Heparin-binding serum protein(s) is required for the protection of sialyltransferase released during the incubation of rat jejunal slices

Sheila NADKARNI, Donna HUNT, Shobhitha RATNAM, Arun NAGPURKAR and Sainen MOOKERJEA*
Department of Biochemistry, Memorial University, St. John's, Newfoundland, Canada A1B 3X9

Incubation of rat jejunal slices in Krebs–Ringer bicarbonate buffer (KRB) required the presence of heat-inactivated horse serum (HHS) in order to show time-dependent release of sialyltransferase into the medium. Sialyltransferase activity could not be detected in the medium when KRB alone or KRB supplemented with either albumin or glycerol was used in the incubations. The viability of the jejunal slices for up to 4 h of incubation was determined by studying the incorporation of glucosamine and leucine into acid-insoluble proteins. Supplementation of KRB with HHS had no beneficial effect on the rate of incorporation of leucine and glucosamine into proteins. KRB medium obtained after different periods of incubation contained higher trypsin-like activity than KRB medium containing HHS. Various antiproteases present as supplements to KRB resulted in the release of sialyltransferase activity from the jejunal slices. Among these antiproteases, α_1 -proteinase inhibitor (α_1 -PI) was the most effective. Also, HHS added to KRB immediately following incubation resulted in partial restoration of sialyltransferase activity in the medium, suggesting the presence of anti-proteolytic factors in HHS. The addition of increasing concentrations of heparin to incubations containing HHS caused a decrease in the medium sialyltransferase activity. The heparin-binding fraction (HBF) from HHS, when added to incubations, was able to protect the sialyltransferase released into medium. However, HHS depleted of its heparin-binding fraction by heparin–agarose affinity chromatography was unable to protect the sialyltransferase. HBF was separated into high- and low-molecular-mass fractions (fractions A and B respectively) by gel-filtration chromatography. The capacity to protect the released sialyltransferase was contained in fraction B. Fraction A contained multiple bands on SDS/PAGE and did not protect the enzyme. Fraction B contained a major protein band on the gel which corresponded to the migration of a similar band in human α_1 -PI. HBF as well as fraction B isolated from HHS showed anti-trypsin-like activity. The results presented indicate that HHS contains a heparin-binding protein(s) similar to human α_1 -PI which plays a role in the protection of sialyltransferase released from jejunal slices.

INTRODUCTION

Sialyltransferases are membrane-bound enzymes which catalyse the transfer of sialic acid, usually to terminal positions on the carbohydrate chains of glycoconjugates (Beyer *et al.*, 1981). Soluble forms of sialyltransferases occur in tissue fluids such as blood, breast milk, colostrum (Hudgin & Schachter, 1972; Bartholomew *et al.*, 1973) and lymph (Ratnam *et al.*, 1981). Liver (Van Dijk *et al.*, 1986), platelets (Beufois *et al.*, 1982), kidney (Bally *et al.*, 1988) and intestine (Fraser *et al.*, 1980) are likely sources of the enzymes found in serum and other tissue fluids. In the rat, increases in serum sialyltransferase activity have been observed in turpentine-induced inflammation (Kaplan *et al.*, 1983; Fraser *et al.*, 1984) and after administration of colchicine (Fraser *et al.*, 1980; Ratnam *et al.*, 1981). Increased levels of sialyltransferase have also been detected in the serum of patients with tumours (Frithz *et al.*, 1985). However, the role of these soluble enzymes and their relationship to the membrane-bound sialyltransferase is unclear. Also, very little is known regarding the physiological regulations by which the membrane-bound sialyltransferases are released into the extracellular fluids as soluble enzymes.

The present study provides a model for studying the release of a soluble form of sialyltransferase during the incubation of rat jejunal slices in Krebs–Ringer bicarbonate buffer (KRB) supplemented with heat-inactivated horse serum (HHS). HHS was required in the incubation medium to protect the released enzyme against proteolytic degradation. Furthermore, an anti-proteolytic

heparin-binding protein(s) present in HHS mimics the action of HHS and is able to protect the released sialyltransferase.

