ELSEVIER

Comparative Biochemistry and Physiology Part B 134 (2003) 189–197

CBP

www.elsevier.com/locate/cbpb

Turkey intestine as a commercial source of heparin? Comparative structural studies of intestinal avian and mammalian glycosaminoglycans

Mohamad Warda[a], Wenjun Mao[b], Toshihiko Toida[c], Robert J. Linhardt[b,*]

[a]*Department of Biochemistry Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt*
[b]*Department of Chemistry, Department of Chemical and Biochemical Engineering, Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, Iowa, USA*
[c]*Graduate School of Pharmaceutical Science, Chiba University, Chiba, Japan*

Received 26 October 2002; received in revised form 30 October 2002; accepted 1 November 2002

Abstract

Heparin is a glycosaminoglycan (GAG) that is extracted primarily from porcine intestinal tissues and is widely used as a clinical anticoagulant. It is biosynthesized as a proteoglycan and stored exclusively in mast cells and is partially degraded to peptidoglycan and GAG on immunologically activated mast cell degranulation. In contrast, the structurally related heparan sulfate, is the polysaccharide portion of a ubiquitous proteoglycan, localized on cell surface and in the extracellular matrix of all animal tissues. Heparin and heparan sulfate are made in the Golgi through a similar biosynthetic pathway. The current study was undertaken in a search for alternative, non-mammalian, sources of anticoagulant heparin. The heparin/heparan sulfate family of GAGs, prepared and purified from turkey intestine, were assayed for anticoagulant activity and structurally characterized. The resulting GAGs displayed a very low anticoagulant activity when compared to those obtained from porcine intestine using an identical procedure. Structural characterization studies clearly demonstrate that heparan sulfate is the major GAG in the turkey intestine. This observation is rationalized based on differences in the mammalian and avian coagulation and immune systems.

Keywords: Heparin; Heparin sulfate; Avian; Mammalian; Intestine

1. Introduction

Commercial manufacture of heparin relies on either porcine intestinal or bovine lung tissue as the raw material. These tissues are rich in mast cells, presumably resulting from the high foreign parasite burden in these tissues, e.g. bacteria and viruses (Vaheri, 1964; Regelson, 1968; Weiss, 1977). 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). Heparin exhibits anticoagulant activity primarily from its binding to the serine protease inhibitor (SERPIN) antithrombin III, which 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).

*Corresponding author. Tel.: +1-319-335-8834; fax: +1-319-335-6634.
E-mail address: Robert-linhardt@uiowa.edu (R.J. Linhardt).

1096-4959/03/$ - see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S1096-4959(02)00250-6

Porcine intestinal heparin has an antithrombin III pentasaccharide binding site containing an *N*-acetylated glucosamine residue, while the same residue in bovine heparin is substituted with an *N*-sulfo group (Loganathan et al., 1990). Although disaccharide compositional differences, between bovine and porcine heparin, can be used to distinguish the source of a batch of heparin, they cannot be used to determine if porcine heparin has been adulterated with small amounts of bovine heparin (Linhardt and Gunay, 1999). Porcine heparin also has problems associated with religious restrictions on its use. 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, non-mammalian sources for heparin. Avian species, such as chicken, have been shown to contain the heparin family of GAGs (Loganathan et al., 1990). Because of the widespread availability of turkey and the absence of any apparent use for turkey intestine, we turned our attention to the study of turkey intestine as potential new source of heparin.

2. Materials and methods

2.1. Materials

Fresh turkey and porcine small intestines were collected at the slaughter house and immediately preserved with 1.5% 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 and heparan sulfate, obtained from porcine intestine, were purchased from Celsus Laboratories Inc. (Cincinnati Ohio). 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, modified from that reported by Van Gorp (1995), was performed for the recovery of intestinal GAGs from both turkey and porcine intestines. Briefly, small intestine (600 g) was cut into small pieces and homogenized in a blender. The homogenate was adjusted to pH 9.0 by addition of 0.05 M sodium carbonate. Proteolytic digestion of homogenate was performed using alkalase (2.7 AU/ 100 g wet weight) at 50 °C for 12 h (Linhardt et al., 1992). 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 45 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 3 wt.% sodium chloride to elute residual peptides and low molecular weight contaminants. The resin was then eluted by 16 wt.% 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 1000) against deionized water overnight at 4 °C. The dialyzed material was freeze-dried then re-suspended in 20 mM Tris–HCl buffer (pH 8) containing 2 mM magnesium chloride and digested with endonuclease (2500 units/g) for 12 h at 37 °C (Furukawa and Terayama, 1977). After endonuclease digestion, sodium chloride concentration was brought to 16 wt.% and the GAGs were precipitated by adding methanol to 80 vol.%. The recovered precipitate was dissolved in 10 ml of de-ionized water and dialyzed overnight at 4 °C and freeze-dried. The dried GAG was then subjected for structural and functional characterization.

