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Electrophoretic separation and characterization of urinary glycosaminoglycans and their roles in urolithiasis

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Abstract—Urinary polyanions recovered from the urine samples of kidney stone-formers and normal controls were subjected to preparative agarose gel electrophoresis, which yielded fractions 1–5 in a decreasing order of mobility. In both groups, chondroitin sulfates were identified in the fast-moving fractions and heparan sulfates in the slow-moving fractions. Furthermore, two types of heparan sulfates were identified based on their electrophoretic mobility: slow-moving and fast-moving. The fractionated urinary polyanions were then tested in an in vitro calcium oxalate crystallization assay and compared at the same uronic acid concentration, whereby, the chondroitin sulfates of stone-formers and heparan sulfates of normals enhanced crystal nucleation. Fraction 5 of the normals, containing glycoproteins (14–97 kDa) and associated glycosaminoglycans, were found to effectively inhibit crystallization. Papainization of this fraction in stone-formers revealed crystal-suppressive effects of glycoproteins, which was not seen in similar fractions of normals. It was concluded that glycoproteins could modulate the crystal-enhancing glycosaminoglycans such as chondroitin sulfates of stone-formers but not in normals. The differing crystallization activities of electrophoretic fraction 1 of normals and stone-formers revealed the presence of another class of glycosaminoglycan–hyaluronan. Hence, in the natural milieu, different macromolecules combine to have an overall outcome in the crystallization of calcium oxalate.

Keywords: Crystallization; Electrophoresis; Glycoproteins; Glycosaminoglycans; Kidney stones; Urolithiasis

1. Introduction

The urinary environment, in which the salts of kidney stones can be found, also contain macromolecules that are considered to be important in the pathogenesis of urinary stone disease. These macromolecules range from ribonucleic acids, glycosaminoglycans (GAGs), proteins of few kilodaltons to the most widely studied protein inhibitors of crystallization, osteopontin, bikunin and prothrombin fragment 1.[1] The role of the macromolecules found in the urine remains unresolved, although several studies have been done to observe the effects of crude extracts of urinary macromolecules on the crystallization of calcium oxalate,[2–5] a salt most commonly found in human urine. Certain glycoproteins (Gps)[6–10] and GAGs[11–14] have also been purified from urine samples to study their effects on in vitro calcium oxalate crystallization systems.

In these studies, there is much controversy regarding the possible roles (promotion/inhibition of nucleation, growth and aggregation) played by individual macromolecules be it glycoproteins or GAGs and these have been adequately reviewed by Rodgers and Khan.[15,16]

Abbreviations: CPC, cetylpyridinium chloride; CS, chondroitin sulfate; CsCl, caesium chloride; DS, dermatan sulfate; GAG, glycosaminoglycan; Gp, glycoprotein; HA, hyaluronan; HPLC, high pressure liquid chromatography; HS, heparan sulfate (*f*—fast; *s*—slow); kDa, kilodalton; NC, normal control; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SF, stone-former.

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It is problematic to establish what individual macromolecule is most active in urine as it appears that there is an overabundance of crystal modulators.[17] The different methods employed for the isolation of the urinary macromolecule and crystallization assays may lead to a distinctive role of the individual macromolecule.

Earlier studies on GAGs relied largely on commercial products, which were from non-human sources.[13,18–21] Proteoglycan metabolites in urine include chondroitin sulfates (CS) and heparan sulfates (HS) both as free GAGs and as peptide-linked forms bearing the serine–xylose link.[22] Recently, urinary GAGs have been implicated in the modulation of calcium oxalate crystallization[15,17,23] and studies have also shown that urinary HS is incorporated in the stone matrix.[24] Both urinary HS and CS have been observed to inhibit crystal growth.[25] However, these studies were done with crude urinary GAG extracts, which had not been purified or separated. Thus the relative importance of the purified GAG-containing macromolecules to calcium oxalate crystallization is not yet clear.

Our previous report[14] on the separation of *papainized* urinary GAGs into CS and HS by the application of agarose gel electrophoresis on a preparative scale revealed that when tested in an in vitro calcium oxalate crystallization system[26] the electrophoretically fast-moving fractions of stone-formers more actively caused crystal nucleation as compared to normals. These fractions, in stone-formers, were subsequently identified to be hyaluronic acid (HA).[27] Urinary HS (slow-moving fractions) were found to be similarly effective in enhancing crystal nucleation in normals. These studies indicate the importance of fractionation in identifying urinary GAGs important to crystallization events.