MATERIALS AND METHODS

Materials

CMP[14 C]NeuAc (1.8 mCi/mmol), [14 C]glucosamine hydrochloride (54.2 mCi/mmol) and [14 C]leucine (52 mCi/mmol) were from New England Nuclear (Lachine, Quebec, Canada); Ready Safe scintillation cocktail was from Beckman (Mississauga, Ontario, Canada); α_1 -acid glycoprotein, antipain, human α_1 -proteinase inhibitor (α_1 -PI), bovine pancreatic trypsin inhibitor (BPTI), leupeptin, heparin–agarose, BSA, α -N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), trypsin and chymotrypsin were from Sigma (St. Louis, MO, U.S.A.). CNBr-activated Sepharose 4B was from Pharmacia (Baie D'Urfe, Quebec, Canada). All other chemicals and solvents were of reagent grade. Horse serum was from Gibco Diagnostics (Burlington, Ontario, Canada). Serum was heated by incubating in a water bath at 56 °C for 30 min, which resulted in complete inactivation of sialyltransferase activity. α_1 -Acid glycoprotein was desialylated by mild acid hydrolysis by the method of Spiro (1964). Heparin was from pig intestinal mucosa (low molecular mass, Na salt, 4–6 kDa); trypsin (type XI) was from bovine pancreas; chymotrypsin (type I) was from bovine pancreas; heparin–agarose contained 410 μ g of heparin/ml of packed gel. KRB was prepared according to the method of Krebs & Egglestein (1945) and contained NaCl (0.154 M), KCl (0.154 M), CaCl_2 (0.11 M),

Abbreviations used: HHS, heat-inactivated horse serum; KRB, Krebs–Ringer bicarbonate buffer; HBF, heparin-binding fraction of HHS; unbound-heparin, non-heparin-binding fraction of HHS; unbound-Sepharose, fraction of HHS passed down a Sepharose column; α_1 -PI, α_1 -proteinase inhibitor; BPTI, bovine pancreatic trypsin inhibitor; BAPNA, α -N-benzoyl-DL-arginine-*p*-nitroanilide.

* To whom correspondence and reprint requests should be addressed.

KH_2PO_4 (0.154 M), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.15 M), NaHCO_3 (0.15 M) and sucrose (25 mM) at pH 7.4.

Isolation of the heparin-binding fraction (HBF) from HHS

HHS (100 ml) was applied to a heparin-agarose affinity column (bed volume 50 ml) equilibrated with 5 mM-Tris/HCl/0.15 M-NaCl, pH 7.4, at a flow rate of 24 ml/h. Absorbance was monitored at 280 nm. The unbound fractions of HHS [(unbound-heparin); approx. 85 % of protein] were pooled and concentrated by ultrafiltration to the original volume (100 ml). The HBF (approx. 14 % of protein) was eluted by 1 M-NaCl in 5 mM-Tris/HCl, pH 7.4. The bound fractions from two or more chromatographic runs were pooled, dialysed and lyophilized.

HHS was also passed down a Sepharose column using the above procedure. The unbound fractions [(unbound-Sepharose); approx. 98 % of protein] were pooled and concentrated by ultrafiltration.

Gel-filtration separation of HBF by h.p.l.c.

The proteins in HBF were separated by gel-filtration h.p.l.c. using an LKB Ultropak column (TSK 3000 SWG; 21.5 mm $\times$ 600 mm) connected to an LKB Ultropak pre-column (TSK SWPG; 21.5 $\times$ 75 mm). Chromatography was carried out on a Perkin-Elmer Series 4 liquid chromatograph connected to a Perkin-Elmer LC-95 UV/Visible Detector. The column was equilibrated with 0.5 M- Na_2SO_4 /0.02 M- NaH_2PO_4 , pH 6.8, at a flow rate of 3 ml/min. HBF (20–60 mg, dissolved in equilibrating buffer) was applied to the column and the elution profile obtained was compared with that of known protein molecular mass standards (Bio-Rad gel-filtration standard). Absorbance was monitored at 280 nm. Fractions under each protein peak resulting from the injection of HBF were collected and pooled. The pooled fractions were dialysed, lyophilized, suspended in KRB and stored at -20°C . Protein concentration was measured by the method of Lowry *et al.* (1951) using BSA as a standard.

