Heparin binding peptides co-purify with glycosaminoglycans from human plasma

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Abstract Glycosaminoglycans (GAGs) are complexed with plasma proteins and proteolysis of plasma reduced the protein–GAG ratio about 140-fold. After dialysis, analysis by gradient PAGE revealed heparinase-1-sensitive GAGs, thus suggesting that heparin could be among the plasma GAGs. However, after dialysis most of the plasma GAGs were still not 'free'. PAGE of peptides resistant to proteolysis showed high molecular weight bands on the two sides of the dialysis membrane despite the 3.5 kDa molecular weight cut-off. Progressive dilution of the sample allowed passage of peptides appearing as high molecular weight bands in the diffusate. We interpret this phenomenon as the presence of low molecular weight peptides that aggregate when concentrated. Peptides on both sides of the membranes bound heparin.

Key words: Heparin; Glycosaminoglycan; Peptide; Human plasma

1. Introduction

A major hindrance in characterizing plasma glycosaminoglycans (GAGs) is their interaction with proteins [1]. Purification of GAGs from plasma is quite tedious and controversy exists about the molecular species actually present and their physiological role [2–4]. Heparin was detected in normal human plasma [5,6] and under pathological conditions [7–9]. Thus, it would be interesting to characterize the proteins that bind GAGs in human plasma in order to ascertain whether this binding might have a role in the maintenance of hemostatic balance. Purification of GAGs from plasma requires extensive proteolysis which yields 100% of circulating GAGs in the supernatant of digested plasma [10]. However, some peptides still co-purify with plasma GAGs as if they were protected from proteolysis by association with GAGs [6]. We hypothesize that these peptides may represent those naturally interacting with plasma GAGs and we examined their characteristics. Thus, the aim of this research is to begin the characterization of those peptides that complex plasma GAGs under physiological conditions and make their purification from plasma quite difficult to reproduce.

2. Materials and methods

[^{3}H]Heparin (specific activity 440 μC/mg, MW 6.0–20.0 kDa) was from New England Nuclear (Boston, MA, USA). Heparin EP 756 from bovine intestinal mucosa, low molecular weight heparin and oligo-heparin were from Opocrin Research Laboratories (Modena, Italy). Properties of heparins are reported in Table 1. The degree of sulfation, expressed as molar ratio (Mr) of sulfate and carboxyl groups, was determined by potentiometric analysis [11]. Activated partial thromboplastin time (APTT) was measured as described [12]. Anti-activated coagulation factor X (AXa) activity was tested in a chromogenic assay [13]. Chondroitin sulfate A, heparinase I (from *Flavobacterium heparinum*, EC 4.2.2.7), heparitinase (heparan sulfate lyase from *F. heparinum*, EC 4.2.2.8.) and chondroitinase ABC (EC 4.2.2.4) were from Sigma Chem. Co., St. Louis, MO, USA. Titan III Zip Zone cellulose acetate plates were from Helena Laboratories (Beaumont, TX, USA). Silver nitrate was from Merck (Darmstadt, Germany), Azur A from BDH (Poole, UK) and Alcian blue from Fisher Scientific Co. (Pittsburgh, PA, USA). Reagents for SDS-PAGE were from Bio-Rad (Hercules, CA, USA). Centricon concentrators (MW cut-off 3.0 kDa) were from Amicon, Inc., Beverly, MA, USA; dialysis membranes (MW cut-off 3.5 kDa) were from Spectrum (Breda, The Netherlands). Human plasma from the local blood bank was subjected to proteolysis as described [6]. Protein concentration was determined by the method of Bradford [14] using bovine serum albumin as a standard. Hexuronic acid was measured as described [15]. Cellulose acetate electrophoresis was performed at pH 1.0 [16]. 2 μl of aqueous solution of heparin of 13.0 kDa and chondroitin sulfate at a concentration of 200 μg/ml was used as standard. Aliquots of 20 μl of the plasma-derived samples were 10-fold concentrated and analyzed by electrophoresis. SDS-PAGE was performed according to Laemmli [17] and gels were stained with Coomassie brilliant blue. Gradient PAGE was performed as described [18] and staining was performed with Azur A and silver nitrate. 0.5 ml of the dialyzed supernatant of digested plasma was lyophilized, solubilized in 100 μl of 0.1 M sodium acetate, 0.1 mM calcium acetate, pH 7.0 and incubated with heparinase I (10 U) for 24 h at 37°C. The sample was dried, dissolved in 20 μl of distilled water and analyzed on gradient PAGE. The GAG content in the incubation mixture of digested plasma with heparinase I, as determined according to Volpi et al. [5], was approximately 5 μg. Aliquots of 100 μl of the dialyzed supernatant of digested plasma were incubated at 37°C for 24 h with the following enzymes: chondroitinase ABC (0.1 U), heparinase I (2 U) and heparitinase (0.1 U). Afterwards, the samples were dried, dissolved in 10 μl of distilled water and 2 μl was analyzed on cellulose acetate electrophoresis. Aliquots of clear supernatant of digested plasma were dialyzed against 10 or 100 volumes of distilled water using a Spectrapore membrane (cut-off 3.5 kDa) for 24 h at 4°C, or, alternatively, were subjected to ultrafiltration on Centricon membranes (cut-off 3.5 kDa). The sample was diluted with 10 or 100 volumes of distilled water before centrifugation. The diffusate, the filtrate and the retentate were collected, concentrated and analyzed on SDS-PAGE. After dialysis of supernatant of digested plasma against 100 volumes of distilled water, [^{3}H]heparin binding experiments were performed on