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 absorb-

ance 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) ^{1}H-NMR experiments were performed on a Bruker DRX-400 equipped with NMR Nuts (PC computer) processing and plotting software.

2.5. Enzymatic depolymerization of glycosaminoglycans

Turkey and porcine GAGs (20 mg/ml) were treated with chondriotin 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 5 min and the digested samples were desalted using microanalysis desalting spin column (Amika Corp®) and freeze dried. The chondroitinase digested samples were next heparinase lyase I, II, III digested (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 weight and the reaction was incubated in sealed tubes at 37 °C over a period of over 24 h. Standard porcine heparin and heparan sulfates were treated in similar manner to serve as controls. The reaction was boiled, desalted and freeze-dried as previously described in chondriotin lyase ABC digestion step.

2.6. Capillary electrophoresis mapping of resulting oligosaccharides

The experiments were performed with a capillary electrophoresis PACE 5500 system (Beckman Instruments, Fullerton, CA) at a constant capillary temperature of 18 °C with a potential of −15 kV and detected by UV absorbance at 232 nm. The electropherograms were acquired using the manufacturer's system Gold software package. The CE system was operated in the reverse polarity mode by applying the sample at the cathode and run with 20 mM phosphoric acid adjusted to pH 3.5 with saturated dibasic sodium phosphate as described previously (Pervin et al., 1994). Separation and analysis were carried out in a fused-silica capillary tube. This capillary was 75 μm inner diameter, 375 μm outer diameter, and 57 cm long, with a 50 cm effective length and was externally coated except where the tube passed through the detector. Prior to every run, the capillary was conditioned with 0.1 M NaOH (1 min, 20 psi) and rinsed (1 min, 20 psi) with running buffer. Samples were applied by pressure injection 5 s at 0.5 psi.

2.7. 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, 12% acrylamide) was prepared and run as described previously (Toida et al., 1997). 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. The gel was visualized by silver staining (Al-Hakim and Linhardt, 1991). After staining with alcian, blue gel was soaked several hours in 50% (v/v) aqueous methanol followed with distilled water for 2 h. Silver staining solution was freshly prepared by adding 2 ml of 4 M silver nitrate, 2 ml of 7.6 M sodium hydroxide solution and 2.8 ml of ammonium hydroxide solution to 193.8 ml of degassed glass distilled water. The gel was stained with gentle shaking for 1 h followed by 3 washes with distilled water over 30 min. The gel was developed in freshly prepared developing buffer prepared by adding 1 ml of 2.5% (w/v) citric acid and 250 μl of 38% (v/v) formaldehyde solution to 500 ml of distilled water. Bands start to develop within 1–15 min. The reaction was stopped by placing the gel in 5% (v/v) aqueous acetic acid solution containing 20% (v/v) methanol.

2.8. Anticoagulant activity assay

Heparin readily catalyzes the inactivation of factor Xa by antithrombin III. Factor Xa inactivation was used in this study to assess the anticoag-

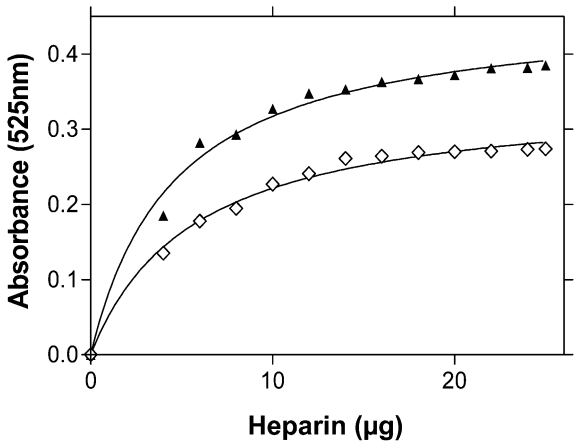

Fig. 1. Azure A analysis of GAG samples. Turkey intestinal GAG (◇) and standard porcine intestinal heparin (▲).