Preparation of jejunal slices and medium samples

Male Sprague-Dawley rats (200–350 g body weight) were fasted for 18 h and exsanguinated under light ether anaesthesia. The jejunum was removed, rinsed twice with ice-cold saline (0.9 % NaCl) and cut into 0.5 cm rings or slices (Fraser *et al.*, 1980). Jejunal slices (10–15 slices; 0.5 g of tissue) were incubated in oxygenated 25 ml stoppered Erlenmeyer flasks containing KRB or KRB supplemented with 20 % (v/v) HHS, 20 % (v/v) glycerol or 12 % (w/v) albumin (20 % of total albumin present in HHS) in a final volume of 2 ml at 37°C for up to 6 h. At specified time intervals between 0 and 6 h, the clear medium obtained by decanting the incubation mixture was centrifuged at 12000 g for 10 min and the supernatant was assayed for sialyltransferase activity.

In experiments carried out to study the effect of heparin on medium sialyltransferase, heparin was added to the incubation medium at concentrations between 0 and 200 $\mu\text{g}/2\text{ ml}$.

In studies carried out to examine the effect of HHS and its fractions on the release of sialyltransferase, jejunal sections were incubated as above in KRB or KRB containing HHS, HBF, fraction A or fraction B (20 %, v/v; each of the HHS fractions, 200–1000 $\mu\text{g}/2\text{ ml}$ was used). Similarly, jejunal sections were also incubated in KRB, supplemented with the unbound-heparin or the unbound-Sepharose fraction (20 %, v/v).

In some experiments, jejunal slices were incubated as above in KRB containing 200 or 400 μg of antiprotease (leupeptin, antipain, BPTI or α_1 -PI). Increasing concentrations of chymotrypsin and trypsin were also added to assays for sialyltransferase activity in 4 h medium samples containing HHS.

Assays for sialyltransferase

Sialyltransferase assays were carried out as previously described by Ratnam *et al.* (1987). Briefly, the assay mixture (50 μl) contained 20 μl of incubation medium, CMP-[^{14}C]sialic acid (6 nmol; 22000 d.p.m.), asialo α_1 -acid glycoprotein (250 μg) and Mes buffer (6.25 μmol , pH 6.8). Assays were incubated at 37°C for 3 h, after which the reaction was stopped by the addition of 1 ml of an ice-cold mixture of 5 % trichloroacetic acid and 1 % phosphotungstic acid (1:1, v/v). The precipitate formed was filtered under suction through 2.4 cm diameter glass fibre filters (Whatman 934 AH), which were then dried and counted for radioactivity in Ready Safe in a Beckman LS 7500 counter. All assays were done in duplicate. Control incubations were done without any exogenous acceptor. The optimum incubation time and sample volume required were previously determined so that the rate of product formation was linear with incubation time (0–6 h) and medium volume (0–35 μl).

Studies on the viability of jejunal slices

The rate of protein and glycoprotein synthesis by jejunal slices during incubation was determined by measuring the incorporation of either [^{14}C]leucine or [^{14}C]glucosamine into trichloroacetic acid/phosphotungstic acid precipitable cellular proteins. Jejunal slices were incubated in oxygenated KRB or KRB plus 20 % (v/v) HHS as described above. [^{14}C]Glucosamine hydrochloride (0.5 μmol , 0.5 $\mu\text{Ci}/\text{mmol}$) or [^{14}C]leucine (0.5 μmol , 0.5 $\mu\text{Ci}/\text{mmol}$) was added to each set of five flasks at 0, 2, 3 and 4 h. Radioactivity incorporation into jejunal proteins in each flask was monitored at 0, 10, 20, 60 and 120 min by the method of Seidler *et al.* (1988). After incubation, the jejunal slices were removed and washed with KRB containing a 10-fold excess of unlabelled leucine or glucosamine. The washed slices were homogenized in KRB using a Polytron homogenizer (setting 7 for $2 \times 10\text{ s}$). Protein was precipitated by leaving the homogenates overnight in an ice-cold mixture of 10 % trichloroacetic acid/1 % phosphotungstic acid. The mixture was centrifuged three times at 12000 g for 15 min. The supernatant was removed and the pellet was suspended in chloroform/methanol (3:1, v/v) and centrifuged twice (12000 g , 10 min). The pellet was dried under