This paper studies the separation of the different classes of GAG-containing macromolecules in their native forms from each other and from the co-extracted glycoproteins by a preparative agarose gel electrophoresis.[14] The fractions so obtained are then tested for effects on both nucleation and the growth of crystals of calcium oxalate arising from endogenous ions in urine. The crystal active roles of GAG-containing macromolecules are confirmed and crystal-suppressive roles of co-extracted glycoproteins are suggested. The results not only show the crystallization activities of isolated GAGs, but also of co-existing glycoproteins, just as they are in the native state in the kidney.

2. Results and discussion

2.1. Recovery of urinary GAGs

Some studies use commercially available GAGs and these, from non-human and non-kidney sources, can be very different from those found in the urinary environment. However, the role of GAGs in urolithiasis is almost always implied or extrapolated from studies that rely on these commercially available GAGs, or other non-purified and non-defined extracts or urinary macromolecules. Glycosaminoglycans in urine may be from two sources, one, from the metabolic turnover of connective tissue, which consists of smaller fragments, the larger ones being degraded by the liver[28] and secondly, endogenous turnover in the kidney itself.[29] Within the kidney, HS is the major component of the glomerular basement membrane. The mesengial matrices have also been found to contain CS and HS.[30,31]

In this study, we studied clearly defined and purified classes of urinary GAGs in their native forms and explored the possibility that GAGs from normals and stone-formers are different. The non-papainized urinary polyanions were obtained from the pooled urine samples of age-matched (22–56 yrs) normal controls (NC, $n = 51$) and stone-formers (SF, $n = 55$). The average recovery from urine for the urinary polyanions was 227 and 149 μg/mL, respectively. Compared to NC, the lower average concentration of urinary polyanions among stone-formers was significant ($P < 0.01$) and may be due to an increased fluid intake and hence urinary output. Preliminary identification of the crude GAGs on cellulose acetate electrophoresis showed Alcian-blue stainable materials in the mobility range of standard GAGs and one band of mobility lower than that of standard heparin for both NC and SF (Fig. 1). The full identification of GAG classes in the urinary polyanions was done on fractions recovered from preparative agarose gel electrophoresis.

2.2. Preparative electrophoresis of non-papainized urinary polyanions

Both normal controls (Fig. 1a) and stone-formers (Fig. 1b) showed a major A_{400} peak within 5 h of electrophoresis, whereas the A_{280} peak was slightly out of phase. The electrophoretic profiles shown here are representative though variations in terms of GAG and protein content were observed from one pooled urine extract to another. The materials from repeated electrophoretic separations were then pooled into fractions 1–5 in the decreasing order of mobility to standard GAGs (Fig. 1, inset). The pooled fractions were then tested for GAGs and Gp composition based on their susceptibilities to enzyme and chemical treatments. Overall, chondroitin sulfates (CS), which appeared in fraction 1 of NC and SF, were separated from the heparan sulfates (HS) in fraction 4 (Fig. 2, lane 3 of a and d, top and bottom). For the intermediate fractions, HS appears as early as in pooled fraction 2. Two distinct sub-populations of HS were identified (based on their electrophoretic mobilities on cellulose acetate), a *fast*-moving HS (*f*HS) with a mobility comparable to standard dermatan