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Abbreviations: GAG, glycosaminoglycan; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; MW, molecular weight; EP 756, heparin; LMW 2123/850, low molecular weight heparin; Oligo-H 2000, oligo-heparin

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both diffusate and retentate. Aliquots of 20 μl of 0.1% human serum albumin, 20 μl of human plasma, 20 μl of supernatant of digested plasma, 20 μl of the retentate, 2 ml of the diffusate, were lyophilized, dissolved in 100 μl of phosphate buffered saline (PBS), pH 7.4 and incubated on 96-well microtiter plates overnight at 37°C. The next day, PBS was removed and the wells were preincubated with 100 μl of PBS, pH 7.4 containing 1% (w/v) human serum albumin for 30 min at room temperature. We used human serum albumin instead of the detergents to minimize non-specific binding. The wells were rinsed three times with the same buffer. [^{3}H]Heparin binding was with 1×10^6 dpm/dish in 100 μl of the binding buffer (1 μM [^{3}H]heparin (440 μC/mg) in PBS) containing 1% human serum albumin and 0.01% sodium azide in the absence or presence of a 100-fold excess of unlabelled ligand. Non-specific binding was negligible (5% of total binding). After 2 h incubation at 37°C, the coated plates were washed five times with cold PBS (100 μl), then solubilized in 4 M GdHCl overnight at 37°C. Radioactivity was determined by liquid scintillation counting. Utilizing a Bio-Rad Model 422 Electro-Eluter, peptides were electroeluted at room temperature from gel slices into 0.25 ml of 0.025 M Tris, 0.192 M glycine, pH 8.3, 0.1% (w/v) SDS at the cathode. Aliquots of the electroeluates were analyzed for amino acid composition and sequencing. Amino acid analysis was performed on 6 M HCl hydrolysate using a Carlo Erba 3A29 Amino Acid Analyzer with a computing integrator. Hydrolysis was carried out in vacuum-sealed vials at 110°C for 24 and 48 h. Values for Ser and Thr were corrected for loss during hydrolysis. The Milligen Protein Sequencer model 6600 was used for sequence determination.

3. Results and discussion

We previously reported that exhaustive proteolysis of murine plasma with a cocktail of proteases, released 100% of ^{35}S radioactivity from ^{35}S-labelled plasma, thus demonstrating that GAGs are complexed with plasma proteins [10]. Here, we performed the same procedure using normal human plasma. Results of a typical procedure of plasma digestion are: 20 ml of plasma containing 69.5 mg/ml of protein yielded 15 ml of supernatant of digested plasma containing 0.66 mg/ml of protein: the protein–GAG ratio was reduced about 140-fold. This protein–GAG ratio derives from quantification of GAGs as release of ^{35}S radioactivity [10]; thus, classical determination of uronic acid content [15] was not feasible as the amount of proteins prevented development of a reproducible colorimetric reaction. However, these GAGs were not pure and electrophoresis on cellulose acetate did not reveal GAGs co-migrating with appropriate GAG standard (Fig. 1A). Gradient PAGE (24–30%) was not feasible because of the presence of large amounts of small plasma molecules. Some of these molecules could be removed by dialysis, which reduced the protein content of the supernatant from 0.66 to 0.27 mg/ml: gradient PAGE after dialysis revealed heparinase-1-sensitive GAGs, suggesting that heparin could be among these GAGs (Fig. 1B). However, even after dialysis, most of the plasma GAGs were not 'free' as evidenced by cellulose acetate electrophoresis (Fig. 1A). This figure shows Alcian blue-positive bands migrating more slowly than standard GAGs, but does not reveal any compound co-migrating with standard heparins. These results could be explained considering that