Fig. 1. Effect of the presence of HHS in the incubation medium on sialyltransferase release from jejunal slices

Jejunal slices (500 mg) were incubated in KRB alone (□), or in KRB plus 20 % (v/v) HHS (△), 12 % (w/v) albumin (○) or 20 % (v/v) glycerol (◇). At the indicated times, incubations were stopped, and the medium was decanted, centrifuged and assayed for sialyltransferase activity as described in the Materials and methods section. Sialyltransferase activity is expressed as nmol of sialic acid transferred to desialylated α_1 -acid glycoprotein/h per ml of incubation medium. Results represent means $\pm$ S.D. from four experiments.

Fig. 2. Incorporation of [^{14}C]glucosamine and [^{14}C]leucine into proteins by jejunal slices

Jejunal slices were incubated in either KRB (a and c) or KRB plus 20% (v/v) HHS (b and d) as in Fig. 1. [^{14}C]Glucosamine (a and b) or [^{14}C]leucine (c and d) was added after 0 h (○), 2 h (▲), 3 h (◆) or 4 h (□) to incubations which were further continued for a maximum of 120 min. At the indicated times these incubations were stopped, homogenates of the slices were prepared and the incorporation of radiolabelled glucosamine or leucine into acid-insoluble proteins was measured as described in the Materials and methods section. Results are expressed as d.p.m./mg of protein and represent mean values from two experiments.

nitrogen and suspended in 6 M-KOH, and aliquots were used for measurement of radioactivity and protein.

Measurement of trypsin inhibitory activity

The inhibition of hydrolysis of the substrate by trypsin was measured according to the method of Dietz *et al.* (1974). HHS and unbound-heparin fraction were diluted 100-fold, whereas HBF, fraction A and fraction B were used at concentrations of 70 $\mu\text{g/ml}$ and $\alpha_1\text{-PI}$ was used at a concentration of 40 $\mu\text{g/ml}$. For all assays the conditions were such that product formation was linear with respect to sample volume and incubation time.

SDS/PAGE of HHS and its fractions

SDS/PAGE was carried out with HHS, unbound-heparin fraction, HBF, fraction A, fraction B and human $\alpha_1\text{-PI}$ using the Phast system (Pharmacia). SDS/PAGE in a PhastGel 8–25% gradient was performed in accordance with the instructions of the manufacturer, and the protein bands were stained with Coomassie Blue. The molecular masses of SDS-denatured pro-

teins were determined by comparison with low-molecular-mass standards (Pharmacia).

RESULTS

Effect of HHS on sialyltransferase release

Fig. 1 shows the release of sialyltransferase from jejunal slices into KRB supplemented with HHS. In the presence of 20% (v/v) HHS the sialyltransferase activity in the medium progressively increased with time up to 6 h. However, when the jejunal slices were incubated in KRB or KRB supplemented with either 12% albumin or 20% glycerol, only trace amounts of sialyltransferase activity could be detected in the medium (Fig. 1). The progressive increase in sialyltransferase activity released into the medium was dependent on the concentration of HHS used in the incubation, up to 20% (results not shown).

Addition of 0.3% (v/v) Triton X-100 to the assays for sialyltransferase in the medium (prepared after centrifuging at

12000 *g* for 10 min or at 100000 *g* for 60 min) did not result in significant increase in enzyme activity (results not shown). This suggested that the sialyltransferase released into the medium was soluble in nature. Release of the enzyme was observed at 37 °C, but incubation of jejunum slices in KRB supplemented with HHS at 4 °C did not result in any release of enzyme activity into the medium (results not shown).

