycoproteins. This contrasts with the numerous studies on neuraminidases which remove the other negatively charged groups from glycoproteins. Rhodes et al. (1985) incubated biopsy specimens of mucosa with filter-sterilized faecal extracts and showed that sialo-mucus and sulpho-mucus could be changed to neutral mucus, implying that bacterial neuraminidase and sulphatase were removing neuraminate and sulphate from the mucus glycoprotein. Corfield et al. (1987) described the presence of carbohydrate sulphatase in normal faecal extracts. Tsai et al. (1991) demonstrated a glycosulphatase in *Bacteroides melaninogenicus*, and a mucin-degrading sulphatase was recently purified from human faeces (Tsai et al., 1992). During studies on the breakdown of mucus glycopeptide by anaerobic bacteria, one of our isolates (*Bacteroides* strain RS2), which has since been renamed as a *Prevotella* (see below), was shown to remove 63% of the sulphate from pig gastric glycopeptide (Stanley et al., 1986). This bacterium was later shown to have a glycosulphatase which cleaved the sulphate ester from glucose 6-sulphate (Wilkinson and Roberton, 1988). However, as pointed out by Dodgson et al. (1982), there are many sulphated substrates in the bowel, including sulphated glycosides, choline sulphate, glycosaminoglycans, sulphated glycoproteins, sulpholipids, sulphated polysaccharides from certain foods, steroid sulphates, bilirubin sulphate and sulphated drug metabolites. Thus the detection of sulphatases using a sulphated model substrate gives no guarantee that the enzyme can partially desulphate mucus glycoproteins.

In the present study, the glycosulphatase from strain RS2 has been isolated and characterized, and the pure enzyme has been

Abbreviations used: SPGM, Sigma pig gastric mucus glycoprotein; GOR, glucose oxidase reagent; ΔDi-6S, α-Δ4,5-glucuronyl-[1–3]-*N*-acetylgalactosamine 6-sulphate; ΔDi-4S, α-Δ4,5-glucuronyl-[1–3]-*N*-acetylgalactosamine 4-sulphate; ΔDi-0S, α-Δ4,5-glucuronyl-[1–3]-*N*-acetylgalactosamine.

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tested for its ability to remove a portion of the sulphatase esters from native mucus glycoprotein.

MATERIALS AND METHODS

Materials

Sodium glucose 6-sulphate, glucosamine 2-sulphate, glucosamine 3-sulphate, glucosamine 6-sulphate, *N*-acetylglucosamine 6-sulphate, galactose 6-sulphate, dehydroisoandrosterone 3-sulphate, dehydroisoandrosterone, testosterone, the unsaturated disaccharide breakdown products from chondroitin sulphate α-Δ4,5-glucuronyl-[1–3]-*N*-acetylgalactosamine 6-sulphate (ΔDi-6S), α-Δ4,5-glucuronyl-[1–3]-*N*-acetylgalactosamine 4-sulphate (ΔDi-4S) and α-Δ4,5-glucuronyl-[1–3]-*N*-acetylgalactosamine (ΔDi-0S), pig gastric mucin (type II), egg white lysozyme (grade III, 600 units/g), DEAE-Sepharose CL-6B, calcium ionophore A23187, carbachol, prostaglandin E_1 and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. Testosterone 17-sulphate was obtained from E. Merck, Darmstadt, Germany. Lactose 6-sulphate was prepared by the method of Mian et al. (1979). Glucose oxidase and horseradish peroxidase were purchased from Boehringer, Mannheim, Germany. Yeast extract was from Difco Laboratories, Detroit, MI, U.S.A., and Trypticase was from BBL Microbiology Systems, Cockeysville, MD, U.S.A. Sephacryl S-300 was purchased from Pharmacia, Uppsala, Sweden. Serva Blue R stain was obtained from Serva Feinbiochemica, Heidelberg, Germany. ^{35}S-labelled inorganic sulphate was purchased from Du Pont, Boston, MA, U.S.A. Minimal essential medium (S-MEM) (Joklik modified) was obtained from Gibco Laboratories Life Technologies, Grand Island, NY, U.S.A.

Identification of bacterium

The strain used in this and previous work (Stanley et al., 1986; Wilkinson and Roberton, 1988), formerly known as *Bacteroides* strain RS2, was sent to Dr A. B. Onderdonk (Channing Laboratory, Boston, MA, U.S.A.), who confirmed that the bacterium was indeed a *Bacteroides* under an earlier classification system. However, the genus has recently been renamed *Prevotella*. In this work the isolate will therefore be referred to as *Prevotella* strain RS2.

Growth of bacteria

Prevotella strain RS2 was originally isolated from pig colonic mucosa using pig colonic mucus glycopeptide as the energy source (Stanley et al., 1986).

