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Isolation and characterization of raw heparin from dromedary intestine: evaluation of a new source of pharmaceutical heparin

Mohamad Warda[a], Eman M. Gouda[a], Toshihiko Toida[b], Lianli Chi[c], Robert J. Linhardt[c,*]

[a]*Faculty of Veterinary Medicine, Department of Biochemistry, Cairo University, Cairo, Egypt*
[b]*Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan*
[c]*Departments of Chemistry, Biology and Chemical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, USA*

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Abstract

Heparin, a heterogeneous anionic polysaccharide, is the glycosaminoglycan (GAG) used clinically an anticoagulant. This anticoagulant activity is primarily derived from its binding to the serine protease inhibitor antithrombin III, a potent inhibitor of thrombin (factor IIa) and factor Xa. Heparin is a complex natural product and its in vitro synthesis is not yet possible due to the difficulty of organizing the many biosynthetic enzymes required for its synthesis. The principle natural sources for heparin include porcine intestine and bovine lung. These two sources pose concerns for religious and health reasons, respectively. To circumvent these concerns, GAG from the intestinal tissue of one humped camel was isolated. Chemical characterization of this newly isolated GAG and spectroscopic analysis by 1D and 2D ^{1}H-NMR were undertaken. Unsaturated disaccharide compositional analysis was performed on the enzymatically depolymerized GAG and the molecular weight of the isolated GAG was determined by gradient polyacrylamide gel electrophoresis. Anticoagulant activity of the newly isolated GAG was tested by using an anti-factor Xa assay. The results of these studies suggest that the GAG from one humped camel intestine is a mixture of heparin and heparan sulfate and represents an alternative source of heparin.

Keywords: Heparin; Glycosaminoglycan; Heparan sulfate; One humped camel; Intestine; Disaccharide analysis; Anticoagulant

1. Introduction

Bovine lung or porcine intestine tissues are currently the only raw materials used to prepare commercial, pharmaceutical heparins (Linhardt and Gunay, 1999). The appearance of bovine spongiform encephalopathy, 'mad cow disease', and its apparent link to the similar prion-based Creutzfeldt–Jakob disease in humans (Schonberger, 1998), has limited the use of bovine heparin. Moreover, it is not easy to distinguish bovine and porcine heparins, making it difficult to ensure the species source of heparin (Linhardt and Gunay, 1999). Porcine heparin also has problems with its use, associated with religious restrictions among members of the Muslim and Jewish faiths. Heparin exhibits anticoagulant activity primarily from its binding to the serine protease inhibitor, antithrombin (AT). On binding to heparin, AT undergoes a conformational change becoming a potent inhibitor of thrombin (factor IIa) and factor Xa, serine proteases of the coagulation cascade (Jordan et al., 1980a,b, 1982). Porcine intestinal heparin has within its structure a unique pentasaccharide sequence capable of acting as an AT binding site

*Corresponding author. Tel.: +1-518-276-3404; fax: +1-518-276-3405.
E-mail address: linhar@rpi.edu (R.J. Linhardt).

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(Choay et al., 1983; Quinsey et al., 2002). Bovine lung heparin has a similar unique pentasaccharide sequence, which differs from that found in porcine intestine in that the *N*-acetylglucosamine in the porcine intestinal heparin AT binding site is replaced by an *N*-sulfoglucosamine residue in the bovine lung heparin AT binding site (Loganathan et al., 1990). These nearly identical binding sites make porcine intestinal and bovine lung heparins pharmacologically equivalent. Although the disaccharide compositions of a bovine and porcine heparin differ, the composition of a heparin sample cannot be used to determine if heparin from porcine source has been adulterated with small amounts of bovine heparin (Linhardt and Gunay, 1999).

Low molecular weight (LMW) heparins have been rapidly replacing heparin as the clinical anticoagulant/AT agent of choice (Linhardt and Gunay, 1999). Currently all approved LMW heparins are prepared from porcine intestinal heparin, limiting their use in countries restricting porcine products. Non-animal sources of heparin, such as chemically synthesized, enzymatically synthesized, or recombinant heparins are currently not available.