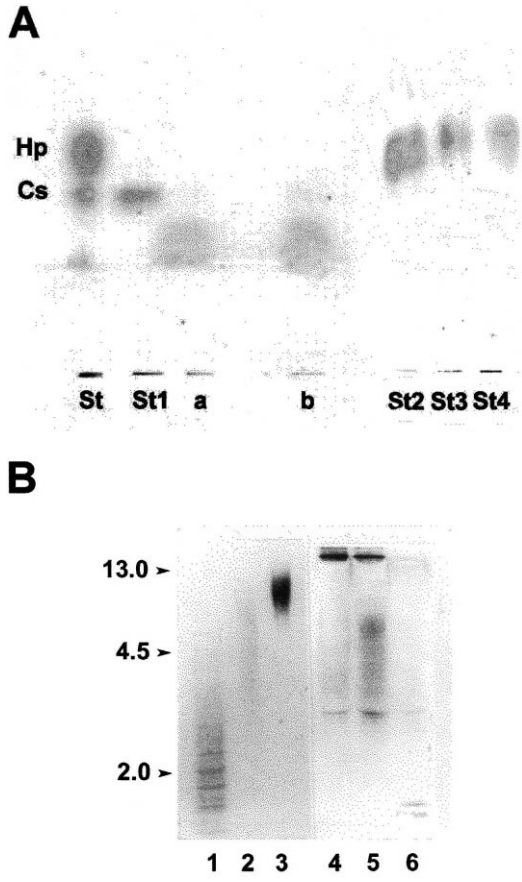

Fig. 1. A: Cellulose acetate electrophoresis at pH 1.0 of the supernatant of digested plasma before (a) and after dialysis (b) (the starting samples were 10-fold concentrated before electrophoresis); standard heparin (Hp) and chondroitin sulfate A (Cs) are run together in St; chondroitin sulfate A alone is in lane St1; St2, heparin HP 756 (13.0 kDa); St3, low molecular weight heparin (4.5 kDa); St4, oligo-heparin (2.0 kDa). B: Gradient PAGE (24–30%) and silver staining of standard heparins of different molecular weights (lanes 1–3) and of supernatant of digested plasma after dialysis (lane 5) (0.5 ml was concentrated at 20 μl for suitable application on the gel). Lane 1: standard heparin-derived oligosaccharides (4 μg), mean MW 2.0 kDa; lane 2: standard low molecular weight heparin (4 μg), mean MW 4.5 kDa; lane 3: standard heparin EP 756 (10 μg), mean MW 13.0 kDa; lane 4: sample 5 treated with heparinase I; lane 6: standard heparin EP 756 treated with heparinase I.

GAGs co-purify with peptides that impair migration on cellulose acetate electrophoresis; however, the selective entrapping properties of 24–30% acrylamide separated peptides from GAGs enabling indirect detection of heparin (Fig. 1B). A silver stain-positive band at about 13 kDa was positive to Coomassie blue (not shown) and it could represent peptides stopped in acrylamide at the top of the running gel. These results suggest that GAGs are non-covalently bound to plas-

Table 1
Properties of heparins used in the experiments

	MW (kDa)	SO_3/COO^- (Mr)	APTT (IU/mg)	AXa (IU/mg)
EP 756	12.9	2.15	164	114
LMW 2123/850	4.5	2.19	37	86
Oligo-H 2000	2.1	2.03	1	50

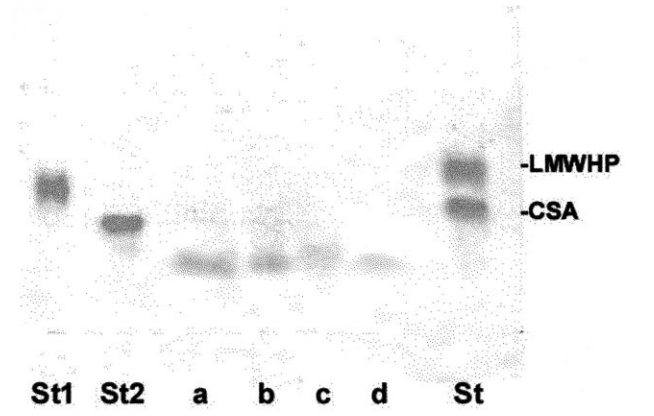

Fig. 2. Cellulose acetate electrophoresis at pH 1.0. St1, St treated with chondroitinase ABC; St2, St treated with heparinase I; a, the dialyzed supernatant of digested plasma; b, a treated with chondroitinase ABC; c, a treated with heparitinase; d, a treated with heparinase I; St, low molecular weight heparin (LMWHP) and chondroitin sulfate A (Cs).

