Gastric and salivary mucins inhibit angiotensin-converting enzyme

Inhibition is partly due to oligosaccharides

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Pig gastric mucin, a highly glycosylated glycoprotein, inhibits angiotensin-converting enzyme (ACE) with an IC_{50} of 2 mM-neutral hexose content. Pig submaxillary mucin at 2.3 mM inhibits by 73%. To determine whether the oligosaccharide moieties of the mucins contribute to this inhibition, oligosaccharides were prepared from each mucin by reductive β -elimination and their effects on enzyme activity determined. Total oligosaccharides from gastric mucin inhibited enzyme activity with an IC_{50} of 0.3 mM based on the neutral hexose content of the oligosaccharide solution. Fractions isolated from gastric mucin by chromatography on DEAE-cellulose and Bio-Gel P-2 inhibited ACE with IC_{50} values ranging from 2 to 16 mM-oligosaccharide. Larger oligosaccharides inhibited with lower IC_{50} values than did smaller oligosaccharides. Fractions of average molecular mass 1100 and 740 Da prepared from submaxillary mucin inhibited with IC_{50} values of 40 and 80 mM-oligosaccharide respectively. Monosaccharides commonly present in serum and membrane glycoproteins were also tested for their effect on ACE. Galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and glucosamine were inhibitory. *N*-Acetylneuraminic acid stimulated the activity of ACE. Fucose, ethylene glycol and sucrose had no effect on the activity of the enzyme. The influences of different buffers, ion concentrations, pH and substrate structure on the effect of carbohydrate on enzyme activity were also evaluated. The extent of inhibition by the monosaccharide galactose was strongly influenced by buffer ion and substrate concentration. The effects of the oligosaccharide moieties and intact mucins were less sensitive to assay conditions.

INTRODUCTION

Glycosylation of proteins is a post-translational modification, the functions of which are only partly understood. It is well known that covalently bound oligosaccharides influence such physicochemical parameters of a glycoprotein as thermal stability and solubility [1]. Differential glycosylation can also create a pool of different glycoforms of a protein, each with a unique spectrum of biological activities [2]. Recently it has been shown that differential *N*-glycosylation of the same sites in tissue plasminogen activator modifies the fibrinolytic activity of this serine proteinase [3,4], demonstrating that endogenous carbohydrates on a proteinase can influence the activity of the enzyme.

We have reported that angiotensin-converting enzyme (ACE; EC 3.4.15.1), a zinc proteinase, is inhibited by the soluble lectin *Ricinus communis* and binds to lectin that has been immobilized to agarose [5]. When the enzyme is eluted from the column with galactose, total recovery of the activity requires removal of the galactose from the solution. These data led us to consider the possibility that carbohydrate, either supplied by its own side chains or exogenously, may participate in regulating the activity of this enzyme.

ACE is a dipeptidyl carboxypeptidase that catalyses the hydrolytic release of dipeptides from the C-terminus of oligopeptide substrates [6,7]. The enzyme has a broad specificity. Its best-known physiological roles are the formation of angiotensin II, a potent vasopressor, and the inactivation of the vasodilator bradykinin. ACE is a glycoprotein that has been reported to contain as much as 25% carbohydrate by weight [8,9]. It is associated with the endothelium, the inner lining of the vascular system [10–12]. It is also found on macrophages [13], kidney

epithelial cells [14,15], the mucosal brush border of the small intestine [16] and, in soluble form, in the plasma [17]. It may have more functions than those documented [18].

In vitro, the activity of the enzyme is sensitive to a wide range of substances such as chloride, peptides, carboxylates, chelators and phosphates [19]. Therefore it is important to study the influence of different components of the enzyme's native environment on enzyme activity to gain insight into the mode(s) of its physiological regulation, as Shapiro *et al.* [20] and Bunning & Riordan [21] have emphasized in their detailed studies of the interaction of the enzyme with chloride and different substrates.

Gastric and submaxillary mucins provide a convenient source of oligosaccharide groups that are similar in structure to those found in serum and membrane glycoproteins. Here we present evidence that pig gastric and salivary mucins, as well as oligosaccharides prepared from them and monosaccharides found in them, influence the activity of ACE.

EXPERIMENTAL

Materials

Pig kidneys, lungs and submaxillary glands were obtained from a local slaughterhouse. Pig gastric mucin (crude type) and hippurylhistidyl-leucine (Hip-His-Leu) were purchased from Sigma (St. Louis, MO, U.S.A.). Furan-acryloylphenylalanyl-glycylglycine (FA-Phe-Gly-Gly) was obtained from Bachem (Philadelphia, PA, U.S.A.). Other commercial products used for the mucin and oligosaccharide purification include Dowex 50 (H^+ form) (Sigma), cetyltrimethylammonium bromide (Serva, Westbury, NJ, U.S.A.), hydroxyapatite and Bio-Gel P-2 (Bio-Rad, Rockville Centre, NY, U.S.A.), DEAE-cellulose (Whatman, Hillsboro, OR, U.S.A.) and DEAE-Sephadex, Sephadex

Abbreviations used: ACE, angiotensin-converting enzyme; PAS, periodate-Schiff's reagent; FA-Phe-Gly-Gly, furan-acryloylphenylalanyl-glycylglycine.