These concerns have motivated us to look for alternative, tissue sources for heparin. We recently examined avian tissues as a source of heparin but found that the major intestinal glycosaminoglycan (GAG) isolated from intestinal tissue of turkey was instead heparan sulfate having low level of sulfation and virtually devoid of anticoagulant activity (Warda et al., 2003). Dromedary camel is a domestic ungulate species of great economic importance to the pastoral nomadic communities that inhabit hot arid areas of the Middle East. Camel has not been demonstrated to carry any prion based disease. Thus, we turned our attention to the study of camel intestine a currently unused new material, as potential new source of heparin.

2. Materials and methods

2.1. Materials

One humped camel *Camelus dromedarius* small intestine was collected at the slaughter house (Nahya Slaughter House, Giza District, Egypt) and immediately preserved with 0.125 M sodium bisulfite as antioxidant, placed on dry ice and stored in −70 °C until processing. Alkalase from *Bacillus subtilis*, chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris*, endonuclease (EC 3.1.30.2) from *Serratia marcescens* and heparin lyase I (heparinase EC 4.2.2.7), heparin lyase II (heparitinase II), heparin lyase III (heparitinase I EC 4.2.2.8) from *Flavobacterium heparinum* and Dowex macroporous resin as strong basic anion exchanger (SAX) were purchased from Sigma Chemical Co. (St. Louis, MO). Spectra/Por® dialysis tubing MWCO 3500 was from Spectrum Medical Industries, Inc. (Los Angeles, CA). Standard heparin obtained from porcine intestine, were purchased from Celsus Laboratories, Inc. (Cincinnati, OH). Heparin assay kit used for the quantitative determination of heparin was purchased from Sigma Diagnostics (St. Louis, MO). All other reagents used were analytical grade.

2.2. Preparation of intestinal GAGs

A method of intestinal GAG preparation was previously described (Warda et al., 2003). Briefly, small intestine (300 g, fresh mass once emptied of its contents) was cut into small pieces and homogenized in a blender. The homogenate was digested by actinase E (10 mg/g) using 0.05 M Tris acetate buffer (pH 8.0) at 50 °C for 12 h. The proteolytic homogenate was put in boiling water bath for 30 min to deactivate the protease then centrifuged ($1500 \times g$) at 4 °C for 30 min. The recovered supernatant was added to a pre-activated (washed extensively with methanol, H_2O, 2 M NaCl, H_2O) SAX Dowex macroporous resin to bind GAGs in a chromatographic batch separation. The resin was washed with water followed by 0.5 M sodium chloride to elute residual peptides and LMW contaminants. The resin was then eluted by 2.7 M sodium chloride to elute GAGs. The GAGs eluted from the SAX resin were precipitated by addition of methanol 80 vol.% (v/v). The precipitated material was recovered by centrifugation and dialyzed in cellulose membrane tubing (MWCO 3500) against deionized water overnight at 4 °C. The retentate was freeze-dried then resuspended in 20 mM Tris–HCl buffer (pH 8) containing 2 mM $MgCl_2$ and digested with endonuclease (2500 units/g) for 12 h at 37 °C. After endonuclease digestion, NaCl concentration was brought to 2.7 M and the GAGs were precipitated by adding methanol to 80 vol.%. The recovered precipitate was dissolved in 10 ml of deionized water and dialyzed overnight at 4 °C and freeze-

dried. The dried GAG was then subjected for structural and functional characterization. In same steps, parallel experiment of porcine intestinal GAG preparation was performed as control.

2.3. Chemical characterization

Azure A assay was performed to estimate the level of sulfo group substitution of the purified GAGs. Metachromasia of the blue dye on addition of negatively charged GAGs results in a concentration-dependent increase in absorbance at 530 nm (Grant et al., 1984). Carbazole assay was performed to determine content of uronic acid in the GAG preparation by determining the absorbance at 525 nm (Bitter and Muir, 1962). Porcine intestinal heparin standard was used in both cases to prepare standard curves.