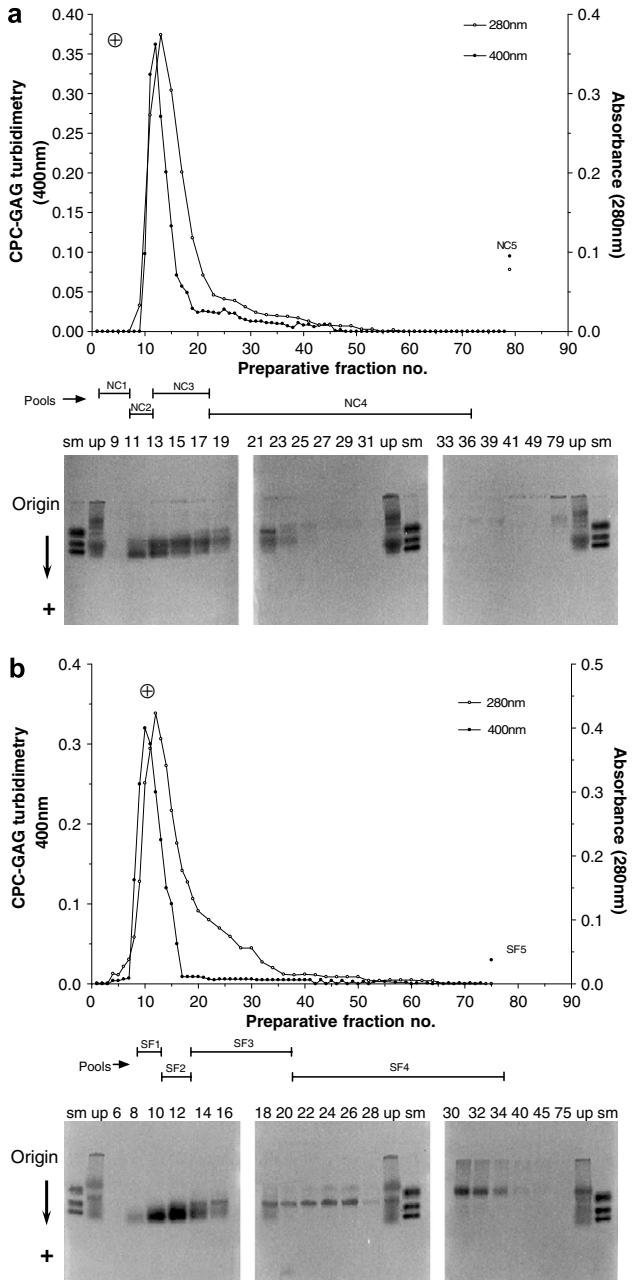

Figure 1. Preparative agarose gel electrophoresis of non-papainized urinary polyanions, from (a) normal controls and (b) stone-formers were monitored for cetylpyridinium chloride (CPC) turbidimetry at A_{400} and proteins at A_{280}. The relative electrophoretic mobilities of alternate fractions were reproducible as shown in cellulose acetate electrophoresis. The electrophoretic fractions were pooled as shown by horizontal bars into fractions (1–4) and residual polyanions at the origin are indicated as fraction 5. Fractions, 4 mL each, sm, standard reference material; up, non-fractionated urinary polyanions.

sulfate (DS) and a *slow*-moving HS (*s*HS) with a mobility comparable to standard heparin (Hp). Comparing the composition of GAGs and Gps in the pooled fractions 1–5, the trend is that the early fractions comprise mainly of CS and the latter fractions contain increasing proportions of HS and Gp–GAGs. These observations are mainly from enzyme and/or the chemical treatment of the extracts. DS was not detected in the SF and was barely discernable in the cellulose acetates of NC after chondroitinase-ABC digestions. Fractions 3–5 of NC (Fig. 2, lane 6 of b–d, top) and fractions 2–4 of SF (Fig. 2, lane 8 of a, lane 6 of b and c, bottom) retained an alcian-blue stainable band after sequential digestion with chondroitinase-AC, chondroitinase-ABC and nitrous acid treatment. This material was resistant also to heparitinase, hyaluronidase, neuraminidase, papain and alkaline treatment.

One GAG sub-class that was not distinguished in our early studies using papainized extracts of urinary GAGs[14] was *f*HS (Fig. 2). This *f*HS had a mobility comparable to the standard DS. The molecular weight of urinary HS ranges from 8 to 30 kDa and those HS polysaccharide chains that were less sulfated were also smaller in molecular size.[32] It is conceivable that this *f*HS is precipitable with CPC only with its protein subunit. In the papainized sample, being less sulfated and smaller, it falls below its critical electrolyte concentration[33] for precipitation with cetylpyridinium chloride.

2.3. Crystallization characteristics of the preparative electrophoretic fractions

Among NC samples, fractions 1 and 2 containing predominantly CS and *fast*-moving HS showed low crystal-nucleating activities with a ratio of 1–2.5 to the basal activity of the neat urine ultrafiltrate (Fig. 3a). Fractions 3 and 4, containing mainly CS and fast- and slow-moving HS, showed activities 8 and 20 times that of the ultrafiltrate. Fraction 5 containing mainly Gp associated with GAGs and HS showed a marked crystal-inhibitory activity towards nucleation. Fractions 1–4 obtained from SF indicated crystal-nucleating activities nearly 5–10 times that of neat ultrafiltrates except for fraction 5, which showed activities similar to that of ultrafiltrates.