ma peptides. Fig. 2 shows sensitivity to chondroitinase ABC and heparinase I of the band migrating more slowly than standard chondroitin sulfate in cellulose acetate electrophoresis. Densitometric analysis revealed an approximately 40% decrease after treatment with each enzyme suggesting that the highly sulfated GAGs in human plasma [5] were slowed by interacting peptides, thus co-migrating together with undersulfated chondroitin sulfate. A material positive to Ponceau red staining was detectable in cellulose acetate electrophoresis in proximity of the starting point of application (not shown).

We analyzed on SDS-PAGE the protein material of digested plasma, the material retained by the dialysis membrane and the dialyzed material (Fig. 3A); high MW bands were evident on the two sides of the membrane despite the 3.5 kDa MW cut-off. The protein content ratios between retentate and diffusate were 3 and 0.4 respectively in sample dialyzed against 10 or 100 volumes of water indicating that dilution favored diffusion of peptides. The band of supposedly 56 kDa decreased in the retentate of the diluted sample and was detectable in the diffusate where no peptides of low MW could be detected; the band at 20 kDa did not appear to cross the membrane. We repeated the same experiment using a Centricon concentrator with the same MW cut-off, but at different dilutions of the sample. Fig. 3B shows that dilution allowed

Table 2
Amino acid composition

Amino acid	Residue %
Aspartic acid	10.8
Serine	2.8
Proline	n.d.
Alanine	4.5
Methionine	1.4
Leucine	9.1
Phenylalanine	5.8
Histidine	2.8
Tryptophan	n.d.
Threonine	2.3
Glutamic acid	17.0
Glycine	21.1
Valine	4.6
Isoleucine	5.7
Tyrosine	2.8
Lysine	6.2
Arginine	3.4
Total	100.3

Results are expressed as amino acid residue %. Values for Ser and Thr were determined by hydrolysis at 110°C for 24 and 48 h in duplicate and extrapolated to zero time.