2.4. ^{1}H-NMR analysis

NMR spectroscopy was performed on samples (~5 mg) dissolved in D_2O (99.96 at.%), filtered through a 0.45 μm syringe filter, freeze-dried twice from D_2O to remove exchangeable protons and transferred to Shigemi tubes. One-dimensional (1D) and two-dimensional (2D) ^{1}H-NMR experiments were performed on a Bruker DRX-400 instrument.

2.5. Enzymatic depolymerization of GAGs

Camel intestinal GAG (5 mg/ml) was treated with chondroitin lyase ABC (0.2 unit/100 mg in 50 mM sodium acetate, pH 8) at 37 °C for 24 h in sealed tubes. After digestion, the reactions were terminated by heating in boiling water bath for 5 min and the digested samples were desalted using microanalysis desalting spin column (Amika Corp®) and freeze-dried. The chondroitinase digested samples were next digested with heparin lyase I, II, III (Griffin et al., 1995). Dried samples were dissolved in buffer (50 mM sodium phosphate buffer, pH 7.1 and 100 mM NaCl) at a concentration of 10 mg/ml. Each heparin lyase was added at 0, 8 and 16 h to a final concentration of 0.02 mU/mg GAG dry wt. and the reaction was incubated in sealed tubes at 37 °C over a period of over 24 h. Standard porcine heparin was treated in similar manner to serve as controls. The reaction was boiled, desalted and freeze-dried as previously described in chondroitin lyase ABC digestion step. Disaccharide analysis was performed on the obtained freeze-dried materials.

2.6. Gradient polyacrylamide gel electrophoresis

Gradient polyacrylamide gel electrophoresis (PAGE) was performed on a 32 cm vertical slab gel Bio-Rad unit equipped with model 1420B power source from Bio-Rad (Richmond, CA). Polyacrylamide linear gradient resolving gels (14×28 cm^2, 12% acrylamide) were prepared and run as described previously (Edens et al., 1992). For the polyacrylamide gradient gel electrophoresis disaccharide mapping, the chondroitinase-digested samples were further digested with heparin lyase, in the same way as described in Section 2.5 except only heparin lyase I was used. The molecular sizes were determined by comparing with a banding ladder of heparin oligosaccharide standards prepared from bovine lung heparin and tetrasaccharide marker was added to identify the bands (using UNSCAN-IT GEL, automated digitizing system, version 4.3 for Macintosh, Silk Scientific Corp., Orem, UT).

2.7. Determination of unsaturated disaccharides from heparin

Unsaturated disaccharides, produced enzymatically from camel intestinal raw heparin and standard porcine intestinal heparin, were determined by reverse phase ion-pairing (RPIP) high-performance liquid chromatography (HPLC) with UV detection (Thanawiroon and Linhardt, 2003). The system included Shimadzu LC-10Ai pumps and a Shimadzu SPD-10Ai UV/Vis detector. The data acquisition and processing were done with CLASS-VP 4.2 software (Shimadzu Corp., Japan). The separation was performed on the Discovery C-18 column (5 μm, 4.6×250 mm^2) from Supelco Company. An isocratic elution was applied at the flow rate of 1 ml/min. The eluent is a mixture of 15 mM tributylamine, 50 mM ammonium acetate, and 15 mM acetic acid in acetonitrile:water (20:80, v:v). Sample (5 μl) was injected and detected with UV at 232 nm.

2.8. Anticoagulant activity assay

Heparin readily catalyzes the inactivation of factor Xa by AT. Factor Xa inactivation was used in this study to assess the anticoagulant activity of

the raw heparin prepared from camel intestines using a Heparin Assay Kit (Sigma). In this assay, when both factor Xa and AT are present in excess, the inhibition of factor Xa is directly proportional to the limiting concentration of heparin. Thus, residual factor Xa activity, measured with factor Xa-specific chromogenic substrate, is inversely proportional to the heparin concentration (Teien et al., 1976; Teien and Lei, 1977).