Crystal growth-inhibitory activities were evident in fractions 1–4 of both NC and SF except for fraction 5 (Fig. 3b). The most marked growth-inhibitory activities were found in fraction 4 for both the NC and SF, which also corresponded to the fraction containing the greatest crystal-nucleating activities. It was however noticed that in the SF the growth-inhibitory activities were similar (ratio of 0.5–0.65 to that of ultrafiltrate crystal size), however, when compared to NC, the crystal sizes were dissimilar (30–40%), which was statistically different between the two populations in fractions 1, 2 and 4. In NC there is a discerning growth-inhibitory activity due to fraction 4 (40%), which corresponded to a nucleation ratio of 22 times that of urine ultrafiltrates.

The crystallization studies revealed that polyanions extracted from SF and fractionated by preparative elec-

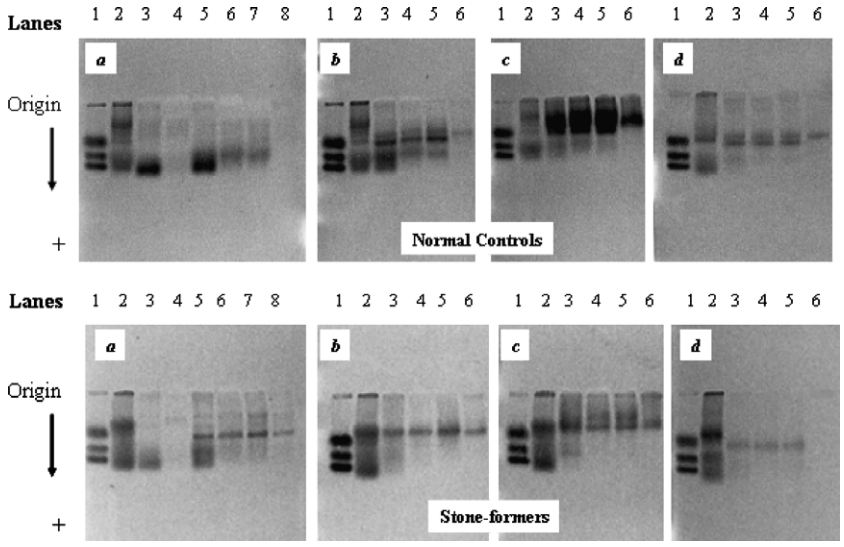

Figure 2. Identification of GAG classes in the pooled preparative electrophoretic fractions of normal control subjects (top) and stone-formers (bottom). Cellulose acetate electrophoresis (a–d; Alcian blue stained) was performed after sequential enzymatic/chemical treatment of the pooled fractions. Lanes 1 of (a)–(d) show the standard reference mixture of heparin, DS and CS, in an increasing order of mobility. Lanes 2 of (a)–(d) show the non-fractionated urinary polyanions. CS in fraction 1 (lane 3 of a), fraction 2 (lane 5 of a), fraction 3 (lane 3 of b), fraction 4 (lane 3 of c) and fraction 5 of NC (lane 3 of d) was digestible by chondroitinase AC (lane 4 of a, lane 6 of a, lane 4 of b and c and lane 4 of d (top)). DS removal by chondroitinase ABC was virtually indiscernible in fraction 2 (lane 7 of a), fractions 3–5 (lanes 5 of b–d). *f*HS and *s*HS was removable by nitrous acid treatment in fraction 2 (lane 8 of a), fraction 3 (lane 6 of b), fraction 4 (lane 6 of c) and fraction 5 (lane 6 of d). Electrophoresis was performed at 5 mA (constant current) for 30 min and an equal amount was applied in each lane.

trophoresis reached 8–12 times that of urine ultrafiltrates alone. In the normal controls, the late fractions containing increasing proportions of HS, showed crystal activities as high as 20 times that of urine ultrafiltrates. The level of enhancement so observed was however consistent but lower than that due to papainized extracts of urinary polyanions studied earlier.[14] Mainly, it is observed that fraction 1 of SF is active in crystal nucleation mainly due to the presence of HA. The material that did not electrophorese and remained at the origin after 40 h was recovered and revealed to contain glycoproteins associated with GAGs. It is suggested that this material at the origin (for NC only) is aggregated as it would migrate into SDS-PAGE only after it had been treated with 4 M guanidinium hydrochloride and separated by ultracentrifugation in a CsCl gradient. SDS-PAGE analysis (not shown) suggests that the major glycoproteins observed were in the range of 14–97 kDa. Analyses by specific antibodies revealed it to be positive for osteopontin, Tamm-horsfall glycoprotein, albumin and human prothrombin fragment 1. NC5 fraction was highly inhibitory towards crystal nucleation. As the NC5 fraction contains a mixture of glycoproteins, further purification and analyses would be required to assess as to which particular glycoprotein is important. Most studies have indicated that much of the inhibitory effect of crystallization is attributable to urinary proteins such as prothrombin fragment 1, osteopontin, bikunin and Tamm-horsfall glycoprotein[1,15,34] and we reveal the similar proteins in our crystal-suppressive fraction 5.