Viability of jejunal slices during incubation: incorporation of radiolabelled leucine and glucosamine into proteins in the slices

Jejunal slices were incubated in either KRB or KRB supplemented with 20% (v/v) HHS for 0, 1, 3 or 4 h. In these incubations the rates of incorporation of [¹⁴C]glucosamine (Figs. 2*a* and 2*b*) and [¹⁴C]leucine (Figs. 2*c* and 2*d*) into acid-insoluble proteins were measured for up to 120 min. The maximum incorporation of leucine into proteins occurred within 30 min, whereas incorporation of glucosamine continued to increase for up to 120 min. Also, the overall rates of incorporation (up to 120 min) of both labelled glucosamine and leucine decreased progressively from 0 to 4 h of incubation. This suggested that cells were less efficient in synthesizing glycoproteins and proteins at 4 h than at 0 h. This decreasing rate of glycoprotein and protein synthesis occurred whether the incubations were carried out in KRB alone or in KRB supplemented with HHS.

Function of HHS in the release of sialyltransferase

To investigate the role of HHS in the incubation, experiments were done wherein: (1) 20% (v/v) HHS was added to KRB containing the jejunal slices throughout the incubation, and (2) 20% (v/v) HHS or 0.9% NaCl was added immediately to the decanted KRB after 0, 1, 2 and 4 h of incubation. Sialyltransferase activity was then measured. The results show that the immediate addition of HHS to KRB partially restored sialyltransferase activity (Fig. 3). In further experiments where HHS was added at different times to KRB medium on ice, there was a gradual loss of enzyme activity with time (Fig. 3 inset), compared with when HHS was added immediately.

Measurement of trypsin activity in the medium

Trypsin activity in the medium was increased for up to 2 h, when jejunal slices were incubated in KRB alone, whereas in incubations with KRB plus HHS the proteolytic activity increased up to 1 h and the overall activity remained much lower. Also, addition of increasing concentrations of trypsin or chymotrypsin directly to the assay mixture inhibited sialyltransferase activity of the 4 h KRB+HHS medium in a dose-dependent manner. Similarly, addition of increasing concentrations of KRB medium obtained after a 4 h incubation of jejunal slices, which presumably contained proteolytic activity, inhibited the sialyltransferase activity of the 4 h KRB+HHS medium (results not shown).

Effect of antiproteases on the sialyltransferase released from jejunal slices

Addition of antiproteases (antipain, BPTI or leupeptin) to KRB during 2 and 4 h incubations of jejunal slices resulted in a 2.5–5-fold (at a 200 µg dose) increase of sialyltransferase activity in the medium, whereas the addition of human α_1 -PI inhibitor to KRB resulted in 11–20-fold (at a 200 µg dose) increases in sialyltransferase activity (Table 1).

Effect of heparin on the medium sialyltransferase activity

Heparin, in a dose-dependent manner, inhibited the activity of sialyltransferase released from jejunal slice incubations in the presence of HHS (Fig. 4). The inhibitory effect of heparin is also shown as a function of the incubation period (Fig. 4 inset). The

Fig. 3. Effect of immediate addition of HHS to KRB medium on sialyltransferase released from jejunal slices

Jejunal slices were incubated in KRB (□) or in KRB plus 20% (v/v) HHS (△) as described in Fig. 1. At the indicated times the incubations were stopped, the medium was decanted, 20% (v/v) saline (0.9% NaCl) was added and sialyltransferase activity was measured. In one set of flasks containing jejunal slices and KRB, 20% (v/v) HHS (◆) was added immediately to the decanted medium at the specified times of incubation, and the medium samples were then assayed for enzyme activity. Results represent means $\pm$ S.D. from three experiments. Inset: jejunal slices were incubated in KRB (□) or KRB plus 20% (v/v) HHS (△). After 2 h of incubation the medium samples were decanted into a tube and the tube was kept on ice. At specified times 20% (v/v) saline was added to the KRB+HHS incubations, whereas 20% (v/v) HHS (◆) or 20% (v/v) saline (□) was added to the 'KRB alone' incubations. The medium samples were centrifuged and sialyltransferase activity was measured as described. Results represent means $\pm$ S.D. from four experiments. Enzyme activity is expressed as nmol of sialic acid transferred/h per ml of medium.