3. Results and discussion

While detailed studies on heparin have been performed on many mammalian species (Einarsson and Andersson, 1977; Rosenberg, 1974) there are no reported data about the structure of heparin from one humped camel. Camel is a unique creature that has the ability to survive severe drought in desert habitats (Chandrasena et al., 1979). Plasma hyperosmolality, increased hematocrit and a decreased blood volume follows long lasting famine conditions (Etzion et al., 1984; Warda, 1998) all result in increased blood viscosity during dehydration. Endogenous heparin or other related GAGs may counteract intravascular coagulation occurring under dehydration conditions. Thus we would predict a comparatively higher physiological level of heparin in camel than observed in other mammalian species. In the present study we examined whether heparin was present in one humped camel and characterized its structure.

Raw heparin (~400 mg/kg) was recovered from the small intestine of an adult camel by proteolysis, isolated on an SAX column, and purified by methanol precipitation and endonuclease and chondriotinase treatments. The recovery of raw heparin from camel intestine compares favorably to the 250 mg/kg recovered from porcine intestine.

Chemical evaluation of the raw heparin isolated from camel intestine was undertaken using Azure A assay (Grant et al., 1984) and carbazole assay (Bitter and Muir, 1962) (Fig. 1A and B). Azure A assay, used to determine the charge density of GAGs, gave a line of equation $y=0.015x+0.95$, $r^2=0.95$. Pharmaceutical porcine heparin standard gave a line of equation $y=0.019x+0.01$, $r^2=0.97$ showing a 22% greater slope than camel intestinal raw heparin. Negatively charged groups in GAGs, e.g. carboxyl, *N*- and *O*-sulfo groups, contribute to protein binding. *N*-sulfo groups are located at the C-2 position of the glucosamine (GlcN) residues in heparin (86% *N*-sulfo, 14% *N*-acetyl) and heparan sulfate (10% *N*-sulfo, 90% *N*-acetyl), (Bazin et al., 2002). Azure A data indicate that camel intestinal raw heparin carries approximately 3 negative charges/disaccharide unit compared to 3.7 (2.7 sulfo groups and 1 carboxyl group) for porcine intestinal heparin (Kim and Linhardt, 1989). Carbazole assay, used to determine the uronic acid component of GAGs, gave a line with the equation $y=0.007x-0.01$, $r^2=0.96$ for porcine heparin showing a 10% higher concentration of uronic acids per unit mass than camel raw heparin, which gave a line with the equation $y=0.007x-0.018$, $r^2=0.99$.

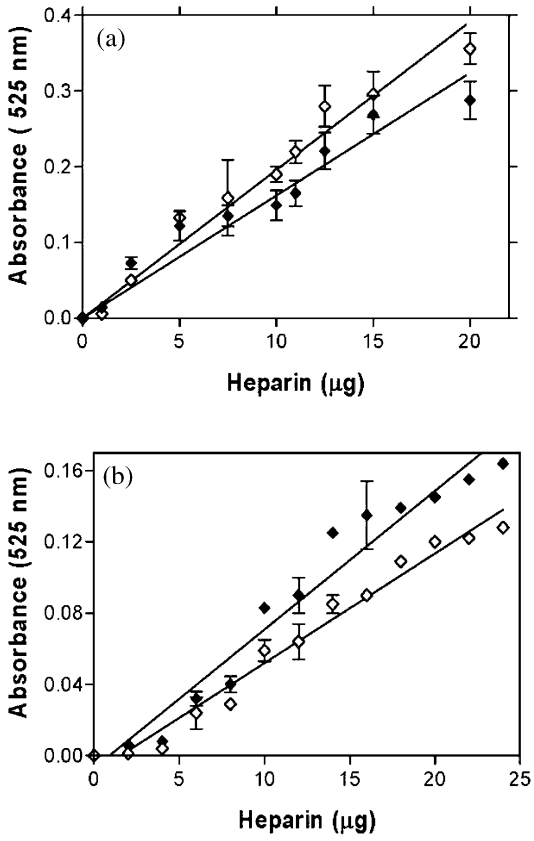

Fig. 1. Panel A: Azure A analysis of camel intestinal raw heparin (♦) and porcine intestinal heparin (◇). Error bars represent S.E.M. of 5 determinations. Panel B: Carbazole assay of camel intestinal raw heparin (◇) and standard porcine intestinal heparin (♦). The two curves shown in both panel A and panel B are significantly different, with camel raw heparin having a reduced content of sulfation and uronic acid compared to porcine intestinal heparin.