2.4. Crystal-suppressive effects of the 'putative' protein

The observation of a strong nucleation–inhibitory activity in NC5 prompted the use of this fraction to test for the suppressive effects on crystal-active fractions. For this purpose, electrophoretic preparations of urinary GAGs—NC-HS (fraction 4) and SF-CS (fraction 1)—were used as they were shown to be particularly crystal active as much as 30 times that of the neat urine ultrafiltrates.[14] Figure 4a shows the inhibitory activity of fraction 5 of NC (*non-papainized* urinary polyanions) on SF-CS (fraction 1) and NC-HS (fraction 4). Fraction 5 of NC showed a marked inhibitory activity towards crystal nucleation, even at the levels of 1 μg hexuronate/mL in the final solution. Correspondingly, crystal-growth inhibition was relieved to reach the levels of those found in urine ultrafiltrates.

To further demonstrate the crystal-suppressive effects of the Gp, fraction 1 of both NC and SF were subjected to papain digestion and the GAGs recovered from the digestion product were assayed for crystallization activity. As shown in Figure 4b, the moderate activity of fraction 1 of SF sample was increased significantly after papainization, but no change was observed in the basal activity of fraction 1 of NC after papainization. In contrast, the basal activity, characteristic of NC-CS was unmodified by Gp, if any that co-migrated in this fraction. The nucleation-promoting activities of SF-CS was apparently recovered after digestive removal of the co-migrating SF-Gp, reaching the levels observed

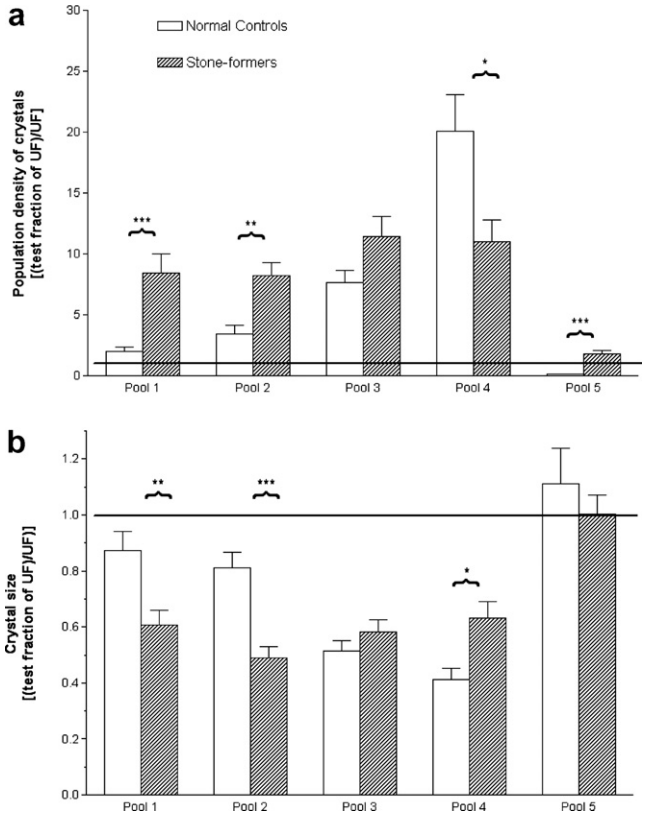

Figure 3. Calcium oxalate crystal nucleation-promoting activities (a) and growth-inhibitory activities (b) of preparative electrophoretic fractions of urinary GAGs at 5 μg hexuronate/mL. The horizontal line depicts the population density (a) and size (b) of the crystals found in urine ultrafiltrates alone. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$ ($n = 15$, mean ± SEM).