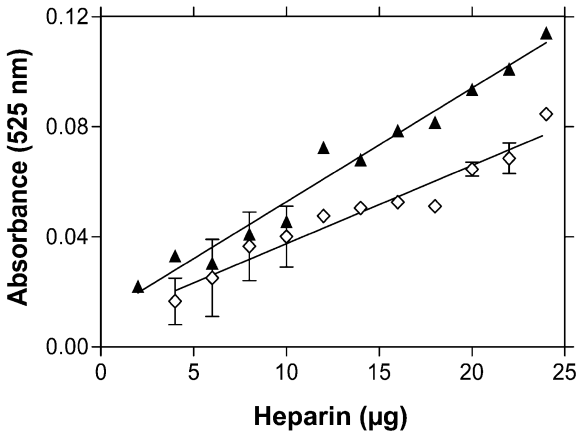

Fig. 2. Carbazole analysis of GAG samples. Turkey intestinal GAG (◇) and standard porcine intestinal heparin (▲).

ulant activity of the GAGs prepared from porcine and turkey intestines using a Heparin Assay Kit (Sigma). In this assay, when both factor Xa and antithromin III is 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 Lie, 1977).

3. Results and discussion

GAG was isolated from both porcine and turkey intestine by precipitation from the 16 wt.% sodium chloride eluent, from the SAX resin to which the GAG had been bound. Turkey small intestine afforded 307 mg GAG/kg small intestine (200 g small intestine/animal) compared to the 638 mg GAG /kg small intestine (4 kg small intestine/animal) obtained from pig.

Chemical characterization by Azure A and carbazol assays (Figs. 1 and 2) showed that turkey GAG contained only approximately half the negative charge that found on a standard porcine heparin. ^{1}H-NMR analysis (Fig. 3a) revealed the presence of signals corresponding to GlcNAc (*N*-acetyl methyl at 2.02 ppm, H-1 at 5.4 ppm) and H-1 and H-2 signals of GlcA at 4.48 and 3.35 ppm, respectively in turkey GAG. This strongly suggests that GAG obtained from turkey intestinal mucosa is heparan sulfate. In contrast, the presence of H-1 and H-2 signals of GlcNS at 5.5 and 3.25 ppm, respectively, and the lack (or small signal) of *N*-acetyl methyl signal of GlcNAc show that GAG isolated from porcine intesitinal mucosa is heparin (Fig. 3b).

In Fig. 4, turkey GAGs (lanes 3 and 4) show an undefined wide diffuse band without definite bands on the silver stained gel. Treatment with heparin lyases shows the disappearance of this diffuse staining without the appearance of new

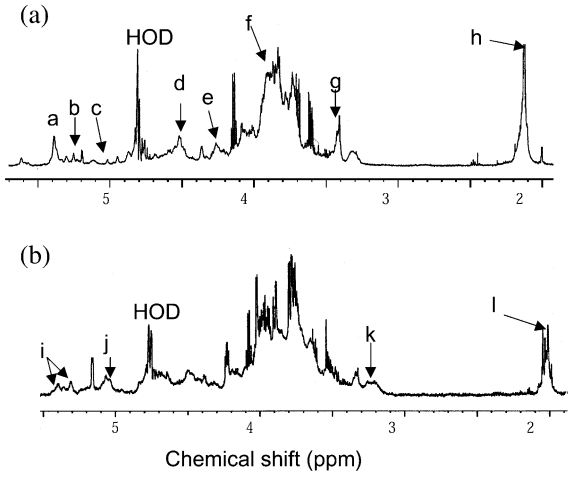

Fig. 3. ^{1}H-NMR spectroscopy of turkey (a) and porcine (b) intestinal GAGs. Intestinal GAGs (5 mg of each) were dissolved in D_2O, filtered and freeze-dried two times. One dimensional (1d) ^{1}H-NMR experiments were performed on a Bruker DRX-400 equipped with NMR Nuts (PC computer) processing and plotting software. In a: (a) H-1 GlcNAc; (b) H-1 IdoA2S; (c) H-1 IdoA; (d) H-1 GlcA; (e) H-6 GlcNAc6S; (f) H-6 GlcNAc; (g) H-2 GlcA; and (h) *N*-acetyl (methyl) GlcNAc. In b: (i). H-1 GlcNS; (j) H-1 Ido2S; (k) H-2 GlcNS; and (l) *N*-acetyl(methyl) GlcNAc.