^{1}H-NMR experiments were performed on camel

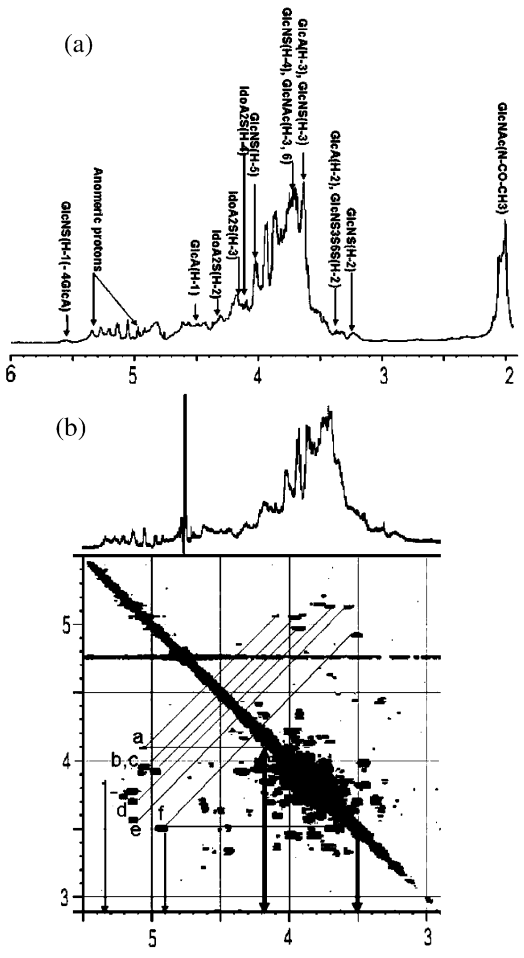

Fig. 2. Panel A: 1D ^{1}H-NMR spectrum of camel intestinal raw heparin. Panel B: Partial 2D ^{1}H-NMR COSY spectrum of camel intestinal raw heparin. The spectral data for porcine heparin and heparan sulfate can be found in Griffin et al., 1995.

intestinal heparin (Fig. 2A). There are many reporter signals indicating the degree of sulfation of camel intestinal raw heparin beside the GlcNAc (*N*-acetyl methyl) signal at 2.02 ppm. The presence of H-1, H-2 and H-5 signals corresponding to GlcNS at 5.5, 3.25 and 4.03 ppm, respectively and the appearance of H-2, H-3 and H-4 signals of 2-sulfo iduronic acid (IdoA2S) at 4.33, 4.2 and 4.1 ppm, respectively are shown. Moreover, *N*-sulfo disaccharide is reported at 3.4 ppm by H-2 signal of GlcNS. Assignment of signals indicates an unusual number of peaks corresponding to anomeric protons between 5 and 5.4 ppm. Based on 2D NMR spectroscopy (Fig. 2B), the anomeric protons at 4.9–5.3 ppm (indicated by the thin arrows) represent seven different types of anomeric proton signals. These anomeric protons show connectivity to the protons on C-2 signals (see the connecting lines in Fig. 2B) located from 4.2 to 3.5 (indicated between the thick arrows). These data are consistent with the sample containing heparin along with significant amounts of various kinds of heparan sulfates (Griffin et al., 1995). The presence of these contaminants results from extracting raw heparin from whole segment of camel small intestine. This small intestine is relatively thick when compared with porcine intestine. It may be possible to recover a purer form of raw heparin by stripping the heparin-rich mucosal layer from camel intestine prior to processing. The raw heparin obtained from camel whole intestine in this study was estimated to be approximately 75% pure based on the average value of purity obtained using Azure A assay, carbazole assay and ^{1}H-NMR spectroscopy.