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G-150, Sephadex G-200, CM Sephadex C-25 and CM Sephadex C-50 (Pharmacia, Piscataway, NJ, U.S.A.). Monosaccharide standards were obtained from Sigma. Dansylhydrazine was purchased from Fluka (Ronkonkoma, NY, U.S.A.). All other chemicals were reagent grade.

Enzyme preparation

All steps of both purification schemes were carried out at 4 °C. From kidney, ACE was purified by affinity chromatography as described by Pantoliano *et al.* [22]. For the final purification, a Sepharose CL-6B column (10 cm × 1 cm) with *N*-carboxy-5-aminopentyl-Ala-Gly as ligand bound by a spacer 2.8 nm in length [23] was used. This ligand binds ACE specifically at pH 6.0, and, by raising the pH to 10.0, the enzyme can be eluted. The column was equilibrated with 20 mM-Mes (pH 6.0)/0.5 M-NaCl/0.1 mM-ZnCl₂/0.2% Nonidet P40. The sample was applied by circulating it over the resin for 6–8 h. The column was washed with the same buffer without Nonidet P40 for 8 h. The bound ACE was eluted with 200 mM-borate, pH 10.0. Enzymically active fractions were pooled and the pH was titrated to 7.5 with 1 M-acetic acid. The enzyme solution was concentrated in an Amicon cell (XM-50 membrane) and stored with 0.02% NaN₃ at –80 °C. This preparation yielded enzyme that gave a single band of approximate molecular mass 160 kDa on SDS/PAGE under reducing conditions [24]. The specific activity was 33 units/mg, measured with FA-Phe-Gly-Gly [25]. The yield was 7%, based on the detergent extract as 100%.

Some experiments were carried out with enzyme that had been purified from lung by a modification of the procedure of Oshima *et al.* [26]. Tissue (200 g) was homogenized for 4 min in a Waring blender with 400 ml of 5 mM-Tris/HCl, pH 7.4, containing 0.25 M-sucrose, 0.1 mM-CoCl₂ and 0.2% Triton X-100. The crude homogenate was centrifuged in a Sorvall GSA rotor at 5000 *g* for 30 min. The resulting supernatant (500 ml) was centrifuged in a Beckman L2-65B centrifuge, Ti 30 rotor, at 50 000 *g* for 2 h. The protein that precipitated from the resulting supernatant between 40 and 70% saturation with (NH₄)₂SO₄ was resuspended in a final volume of 260 ml of 5 mM-Tris/HCl (pH 7.4)/0.1 mM-CoCl₂ and dialysed against several changes of the same buffer. The enzyme solution was then applied to a column (5 cm × 15 cm) of DEAE-Sephadex A-50 that had been equilibrated with the same buffer. The column was eluted stepwise with 500 ml of 5 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl/0.1 mM-CoCl₂, followed by 500 ml of 5 mM-Tris/HCl (pH 7.4)/0.3 M-NaCl/0.1 mM-CoCl₂. The fractions containing activity were concentrated on an Amicon PM 30 filter to 30 ml and applied an hydroxyapatite column (1.5 cm × 6 cm) that had been equilibrated with 1 mM-sodium phosphate (pH 7.4)/1 mM-CoCl₂. The enzyme was eluted with 400 ml of a phosphate gradient at pH 6.8, going from 1 mM- to 50 mM-sodium phosphate. Fractions containing activity (250 ml) were concentrated with the Amicon PM 30 filter to 2 ml and applied to a Sephadex G-150 column (2.5 cm × 80 cm) that was equilibrated with 50 mM-Tris/HCl (pH 7.4)/0.1 mM-CoCl₂/200 mM-NaCl. The active fractions from this column were then concentrated to 2 ml and rechromatographed on a Sephadex G-200 column (2.5 cm × 100 cm) that was equilibrated with 50 mM-Tris/HCl (pH 7.4)/0.1 M-CoCl₂/200 mM-NaCl. The enzyme eluted from this column gave a single band on SDS/PAGE of approximate molecular mass 160 kDa, with a specific activity of 47 units/mg, measured with 2.5 mM-Hip-His-Leu [27], with a yield of 7% from the 50 000 *g* supernatant. The enzyme was stored in small portions at a concentration of 50 µg/ml and thawed and diluted just before assay. The stock enzyme solution was not refrozen; under these conditions activity stored at –80 °C is stable for at least a year.

Measurement of enzyme activity

Enzyme assays were carried out either with Hip-His-Leu as the substrate, followed by formation of the fluorescent *o*-phthaldialdehyde adduct of the product, His-Leu [27], or spectrophotometrically with FA-Phe-Gly-Gly as substrate [25]. The concentration of Hip-His-Leu was 2.5 mM and that of FA-Phe-Gly-Gly 0.5 mM, unless otherwise specified. For the Hip-His-Leu assays the buffer was 0.2 M-borate (pH 8.3)/0.1 M-NaCl, unless otherwise specified. For the FA-Phe-Gly-Gly assay the standard buffer was 50 mM-Hepes (pH 7.5)/0.3 M-NaCl, unless otherwise specified. Mucin, oligosaccharides or monosaccharides were dissolved in 1.5-times concentrated buffer, the pH was adjusted as necessary, and the mixture diluted to its final concentration in the assay. Enzyme was added to the pH-adjusted solution and incubated for 30 min at 25 °C before addition of the substrate to initiate the reaction. The timing of the preincubation was precise, and activity was measured with sampling of at least three time-points to show that product formation remained proportional to time. Especially in the Hip-His-Leu assay, care was needed on this point, because the enzyme is slightly unstable at the dilution at which it was measured and at the assay temperature of 37 °C. The addition of carbohydrate stabilized the enzyme against denaturation with temperature.