In the present study cells were grown in liquid culture on a basal medium modified from medium 10 (Caldwell and Bryant, 1966) in which 0.75% (w/v) Sigma pig gastric mucin (SPGM) supplemented with 0.02% (w/v) galactose was the energy source unless otherwise specified, and replaced the usual carbohydrates. The medium contained, per litre (final volume): K_2HPO_4, 0.23 g; $(NH_4)_2SO_4$, 0.23 g; NaCl, 0.46 g; $MgSO_4,7H_2O$, 0.09 g; $CaCl_2,2H_2O$, 0.04 g; yeast extract, 0.5 g; Trypticase, 2 g; resazurin, 0.001 g; haemin, 1×10^{-5} g; vitamin K, 5×10^{-4} g; pH 6.5. These components were sterilized under O_2-free nitrogen (121 °C for 20 min). Sterile anaerobic solutions of CO_2-equilibrated Na_2CO_3 (4 g) and cysteine/HCl (0.5 g) were subsequently added, and the medium was equilibrated with O_2-free CO_2.

Assay of glycosulphatase activity

Glycosulphatase activity of cell extracts was measured by a modification of the glucose 6-sulphatase assay described previously (Wilkinson and Roberton, 1988), in which glucose was released in an initial incubation and then measured in a second reaction utilizing auxiliary enzymes. Glycosulphatase was incubated with 16.5 mM sodium glucose 6-sulphate, 10 mM 2-mercaptoethanol and 20 mM sodium phosphate buffer, pH 7.4, at 37 °C for 30 min in a final volume of 0.2 ml. The tube was then placed on ice, and 0.3 ml of 0.5 M iodoacetamide was added to inactivate the -SH reagent. Then a second incubation was carried out, in which 2.5 ml of glucose oxidase reagent (GOR; containing 0.75 mg of phenol, 10 mg of sodium phosphate, 1 mg of sodium azide, 0.3 mg of 4-aminophenazone, 50 units of glucose oxidase and 3.7 units of horseradish peroxidase, pH 7.6) was added, and reacted at 37 °C for 15 min. The absorbance at 515 nm was then measured. A standard curve was simultaneously constructed with D-glucose (0–100 nmol), to which 2-mercaptoethanol, phosphate buffer and then iodoacetamide were added, and the second incubation carried out as above. A unit of glycosulphatase was defined as the enzyme activity producing 1 μmol of glucose per min under the standard assay conditions. Protein in enzyme fractions was measured using the dye-binding method of Bradford (1976) as outlined in the Bio-rad protein assay instruction book (Bio-Rad Bulletin 1069, p. 4) using BSA as standard. The protein sample readings were multiplied by a correction factor of 2.29 because the BSA gives an abnormally high colour.

Preparation of cell extracts

Cells were centrifuged at 10000 *g* for 20 min, and the loose precipitate was recentrifuged at 12000 *g* for 10 min. Cell extracts were prepared by one of two methods. Firstly, cells were broken by passage through an Aminco French pressure cell (America Instrument Co., Silver Springs, MA, U.S.A.) at 96600 kPa (14000 lbf/in²), after suspension in 10 ml of 20 mM sodium phosphate buffer containing 10 mM 2-mercaptoethanol and 50 μl of a 2% solution of di-isopropyl fluorophosphate. Secondly, a periplasmic extract was prepared by EDTA/lysozyme treatment (Witholt et al., 1976). Tris/chloride buffer (200 mM, pH 7.4, 7.5 ml/500 ml of original culture) was used to suspend the cells. The suspension was mixed with 7.5 ml of 40% (w/v) sucrose in 200 mM Tris/chloride buffer, pH 7.4. To this was added 75 μl of 100 mM EDTA, pH 7.6, and then 1.3 mg of lysozyme. The mixture was diluted with 15 ml of ice-cold water and incubated at room temperature until spheroplasts formed. When this occurred, 2-mercaptoethanol (10 mM final concentration) and 50 μl of a 2% solution of di-isopropyl fluorophosphate were added. Before pelleting the spheroplasts by centrifugation (10000 *g* for 20 min at 4 °C), $MgCl_2$ (120 mM) was added to stabilize them. The supernatant containing the periplasmic enzyme was retained.

Purification of the glycosulphatase

Periplasmic extract from 2.5 litres of original culture was first fractionated with ammonium sulphate, and protein precipitating between 40% and 90% saturation was retained. This was redissolved in 10–12 ml of 20 mM sodium phosphate buffer, pH 7.4, containing 10 mM 2-mercaptoethanol. The protein was applied to a Sephacryl S-300 ascending gel chromatography column (2 cm × 70 cm) and the above buffer was applied at a flow rate of 15 ml·h^{-1}. The active fractions were then chromatographed on a DEAE-Sepharose CL-6B anion-exchange

column (1 cm × 18 cm). The sample (usually 25–30 ml) was slowly loaded on to the column at approx. 6 ml·h^{-1} in 20 mM sodium phosphate buffer, pH 7.4, containing 10 mM 2-mercaptoethanol. After washing with 10 ml of this buffer, an NaCl gradient (0–200 mM over 80 ml) in the above buffer was used to elute the glycosulphatase. The active fractions were dialysed against 20 mM phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol. A second anion-exchange chromatography was then carried out using similar conditions to the first column, except that the pH was 7.0.

