

Original articles

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GLYCOSULFATASE ACTIVITY OF HELICOBACTER PYLORI TOWARDS GASTRIC SULFOMUCIN: EFFECT OF NITECAPONE

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A glycosulfatase activity towards human gastric sulfomucin was identified in the extracellular material elaborated by *H. pylori*, a pathogen implicated in the etiology of gastric disease. The purified enzyme exhibited maximum activity at pH 5.7 in the presence of 0.3% Triton X-100 and 100mM CaCl₂, and displayed on SDS-PAGE an apparent molecular weight of 30kDa. The *H. pylori* glycosulfatase effectively caused desulfation of N-acetylglucosamine-6-sulfate and galactose-6-sulfate of the carbohydrate chains of mucins, as well as that of glucose-6-sulfate of glyceroglucolipids, but was ineffective towards galactosyl -and lactosylceramide sulfates which contain galactose-3-sulfate. The glycosulfatase activity towards human gastric sulfomucin was inhibited by an antiulcer agent, nitecapone, which at its optimal concentration (100 µg/ml) caused a 61% decrease in mucin desulfation. The results show that *H. pylori* through its glycosulfatase activity causes desulfation of sulfated mucins and glyceroglucolipids of the protective mucus layer, and that nitecapone is able to interfere with this detrimental action.

Key words: *H. pylori*, glycosulfatase activity, mucin desulfation, effect of nitecapone

INTRODUCTION

The maintenance of gastric mucosal integrity under the adverse environment of lumen and the resistance to the bacterial challenge depends upon a delicate balance of factors affecting the synthesis, secretion, and breakdown of the components constituting the elements of mucosal defense (1, 2). Among these protective components are sulfated glycoproteins and glycolipids (3–5). As constituents the mucus perimeter of gastric mucosal defense, the sulfated glycoproteins and glyceroglucolipids impart a strong

negative charge to mucus gel, thus influencing its viscoelastic, hydrophobic and permselective properties, and modulate the corrosive action of pepsin (3, 5—7).

The sulfated glycosphingolipids of gastric epithelial cell membranes play an important role in cellular recognition processes, participate in cell surface receptor interaction with extracellular matrix proteins, and are directly involved in the maintenance of epithelial integrity (4, 8, 9). The importance of sulfated glycoproteins and glycolipids in the preservation of gastric mucosal integrity is evident from the studies showing that a decrease in the synthesis of sulfated glycolipids accompanies the development of gastritis, and a decline in sulfated mucus glycoprotein content is a prominent feature in the etiology of peptic ulcer (9, 10). Furthermore, sulfated glycosphingolipids have been demonstrated to serve as receptors for colonization of gastric epithelium by *H. pylori* (11), a bacteria recognized as a causative factor in gastric disease (12, 13). The sulfomucins of gastric mucus apparently have the ability to interfere with this colonization process and thus play a defensive role in the mucosal resistance to *H. pylori* (14).

In this study, we present evidence that *H. pylori* displays glycosulfatase activity towards sulfated glycoproteins and glycolipids of gastric mucus, but not towards sulfated glycosphingolipids of gastric epithelium, and show the effect of antiulcer drug, nitecapone, on this enzyme.

MATERIALS AND METHODS

Gastric sulfomucin isolation

Sulfomucin was isolated from the purified human gastric mucus glycoprotein (14). For this, human gastric mucus glycoprotein was dissolved in 0.05M Tris-HCl buffer, pH 6.8, containing 1mM EDTA and applied to a DEAE-Sephacel column. The glycoprotein fractions were then eluted in a stepwise manner, first with the above buffer followed by buffer containing 0.1M, 0.2M, and 0.4M NaCl. Each fraction was subjected to dialysis against distilled water and lyophilized. Removal of the sulfate ester groups from the mucin fraction eluted with 0.4M NaCl was accomplished by acid catalyzed solvolysis with 0.5M HCl in dry methanol (15).

³⁵S-Labeled sulfomucin substrate

³⁵S-Labeled sulfated mucus glycoprotein substrate was prepared by subjecting the desulfated human gastric mucin to enzymatic sulfation in the presence of 3'-phospho-adenosine 5'-phospho sulfate ([³⁵S] PAPS) and Golgi-rich fraction of rat gastric mucosa as source of sulfotransferase enzyme (15). The reaction was terminated after 2h at 37°C by addition of ethanol, and the formed precipitate was collected by centrifugation (16). The pellet was dissolved in 2ml of 0.1M citrate buffer, pH 3.6, and the ³⁵S-labeled glycoprotein product was separated from the components of

the reaction mixture by chromatography on Bio-Gel P-30 column (15). The ^{35}S -labeled glycoprotein, eluted in the exclusion volume of the column with 0.1M citrate buffer was collected, and following dialysis and lyophilization used as substrate for *H. pylori* glycosulfatase enzyme.