Preparation of oligosaccharides from pig gastric mucin

Pig gastrin mucin as supplied by Sigma was designated step I. To prepare oligosaccharides, 2 g of gastric mucin was dissolved in 100 ml of 50 mM-NaOH. Then 100 ml of 1 M-NaBH₄ in 50 mM-NaOH was added and the mixture incubated for 17 h at 52 °C [28]. The mixture was then titrated to pH 5.0 with 5 M-acetic acid. A Dowex 50 column (50 cm × 3 cm) was prepared and equilibrated with deionized water. The sample (400 ml) was applied with a pressure of 150 cmH₂O. The column was eluted with 1500 ml of deionized water, and fractions of pH less than 4 were collected. The pH of the pooled eluate was titrated to 7.0 with NaOH. The volume of the eluate was decreased in a rotary evaporator (Hoefer). The residue was dissolved in dry methanol and the solvent evaporated together with methyl borate. This procedure was performed five times to remove borate. The final residue was dissolved in the smallest amount of deionized water possible. Oligosaccharides were freeze-dried and stored in a desiccator at 4 °C (step II).

To prepare subfractions of known size, a Bio-gel P-2 column (225 cm × 0.5 cm) was prepared and calibrated with Blue Dextran, Bromophenol Blue, glucose oligomers and NaCl. Then 0.5 ml of the not-yet-freeze-dried oligosaccharides was applied at a flow rate of 2 ml/h and the column eluted with degassed deionized water. Fractions of volume 0.5 ml were collected and fractions that contained more than 0.5 mg of neutral hexose/ml [29] and had a conductivity in the µS range were pooled. The material from fractions 5–30 was pooled and freeze-dried (step III).

Preparation of neutral oligosaccharides

A DEAE-cellulose column (50 cm × 1 cm) was equilibrated with 0.5 mM-potassium phosphate buffer, pH 7.85. Oligosaccharides (step II; 0.5 g) were dissolved in the same buffer. The sample was applied and eluted with the same buffer. Fractions of volume 1.5 ml were collected and those containing more than 0.5 mg of neutral hexose were pooled, concentrated and separated according to size as described above (step IV).

Preparation of pig submaxillary mucin

The purification followed the method of DeSalegui & Plonska [30]. In the final step, the mucin was precipitated from the

supernatant with 40, 48 and 54% ethanol. For this procedure, a mixture of 94% ethanol, 4% acetone and 1% methyl ethyl ketone was used. After addition of the alcohol, the samples were left for 48 h at 4 °C. Then the mixture was centrifuged at 38 000 g for 30 min in a Sorvall SS34 centrifuge at 4 °C. The supernatants were collected for the next precipitation and the precipitates were dissolved in deionized water and dialysed against it. Finally, the mucin was stored at -80 °C until used.

Preparation of oligosaccharides from pig submaxillary mucin

The dialysed mucin of the submaxillary glands was adjusted to 50 mM-KOH/0.5 M-NaBH₄, and incubated for 20 h at 43 °C. Then the pH was titrated to 4.5 with conc. acetic acid. The sample was applied to a Dowex 50 column (75 cm × 2.5 cm) and eluted with deionized water. Fractions of pH lower than 7.0 were collected and the pH was adjusted to 7.0. Pooled fractions were concentrated in a rotary evaporator. Borate was removed as described above. The residue was dissolved in deionized water and oligosaccharides were separated according to size as described above.

Determination of monosaccharide content of mucin oligosaccharides

Sialic acid was determined spectrophotometrically without hydrolysis by the method of Jourdan *et al.* [31]. To determine the other monosaccharides, 1 mg of oligosaccharides was dissolved in 0.2 ml of 2 M-trifluoroacetic acid. Hydrolysis was carried out under vacuum at 100 °C for 90 min. The acid was removed by drying in vacuum over solid KOH. The residue was dissolved in deionized water and dried in a Speed Vac concentrator (Savant, Hicksville, NY, U.S.A.). This procedure was done twice more with methanol. The monosaccharide composition of the hydrolysate was determined after reaction with dansylhydrazine [32], by silica-gel t.l.c. (Kieselgel 60, F254, Merck). The monosaccharides galactose, glucose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, galactosamine, glucosamine and *N*-acetylneuraminic acid were treated in the same way and spotted as standards. For the separation, the solvent system chloroform/ethyl acetate/1% borate (3:5:2, by vol.) in methanol was used. The spots were marked under long-wave u.v. light and scraped into 1 ml of 1% acetic acid in methanol. The eluted fluorescence was measured at 510 nm at an excitation wavelength of 360 nm. The monosaccharides in the hydrolysate were quantified by comparison with the standards.