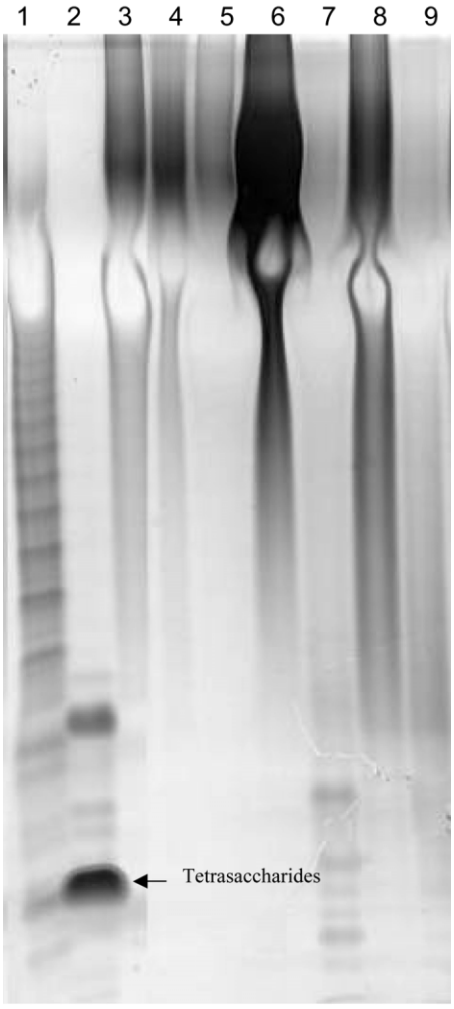

Fig. 4. Silver stained gradient polyacrylamide gel containing turkey and porcine intestinal GAGs. Lane 1 heparin-derived oligosaccharide standards enzymatically prepared from bovine lung heparin (Edens et al., 1992); lane 2 heparin hexasulfated tetrasaccharide standard (Pervin et al., 1995); lane 3 crude turkey intestinal GAG; Lane 4 treatment of crude turkey intestinal GAG with chondroitin ABC lyase; lane 5 treatment of crude turkey intestinal GAG with chondroitin ABC lyase and heparin lyases; lane 6, standard porcine intestinal heparin; lane 7, treatment of standard porcine intestinal heparin with heparin lyases; lane 8, standard porcine intestinal heparan sulfate; lane 9, treatment of standard porcine intestinal heparan sulfate with heparin lyases.

bands, due to an inability to detect undersulfated heparan sulfate oligosaccharides (lanes 5 and 9). In contrast, heparin shows a more intesnse diffuse band (lane 6) that on treatment with heparin lyases shows defined banding attributable to highly sulfated heparin oligosaccharides. Next, both turkey and porcine intestine GAGs were completely digested using a mixture of heparin lyases and analyzed by capillary electrophoresis (Fig. 5). The CE analysis of the resulting disaccharides showed that the turkey intestinal GAG gave an electropherogram (Fig. 5a) nearly identical to that observed when standard porcine intestinal heparan sulfate was digested with the same enzymes (Fig. 5c). As expected, digestion of porcine intestinal GAG gave an electropherogram (Fig. 5b) nearly identical to that obtained on digestion of standard porcine intestinal heparin (Fig. 5d). The most prominent, rapidly migrating peak in the CE analysis of the porcine GAG corresponded to the trisulfated disaccharide (peak 1 in Fig. 5b,d). This disaccharide is known to comprise the major sequence unit in heparin. In contrast, turkey GAG and standard heparan sulfate afforded a prominent slowly migrating broad peak (peak 4 in Fig. 5a,c), corresponding to unsulfated disaccharide product. This disaccharide is known to be the major sequence unit in heparan sulfate. Based on these data, it was clear that turkey intestine primarily contains heparan sulfate while porcine intestine primarily contains heparin. These results on the GAG composition of turkey intestine differ from those reported by Bianchini et al. (1997), who found the most prominent disaccharides in the chicken intestinal GAG is the trisulfated disaccharide ($>50\%$ of the total disaccharide). We can find no reports on the role of mast cells in birds.

The anticoagulant activity of the GAG prepared from turkey, as measured by factor Xa amidolytic assay, showed significantly ($P<0.05$) lower activity (16.6 ± 3.6 U/mg) than that of the GAG prepared under the same conditions from pig (111.5 ± 21.7 U/mg) (Fig. 6). Differences in the recovery and anticoagulant activity of the porcine intestinal GAG in the current study differ slightly from that prepared from porcine by Griffin et al. (1995), due to the differences in the starting material (the intestine used in the current study was not washed and contained fecal material).