Sulfated glycolipid substrates

The sulfated glycosphingolipid substrates, galactosylceramide sulfate (SO_3 , 3Gal β 1, 1Cer) and lactosylceramide sulfate (SO_3H , 3Gal β 1, 4Glc β 1, 1Cer) were prepared from hog gastric mucosa (8,9), triglucosyl monoalkylmonoacylglycerol sulfate (SO_3H , 6Glc α 1, 6Glc α 1, 6Glc α 1, 3-0-alkyl-2-0-acylglycerol) was isolated from human gastric secretion (17).

Glycosulfatase enzyme preparation

Strains of *H. pylori* used for glycosulfatase enzyme preparation were isolated from antral mucosal biopsy specimens of patients undergoing tests for upper abdominal complaints (18). The *H. pylori* isolates were cultured on Skirrow's medium in microaerophilic atmosphere, and the plates with grown colonies were gently washed with 0.15M NaCl. The solution was filtered through sterilization filter (0.2 μm) to retain the bacteria (11), and the filtrate was dialyzed and lyophilized. For enrichment of glycosulfatase activity, the lyophilized powder was dissolved in 0.2M phosphate buffer, pH 7.0, and subjected to acetone fractionation (16). Prior to fractionation, all reagents were chilled to 2°C and the precipitation was started by dropwise addition of acetone under constant gentle mixing. The mixture was allowed to stand for 15 min, centrifuged at 2000xg for 20 min, the precipitate collected, and the supernatant subjected to subsequent acetone fractionation. The precipitates at different acetone concentrations were dried under stream of nitrogen and stored at -70°C until use.

Glycosulfatase activity assay towards ^{35}S -labeled sulfomucin

The *H. pylori* glycosulfatase activity assays towards sulfated mucus glycoprotein consisted of ^{35}S -labeled sulfated human gastric mucus glycoprotein, 50—100 μg ; nitecapone, 0—150 μg ; *H. pylori* enzyme preparation containing 10—50 μg protein; Triton X—100, 0.3%; CaCl_2 , 100mM; and 0.2M sodium acetate buffer, pH 5.7, in a final volume of 500 μl . Incubation was carried out at 37°C for various periods of time up to 3h, the reaction was terminated by boiling (2 min), and the mixture subjected to filtration through Centricon-3 membrane (m. w. cutoff, 3,000) to retain the glycoprotein. Following centrifugation, the filtrate was assayed for the released ^{35}S -sulfate by scintillation spectrometry. The tubes containing the glycoprotein and boiled enzyme served as controls.

Glycosulfatase activity assay towards glycolipids

The *H. pylori* glycosulfatase activity assays towards gastric mucosal sulfated glycolipids were conducted with triglucosyl glyceroglucolipid sulfate, galactosylceramide sulfate, and lactosylceramide sulfate. The individual glycolipids (50—80 μM) were suspended in 0.2M acetate-100mM CaCl_2 buffer, pH 4.0—7.2, and incubated at 37°C for various periods of time with the *H. pylori* enzyme protein (10—50 μg) with and without sodium cholate (0.1mg/0.1ml) as an emulsifying agent. The reaction was terminated by addition of 5 volumes of chloroform-methanol (2:1, v/v), and the mixture was vortexed and centrifuged. The glycolipids contained the organic phase were

then applied to a DEAE-Sephadex column (18). The column was eluted with methanolchloroform-water (60:30:8, by vol.) for neutral glycolipids, and for sulfated glycolipids with 0.4M sodium acetate in methanol-chloroform-water (60:30:8, by vol.). The enzyme activity was assessed by quantitating the neutral glycolipid carbohydrate content by gas-liquid chromatography (8,15).

Specificity of H. pylori glycosulfatase

The glycosulfatase specificity with respect to sulfated sugar units occurring in gastric mucin was assessed using oligosaccharides containing N-acetylglucosamine-6-sulfate and galactose-6-sulfate. The oligosaccharide containing GlcNAc-6-sulfate (SO_3H , 6GlcNAc β 1, 3Gal β 1, 3GalNAc-ol) was obtained from hog gastric mucin (15), and Gal-6-sulfate containing oligosaccharide (SO_3H , 6Gal β 1, 4 GlcNAc β 1, 6(Fuc α 1, 2Gal β 1, 4GlcNAc β 1, 3Gal β 1,3)GalNAc-ol) was isolated from salivary mucin (18). Following incubation with the *H. pylori* enzyme under the conditions described above, the reaction mixtures were applied to small columns ($0.7 \times 5\text{cm}$) of AG1-X2(Cl^-) and the resulting neutral oligosaccharides were eluted with water, while the sulfated compounds were recovered by elution with 0.5M NaCl (15). The enzyme activity was measured by quantitating the neutral sugar content in the water eluates by gas-liquid chromatography (8,15).