The concentrations of amino sugars and galactosaminitol were determined on a Kontron Liquimat III amino acid analyser equipped with a 30 cm Dionex Durum DC 6A column. The Kontron standard programme for physiological solutions was used. The amino sugars, galactosaminitol and amino acids were determined as ninhydrin adducts.

Determination of amino acid content

Samples of 0.01, 0.05 or 0.1 mg of mucin oligosaccharides were hydrolysed in sealed capillaries in 6 M-HCl, under vacuum, at 100 °C for 20 h. After removal of the acid, the samples were applied to a Biotronic amino acid analyser LC 6000 E. The amino acids were determined as fluorescent *o*-phthalaldehyde adducts. Proline was determined with the Kontron Liquimat amino acid analyser with the same column and programme as described for the amino sugars. The limit of sensitivity was 100 nM.

Other determinations

Protein concentrations were determined by the methods of Lowry *et al.* [33] and Bradford [34]. The mucin preparations were tested for residual peptides by mixing a portion 1:1 with 0.5 mg

of fluorescamine/ml of acetone and measuring the fluorescence. His-Leu was used as a peptide standard. Neutral-hexose content was determined by the phenol/H₂SO₄ assay of Dubois *et al.* [29] with galactose as standard.

Gel electrophoresis

Proteins and mucins were subjected to electrophoresis on polyacrylamide by the methods of Laemmli [24] or Weber & Osborn [35]. The Laemmli method was used when the gels were to be stained with Coomassie Blue for detection of proteins, and the Weber & Osborn method when the gels were to be stained with periodate-Schiff's reagent (PAS) [36] for detection of carbohydrates.

RESULTS

Pig gastric mucin, as obtained from Sigma (step I), inhibits ACE activity with an IC₅₀ of 2 mM-neutral hexose (Fig. 1). (For convenience in comparing preparations of unknown or mixed molecular mass, the mucin concentrations throughout this report are expressed by giving the amount of neutral hexose contained in the solution [29].) The same IC₅₀ was found with both 0.8 mM- and 0.1 mM-NaCl in the assay buffer. Concentrations higher than 6 mM neutral hexose could not be tested because at these concentrations the mucin formed a gel with the buffer. To determine whether the mucin preparation contained proteins other than mucin, the preparation was examined by SDS/PAGE and stained with Coomassie Brilliant Blue or PAS (Figs. 2a and 2b). The mucin molecules stayed in the upper gel, and no smaller proteins in the molecular-mass range of 30–200 kDa were detected in the lower gel. The mucin was also dialysed against borate buffer containing EDTA to free it from adventitious metal ions and peptides of molecular mass less than 3500 Da (step I, dialysed). The dialysed mucin inhibited with an IC₅₀ of 3 mM neutral hexose (result not shown).

In the following experiments we tested whether inhibition of the enzyme by mucin is due, at least in part, to oligosaccharides. *O*-Linked oligosaccharides were separated from the peptide backbone by reductive β -elimination. Cationic species were removed from the preparation by treating it with Dowex 50 (H⁺

Fig. 1. Inhibition of ACE activity by pig gastric mucin

Enzyme activity was measured with Hip-His-Leu as substrate in 0.2 M-borate buffer, pH 8.3, containing 0.8 M-NaCl (○, □) or 0.1 M-NaCl (●). The mucin concentration is expressed as its neutral hexose content. ○, ●, Mucin as purchased; □, oligosaccharide prepared by β -elimination, after removal of cationic species with Dowex 50. Each value is the average of two separate experiments done in duplicate. Single results differ in all cases by less than 15% from the mean.

Fig. 2. SDS/PAGE of pig mucin

(a) and (b) Pig gastic mucin: lane 1, molecular-mass markers; lane 2, pig gastic mucin (30 μ g of protein). (c) and (d) Samples from the purification of pig submaxillary mucin: lane 1, molecular-mass markers; lane 2, extract (28 μ g of protein); lane 3, after CM-Sephadex C50 adsorption (27 μ g of protein); lane 4, 40%-ethanol precipitation (20 μ g of protein); lane 5, 48%-ethanol precipitation (16 μ g of protein); lane 6, 54%-ethanol precipitation (18 μ g of protein). Molecular-mass markers: myoglobin (205 kDa), β -galactosidase (116 kDa), phosphorylase (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), α_2 -macroglobulin (179 kDa), α_1 -proteinase inhibitor (51 kDa). (a) and (c) SDS/PAGE (7.5% gel) [24] stained with Coomassie Blue for protein. (b) and (d) SDS/PAGE (5% gel) [35] stained with PAS for carbohydrate.

Table 1. Inhibition of ACE by derivatives of pig gastric mucin

For steps I and II, IC_{50} is reported as neutral hexose because the preparation consists of a mixture of different molecules. For steps III and IV, IC_{50} is reported as oligosaccharides [42]. Each value is the average of two separate experiments done in duplicate. Single results differ in all cases by less than 15% from the mean.