Detailed studies on heparin have been limited to mammalian species (Einnarsson and Andersson, 1977; Rosenberg and Lam, 1979). While heparin resides exclusively in mast cells, anticoagulant heparan sulfate, residues in proteoglycans that line the lumen of the vascular endothelium. These heparan sulfate chains contain the critical antithrombin III pentasaccharide binding site required for antithrombin III-based inhibitor of thrombin

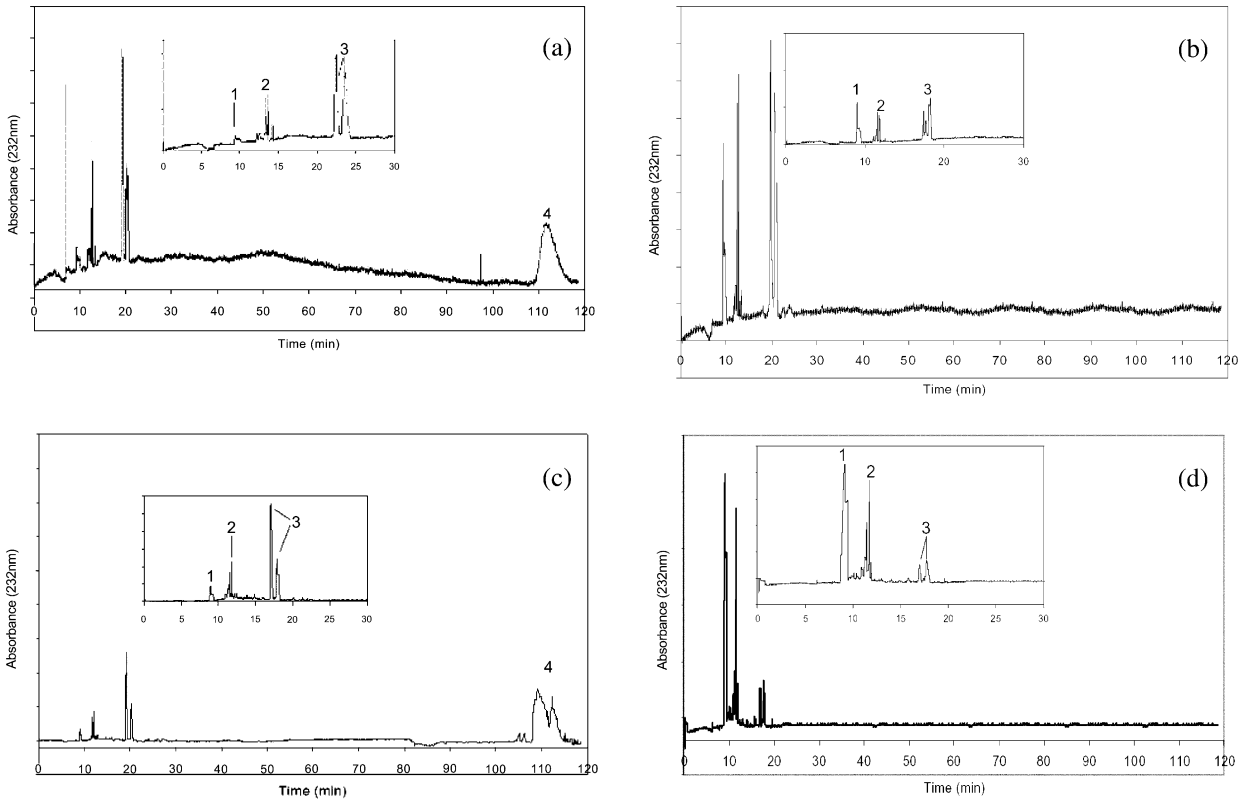

Fig. 5. Capillary electrophoresis disaccharides prepared by exhaustive treatment of GAG with heparin lyases: (a) turkey intestinal GAG, (b) porcine intestinal intestinal GAG, (c) standard porcine intestinal heparan sulfate, (d) standard porcine intestinal heparin. The inset shows a second electropherogram expanded over the first 30 min of analysis. Note that there is a slight variation in migration times and intensities between runs, which is commonly observed in CE. Peak 1 is highly sulfated disaccharide (ΔUA2S-(1→4)-α-D-GlcNS6S), (where ΔUA is 4-deoxy-a-L-*threo*-hexo-4-enopyranosyluronic acid), peaks labeled 2 correspond to disulfated disaccharides (ΔUA2S-(1→4)- α-D-GlcNAc6S, ΔUA-(1→4)- α-D-GlcNS6S, ΔUA2S-(1→4)- α-D-GlcNS), peaks labeled 3 correspond to monosulfated disaccharides (ΔUA-(1→4)- α-D-GlcNAc6S, ΔUA2S-(1→4)- α-D-GlcNAc, ΔUA-(1→4)- α-D-GlcNS), peak 4 is the unsulfated disaccharide (ΔUA-(1→4)- α-D-GlcNAc).