Antiulcer drug

Nitecapone, 3-(3,4-dihydroxy-5-benzylidene)2,4-pentanedione, Lot No. 260, was donated by Dr. E. Nissinen, Orion-Farmos Pharmaceuticals, Espoo, Finland. The drug was stored at 4°C in the dark and was suspended in 0.2M acetate buffer, pH 5.7, shortly before each experiment.

Analytical methods

The protein content of samples was measured by the method of Lowry et al. (19), and the sulfate was assayed turbidimetrically (20). The content and composition of carbohydrates were determined by gas-liquid chromatography following methanolysis, re-N-acetylation and derivatization with silylating reagent (15). Gel electrophoresis in 1% SDS was performed with 10% polyacrylamide gels, and the protein bands visualized with silver stain. All experiments were carried out in duplicate and the results are expressed as means \pm SD.

RESULTS

Helicobacter pylori, a pathogen in gastric disease, was found to display glycosulfatase activity towards human gastric mucus glycoprotein. The activity was identified in the extracellular material elaborated by this bacteria employing ^{35}S -labeled glycoprotein prepared from gastric sulfomucin. The chemical composition of the intact and sulfated mucin preparations is given in *Table 1*. In comparison to intact mucin, the sulfated mucus glycoprotein was found to be 4-fold enriched in sulfate and contained 14% more sialic acid, but did not differ with respect to other constituents. Following desulfation, the glycoprotein was subjected to enzymatic sulfation in the presence of [^{35}S] PAPS and such ^{35}S -labeled glycoprotein was used as substrate.

Table 1. Chemical composition of the intact and sulfated human gastric mucus glycoprotein

Component (mg/100 mg)	Mucus glycoprotein	
	Intact	Sulfated
Fucose	14.9	14.3
Galactose	25.6	25.0
N-Acetylgalactosamine	12.7	12.5
N-Acetylglucosamine	22.8	22.7
Sialic acid	5.7	6.5
Sulfate	1.3	5.1
Protein	14.8	14.6

Values represent the means of triplicate analyses.

Table 2. *H. pylori* glycosulfatase activity towards sulfated gastric mucus glycoprotein

Type of preparation	Glycosulfatase activity (pmol/mg protein/60 min)
Extracellular filtrate	17.2 ± 3.1
Acetone fraction	
45% precipitate	18.2 ± 1.9
63% precipitate	314.8 ± 36.5
85% precipitate	20.6 ± 2.7

Values represent the means ±SD of six experiments performed in duplicate.

Fractionation of the *H. pylori* extracellular material containing glycosulfatase activity employing low temperature acetone precipitation procedure (16) resulted in the enrichment of enzyme activity in the protein fraction precipitated at 63% acetone concentration (*Table 2*). The glycosulfatase activity of this fraction towards sulfated human gastric mucin was found to be at least 18-fold higher than that of the original extracellular filtrate. The enzyme-enriched 63% acetone fraction, when subjected to SDS-PAGE, gave on silver staining a major protein band of 30kDa. The glycosulfatase displayed optimum activity towards the sulfated mucus glycoprotein at pH 5.7 in the presence of 0.3% Triton X-100 and 100mM CaCl₂ (*Table 3*). Under the assay conditions, the extent of sulfate ester group removal from sulfomucin increased with increasing amounts of enzyme protein up to 40µg, and remained constant with time of incubation for at least 3h. Introduction of nitecapone to the incubation mixture led to a reduction in the rate of mucin desulfation. The rate of inhibition of *H. pylori* glycosulfatase activity was proportional to nitecapone concentration up to 100µg/ml at which point a 61% decrease in mucus glycoprotein desulfation occurred (*Fig. 1*).

Table 3. Requirements for *H. pylori* glycosulfatase activity towards sulfated gastric mucus glycoprotein

Assay mixture	specific activity (%)
Complete (optimal pH 5.7)	100
Minus Triton X-100 (0.3%)	91.4 ± 8.6
Minus CaCl ₂ (100 mM)	87.3 ± 7.5
Complete at pH 4.5	72.6 ± 7.1
Complete at pH 7.0	63.8 ± 5.4
Complete at pH 8.0	61.2 ± 5.5

The assays were conducted with purified glycosulfatase preparation (63% acetone precipitate). Values represent the means ± SD of five separate experiments performed in duplicate.