Preparation	IC_{50} (mM)
Step I, whole mucin	2
Step I, dialysed	3
Step II, mixed oligosaccharides after Dowex 50	0.3
Step III, 1600–3500 Da oligosaccharides	2.7–4.3
Step IV, after DEAE-cellulose	
(a) 2000–3500 Da	8.5
(b) 1600 Da	16

Table 2. IC_{50} for step-III oligosaccharides, measured under different substrate and buffer conditions

Each value is the average of two separate experiments done in duplicate. Single results differ in all cases by less than 15% from the mean.

Buffer	Substrate	IC_{50} (mM)
50 mM-Hepes (pH 7.5)/0.3 M-NaCl	FA-Phe-Gly-Gly	4.3
50 mM-Hepps (pH 8.3)/0.1 M-NaCl	FA-Phe-Gly-Gly	2.7
0.2 M-Borate (pH 8.3)/0.1 M-NaCl	FA-Phe-Gly-Gly	2.8
50 mM-Hepps (pH 8.3)/0.1 M-NaCl	Hip-His-Leu	3.1
0.2 M-Borate (pH 8.3)/0.1 M-NaCl	Hip-His-Leu	2.9

Table 3. Monosaccharides and amino acids found after hydrolysis of the 1600–3500 Da oligosaccharides of step III

Component	Content (nmol/mg dry wt.)
Monosaccharides	
Glucosamine	405
Galactosamine	182
Galactose	442
Fucose	90
Amino acids	
Aspartate	14.2
Threonine	36.7
Serine	14.2
Glutamate	20.7
Glycine	37.2
Isoleucine	40.7
Valine	19.5
Leucine	22.8
Tyrosine	18.8
Phenylalanine	18.3
Lysine	4.2
Histidine	1.4
Arginine	10.6

form) (step II). Separation of the oligosaccharides from the peptide backbone increased their inhibitory effectiveness on a per-mol-of-neutral-hexose basis (Fig. 1). The IC_{50} of the separated oligosaccharides was 0.3 mM neutral hexose.

The oligosaccharide preparation at step II was further characterized by using a Bio-Gel P-2 size-exclusion column. The combined material in the peaks containing oligosaccharides of molecular mass 2000–3500 and 1600 Da (step III) was tested. The material of step II was also subjected to chromatography on

DEAE-cellulose to remove anionic species (step IV), followed by Bio-Gel P-2 chromatography. Both the void-volume material, molecular mass 2000–3500 Da (step IVa), and the material of molecular mass 1600 Da (step IVb), were tested for their effect on ACE. The stated upper limit of the molecular mass of the material in steps III and IVa is based on the observation that all of this material passes through a dialysis membrane of molecular-mass cut-off 3500 Da. Table 1 shows the IC_{50} values of the mucin and the oligosaccharides of steps II–IVb.

The activity of ACE is known to be affected by several buffer ions. The effects of borate, Hepps and Hepes, as well as the substrates Hip-His-Leu and FA-Phe-Gly-Gly, were compared with respect to their effects on inhibition of ACE by oligosaccharides. The measurements shown in Fig. 1 were made in borate buffer. In Hepps the same IC_{50} values were found. The IC_{50} of the material at step III was measured in the three different buffers and at two different H^+ concentrations, and Hip-His-Leu or FA-Phe-Gly-Gly as substrate. The oligosaccharide preparation was equally effective as an inhibitor under all these conditions (Table 2).

The preparations from steps II–IV were free of protein detectable by the Lowry and Bradford methods. Step-II and -III material was also found to be free of primary amino groups detectable by fluorescamine. However, when the material from step III was subjected to amino acid analysis after hydrolysis in 6 N-HCl, it was found to contain amino acids. Table 3 shows the amino acid composition of the material from step III. The average molecular size of the material that was used for analysis and for enzyme inhibition was approx. 2000 Da. If one assumes an average molecular mass for each peptide residue of 105 Da (based on the amino acid composition found), the step-III material contained approx. 0.03 mol of peptide/mol of neutral hexose. Neither fraction contained measurable amounts of proline. The threshold of the procedure used for the detection of proline was 20 pmol/mg dry wt.

The monosaccharide composition observed after acid hydrolysis of the material in step III is also shown in Table 3. The content of galactosamine is underestimated by the number of residues that serve to attach the oligosaccharide to the protein, because these residues are converted into the corresponding sugar alcohol during β -elimination and no separate analysis of galactosaminitol was made for these samples. The composition is reported in terms of the amounts of galactosamine and glucosamine found after the hydrolysis procedure; presumably these sugars are principally *N*-acetylated in step III.

Pig submaxillary mucin, which has a different monosaccharide composition from stomach mucin, was prepared from pig submaxillary glands, and the whole mucin and the oligosaccharides prepared from it were examined for their effect on ACE activity. Figs 2(c) and (d) (lanes 3–6) show that after the CM-Sephadex adsorption steps the preparation of whole mucin was free of protein contaminants of the molecular-mass range 30–200 kDa. Although all the ethanol precipitates were alike in this respect, the fraction precipitated between 48 and 54% ethanol had the highest carbohydrate/protein ratio (Table 4). This mucin was tested for inhibition of ACE activity. The highest mucin concentration that could be tested contained 2.3 mM neutral hexose (higher mucin concentrations formed gels). In either borate or Hepps buffer, submaxillary mucin at 2.3 mM neutral hexose led to 73% inhibition of activity.