Gel electrophoresis

The enzyme sample was dialysed against water overnight and then freeze-dried before electrophoresis. SDS/PAGE was performed using a 10% gel and a 5.7% stacking gel by the method of Laemmli (1970), and the gels were stained with Serva Blue R [four tablets dissolved in 250 ml of methanol/water/acetic acid (4:5:1, by vol.)].

Specificity of cleavage of sulphated sugars and sulphated steroids by glycosulphatase

Glycosulphatase was incubated with sulphated sugars (glucose 6-sulphate, glucosamine 2-sulphate, glucosamine 3-sulphate, glucosamine 6-sulphate, *N*-acetylglucosamine 6-sulphate, galactose 6-sulphate or lactose 6-sulphate) (16.5 mM) in 20 mM phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol. Before enzyme addition and after 3 h at 37 °C, a 0.2 ml aliquot was spotted on to Whatman No. 1 paper. The products were separated from the substrate by ascending chromatography using as solvent butan-1-ol/acetic acid/1 M NaOH (2:3:1, by vol.) (Liau and Horowitz, 1982) together with appropriate standards. After drying the paper, the reducing sugars and sugar sulphates were visualized using an alkaline silver nitrate dip (Mayer and Larner, 1959).

The extent of desulphation of ΔDi-6S and ΔDi-4S was measured by incubating glycosulphatase with 16 mM ΔDi-6S or ΔDi-4S in 20 mM phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol (volume 0.1 ml). Controls containing no enzyme were also carried out. After 2 h and 4 h, samples (50 μl) were freeze-dried, dissolved in 0.01 ml of water and spotted on to Whatman No. 1 paper together with standards, including ΔDi-0S, ΔDi-4S and ΔDi-6S at 5 and 15 μg. Descending chromatography was carried out for 22 h using as solvent butan-1-ol/acetic acid/1 M ammonia (2:3:1, by vol.) (Saito et al., 1968). After drying the paper, the reducing sugars were visualized using the alkaline silver nitrate dip as above.

Desulphation of steroid sulphates was tested by incubating 10 mM steroid sulphate with glycosulphatase in 20 mM phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol (volume 0.1 ml). Additional incubations containing 1 mg·ml^{-1} BSA were carried out to ensure that the steroid sulphate was kept in solution, and controls without enzyme were also performed. After 2 and 4 h, samples (50 μl) were taken and freeze-dried, dissolved in 0.01 ml of ethanol and spotted on to silica gel t.l.c. ready foils (F1500; 20 cm × 20 cm) (Schleicher and Schuell, Dassel, Germany). The free steroids and steroid sulphates were separated by ascending chromatography, using the solvent system of Joseph et al. (1966): upper layer (benzene/acetone/water, 2:1:2 by vol.)/methanol (7:3, v/v). Steroids and steroid sulphates were visualized by spraying the plates with phosphomolybdic acid (10% w/v in methanol) followed by heating at 100 °C for 10 min.

Preparation of ^{35}S-labelled mucus glycoprotein

[^{35}S]Sulphate was incorporated into mucus glycoprotein during tissue culture of rat stomach (corpus region) tissue, essentially as described by Goso and Hotta (1989). The corpus region of the stomach from three male Wistar rats (250 g) was washed in Dulbecco's buffer (Bashor, 1979) and placed in minimal essential medium (S-MEM) (Joklil modified), which is free of inorganic sulphate, to which the following were added (mg·l^{-1}): anhydrous $CaCl_2$, 200; $FeCl_2$, 0.1; sodium pyruvate, 100; sodium bicarbonate, 350; L-alanine, 8.9; L-asparagine, 15; L-aspartate, 13.3; L-glutamate, 15; L-glycine, 50; L-proline, 50; L-serine, 50; L-tyrosine, 36; L-methionine, 140; L-fucose, 100; D-glucosamine, 200; D-galactose, 200. To stimulate mucus glycoprotein production, carbachol (1 mM), prostaglandin E_1 (1 μM) and A23187 (10 μM) were also added. This modified medium was filter-sterilized before use.

The gastric corpus tissue was cut into cubes of less than 2 mm, washed several times with modified medium and finally suspended in 10 ml of modified medium. [^{35}S]Sulphate (150 μl, containing 0.75 mCi) was then added, and the tissue was incubated at 37 °C for 5 h under an atmosphere of 5% CO_2 in air. At the end of the incubation, EDTA (5 mM), phenylmethanesulphonyl fluoride (1 mM) and one grain of sodium azide were added, and the tissue was homogenized and then centrifuged at 10000 *g* for 20 min. The mucus glycoprotein was purified from the supernatant by Sepharose CL-4B chromatography and CsCl density gradient centrifugation, desalted on Sephadex G-25 and freeze-dried (Roberton et al., 1992). It was then suspended in 1.5 ml of 20 mM sodium phosphate buffer and stored at −70 °C.