Raw heparin prepared from camel intestine was next analyzed by PAGE (Fig. 3A). PAGE analysis is useful in both determining the molecular weight of GAGs (Edens et al., 1992) as well as for oligosaccharide mapping. Intact porcine intestinal heparin gave a broad smear, with no apparent banding pattern, consistent with a polydisperse mixture of polysaccharide chains (Fig. 3A, lane 3). A pure, fully sulfated tetrasaccharide standard was analyzed by PAGE to assign the first intense oligosaccharide band (Fig. 3A lane 1) in a mixture of oligosaccharides prepared from bovine lung heparin (Fig. 3A lane 2). This defined oligosaccharide provides a reading frame necessary to identify the intense bands molecular weight of the main components in the mixture of oligosaccharides in lane 2 (Edens et al., 1992). The log MW as a function of migration distance gives line of equation $y = -0.0048x + 4.6$, $r^2 = 0.999$ (Fig. 3B). The average molecular weight (MW_{avg}) of intact porcine intestinal heparin (Fig. 3A, lane 3) was determined as 17 400. Intact porcine heparan sulfate ($MW_{avg} = 26\,200$) and camel intestinal raw heparin ($MW_{avg} = 24\,000$) both showed somewhat higher molecular weights (Fig. 3A, lanes 5 and 7).

Oligosaccharide mapping of porcine heparin, in which each intact GAG sample, is first digested with heparin lyase I and then analyzed by PAGE, gave a characteristic banding pattern associated with products migrating between the fully sulfated disaccharide (labeled 2) and fully sulfated hexasaccharide (labeled 6) (Fig. 3A, lane 4). This