Oligosaccharides were prepared from this mucin and separated into three fractions of average molecular masses 1700, 1100 and 740 Da by gel-filtration chromatography on Bio-Gel P-2. The 1700 Da oligosaccharide activated the enzyme slightly but reproducibly. The amount of material obtained in this fraction did not permit further characterization. Inhibition of activity by the

Table 4. Purification of mucin from pig submaxillary glands

Neutral hexose was measured by the phenol/H₂SO₄ assay [29] with galactose as standard. Protein was measured by the Bradford assay [34] with BSA as standard

Purification step	Neutral hexose (mg/mg of protein)
Extract	0.70
After Sephadex CM50	2.57
Precipitation with 40 % ethanol	2.15
Precipitation with 48 % ethanol	2.15
Precipitation with 54 % ethanol	5.40

Fig. 3. Inhibition of ACE by oligosaccharides from pig submaxillary mucin

△, Fraction of molecular mass 1100 Da; ○, 740 Da. Each value is the average of two separate experiments done in duplicate. Single results differ in all cases by less than 15 % from the mean.

Table 5. Monosaccharides and amino acids found after hydrolysis of the 1100 Da oligosaccharides (step II) and the 740 Da oligosaccharides (step III) of pig submaxillary mucin

Sialic acid was determined as described by Jourdan *et al.* [31]. Galactosaminitol was determined on the amino acid analyser.

Component	Content (nmol/mg content dry wt.)	
	Step II	Step III
Monosaccharides		
Sialic acids	360	318
Galactosamine	396	60
Galactosaminitol	350	316
Galactose	341	327
Fucose	339	297
Amino acids		
Threonine	3.9	17.9
Glutamate	1.2	—
Glycine	10.8	10.6
Alanine	12.6	—
Valine	3.9	—
Tyrosine	22.9	20.2
Phenylalanine	3.3	9.9

fractions of average molecular mass 1100 and 740 Da is shown in Fig. 3. The IC₅₀ of the 1100 Da material was 50 mM neutral hexose, which corresponds to 40 mM oligosaccharide, with either

Fig. 4. Effect of saccharides on kidney ACE activity

Buffer was 0.2 M-borate (pH 8.3)/0.1 M-NaCl. Activity is expressed as percentage of activity in buffer alone. ●, *N*-Acetylneuraminic acid; ▼, fucose; ▽, *N*-acetylglucosamine; ○, galactose; ■, *N*-acetylgalactosamine; ▲, glucosamine. Each value is the average of two separate experiments done in duplicate. Single results differ in all cases by less than 15 % from the mean.

Hip-His-Leu or FA-Phe-Gly-Gly as substrate in borate buffer. The IC₅₀ in Hepps buffer was 62 mM. The IC₅₀ of the 740 Da fraction was 110 mM neutral hexose, which corresponds to 80 mM oligosaccharide. Inhibition of the enzyme by this fraction was the same at 0.1 M- or 1 M-NaCl. Inhibition was also unaffected by the addition of up to 20 mM-CaCl₂. The high concentration of Ca²⁺ was used to counteract negative charges on the oligosaccharide, to exclude the possibility that the negative charges were responsible for inhibition of enzyme activity.

The monosaccharide compositions of the two oligosaccharide fractions, as well as the amino acids found in them, are shown in Table 5. In each case contamination of the oligosaccharide by peptide was approx. 0.02 mol/mol of neutral hexose.

In the next group of experiments, the monosaccharides that were found after hydrolysis were tested for their effect on enzyme activity. Fig. 4 shows the effects of galactose, fucose, glucosamine, *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid measured in 0.2 M-borate (pH 8.3)/0.1 M-NaCl with the pig kidney enzyme. Glucosamine was the most effective inhibitor of the monosaccharides tested, and this was also true in Hepps buffer. *N*-Acetylglucosamine, although inhibitory at high concentrations, was slightly but reproducibly a positive effector at 10–50 mM. *N*-acetylneuraminic acid stimulated enzyme activity at all concentrations tested up to 0.5 M (Fig. 4). This was not an anion effect; matched concentrations of acetate inhibited slightly (results not shown) [19].

The effects of monosaccharides on ACE were also investigated with enzyme purified from pig lung. When the effects of galactose, fucose and *N*-acetylglucosamine were compared, no difference was found between the two enzyme preparations.

Methyl β-galactoside was examined to determine whether a free reducing group was required for inhibition. The effect of methyl β-galactoside was less than that of free galactose, but some inhibition was still observed. Glucose and galactose inhibited the enzyme equivalently.

The concentrations of monosaccharides required to inhibit ACE were high enough that we considered that inhibition might be due to non-specific solvent effects of the high polyol concentration. Polyols with average diameters greater than and less than hexose (0.72 nm) were tested. Neither sucrose (0.94 nm) nor ethylene glycol (0.43 nm) had any effect on activity at 200 mM. As

Fig. 5. Effect of borate on ACE activity
Incubation mixtures contained 15–20 ng of protein and 2.5 mM-substrate in borate as shown, and 0.76 M-NaCl, all at pH 8.3. ○, borate alone; ■, 30 mM-Hepes plus borate. Results are means ± s.d. of six determinations.