Removal of sulphate from ^{35}S-labelled mucus glycoprotein by glycosulphatase

Purified rat gastric ^{35}S-labelled mucus glycoprotein was incubated with purified glycosulphatase or crude periplasmic extract containing 2–3 munits of enzyme, 20 mM sodium phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol at 37 °C (final volume 0.4 ml). At intervals, aliquots (66 μl) were removed from the incubation, and frozen at −70 °C for at least 30 min (this inactivates the enzyme). They were then thawed and spotted on to Whatman No. 3 filter paper. Ethanol (100 μl) was used to rinse the tube, and was also spotted on to the paper. The strips were electrophoresed with 0.5 M ammonium acetate buffer, pH 5.6, as solvent for 1 h at 12.5 V·cm^{-1}. The paper was dried and cut into 1 cm strips for scintillation counting. Inorganic sulphate was well separated from monosaccharide sulphate and from mucus glycoprotein (Liau and Horowitz, 1982).

RESULTS

Enzyme induction

To determine growth conditions that produced a high enzyme yield, glycosulphatase activity was measured in extracts prepared from cells grown on galactose or SPGM, or both (Table 1). A 4.2-fold greater specific activity was produced using the SPGM as growth substrate as compared with the galactose. When both energy sources were present, nearly double the enzyme yield was found associated with an increased cell yield, but the specific activity was only half of that found with SPGM alone. Bacterial growth experiments were also carried out to assess the relationship between SPGM concentration (as sole energy source) and cell growth. A concentration of 0.75 g/100 ml gave maximum cell yield.

Routine conditions for growing cells for enzyme production

Table 1 Activity of glycosulphatase induced by growth on different energy sources

Cells were grown in 500 ml batches of medium using (a) 0.2% (w/v) galactose, (b) 0.2% (w/v) galactose plus 0.75% (w/v) SPGM, or (c) 0.75% (w/v) SPGM as energy sources. At the beginning of the stationary growth phase, cells were harvested, washed and broken using the French pressure cell. After centifugation, cell extracts were assayed for glycosulphatase activity. Data for each energy source refer to cells from 500 ml of medium. The data shown are a typical set of results, and similar results were obtained in other experiments.

Energy source	Specific activity (units · mg of protein^{-1})	Total activity (units)
(a) Galactose	0.0040	0.650
(b) Galactose plus SPGM	0.0091	3.637
(c) SPGM	0.0168	2.020

Table 2 Effect of stage of cell growth on glycosulphatase production

Cells were grown in 500 ml batches of medium using 0.02% (w/v) galactose plus 0.75% (w/v) SPGM as energy source. Cells were harvested at (a) 70% of full growth, (b) full growth, and (c) 4 h after full growth. Cells were harvested, washed and broken using EDTA/lysozyme treatment. After centrifugation, soluble extracts were assayed for glycosulphatase specific activity and total activity. The data shown are a typical set of results, and similar results were obtained in other experiments.

Stage of cell growth	Specific activity (units · mg of protein^{-1})	Total activity (units)
(a) 70% of full growth	0.0066	0.873
(b) Full growth	0.0747	5.556
(c) 4 h after full growth	0.0641	4.865

were then selected on the basis of the above results: the energy source was standardized at 0.75% (w/v) SPGM supplemented with 0.02% (w/v) galactose to stimulate initial growth during the batch culture.

The stage of growth at which the cells were harvested affected enzyme yield (Table 2). The enzyme level was low during growth, but had increased 10-fold by full growth and was maintained during the stationary phase.

Location of glycosulphatase in the cell

Comparison of the specific activities of crude glycosulphatase, prepared by French pressure cell breakage (Table 1) and by periplasmic enzyme release (Table 2), indicated that the latter resulted in a higher glycosulphatase specific activity. To confirm this, cells were grown on 1 litre of medium containing 0.02% (w/v) galactose plus 0.75% (w/v) SPEM as energy source, and divided into two equal portions at the beginning of the stationary phase. The cells were harvested, washed and broken using either the French pressure cell or EDTA/lysozyme treatment. After centrifugation, soluble extracts were assayed for protein yield and glycosulphatase activity. The French pressure cell extracts, comprising cytoplasm plus periplasm, contained 1134 mg of protein with an activity of 0.007 unit/mg of protein. The EDTA/lysozyme-treated extract, composed of periplasm, contained 81 mg of protein with an activity of 0.067 units/mg of protein, and very little enzyme was present in the spheroplasts after breakage. The results indicate that the glycosulphatase is located in the periplasm.

Table 3 Purification of the glycosulphatase from periplasmic extract

The purification data refer to a 500 ml batch of medium.