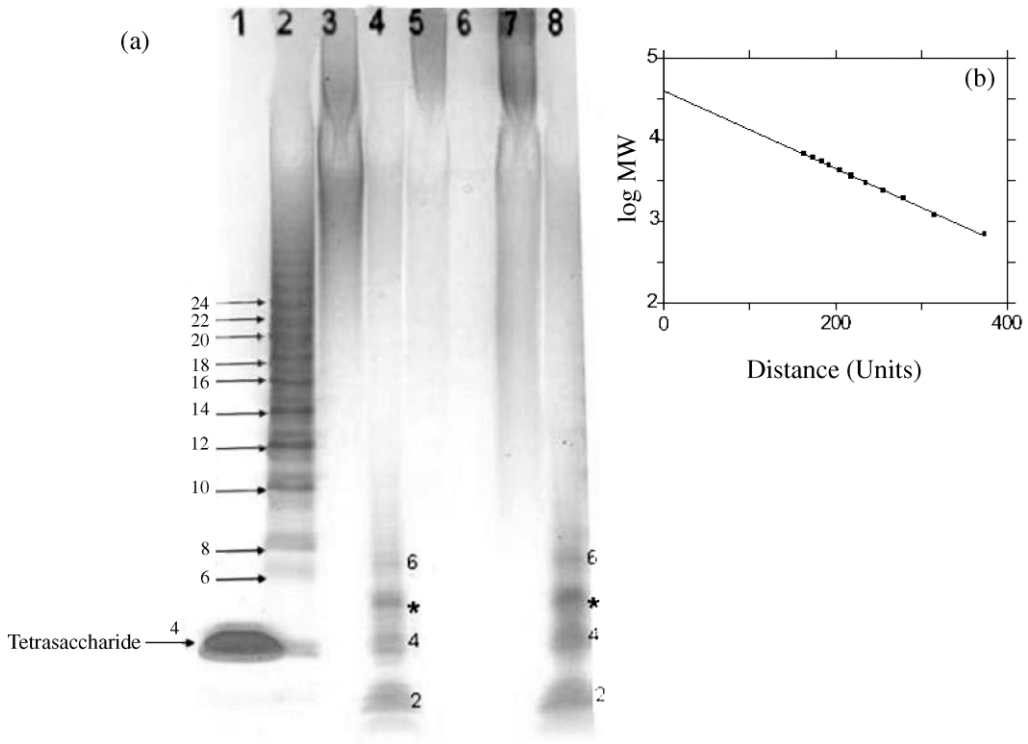

Fig. 3. The gradient PAGE analysis with alcian blue staining of porcine intestinal heparin and camel intestinal raw heparin and their sensitivity towards heparinase. Lane 1 is heparin hexasulfated tetrasaccharide standard, corresponding to ΔUA2S(1→4)GlcNS6S(1→4) IdoA2S (1→4) GlcNS6S (where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) (Linhardt et al., 1990; Merchant et al., 1985), indicated by the arrow (Pervin et al., 1995), lane 2 is bovine lung heparin-derived oligosaccharide standards (Edens et al., 1992); lane 3 is intact porcine intestinal heparin; lane 4 is heparinase depolymerized porcine intestinal heparin; lane 5 is intact porcine intestinal heparan sulfate; lane 6 is porcine intestinal heparan sulfate after heparinase treatment; lane 7 is intact camel intestinal raw heparin; and lane 8 is camel intestinal raw heparin after heparinase treatment. The bands labeled with the asterisk (*) in lane 4 and lane 8 correspond to the fragment of the AT binding site. Panel B is the standard curve of log molecular weight of bovine lung heparin-derived oligosaccharide standards (lane 2 Panel A) from which the MW_{avg} of porcine intestinal heparin and camel intestinal raw heparin can be calculated.

oligosaccharide map for porcine intestinal heparin is characteristic of its structure (Loganathan et al., 1990). A band clearly observed between the one assigned to fully sulfated tetrasaccharide standard (4) and hexasaccharide (6) labeled with an asterisk corresponds to a fragment of the AT binding site (Fig. 3A, lane 4). A nearly identical distribution of products is observed in Fig. 3A, lane 8 when camel intestinal raw heparin treated with heparin lyase I (including the same AT binding site fragment indicated with an asterisk). It should be noted that no such banding observed for porcine intestinal heparan sulfate because of its low level of sulfation and the absence of an AT binding site (Fig. 3A lane 6). Similar results for oligosaccharide map of porcine intestinal heparan sulfate have previously been reported (Warda et al., 2003). Oligosaccharide maps, show camel intestinal raw heparin closely resembles porcine intestinal heparin (Linhardt et al., 1990) and human heparin (Linhardt et al., 1992), but is distinctly different from bovine lung heparin (Loganathan et al., 1990), because of the subtle difference in the structure of its AT binding site. These data suggest that porcine intestinal heparin, camel intestinal raw heparin and human heparin contain identical AT binding sites. Disaccharide analysis of porcine intestinal heparin and camel intestinal raw heparin were next carried out following their complete depolymerization with an equivalent unit mixture of heparin lyases I, II and III, using RPIP HPLC (Thanawiroon and Linhardt, 2003). The results (Table 1 and Fig. 4) show that all the disaccharides, with the exception of ΔUA2S-GlcNAc,

Table 1
Disaccharide composition of camel intestinal raw heparin and porcine intestinal heparin

	(1) ΔUA-GlcNAc	(2) ΔUA-GlcNS	(3) ΔUA-GlcNAc6S	(4) ΔUA2S-GlcNAc	(5) ΔUA-GlcNS6S	(6) ΔUA2S-GlcNS	(7) ΔUA2S-GlcNAc6S	(8) ΔUA2S-GlcNS6S
Camel intestinal heparin	18.4	22	20.9	ND	11.4	12.8	ND	14.5
Porcine intestinal heparin	3.1	3.6	14.5	1.2	24.9	12.4	ND	40.3

observed in porcine intestinal heparin are also found in camel intestinal raw heparin. Disaccharide analysis revealed that GlcNAc% corresponds to less than 20% of the total disaccharides in raw heparin obtained from camel intestines. This clearly distinguishes it from a simple mixture of heparan sulfates, which would contain 51–79% GlcNAc (Toida et al., 1997). The percentage of non-sulfated disaccharide (ΔUA-GlcNAc) and mono-sulfated disaccharides (ΔUA-GlcNS and ΔUA-GlcNAc6S) is higher in camel intestinal raw heparin than that of porcine intestinal heparin. In contrast, the fully sulfated disaccharide (ΔUA2S-GlcNS6S) is in twofold greater abundance in porcine intestinal heparin than in camel intestinal raw heparin. Based on disaccharide analysis, the overall degree of sulfation of camel intestinal raw heparin is approximately two-thirds that of porcine intestinal heparin.