Table 1. Effect of antiproteases on the activity of sialyltransferase released from jejunal slices

Jejunal slices were incubated in KRB containing either 20% (v/v) HHS or antiproteases (200 or 400 µg of antipain, BPTI, leupeptin or α_1 -PI). Medium samples were prepared from 2 h and 4 h incubations as described in the Materials and methods section, and sialyltransferase activity was measured. Sialyltransferase activity is expressed as pmol of sialic acid transferred/h per ml of medium. Results represent means $\pm$ S.D. from four experiments.

Incubation mixture	Sialyltransferase activity (pmol/h per ml)	
	2 h	4 h
KRB	62.3 $\pm$ 26.0	70.4 $\pm$ 23.1
KRB + leupeptin (200 µg)	347.0 $\pm$ 136.5	222.9 $\pm$ 187.3
KRB + leupeptin (400 µg)	328.4 $\pm$ 102.2	464.2 $\pm$ 68.9
KRB + antipain (200 µg)	256.8 $\pm$ 30.1	248.9 $\pm$ 156.7
KRB + antipain (400 µg)	220.6 $\pm$ 156.7	164.2 $\pm$ 117.1
KRB + BPTI (200 µg)	223.9 $\pm$ 87.4	239.0 $\pm$ 116.0
KRB + BPTI (400 µg)	316.2 $\pm$ 93.0	355.3 $\pm$ 59.9
KRB + α_1 -PI (200 µg)	742.0 $\pm$ 124.0	1438.3 $\pm$ 95.6
KRB + α_1 -PI (400 µg)	1378.9 $\pm$ 526.0	2332.6 $\pm$ 193.7
KRB + 20% (v/v) HHS	1444.9 $\pm$ 42.5	2478.5 $\pm$ 123.2

Fig. 4. Effect of heparin on sialyltransferase released from jejunal slices

Increasing concentrations of heparin were added at zero time to jejunal slice incubations supplemented with HHS. The incubations were stopped at either 2 h (○) or 4 h (●) and medium samples were analysed for sialyltransferase activity as described in the Materials and methods section. Results represent means $\pm$ S.D. from three experiments. Inset: jejunal slices were incubated in KRB containing 20% (v/v) HHS. Heparin (100 μ g/2 ml) was added at 0 h to one set of incubations (▲). Control incubations were carried out in the absence of heparin (△). At the specified times, the samples were processed and sialyltransferase activity in the medium was measured. Results represent means $\pm$ S.D. from five experiments. Sialyltransferase activity is expressed as nmol of sialic acid transferred/h per ml of medium.

presence of heparin (100 μ g/2 ml) in the incubations caused 70% and 55% decreases in sialyltransferase activity in the 4 h and 2 h jejunal medium respectively. The addition of heparin (200 μ g/2 ml) further decreased sialyltransferase activity by 79% and 76% respectively in the 4 h and 2 h jejunal medium. When heparin was added directly to the assays, a concentration of 100 μ g/2 ml caused a 10–20% decrease in enzyme activity for the 4 h and 2 h jejunal medium, and addition of 200 μ g/2 ml caused decreases of 25–39% (results not shown). This suggested that when heparin was present during the incubation of jejunal slices it caused greater inhibition of sialyltransferase activity than when it was added directly to the assays. This led to investigations to determine whether heparin binds to specific antiprotease-like proteins in HHS, and if such proteins are actually responsible for the protection of the released sialyltransferase as observed in our studies.

Effect of the HBF of HHS on the release of sialyltransferase by jejunal slices

When the unbound-heparin fraction from HHS was used in the incubations, the activity of sialyltransferase in the medium was decreased compared with when complete HHS was used (Fig. 5). The addition of the unbound-Sepharose fraction (20%, v/v) or HBF (400 μ g) from HHS showed effects similar to those of HHS, presumably due to protection of the released enzyme activity (Fig. 5). Addition of increasing concentrations of the HBF (up to 400 μ g) in the jejunal slice incubations resulted in a progressive increase of enzyme activity in the medium (results not shown). These results suggest that the heparin-bound proteins

Fig. 5. Effect of heparin-bound and heparin-unbound fractions of HHS on the medium sialyltransferase activity

Jejunal sections were incubated in KRB supplemented with 20% (v/v) HHS (△), 20% (v/v) unbound-heparin fraction (○), 20% (v/v) unbound-Sepharose fraction (□) or 400 μ g of HBF (◇). At the indicated times the medium samples were prepared as described in the Materials and methods section and the sialyltransferase activity was measured as described. Results represent means $\pm$ S.D. from four experiments. Sialyltransferase activity is expressed as nmol of sialic acid transferred/h per ml of medium.