Purification	Protein (mg)	Glycosulphatase specific activity (units · mg of protein^{-1})	Total activity (units)
Periplasmic breakage	81.23	0.067	5.427
Ammonium sulphate precipitation (40–90%)	43.1	0.096	4.426
Sephacryl S300 chromatography	16.26	0.268	4.358
DEAE-Sepharose CL-6B chromatography, pH 7.4	1.28	1.13	1.438
DEAE-Sepharose CL-6B chromatography, pH 7.0	0.36	1.9	0.672

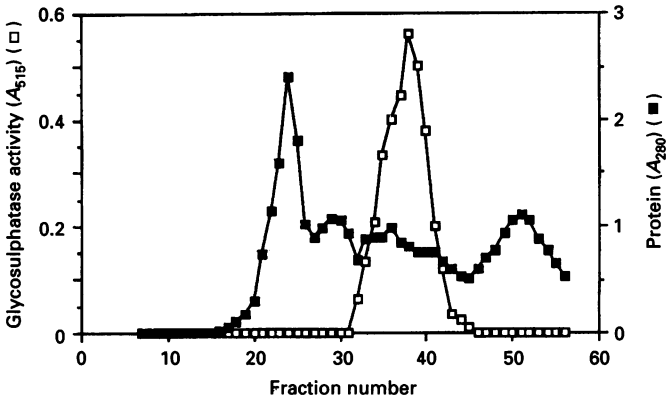

Figure 1 Sephacryl S-300 gel chomatography of the protein, prepared by ammonium sulphate fractionation of the periplasmic extract, during enzyme purification from cells grown in 2.5 litres of medium

■, Protein; □, glycosulphatase. Column fractions were 4 ml.

Purification of the glycosulphatase

The enzyme was purified from a periplasmic extract prepared by EDTA/lysozyme treatment of the cells. Ammonium sulphate was added to the extract and the fraction precipitating between 40 and 90% saturation was retained (Table 3). After redissolving the precipitate in buffer, the enzyme was passed down a Sephacryl S-300 gel-filtration column (Figure 1). The active fraction from this column was then purified on two DEAE-Sepharose anion-exchange columns at slightly different pHs. The enzyme was purified 28-fold during these steps and had a specific activity of 1.9 units · mg^{-1}, though other preparations have had specific activities up to 3.35 units · mg^{-1}. The enzyme gave a single band on an SDS/PAGE gel stained with Serva Blue R (Figure 2). Staining the gel with silver stain also gave a single band, and reverse-phase h.p.l.c. gave a single peak.

Glycosulphatase properties

The apparent molecular mass of the native enzyme was measured by comparison of the elution of the purified enzyme with standards during gel filtration, using conditions similar to those of Figure 1. The K_{av} (elution volume divided by total volume) of standards was plotted against log molecular mass, and the molecular mass of the glycosulphatase obtained by interpolation

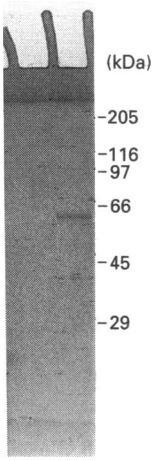

Figure 2 SDS/PAGE of purified glycosulphatase (14 μg of protein)

The molecular masses of standards are shown: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 66 kDa; egg albumin, 45 kDa; carbonic anhydrase, 29 kDa.

Table 4 Specificity of cell extracts and purified glycosulphatase for sulphated sugars

Cells were grown in 500 ml batches of medium using 0.02% (w/v) galactose plus 0.75% (w/v) SPGM as energy source. At the beginning of stationary phase, the cells were harvested, washed and broken (1) using the French pressure cell, or (2) by EDTA/lysozyme treatment to make a periplasmic extract. (3) Glycosulphatase was purified from the latter. Enzyme from the three sources (containing 5.6 munits, 5.0 munits and 7 munits respectively) was incubated with a selection of 16.5 mM sulphated sugars at 37 °C for 3 h. The products were separated from sulphated substrate by paper chromatography and stained for reducing sugar. Product was estimated from the intensity of the product spot, and graded as nil (−), a barely detectable trace (±), and weak (+) to complete (+ + + + +) hydrolysis.

Substrate	(1) French pressure cell extract	(2) Periplasmic extract	(3) Pure enzyme
Glucose 6-sulphate	+ + + +	+ +	+ + + + +
Glucosamine 2-sulphate	+ + +	−	−
Glucosamine 3-sulphate	+ + + +	+ +	+ +
Glucosamine 6-sulphate	+ + + +	±	±
N-Acetylglucosamine 6-sulphate	+ + + +	+ + + +	+ + + + +
Galactose 6-sulphate	+ +	±	±
Lactose 6-sulphate	+ + +	±	±

was 111 kDa (results not shown). This compared with an apparent molecular mass of 58 kDa measured under the dissociating conditions of SDS/PAGE shown in Figure 2.

The pure enzyme was reduced, S-alkylated and then chromatographed on reverse-phase h.p.l.c. A single peak was recovered, indicating that the enzyme is composed of a single subunit type (results not shown). Attempts were made to sequence the N-terminus, but this appeared to be blocked.