Table 6. Reaction of borate with saccharides

The pH value is that observed on adding saccharide to borate initially at pH 8.3. Final net borate concentration, 0.2 M. Free saccharide and free borate concentrations were calculated for the solution after the pH had returned to 8.3, by using association constants determined by Evans *et al.* [38].

Saccharide	pH	Free saccharide (M)	Free borate (M)
Zero saccharide	8.30	0	0.2
0.01 M-saccharide			
Methyl β-galactoside	8.10	—	—
Galactose	7.80	0.0001	0.1949
Ribose	8.17	—	—
0.2 M-saccharide			
Methyl β-galactoside	7.80	—	—
Galactose	7.25	0.0008	0.0997
Ribose	5.93	—	—

fucose also had no effect on activity in concentrations up to 500 mM, it seems unlikely that high polyol concentration had a role in the effects observed.

Borate interacts strongly with many saccharides. Therefore we tried to find a buffer that did not itself affect enzyme activity. Several buffers in the appropriate pH range were tested (Hepes, HEPES, Tris and phosphate). With 2.5 mM-Hip-His-Leu as substrate, the enzyme had greater activity in borate than in any of the other buffers tested. Activity in Hepes was equal to that in Hepes and greater than in Tris or phosphate. The effect of borate concentration on activity was investigated further. Beginning with the smallest amount of borate alone that adequately buffered the reaction, an increase in activity as a function of borate concentration was seen (Fig. 5). Half-maximal activation occurred at 7.5 mM-borate at pH 8.5 with 0.76 M-NaCl. Borate alone does not replace chloride as an activator (results not shown). When Hepes was also present, little increase in activity with borate concentration was seen, and the maximum activity observed in the presence of Hepes was less than in the presence of borate alone. From this it is apparent that Hepes also interacts with the enzyme. Phosphate has been shown to inhibit ACE, presumably by binding the essential zinc atom in the active site [19]. The effects of different buffers did not seem to be exerted by a shift in the pH optimum. Two buffer mixtures were used to test the pH optimum: 0.1 M-sodium phosphate/0.1 M-borate/0.1 M-Tris/0.1 M-NaCl; and 0.1 M-Hepes/0.1 M-Hepes/0.1 M-NaCl. The optimum pH was the same in both cases (8.3), but the rates were greater over the range tested for the mixture of phosphate, boric acid and Tris.

The interaction of buffer ion with the effect of an inhibiting monosaccharide on activity was examined by determining the dependence of activity on substrate concentration in the presence and absence of galactose in three different buffers: 0.2 M-borate, 30 mM-Hepes and 50 mM-phosphate, each at pH 8.3 and containing 0.76 M-NaCl (Fig. 6). Enzyme activity was not affected by galactose when measured in Hepes buffer. Below substrate concentrations of 0.25 mM-Hip-His-Leu, the curves for Hepes in the presence or absence of galactose and for borate in the absence of galactose are similar. Above 0.5 mM substrate the activity in Hepes approached substrate saturation uninfluenced by galactose. Galactose in the presence of borate led to inhibition

Fig. 6. Effect of buffer, the presence of galactose and substrate concentration on Hip-His-Leu hydrolysis
(a) Incubation mixtures contained 15–20 ng of protein and substrate, as shown, in: 0.2 M-borate/0.76 M-NaCl, pH 8.3 (○); 0.2 M-borate/0.76 M-NaCl/200 mM-galactose, pH 8.3 (●); 30 mM-Hepes/0.76 M-NaCl, pH 8.3 (□); 30 mM-Hepes/0.76 M-NaCl (pH 8.3)/200 mM-galactose (■); 50 mM-phosphate/0.76 M-NaCl, pH 8.3 (△); 50 mM-phosphate/0.76 M-NaCl/200 mM-galactose, pH 8.3 (▲). Results are means ± s.d. for six determinations. (b) Data from (a) at low substrate concentrations with the scales expanded for clarity.

of the enzyme, which was not relieved by substrate. Rather, in the presence of galactose, the enzyme was inhibited to a greater degree at high substrate concentrations. As shown in Fig. 6, the activity was lowest in phosphate buffer, and, in the absence of galactose, substrate inhibition was observed. The addition of galactose in phosphate buffer led to almost complete inhibition of activity (> 90%) that was independent of substrate concentration.

We considered the possibility that inhibition caused by monosaccharides in the presence of borate could have a contribution from diminished buffer capacity or a decrease in borate concentration below that needed for maximum activity, caused by the removal of borate through borate-saccharide complexes. Borate has the ability to complex with the 1,2- and 1,3-*cis*-hydroxy groups of saccharides, and the degree to which saccharides are able to react with borate can be estimated by observing the change in pH [37]. The larger the pH fall, the stronger the complex between borate and saccharide. Some of the monosaccharides used in the inhibition studies were tested for reactivity with borate under conditions used for the enzyme assay. The pH changes observed for galactose, ribose and methyl β -galactoside are shown in Table 6. The expected free borate and free saccharide concentrations were calculated using the values of the affinity constants for formation of the 1:1 and 1:2 borate-saccharide complexes with galactose measured by Evans *et al.* [38]. It is clear that, at the highest galactose concentration tested, there was adequate free borate remaining for buffering and for activation of the enzyme.