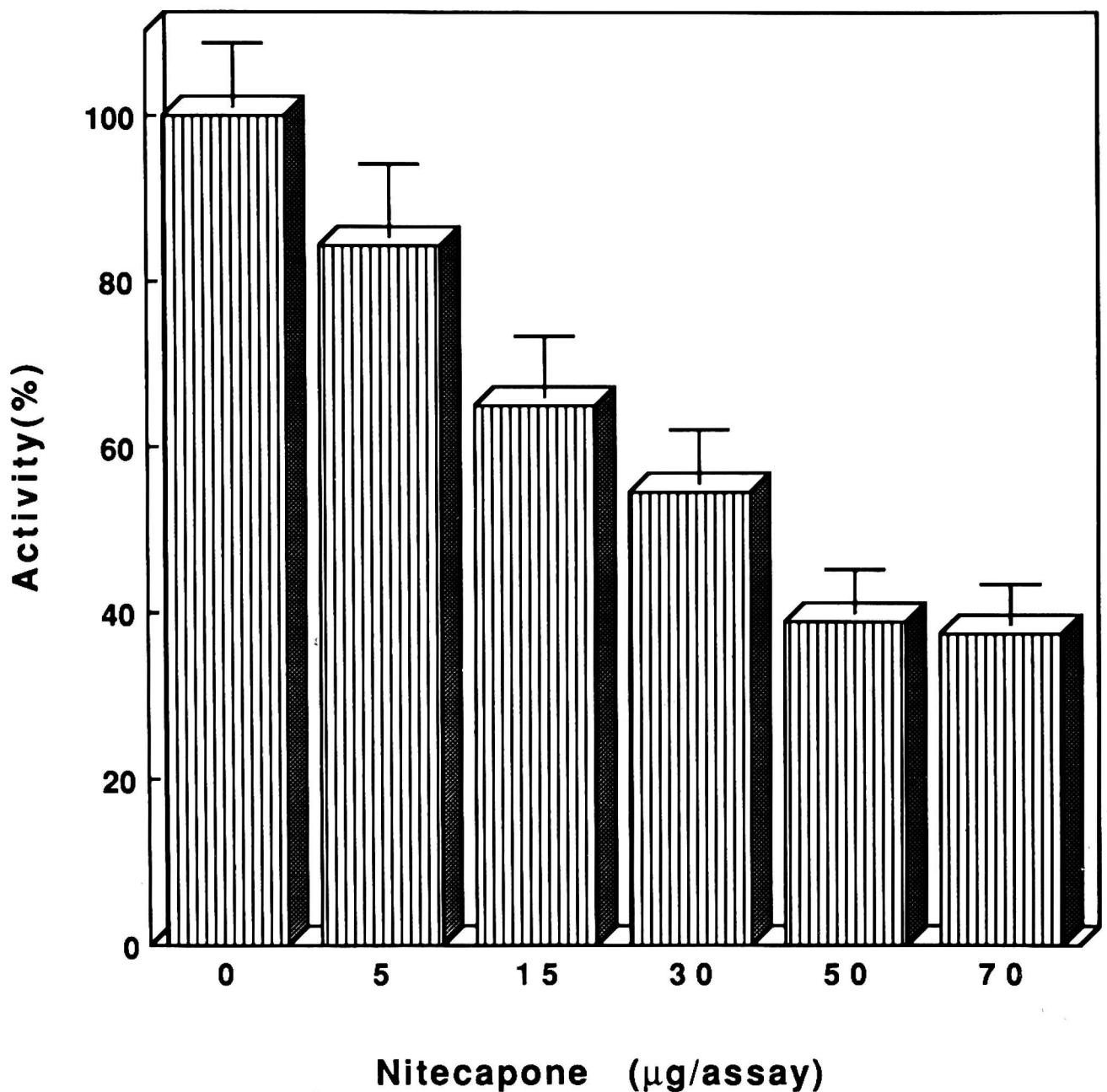


Fig. 1. Effect of nitecapone on the *H. pylori* glycosulfatase activity towards human gastric sulfated mucin. The data show the means ± SD of seven separate experiments performed in duplicate.

Table 4. Substrate specificity of *H. pylori* glycosulfatase

Substrate	Concentration (μg)	Specific activity (pmol/mg protein/60 min)
Sulfated mucus glycoprotein	100	310.5 ± 34.7
Oligosaccharide containing N-acetylglucosamine-6-sulfate	20	142.1 ± 16.5
Oligosaccharide containing galactose-6-sulfate	20	113.8 ± 15.9
Triglucosylglycerolipid sulfate	45	122.5 ± 13.4
Galactosylceramide sulfate	65	2.6 ± 0.8
Lactosylceramide sulfate	40	2.5 ± 0.9

The assays were conducted with purified (63% acetone precipitate) glycosulfatase preparation. Each value represents the mean \pm SD of four experiments performed in duplicate.