The enzyme is very unstable unless kept in the presence of a reducing agent (2-mercaptoethanol). After dialysis for 2 h against 20 mM phosphate buffer, pH 7.4, lacking reducing agent, 73% of the enzymic activity was lost after a further 8 h at 4 °C. In contrast, when pure enzyme was kept at 4 °C in buffer containing 2-mercaptoethanol for 1 and 3 months, 92 and 74% respectively of the activity was still present. It was unstable to freezing.

The glycosulphatase is inhibited by some thiol-modifying reagents but not others. When enzyme, freshly dialysed against 20 mM phosphate buffer, pH 7.4, was assayed under standard conditions except that 2-mercaptoethanol was omitted, concentrations of 1 mM, 0.1 mM and 0.01 mM *p*-hydroxymercuribenzoate inhibited the activity by 97%, 92% and 18% respectively. However, 0.01 mM and 0.001 mM mercuric chloride gave only 14% and 3% inhibition, and iodoacetamide at concentrations up to 10 mM was not inhibitory.

The specificity of the enzyme towards a variety of commercially available sulphated sugars is shown in Table 4. The hydrolysis of the sulphated sugars was measured qualitatively with pure enzyme, crude periplasmic extract and crude French pressure cell extract as enzyme sources. The pure enzyme was very active against glucose 6-sulphate and *N*-acetylglucosamine 6-sulphate, and showed minor activity against glucosamine 3-sulphate. The periplasmic crude extract showed a similar pattern. Interestingly, a different pattern emerged for the French pressure cell extract, which showed activity towards the above sulphated sugars, and in addition towards glucosamine 2-sulphate, glucosamine 6-sulphate, galactose 6-sulphate and lactose 6-sulphate (esterified on the 6-sulphate of the galactose moiety). These results suggest that one or more additional glycosulphatases is present in the cell cytoplasm.

The unsaturated disaccharide products from chondroitin sulphate breakdown, ΔDi-6S and ΔDi-4S, were tested as substrates of the pure glycosulphatase. ΔDi-6S or ΔDi-4S (1.6 μmol) was incubated with 0.006 unit of enzyme in 0.1 ml. After both 2 h and 4 h, no ΔDi-0S was detectable in aliquots containing half of the original incubation. Standards containing 0.012 μmol of ΔDi-0S were clearly visible. The result shows no evidence of desulphation, and the rate was clearly less than 2% of the rate measured with glucose 6-sulphate.

Two steroid sulphates were tested as substrates. The steroid sulphates (0.9 μmol of dehydroisoandrosterone sulphate or 1 μmol of testosterone sulphate) were incubated with 0.0044 unit of purified enzyme in 0.1 ml. After both 2 h and 4 h a small amount of desulphated dehydroisoandrosterone could be detected, but a similar amount was found in the control without enzyme, and this was interpreted as representing non-enzymic breakdown. After 2 h and 4 h the intensity of this dehydroisoandrosterone product was less than that of the 0.013 and 0.027 μmol standards respectively. Thus the rate of desulphation was less than 5% of the rate seen with glucose 6-sulphate, and similar to that in the non-enzymic control. In the other experiment using testosterone sulphate neither enzymic nor non-enzymic desulphation was detected, and the rate was clearly less than 1% of the rate measured with glucose 6-sulphate. Addition of BSA to incubations made no difference to the results.

K_m and $V_{max.}$

The pure enzyme was assayed with a range of substrate concentrations, and the K_m and $V_{max.}$ values were determined (Figure 3). The K_m for glucose 6-sulphate was 60 mM and the $V_{max.}$ was 20.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. This $V_{max.}$ value is higher than the activity measured under the standard assay conditions, in which the glucose 6-sulphate concentration was 16.5 mM.

Removal of sulphate from mucus glycoprotein

Rat gastric mucus glycoprotein (isolated and purified from the corpus region) was labelled with [^{35}S]sulphate. Purified glycosulphatase was incubated with the labelled mucus glycoprotein and released a portion of the sulphate from this

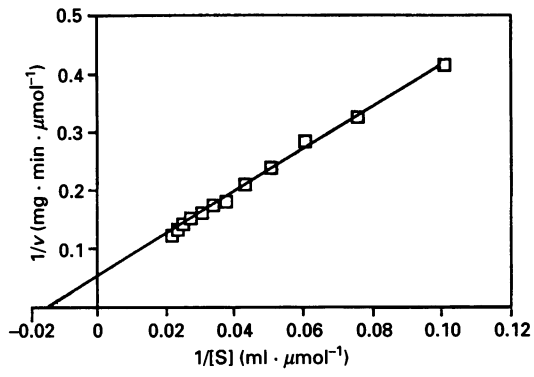

Figure 3 Lineweaver–Burk plot showing the relationship between glycosulphatase activity and glucose 6-sulphate concentration

A range of glucose 6-sulphate concentrations was incubated with purified glycosulphatase (0.54 munit), 20 mM sodium phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol for 30 min at 37 °C, in a final volume of 0.2 ml. Glucose production was determined using the standard method, and $V_{max.}$ and K_m values were calculated. Measurements were made in five separate experiments.