DISCUSSION

We show here that ACE is inhibited by mucins from pig stomach and submaxillary glands, by oligosaccharides prepared from them, and by some, but not all, of the component monosaccharides of the mucins. The concentrations of the saccharides studied here required to alter enzyme activity were higher than ordinarily viewed as physiologically significant. Because comparable concentrations of fucose, sucrose and ethylene glycol had no effect on enzyme activity, it is clear that the inhibition by saccharides could not be due simply to non-specific polyol effects. 'Physiologically significant' should be defined in terms of what may be present in the microenvironment of an enzyme and not by arbitrary standards of concentration. Tissue ACE is an ectoenzyme and resides *in vivo* on the cellular plasma membrane in a microenvironment very rich in oligosaccharide chains. Ryan *et al.* [10] have reported that on the surface of vascular endothelium, ACE is embedded below the surface of the glycocalyx. Further, on the cell surface, at least some and perhaps all of the enzyme is located so that its catalytically active site and its own oligosaccharide chains are exposed to the extracellular fluids, which contain substantial concentrations of glycoproteins.

The enzyme itself is highly glycosylated. If the binding of one of its own carbohydrate chains to a site on the enzyme acts to maintain it in its most active form, exogenous saccharides could inhibit by competing for this binding. The notion that binding to its own carbohydrate chains is important to enzyme function is reinforced by the findings that borate, which would be expected to stabilize conformations in which carbohydrate chains are near enough to be cross-linked, activates the enzyme independently of anion effect and that no apparent substrate inhibition is observed in the presence of borate.

Determination of the primary amino acid sequence from the cDNA has shown that human somatic ACE consists of two domains, each containing a possible active site [39]. The somatic enzyme binds 2 mol of zinc and 2 mol of the active-site inhibitor

lisinopril/mol of protein [40]. Expression of each domain separately in Chinese-hamster ovary cells shows that each is catalytically active, although with different efficiencies and different dependence on activation by chloride [41]. The somatic enzyme is able to hydrolyse one peptide substrate on which the testis enzyme, containing only the C-terminal domain, is inactive [40]. The two domains are very similar in amino acid sequence. Possibly differential glycosylation contributes to the kinetic differences in the behaviour of the two domains.

The data of Ehlers & Riordan [40] also suggest some degree of interaction between the domains. This may explain the tendency of this enzyme to substrate inhibition and its sensitivity to amine-containing buffers. The observations shown in Figs. 5 and 6 could all be accommodated by a model in which borate stabilizes the most active conformation of the enzyme by cross-linking endogenous oligosaccharide groups. Binding of Hepes or Heppps to the second substrate site or another site could prevent formation of the borate-stabilized conformation. In the presence of borate, added galactose or oligosaccharide could compete with endogenous saccharides and disrupt cross-linking.

The extent of inhibition by intact mucins or oligosaccharide fractions was relatively independent of buffer-ion effects, whereas the effectiveness as an inhibitor of the monosaccharide galactose was very much dependent on the nature of the buffer ion. Also, when the oligosaccharides from either gastric or submaxillary mucin were separated according to size, the larger oligosaccharide inhibited at lower concentrations than did the smaller in both cases. Together, these findings suggest that multiple saccharide units are needed for optimum interaction with the binding site.

As shown in Fig. 4, *N*-acetylneuraminic acid causes an increase rather than a decrease in enzyme activity, and the oligosaccharides that have a high content of sialic acids such as those from steps II and III from pig submaxillary mucin are weaker inhibitors than are the oligosaccharides from pig stomach mucin. Glucosamine, which would be partially positively charged at the pH of the enzyme assay, was the most effective inhibitor of the monosaccharides. These findings suggest a concentration of negative charge in the region of the saccharide-binding site(s).

The monosaccharide composition of the material obtained at step III from pig gastric mucin is very close to that predicted for blood-group substances of molecular mass 2800 Da (blood-group substance H) and 3200 Da (blood group substance A) [42]. Binding studies of the material in step III to an affinity resin containing the soya-bean lectin that specifically binds *N*-acetylgalactosamine showed that most of this material bound to the lectin and could be eluted with *N*-acetylgalactosamine (results not shown). The presence of *N*-acetylgalactosamine on the non-reducing ends of most of the carbohydrate chains corresponds to the structure of the blood-group substance A [36]. The 1600 Da oligosaccharides observed are presumably products of incomplete synthesis of the carbohydrate moiety. This is similar to the microheterogeneity of mucin oligosaccharides reported by Allen [43]. The monosaccharide composition and the molecular masses of the oligosaccharides from submaxillary mucin correspond well to those reported by Carlson [44] and Baig & Aminoff [45].

The oligosaccharides prepared by β -elimination from both gastric and submaxillary mucins were subjected to either batch binding or column chromatography on the cation-exchange resin Dowex 50 and were free of amino groups detectable by fluorescamine. Yet amino acids were detected after acid hydrolysis. This suggests that the various fractions contained peptides with blocked *N*-terminal amino groups. It is possible that this could occur by the rearrangement shown in Fig. 7.

It cannot be ruled out that contaminating peptides contribute to the inhibition of enzyme activity by some of the oligosaccharide preparations. ACE is known to be inhibited by a number of

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Vol. 285