Fig. 6. Comparison of the protective effects of HHS, HBF and fraction B

Jejunal slices were incubated in KRB (□), or KRB supplemented with 20% (v/v) HHS (■), 400 μ g of HBF (▨), 500 μ g of fraction A (▧) or 500 μ g of fraction B (■). Medium samples were prepared after 4 h of incubation and sialyltransferase activity was measured as described in the Materials and methods section. Results represent means $\pm$ S.D. from four experiments. Sialyltransferase activity is expressed as nmol of sialic acid transferred/h per ml of medium.

of HHS are required in the incubation medium in order to observe the release of sialyltransferase activity from the incubated slices. It appears that the sialyltransferase released from the jejunum is susceptible to degradation, probably by proteolytic agents, and that this process is halted by addition of either HHS or HBF.

By gel-filtration h.p.l.c., HBF was separated into two distinct fractions, A (V_e 102 ml) and B (V_e 173 ml). Addition of fraction B (500 μ g/2 ml) to the incubations resulted in a marked increase in sialyltransferase activity in the medium. However, similar addition of fraction A did not have any such effect. A comparison of the protective effects of different fractions showed that fraction B was less effective than HBF (Fig. 6). There was no increase in the enzyme activity when fractions A and B were mixed together and added to the incubation (results not shown). This might suggest that some of the protective activity of HBF was lost when it was chromatographed to obtain fraction B.

Fig. 7. SDS/PAGE profiles of HHS, unbound-heparin fraction, HBF, fraction A and fraction B

Electrophoretic profiles are shown of low-molecular-mass standard mixture (lanes 1 and 8), HHS (lane 2), unbound-heparin fraction (lane 3), fraction A (lane 4), HBF (lane 5), fraction B (lane 6, arrow) and human α_1 -PI (lane 7). Samples were processed for SDS/PAGE as described in the Materials and methods section. The standards indicated were phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and sucrose (14.4 kDa).

Fig. 8. Trypsin-inhibitory effect of HHS and its fractions

Inhibition of trypsin activity is compared between samples of HHS (■), unbound-heparin fraction (▨), HBF (▤), Fraction A (▥), Fraction B (▧) and human α_1 -PI (▩). Assays were carried out as described in the Materials and methods section. Trypsin-inhibitory capacity is expressed as $\mu\text{mol}/\text{min}$ per ml of serum for HHS and the unbound-heparin fraction, and as nmol/min per mg of protein for HBF, fraction A, fraction B and α_1 -PI. Results represent means $\pm$ S.D. from five experiments.

SDS/PAGE of HHS and its fractions

Fig. 7 shows the SDS/PAGE profiles of HHS, unbound-heparin, HBF, fraction A and fraction B. The migration profiles of HHS (lane 2), unbound-heparin (lane 3), fraction A (lane 4), HBF (lane 5), fraction B (lane 6) and human α_1 -PI (lane 7) were compared with those of low-molecular-mass standards (lanes 1 and 8). Multiple bands were observed for HHS, unbound-heparin fraction, HBF and fraction A. Interestingly, fraction B showed one major band, with an apparent molecular mass of 55 kDa, which migrated to a position corresponding to that of the major band observed for human α_1 -PI.

Inhibition of trypsin activity by HHS and HBF

The inhibition of trypsin activity was measured for HHS, unbound-heparin fraction, HBF, fraction A, fraction B and

human α_1 -PI (Fig. 8). HBF and fraction B showed marked trypsin inhibitory-activity, whereas the unbound-heparin fraction was unable to inhibit trypsin. This suggested that HBF contained the major anti-tryptic proteins present in HHS. Fraction A showed almost no inhibition of trypsin activity, whereas the trypsin-inhibitory capacity of fraction B was very similar to that observed for HBF.