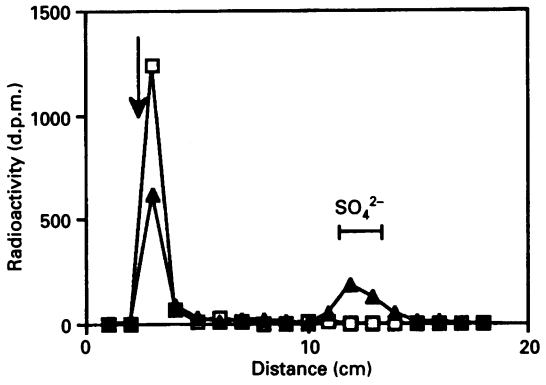

Figure 4 Paper electrophoresis of products liberated during incubation of ^{35}S-labelled mucus glycoprotein with purified glycosulphatase

Purified rat gastric ^{35}S-labelled mucus glycoprotein (8600 d.p.m.) was incubated with purified glycosulphatase (2.8 munits), 20 mM sodium phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol at 37 °C, in a final volume of 0.4 ml. Samples (66 μl) were removed and electrophoresed on Whatman No. 3 paper, and the distribution of radioactivity in products was measured in the zero time sample (□) and the 180 min sample (▲). The origin, indicated by the arrow, was at 2.5 cm on the axis. Inorganic sulphate standard migrated to 10–11 cm from the origin, as indicated by the horizontal bar.

macromolecule as inorganic sulphate (Figure 4). Within 10 min, 7 munits of enzyme/ml removed 19% of the sulphate, and this only increased to 33% after 3 h (Figure 5).

In other experiments (results not shown), the release of inorganic sulphate from another preparation (from rat stomach corpus region) of ^{35}S-labelled mucus glycoprotein by crude periplasmic extract (prepared by the EDTA/lysozyme method) or by purified enzyme was measured. The former released 79% of radioactivity as sulphate, and the latter 35%. With the crude enzyme a new radioactive peak was seen midway between the origin and the inorganic sulphate, which was not observed with the pure enzyme. The nature of this product has not been investigated further, but it may be sulphated carbohydrates (the

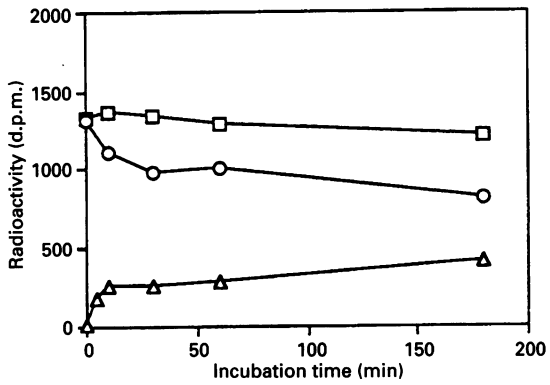

Figure 5 Time course of removal of inorganic sulphate from ^{35}S-labelled mucus glycoprotein

Conditions were the same as in Figure 4. △, Radioactivity present as sulphate; ○, mucus glycoprotein; □, total radioactivity recovered.

glucose 6-sulphate standard ran to this position) or even labelled peptides or glycopeptides.

DISCUSSION

Glycosulphatases are thought to play a significant role in the degradation of mucus glycoproteins, which are the major structural constituents of the digestive tract mucus layer. Several pieces of evidence suggest that the glycosulphatase purified from *Prevotella* strain RS2 fulfils the role of removing sulphate from oligosaccharide chains during the degradation of native mucus glycoprotein. The enzyme was inducible by gastric mucus glycoprotein, which is consistent with the enzyme playing a part in the degradation of this component. The purified enzyme was able to remove a portion of the sulphate groups (33% of the total radioactivity) from native mucus glycoprotein prepared from an area of rat stomach rich in sulphated mucus. The purified enzyme was highly active against monomer *N*-acetylglucosamine 6-sulphate. This sulphated sugar predominates in the oligosaccharide chains of certain mucus glycoproteins such as rat gastric mucus glycoprotein (Liau and Horowitz, 1982). It therefore seems likely that the enzyme will be active against such residues in the oligosaccharide chains of mucus glycoproteins.

The amount of inorganic sulphate released from mucus glycoprotein increased to over 75% when crude periplasmic extract containing the glycosulphatase was used. The most likely reason for the difference in desulphation by pure glycosulphatase and crude extract is that steric hindrance prevents access of the purified enzyme to parts of the oligosaccharide chains. In the crude extract, glycosidases are likely to be present (Berg et al., 1978; Hoskins et al., 1985; Stanley et al., 1986; Tsai et al., 1991) which can sequentially remove outer sugars and expose sulphate groups. An additional possibility is that the enzyme may have the wrong specificity for some of the remaining sulphated monosaccharides in the chain. Galactose 6-sulphate, which is present in smaller quantities in the oligosaccharide chains of rat gastric mucus glycoprotein (Liau and Horowitz, 1982), is unlikely to be desulphated by the enzyme studied in this work, judging by the purified enzyme's lack of activity against the monomer sulphated sugar. Evidence for the existence of an enzyme that will desulphate galactose 6-sulphate was found in cell extracts obtained by French press breakage.