The substrate specificity of *H. pylori* glycosulfatase is presented in Table 4. The results obtained with sulfated oligosaccharides derived from mucus glycoproteins revealed that the enzyme effectively caused removal of sulfate ester group situated at C-6 of N-acetylglucosamine, as well as that at C-6 of galactose. The *H. pylori* glycosulfatase also caused the desulfation of sulfated glyceroglucolipid of gastric mucus which contains the sulfate ester group at C-6 of terminal glucose residue. However, the glycosulfatase was ineffective towards sulfated glycosphingolipids of gastric epithelium which contain the sulfate ester group on C-3 of galactose. This suggests that *H. pylori* glycosulfatase, while effective towards sulfated mucin and glyceroglucolipids of gastric mucus, is not capable of gastric epithelial sulfated glycosphingolipids degradation.

DISCUSSION

Among the aggressive factors implicated in the etiology of gastric disease is infection with *H. pylori* (12, 13, 21). Studies indicate that the bacteria gains attachment to gastric epithelium through the cell surface sulfated glycosphingolipid receptors, and hence is capable of exerting its pathogenic action on both mucus layer and the mucosal epithelial integrity (22). The integrity of the protective mucus layer is undermined through the elaboration of the extracellular protease and lipase enzymes capable of rapid degradation of its protein, glycoprotein and lipid components (22), while the gastric epithelial defense perimeter is being weakened by disruption of the interaction between the mucosal cells and the components of extracellular matrix through *H. pylori* surface membrane lipopolysaccharide (23). Evidence has also been obtained

that sulfated mucus glycoproteins and glyceroglucolipids interfere with *H. pylori* mucosal attachment, and that the resistance to this bacteria depends upon the gastric mucosal content of sulfated glycoproteins and glycolipids (2, 14).

The results presented herein demonstrate that *H. pylori* is capable of overcoming the aggregating activity of sulfated gastric mucin and glyceroglucolipids, as the extracellular material elaborated by this bacteria displays glycosulfatase activity towards these mucus constituents. The enzyme was found to exhibit maximum activity at pH 5.7 in the presence of Triton X-100 and CaCl_2 , and has an apparent molecular weight of 30kDa. The data on the *H. pylori* glycosulfatase specificity showed that the enzyme was capable of removal of the sulfate ester groups from oligosaccharides containing N-acetylglucosamine-6-sulfate as well as that with galactose-6-sulfate. Both these sulfated sugars are known to be present in the carbohydrate chains of gastric mucus glycoproteins (3, 5, 24). The glycosulfatase activity towards gastric sulfomucin was inhibited by nitecapone, a non-antisecretory antiulcer agent (25). This peripherally acting catechol-O-methyltransferase inhibitor, known for its ability to interfere with *H. pylori* proteolytic and lipolytic activities (2), also proved to be a potent inhibitor of glycosulfatase enzyme elaborated by *H. pylori*. This inhibitory activity of nitecapone was found to be dose dependent and effective well below the therapeutic doses (30—100mg/kg) used in ulcer treatment (26). Maximum inhibition (61%) of *H. pylori* glycosulfatase activity towards human gastric sulfomucin was achieved at nitecapone dose of 100 $\mu\text{g}/\text{ml}$. Our findings attest further to the value of nitecapone in the treatment of gastric disease associated with *H. pylori* infection. This property of nitecapone is of particular importance, since a decrease in gastric sulfomucin levels has for long been suggested to signal the onset of peptic ulcer (3, 5), and the resistance to *H. pylori* colonization appears to be linked to sulfomucin aggregating activity (23).

The *H. pylori* glycosulfatase was also effective in the desulfation of sulfated glyceroglucolipid, a constituent of gastric mucus which contains the sulfate ester group on the C-6 position of terminal glucose residue (4), but was ineffective towards galactosylceramide and lactosylceramide sulfates which contain the sulfate ester group on C-3 of galactose (8). These latter two glycosphingolipids are the major sulfated glycosphingolipid species of the cell membranes of gastric epithelium (4), and, as found recently, mediate the *H. pylori* attachment to gastric mucosa (11). Thus, *H. pylori* glycosulfatase because of its specificity, while not interfering with the bacterial attachment, is able to overcome the aggregating action of sulfomucin component of the protective mucus layer.

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