and factor Xa. There is also evidence suggesting major differences in the coagulation mechanisms of avian and mammalian species. While there are no reports on the mechanism of blood homeostasis or the presence of heparin in turkey, heparin is reportedly absent in seven species of fish and two different species of birds (Hovingh et al., 1986). There are some studies on chicken that can serve as an avian reference model for our discussion. Bianchini et al. (1997) reported considerable heparin in chicken intestine, however, the anticoagulant activity of this isolated heparin was not reported. Analysis of chicken intestinal heparin provided to our laboratory showed both anticoagulant activity and oligosaccharide sequences consistent with the presence of an antithrombin III pentasaccharide binding site (Loganathan et al., 1990)

Antithrombin III and heparin form an interdependent regulatory mechanism in coagulation processes. Avian antithrombin III was first isolated and studied by Koide et al. (1982). Their data showed the sequence of chicken antithrombin III amino-terminal region is homologous with those of mammalian antithrombin III. The authors reported that chicken antithrombin III had no immunological cross-reactivity with mammalian antithrombin III (Kurachi et al., 1976). However, chicken antithrombin III inhibits clotting activity of human thrombin in time dependent manner in absence of heparin and inhibition is markedly accelerated in presence of heparin, suggesting that

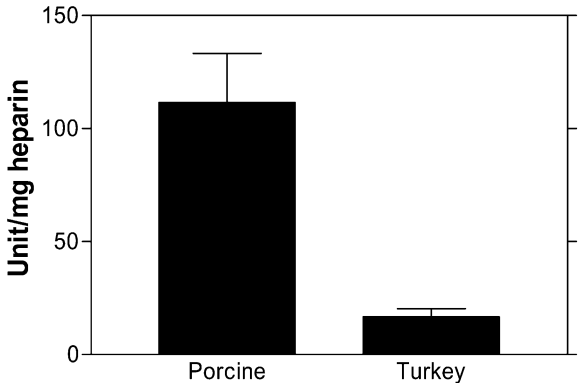

Fig. 6. Comparison of the anti-factor Xa activities of porcine intestinal and turkey intestinal GAGs. The anti-factor Xa of porcine GAG is 111.5±21.7 (mean±S.E.M.). This value is significantly higher ($P<0.5$) than that reported in turkey GAG (16.6±3.6). In the assay standard intestinal porcine heparin (180 unit/mg) was used for preparation of standard curve. The experiment was repeated at least 5 times and S.E.M. is the standard errors of the mean values.

chicken antithrombin III is functionally similar to mammalian antithrombin III in both mechanism of action of action on heparin binding. Hen antithrombin III was isolated (Jordan, 1983) by affinity chromatography on immobilized porcine heparin from birds. The purified avian inhibitor (Mr ~60 000) showed physical and functional homologies to human antithrombin III-like heparin-enhanced inhibition of both bovine thrombin and human Factor Xa. Also, the heparin-binding interaction of avian antithrombin III is highly selective demonstrating the same rigid specificity for heparin species fractionated on the basis of their affinity for human antithrombin.

There are also remarkable difference between mammal and avian coagulation system. Sørbye (1962) identified 19 separate clotting factors in chicken plasma and associated 11 of them with extrinsic functions. Thus, an activity associated with a single factor in mammalian coagulation may be several factors in birds or vice versa. Coagulation of blood in chicken is considered the result of an extrinsic clotting system, initiated, as in mammals, by tissue thromboplastin released from injured tissues. Older literature concerning intrinsic hemostasis has generally described as having little if any negligible importance, in avian species (Ratnoff and Rosenblum 1958; Didisheim et al., 1959; Bigland and Triantaphyllopoulos, 1960; Bigland, 1964; Stopforth, 1970; Archer, 1971). More recent evaluation of intrinsic coagulation in chicken (Doerr and Hamilton, 1981) provided evidence of a functioning intrinsic clotting mechanism, however these intrinsic activities were lower than those of mammals whose coagulation system is primarily intrinsic. It is generally accepted that the intrinsic system in birds plays a subsidiary role in the overall coagulation function in comparison to the extrinsic system. The requirement of heparin/heparan sulfate to counteract the intravascular coagulation process in birds may not be as essential as in mammals, as the avian coagulation cascade depends mainly on the extrinsic system. These findings in addition to low blood hematocrit value reported in some birds (Didisheim et al., 1959) could explain the minor role of heparin/heparan sulfate as an endogenous regulator of anticoagulation. The more effective tissue thrompoplastin (extrinsic factor) operates in the presence of a very low level of heparin/heparan sulfate to preserve the overall coagulation mechanism and compensate the slow reacting intrinsic mechanism in birds. This gives protection in birds, having fast heart rates, against possibility of intravascular rupture.