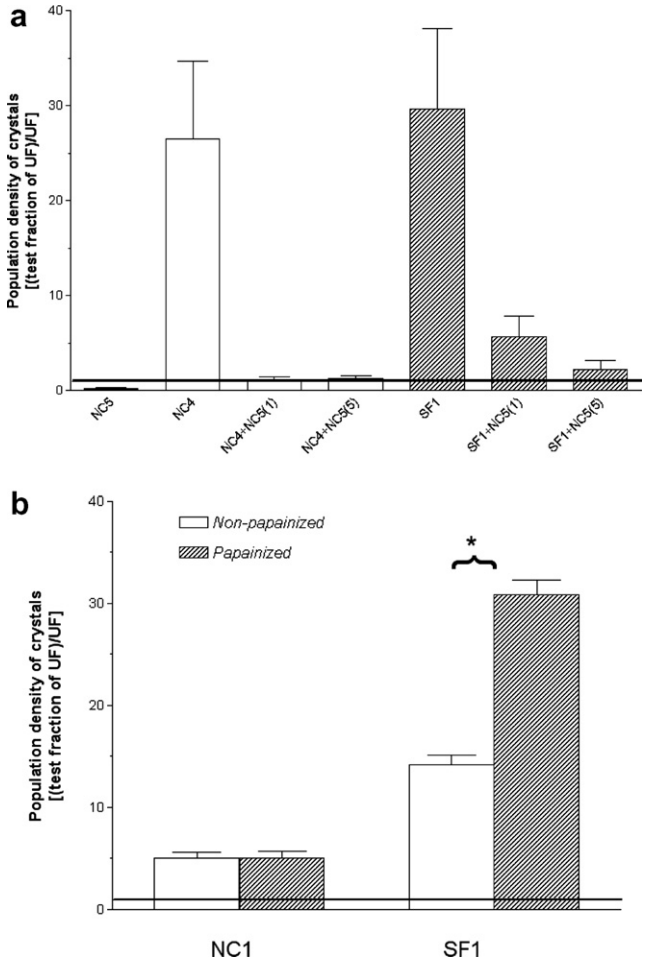

Figure 4. Crystal-suppressive effects of the putative glycoprotein is shown by the (a) modulatory effects of NC5 on crystallization activities of NC-HS (fraction 4) and SF-CS (fraction 1) ($^{*}P < 0.001$, $n = 6$; mean ± SEM) and (b) papainization of SF1 and NC1 showing the suppressive effects of glycoproteins on crystallization activities ($^{*}P < 0.05$; $n = 16$; mean ± SEM). All crystal studies were performed at 5 μg hexuronate/mL and values in brackets indicate μg hexuronate/mL for fraction NC5. The solid horizontal line indicates a ratio equals to 1 (i.e., crystallization activity equivalent to urine ultrafiltrates).

in the electrophoretic fractions of the *papainized* counterpart.[14] This confirmed our original crystallization activities on the fraction 1 of SF and fraction 4 of NC in Figure 3.

As glycoproteins co-migrate with the GAGs during preparative electrophoresis, the crystal activities observed would be the interplay of both the crystal-inhibitory activities of the glycoproteins and the crystal-promoting activities of the GAGs, which is proven above. It is possible that the free chains of HA and HS in the absence of glycoproteins (NC5) could provide the template for crystal nucleation, whereas in the presence of glycoproteins, the free GAGs may aggregate with the GPs thus removing the template from the solution.[35] The effects of glycoproteins were further corroborated by papainizing fractions NC1 and SF1 and studying the crystal properties before and after papainization (Fig. 4b). The crystal-nucleating promoting properties of NC1 was unaffected suggesting that the basal level (above that of urine ultrafiltrates alone) of crystal nucleation is solely due to CS. However, for the SF1, the crystal-nucleating promoting properties was enhanced after papainization, confirming our earlier observation by crystallization[14] that SF GAG class in fraction 1 was distinctly different from that of fraction 1 of NC.

Murata et al.[36] has shown that several chondroitin sulfate isomers exist in normal human urine. When the CS were digested by chondroitinase AC and/or ABC enzymes, the products were mainly disaccharides including non-sulfated-, C4-sulfated-, C6-sulfated-, di-sulfated species. Indeed, our HPLC analysis of fractions 1 of NC and SF revealed that in addition to disaccharides of CS, hyaluronan isomers were found in both the groups (Fig. 5).[27] Whether the difference in the crystallization activities of the HA in NC and SF lies in the composition of HA will only be revealed when we have studied the nature of urinary HA for their molecular mass and any associated peptides. This will then shed light as to