The *Prevotella* enzyme is different from a sulphatase involved

in chondroitin sulphate catabolism isolated from a *Bacteroides* (Salyers and O'Brien, 1980). Unlike the *Prevotella* enzyme, the latter could desulphate ΔDi-4S and ΔDi-6S and was located in the cytoplasm. The *Prevotella* enzyme could not desulphate dehydroisoandrosterone sulphate or testosterone sulphate with the sulphate groups at the 3 or 17 position respectively, indicating that the *Prevotella* enzyme is not a steroid sulphatase. We have shown previously (Wilkinson and Roberton, 1988) that it cannot desulphate *p*-nitrophenyl sulphate, an arylsulphatase model substrate.

The glycosulphatase was unstable in the absence of reducing agent. In previous studies (Wilkinson and Roberton, 1988) the presence of 2-mercaptoethanol appeared to quickly inactivate the enzyme. However, subsequently it has been found that the 2-mercaptoethanol reacted with the hydrogen peroxide produced in the second part of the assay by the glucose oxidase. The assay was therefore modified so that the 2-mercaptoethanol, present to stabilize the glycosulphatase, was removed by adding iodoacetamide, and a glucose oxidase/peroxidase system could then be used to assay the released glucose. The purification of the glycosulphatase was easily achieved once the enzyme could be stabilized.

The thiol-modifying reagent *p*-hydroxymercuribenzoate inhibited the glycosulphatase, but two other thiol-modifying reagents had little effect. One possible explanation is that the sulphydryl anion is the reactive species, and if it is present in a hydrophobic environment apolar organomercurials such as *p*-hydroxymercuribenzoate would be much more effective inhibitors than hydrophilic reagents such as iodoacetamide or mercuric ion (Means and Feeney, 1971). The organomercurial inhibition of activity and the stabilization conferred by 2-mercaptoethanol both suggest that a thiol group is critical for enzyme stability or activity.

The glycosulphatase was inducible by gastric mucus glycoprotein, and the full activity of glycosulphatase only appears at full growth. This is in contrast to many of the glycosidases involved in mucus glycoprotein breakdown, which are constitutive (Hoskins et al., 1985). The enzyme has a very high K_m (60 mM) for glucose 6-sulphate, which indicates that this is unlikely to be a natural substrate during mucus glycoprotein breakdown. Glucose 6-sulphate has been used in this work as a model substrate, providing a convenient substrate for enzyme activity assays. The glycosulphatase has an apparent molecular mass of 58 kDa on SDS/PAGE, compared with an apparent molecular mass measured on a gel-filtration column of 111 kDa. The enzyme is thus likely to be a dimer.

The *Prevotella* enzyme has some similarities to and some differences from the sulphatase which Tsai et al. (1992) recently isolated from human faeces. The faecal enzyme has the following properties (values for the *Prevotella* enzyme are given in parentheses): K_m for glucose 6-sulphate 41.9 mM (60 mM), $V_{max.}$ 70.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (20.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$), apparent molecular mass on SDS/PAGE 15 kDa (58 kDa), pH optimum 4.5 (7.5) (Wilkinson and Roberton, 1988). If a correction is made for the different sizes of the proteins, then the $V_{max.}$ values are similar. A mucus glycoprotein desulphating enzyme has also been found in *Helicobacter pylori* (Murty et al., 1992), but the activities of the partially purified enzyme against sulphated mucus glycoprotein and sulphated oligosaccharides were very much lower [$(1.9–5.2) \times 10^{-6}$ $\mu mol \cdot min^{-1} \cdot mg^{-1}$] than the rates reported here for the *Prevotella* enzyme with glucose 6-sulphate as substrate.

The glycosulphatase should prove useful in future studies on the contribution of sulphate groups to the properties of mucus glycoproteins. Sulphate esters together with sialic acid will affect the surface charge, the cation binding, the balance between hydrophilicity and hydrophobicity, and the expansion of the molecule by charge repulsion. It should be possible to test whether physical properties are altered, and whether the mucus glycoprotein becomes more susceptible to bacterial degradation, after the enzymic removal of some of the sulphate groups. Also, a possible role of sulphated mucus glycoprotein in inhibiting colonization of the mucosa could be affected (Piotrowski et al., 1991).

The presence of glycosulphatases in digestive tract bacteria may play an important part in the breakdown of mucus glycoproteins, especially if these enzymes prove to be a limiting step in degradation. A high content of sulphated mucus occurs in regions of the digestive tract exposed to significant bacterial populations, such as in the colon (Filipe, 1979). It will be important to determine whether the integrity of the mucus barrier can be affected by the action of bacteria that contain such enzymes.

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