One suggested role for heparin in mammalian mast cell rich tissues, such as intestine and lung, is to fight external parasites (Straus et al., 1982a,b). The absence of heparin in intestinal mucosa of turkey may be due to the different types of parasites that infect avian species. Heparin may also be a factor enhancing the killing of parasites by dendritic cells in mammalian hosts. The defense system for avian species may simply rely on different mechanisms to handle parasitic infections.

From this study we conclude that predominant GAG in turkey intestine is heparan sulfate, which closely resembles heparan sulfate isolated as a major side product in the production of porcine intestinal heparin. The low levels of heparin in turkey, and its greatly reduced anticoagulant activity, may result from differences in the importance of intrinsic mechanism of blood coagulation in avian species. The absence of heparin in intestinal tissue may also reflect characteristic differences in turkey and mammalian intestine. Differences in mast cell types and distribution in avian and mammals might also explain the resistance of avian species to intestinal parasites that infect mammals as these might require heparin for proper binding preceding infection.

Acknowledgments

The authors thank the National Institutes of Health for supporting this research in the form of grants HL 52622 and GM38060.

References

Al-Hakim, A., Linhardt, R.J., 1991. Electrophoresis and detection of nanogram quantities of exogenous and endogenous glycosaminoglycans in biological fluids. Appl. Theoret. Electrophoresis 1, 305–312.

Archer, R.K., 1971. Blood coagulation. In: Bell, D.J., Freeman, B.M. (Eds.), Physiology and biochemistry of domestic fowl, Vol. 2. Academic Press, New York, pp. 897–911.

Bianchini, P., Liverani, L., Mascellani, G., Parima, B., 1997. Heterogeneity of unfractionated heparins studied in connection with species, source and production processes. Sem. Thromb. Hemost. 23, 3–10.

Bigland, C.H., Triantaphyllopoulos, D.C., 1960. A re-evaluationof clotting time of chicken blood. Nature 186, 644.

Bigland, C.H., 1964. Blood clotting times of five avian species. Poult. Sci. 43, 1035–1039.

Bitter, T., Muir, H.M., 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4, 330–334.

Didisheim, P., Hattori, K., Lewis, J.H., 1959. Hematologic and coagulation studies in various animal species. J. Lab. Clin. Med. 53, 866–875.

Doerr, J.A., Hamilton, P.B., 1981. New evidence for intrinsic blood coagulation in chickens. Poult. Sci. 60, 237–242.

Edens, R.E., Al-Hakim, A., Weiler, J.M., Rethwisch, D.G., Fareed, J., Linhardt, R.J., 1992. Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low-molecular-weight heparin derivatives. J. Pharm. Sci. 81, 823–827.

Einnarsson, R., Andersson, L.O., 1977. Binding of heparin to human antithrombin III as studied by measurements of tryptophan fluorescence. Biochim. Biophys. Acta 490, 104–111.

Grant, A.C., Linhardt, R.J., Fitzgerald, G., Park, J.J., Langer, R., 1984. Metachromatic Activity of Heparin and Heparin Fragments. Anal. Biochem. 137, 25–32.

Griffin, C.C., Linhardt, R.J., Van Gorp, C.L., Toida, T., Hileman, R.E., Schubert, R.L., Brown, S.E., 1995. Isolation and characterization of heparan sulfate from crude porcine intestinal mucosa peptidoglycan heparin. Carbohydr. Res. 276, 183–197.

Furukawa, K., Terayama, H., 1977. Isolation and identification of glycosaminoglycans associated with purified nuclei from rat liver. Biochim. Biophys. Acta 499, 278–289.

Hovingh, P., Piepkorn, M., Linker, A., 1986. Biological implications of the structural, antithrombin affinity and anticoagulant activity relationships among vertebrate heparins and heparan sulfates. Biochem. J. 237, 573–581.

Jordan, R.E., 1983. Antithrombin in vertebrate species: conservation of the heparin-dependent anticoagulant mechanism. Arch. Bioch. Biophys. 227, 587–595.

Jordan, R.E., Favreau, L.V., Braswell, E.H., Rosenberg, R.D., 1982. Heparin with two binding sites for antithrombin or platelets factor 4. J. Biol. Chem. 257, 400–4006.