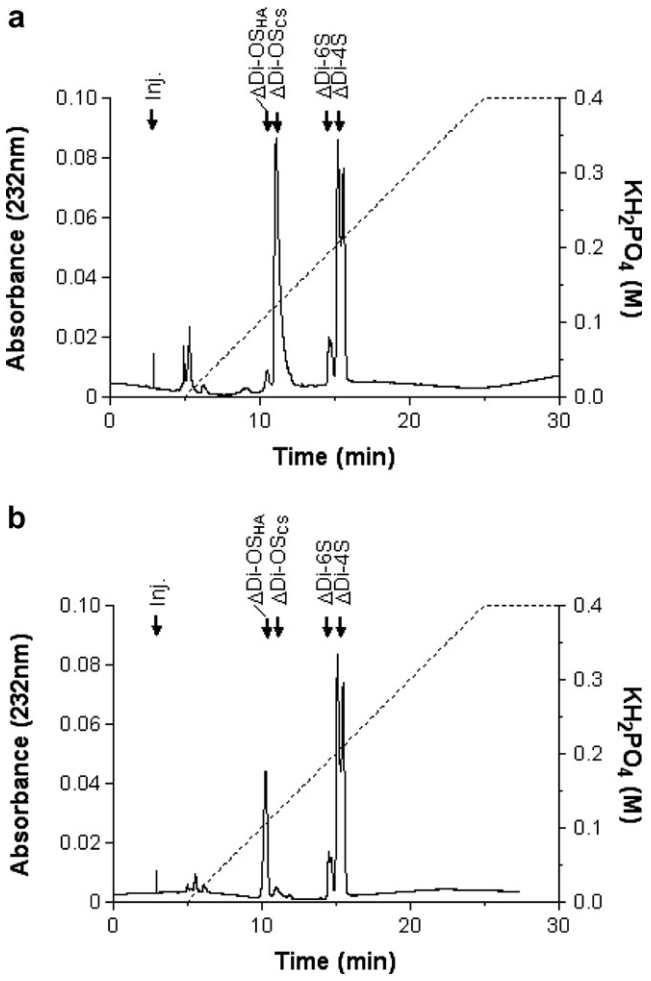

Figure 5. SAX-HPLC chromatograms of the unsaturated disaccharides from chondroitinase digestion of papainized urinary glycosaminoglycan of fraction 1 from (a) normal controls and (b) stone-formers.

the importance of the isomers of HA within which the composition may differ between the two populations. Differential proteoglycan turnover, control and excretion in urine has been implicated in other diseases such as diabetes,[37] bladder carcinoma,[38] pseudoxanthoma elasticum[39] and progressive systemic sclerosis.[40] These have also reported HA to be produced as a secondary product of injury or inflammation, akin to an injury due to a growing stone.

To conclude, there is an extensive amount of literature that has implicated glycosaminoglycans as an active participant in the crystallization processes of calcium oxalate.[41–46] Preparative agarose electrophoretic separation of urinary GAGs and co-existing glycoproteins revealed that CS are predominant in the early fractions, whereas the heparan sulfates and the glycoproteins-associated-GAGs appear in the later fractions. Apparent differences between NC and SF in the separation of urinary GAGs is observed following enzymatic and chemical treatment to analyze the GAG sub-classes by HPLC. The inhibitory glycoproteins merits further investigations as to its identity, structure and function. Thus, an overall crystallization characteristics (nucleation and inhibition of growth) in the urine is achieved through the relative contributions of the different GAG classes and Gps in the natural milieu.

3. Experimental

3.1. Enzymes

Chondroitinase AC (chondroitin AC lyase, EC 4.2.2.5; from *Flavobacterium heparinum*), chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4; from *Proteus vulgaris*), heparitinase (heparan sulfate lyase, EC 4.2.2.8; from *F. heparinum*), hyaluronidase (hyaluronate lyase, EC 4.2.2.1; from *Streptomyces hyalurolyticus*) were obtained from Seikagaku Corporation (Tokyo, Japan) and papain (EC 3.4.22.2) was from Sigma (St. Louis, MO, USA).

3.2. Recovery of urinary GAGs

The procedure is as previously described[14] except that the recovered urinary polyanions were not further digested with papain. Briefly, urinary proteoglycan metabolites and glycoproteins were recovered as polyanions from pooled early morning urine of age-matched healthy controls and stone-formers by sequential precipitation, first as the cetylpyridinium salt and subsequently as the sodium salt.[3,47] The urine samples of stone-formers were treated separately from those of normal control subjects. The stone-formers recruited for this study were all calcium stone-formers with calcium oxalate and/or admixed with calcium phosphate. The subjects were on a free diet and there was no statistical difference between the two populations in terms of the urinary concentrations of sodium, potassium, calcium, chloride, creatinine, oxalate, citrate and phosphate.