DISCUSSION

Jejunal slices incubated in KRB progressively released soluble sialyltransferase into the medium, but the enzyme released was susceptible to proteolytic degradation. Activity of the released enzyme was observed only when the KRB medium was supplemented with HHS, or when HHS was added to the medium immediately after incubation. The medium obtained after incubation with KRB alone contained much more trypsin-like activity compared with the medium obtained after incubation with HHS. Also, the addition of human α_1 -PI to the incubation medium containing KRB resulted in the protection of the released sialyltransferase activity. We have presented evidence that this protective function of HHS is due to a specific heparin-binding protein(s) in HHS, isolated as fraction B from HBF (Fig. 6). Indeed, the removal of the heparin-binding protein(s) from horse serum resulted in loss of the protection of the released sialyltransferase (Fig. 5).

Our results also showed that the jejunal slices were physiologically viable during incubation in both KRB alone or KRB supplemented with HHS, and the rates of protein and glycoprotein synthesis by the slices were similar in both cases. This suggested that the addition of HHS to KRB had no beneficial effect on the viability of the slices during incubation.

Sialyltransferases are present in cells in the membrane-bound form, but are also found in the soluble form in blood and other extracellular fluids. Studies using antibodies against sialyltransferase have shown that in intestine the enzyme is present throughout the Golgi cisternal stack, as well as in other membrane structures of the secretory vesicles and the plasma membrane (Taates *et al.*, 1988; Taates & Roth, 1988). In rats, it has been shown that sialyltransferase is released from its membrane anchor in liver by a cathepsin D-like protease (Lammers & Jamieson, 1988). A similar proteolytic cleavage may be occurring in the intestine, whereby the membrane-bound enzyme is released in the soluble form into the medium. Our results suggest that the sialyltransferase in the jejunal tissue, once released from its membrane anchor into the medium, is unstable and susceptible to proteolytic degradation. We clearly show that HHS, as well as HBF and fraction B obtained from HHS, can prevent the proteolytic degradation of the released sialyltransferase.

In the present study we have also shown that addition of heparin to the jejunal slice incubations containing HHS inhibited sialyltransferase activity in the medium. This led to our search for factor(s) in HHS which might bind to heparin, and the removal of such factor(s) from HHS was the reason for inhibition of the enzyme by heparin. Heparin is known to bind and interact with a number of proteins, including lipoproteins (Sharon, 1975), soluble lectins from human brain (Eloumani *et al.*, 1990), endothelial cell growth factors (Klagsbrun & Shing, 1985), lectin from human placenta (Kohne-Godt & Gabius, 1989) and polypeptide growth factor for hepatocytes (Zarnegar *et al.*, 1990). We have shown that heparin binds to fraction B from HBF and that this fraction has anti-proteolytic activity. We have also demonstrated by SDS/PAGE that fraction B has a migration profile very similar to that of human α_1 -PI. On the basis of these results, we have concluded that the protective effect of HHS on

sialyltransferase released during the incubation of jejunal slices is due to fraction B, a constituent of HHS.

During the acute phase response there is an increase in the levels of certain plasma proteins, generally known as the acute phase reactants (Koj, 1974). Lammers & Jamieson (1988) have shown that during the acute phase response there is an increase in the cathepsin D-like activity, which in turn leads to the increased serum levels of sialyltransferase. They have further hypothesized that sialyltransferase itself is an acute phase marker. Increases in soluble sialyltransferases in serum have been observed in inflammation (Kaplan *et al.*, 1983; Fraser *et al.*, 1984; Woloski *et al.*, 1986), as well as in a number of neoplastic conditions (Mookerjee *et al.*, 1972; Weiser *et al.*, 1982; Martinez & Barsigian, 1987). The factors which control the release of sialyltransferases in physiological and pathological conditions are not clearly understood. It will therefore be of interest to investigate further whether serum proteins such as α_1 -PI and heparin-binding protein(s) play any role in modulating the activity of sialyltransferase in serum and other tissue fluids.

The Medical Research Council of Canada and the Heart and Stroke Foundation of Canada are gratefully acknowledged for financial support.

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Received 3 January 1991/31 July 1991; accepted 9 August 1991