Jordan, R.E., Oosta, G.M., Gardner, W.T., Rosenberg, R.D., 1980a. The binding of low-molecular-weight to hemostatic enzymes. J. Biol. Chem. 255, 10073.

Jordan, R.E., Oosta, G.M., Gardner, W.T., Rosenberg, R.D., 1980b. The kinetics of hemostatic enzyme-antithrombin interactions in the presence of low molecular-weight heparin. J. Biol. Chem. 255, 10081.

Koide, T., Ohta, Y., Odani, S., Ono, T., 1982. Chicken antithrombin. Isolation, characterization, and comparison with mammalian antithrombins and chicken ovalbumin. J. Biochem. 91, 1223–1229.

Kurachi, K., Schmer, G., Hermodson, M.A., Teller, D.C., Davie, E.W., 1976. Biochemistry 15, 368–373.

Linhardt, R.J., Ampofo, S.A., Fareed, J., Hoppensteadt, D., Mulliken, J.B., Flokman, J., 1992. Biochemistry 31, 12441–12445.

Linhardt, R.J., Gunay, N.S., 1999. Production and chemical processing of low molecular weight heparins. Sem. Thromb. Hemost. 25, 5–16.

Loganathan, D., Wang, H.M., Malli, L.M., Linhardt, R.J., 1990. Structural variation in the antithrombin III binding site region and its occurance in heparin from different sources. Biochemistry 29, 4362–4368.

Pervin, A., Al-Hakim, A., Linhardt, R.J., 1994. Separation of glycosaminoglycan-derived oligosaccharide by capillary electrophoresis using reverse polarity. Anal. Biochem. 221, 182–188.

Pervin, A., Gallo, C., Jandik, K.A., Han, X., Linhardt, R.J., 1995. Preparation and structural characterization of large heparin-derived oligosaccharides. Glycobiology 5, 83–95.

Ratnoff, O.D., Rosenblum, M., 1958. Role of Hageman factor in the initiation of clotting by glass. Am. J. Med. 25, 160–168.

Regelson, W., 1968. The antimitotic activity of polyanions (antitumor, antiviral and antibacterial action of heparin, heparinoids, anionic dyes, and synthetic polymers). In: Goldin, A., Hawing, F., Schmitzer, R.J. (Eds.), Advances in Chemotherapy 3. Academic Press, NewYork, pp. 303–307.

Rosenberg, R.D., Lam, L.H., 1979. Correlation between structure and function of heparin. Proc. Natl. Acad. Sci. USA 76, 1218–1222.

Schonberger, L.B., 1998. New variant Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. Infect. Dis. Clin. North Am. 12, 111–121.

Sørbye, O., 1962. Studies on the coagulation of chicken blood. IX. Labile factor activity of fresh oxalated plasma: the combined activity of sixteen discrete labile coagulation factors. Acta Chem. Scand. 16, 2411–2420.

Stopforth, A., 1970. A study of coagulation mechanisms in domestic chickens. J. Comp. Pathol. 80, 525–533.

Straus, A.H., Nader, H.B., Dietrich, C.P., 1982a. Absence of heparin or heparin-like compounds in mast-cell-free tissues and animals. Biochim. Biophy. Acta 717, 478–485.

Straus, A.H., Nader, H.B., Takahashi, H.K., Dietrich, C.P., 1982b. Ontogeny of heparin in mammals: a correlation with the appearance of mast cells in tissues. Anais da Academia Brasileira de Ciencias 54, 439.

Toida, T., Shima, M., Azumaya, S., Maruyama, T., Toyoda, H., Imanari, T., Linhardt, R.J., 1997. Detection of glycosaminoglycans as a copper (II) complex in HPLC. J. Chromat. 787, 266–270.

Teien, A.N., Lie, M., Abildgaard, U., 1976. Assay of heparin in plasma using a chromogenic substrate. Throm. Res. 8, 413–416.

Teien, A.N., Lie, M., 1977. Evaluation of an amidolytic heparin assay method: increased sensitivity by adding purified antithrombin III. Throm. Res. 10, 399–410.

Vaheri, A., 1964. Heparin and related polyionic substances as virus inhibitors. Acta Pathol. Microbiol. Scand. Suppl. 171, 7.

Van Gorp, C.L., 1995. Clin. Hemost. Rev. 9, 17–18.

Weiss, L., 1977. The thymus. The Blood Cells and Hematopoietic Tissues. McGraw Hill, USA, pp. 503–507.