The anticoagulant activity of camel intestinal raw heparin was next evaluated. The anti-factor Xa activity assay of standard pharmaceutical heparin obtained from porcine intestine shows an activity of 170 IU/mg (Fig. 5). The United States Pharmacopoeia (USP) describes a required activity for USP heparin of >120 IU/mg. Raw heparin isolated from porcine intestine in our lab showed an anti-factor Xa activity of ~ 120 units/mg. Raw heparin isolated from camel intestine showed a somewhat reduced activity of $\sim 50-60$ units/mg and porcine intestinal heparan sulfate showed a minimal anti-factor Xa activity of <20 units/mg. These results suggest that by utilizing camel intestinal mucosa and incorporating additional processing steps, aimed at removing contaminating heparan sulfate, a pharmaceutical grade heparin might be prepared from camel intestine. Moreover, a heparin prepared from camel intestinal mucosa might represent a commercially viable product in countries that restrict the use of porcine products or where there is concern regarding prion-based contamination of bovine products.

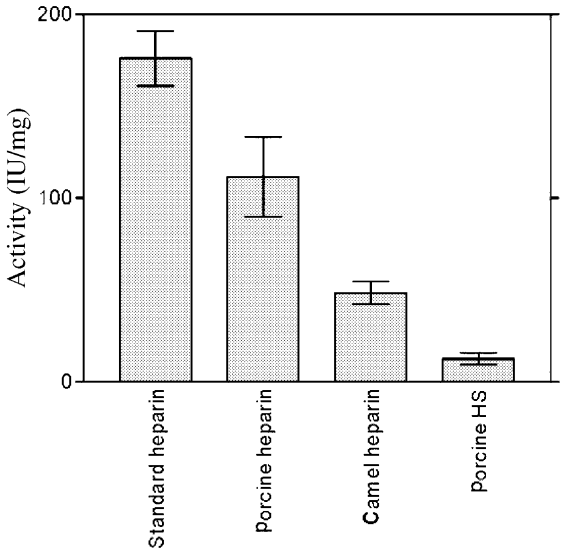

Fig. 5. Anti-factor Xa activity of commercial porcine intestinal heparin, camel intestinal raw heparin and porcine intestinal raw heparin and commercial porcine intestinal heparan sulfate (HS). Error bar is mean ± S.E.M. of at least five times determinations).

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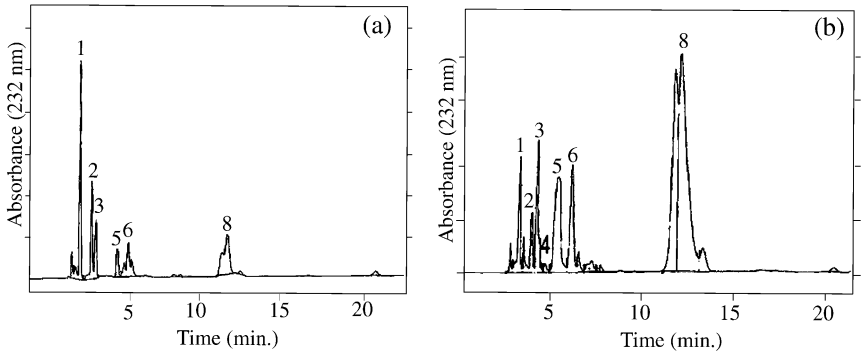

Fig. 4. Unsaturated disaccharide analysis of heparinase treated camel intestinal raw heparin and porcine intestinal heparin using RPIP HPLC. Panel A shows camel intestinal raw heparin derived disaccharides peaks. Panel B is the corresponding peaks of porcine intestinal heparin derived disaccharides. The numbers of peaks represent the different disaccharides (Table 1).

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