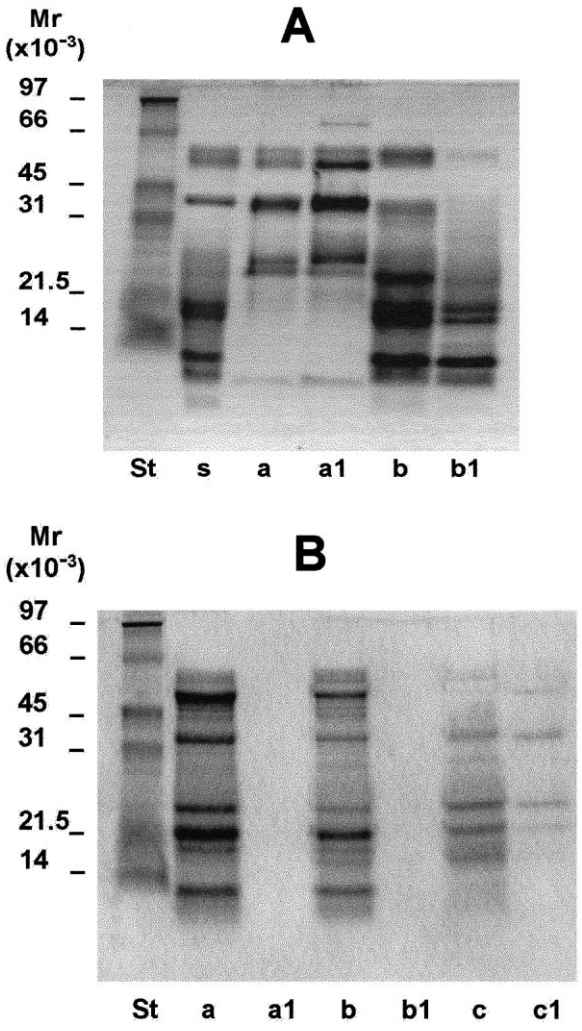

Fig. 3. A: SDS-PAGE (15%) of supernatant of digested plasma before (s) and after dialysis against 10 volumes (b) or 100 volumes (b1) of distilled water. Lanes a and a1 show the peptides in diffusate of samples b and b1. Lanes s, a, a1, b and b1 contained respectively 20 μg, 16 μg, 20 μg, 50 μg and 13 μg of protein in the following volumes: 30 μl, 1.0 ml, 5 ml, 0.1 ml and 50 μl. Each sample was lyophilized and dissolved in 20 μl of distilled water for application on the gel. B: SDS-PAGE (15%) of supernatant of digested plasma subjected to ultrafiltration on a Centricon membrane. Lanes a, b and c show the electrophoretic pattern of peptides in the retained fraction of undiluted sample (a), and of sample diluted 10-fold (b) and 100-fold (c). Lanes a1, b1 and c1 show the peptides in the filtrate fraction of undiluted sample (a1), and of the sample diluted 10-fold (b1) and 100-fold (c1). After centrifugation, the volume of the retentate was reduced to 50% of the starting sample. 15 μl of starting sample was used in lanes a and a1, 100 μl in lanes b and b1, 1 ml in c and c1. Each sample was lyophilized and dissolved in 20 μl distilled water for application on the gel. Lanes a, b, c and c1 contained respectively 20 μg, 15 μg, 9 μg and 6 μg of protein. In samples loaded on lanes a1 and b1 protein content was undetectable. St, standard proteins: phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14 kDa).

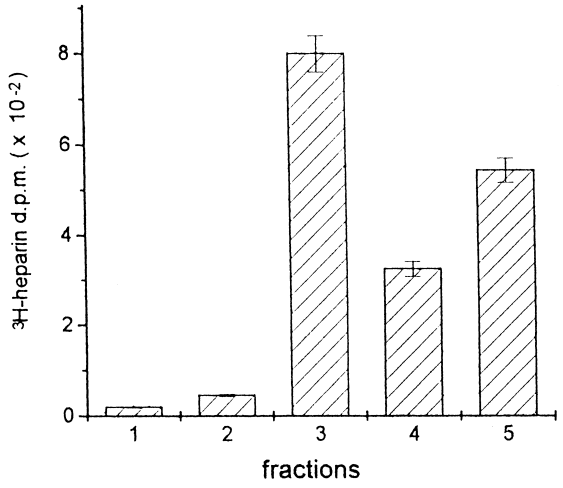

Fig. 4. [^{3}H]Heparin binding to plasma-derived fractions. Binding experiments were performed on 96-well plastic cell culture dishes coated with 20 μl of 0.1% human serum albumin (1), 20 μl of undigested plasma (2), 20 μl of the supernatant of the digested plasma (3), 20 μl of the retentate (4) and 2 ml of the diffusate against 100 volumes of distilled water for 24 h at 4°C, which was previously lyophilized and then dissolved in 20 μl (5). Samples 3, 4 and 5 contained respectively 15 μg, 6 μg and 9 μg of protein. Results are means ± S.D. of triplicate samples. This experiment is representative of three others which gave qualitatively identical results.

passage of peptides that appeared as high MW bands on SDS-PAGE. We interpret these data as the presence of low MW peptides that aggregate when concentrated; they pass through the dialysis membrane, but they pass through Centricon only if diluted. Because SDS-PAGE required concentration, these peptides passed through the membrane might re-aggregate and appear as high MW bands. Peptides could aggregate despite denaturing conditions because they are 'protected' by highly negatively charged GAGs of low MW passing through the membrane (not shown). Peptides on both sides of the dialysis membrane bound heparin (Fig. 4). We suggest that aliquots of peptides bound to heparin retained by the membrane were not removed by dialysis and were responsible for the impairment of heparin migration in cellulose acetate electrophoresis (Fig. 1A). The band of apparent high MW (56 kDa), able to 'pass through' the dialysis membrane, was electroeluted and analyzed in terms of amino acid content and sequence; Table 2 shows the amino acid composition of this material. Sequence determination was unsuccessful: no specific pattern was observed as if there was a variety of peptides with different MW

In conclusion, we demonstrate that GAGs from normal human plasma associated with peptides that aggregate when concentrated. Some of these peptides were removed by dialysis, as if their assembly was dependent on concentration and appeared resistant to SDS. We hypothesize that they are part of native supramolecular structure(s) in which GAGs, including heparin, are vehicled in human plasma; alternatively, they could be generated by proteolysis of plasma proteins. Dilution of these peptides with loss of aggregation might play a role in the interaction between GAGs and endothelial cells [16] or platelets where dilution causes the release of heparin [19].

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