3.3. Electrophoretic methods

3.3.1. Cellulose acetate electrophoresis. This was performed on cellulose acetate in 0.05 M barium acetate buffer (pH 5.8), using a Microzone apparatus (Beckman) at 5 mA (constant current) and 30 min. Each sample was applied equivalently three times. The membranes were stained with 0.2% Alcian Blue.[48]

3.3.2. Preparative agarose gel electrophoresis. A preparative apparatus[49] with 1% agarose gel was used to perform electrophoresis (11 V, constant voltage, 40 h) of the urinary polyanions. The fractions were collected at 30 min intervals and monitored for GAGs and glycoproteins by turbidimetry with cetylpyridinium chloride[47] and for 280-nm-absorbing glycoproteins. Urinary

polyanions in the electrophoretic fractions as well as residual material of application were well recovered by sequential precipitation procedure.[3] The fractions were assayed for hexauronic acid contents by the Carbazole reaction[50] and proteins contents by Bradford protein assay (Biorad). The GAG classes therein were identified by their respective susceptibilities to digestion by chondroitinase AC, chondroitinase ABC,[51] and treatment with nitrous acid.[52] The GAG/glycoprotein associates remaining in the application were well dissociated in 4 M guanidinium hydrochloride and were separated by ultracentrifugation (48 h, 110,000*g*, 4 °C) through a caesium chloride density gradient (1.435 g/mL) followed by dialysis. The separated polyanions were recovered through sequential precipitation procedure as described earlier. These separated GAG/Gp associates were then tested for susceptibility to digestion with enzymes as above and in addition—heparitinase,[53] hyaluronidase,[54] papain[47] and alkaline treatment.[55]

3.3.3. SDS-polyacrylamide gel electrophoresis. This was performed in a mini-gel apparatus (Hoeffer Sciences) (12% gel, $8 \times 10 \times 0.075$ cm, Tris–glycine–SDS buffer, pH 6.8 for stacking gel, pH 8.8 for separating gel) to monitor and determine the molecular weights of proteins that co-migrated with GAG-containing forms in the fractions. The fractions were all standardized with 5 μg protein application onto the gel and mixed with a sample buffer (SDS–glycerol-2-mercaptoethanol in Tris, pH 6.8 buffer, 0.002% bromophenol blue) (1:1 v/v, 20 μL maximum volume). Both high molecular (29–205 kDa) and low molecular (14.2–66 kDa) weight protein standards (100 μg) were applied (Sigma). The gels were pre-run for 15 min at 40 V. Following sample application, the gels were electrophoresed at 40 V for 20 min, 80 V for 30 min and thereafter at 150 V until the tracking dye reached 1 cm from the bottom of the plate. A cooling system was attached to the electrophoretic unit. The gels were stained with Coomassie Brilliant Blue R-250 (0.1% in 50% methanol/10% acetic acid/water) and destained in the same solvent without the dye.

3.4. Strong anion exchange high pressure liquid chromatography (SAX-HPLC)

SAX-HPLC was performed to study the disaccharide composition of the purified extracts following an exhaustive digestion with chondroitinases. Chromatography was performed with an ISCO-HPLC (Model 2350, USA) system equipped with a V^4 absorbance detector and connected to a computer for running of the HPLC pumps (isocratic or gradient) and analyses of the data. The digested urinary GAGs were separated on a Whatman Partisil-10 (SAX) column (250×4.6 mm) at a flow rate of 1.0 mL/min, 25 °C. The column elute was monitored at 232 nm and the peak heights and area determined. The unsaturated disaccharides were eluted with a linear gradient, which was begun at 5 min (post-injection) (0 M KH_2PO_4) to 25 min (0.4 M KH_2PO_4). The integrity and efficiency of the column was tested by measuring the linearity of the disaccharide standards at three different concentrations (2.5, 5 and 10 μg/mL).

3.5. Crystallization assay

GAG-containing macromolecules recovered from the electrophoretic fractions were tested for crystallization activity according to the method of Gohel et al.[26] Briefly, this involved the addition of test GAGs at a final concentration of 5 μg hexuronate/mL to urine ultrafiltrates (pH 5.3, 1250 mOs mol/kg), which had been prepared at a nominal cut-off of 10 kDa (YM-10 membranes; Amicon) from urine of normal control subjects.[3] The concentration of GAGs being used for the crystallization studies are those found in the urinary range (2–6 μg hexuronate/mL of urine) as determined by us[27,56] and others.[57,58] Envelope crystals of calcium oxalate dihydrate were assessed for the population density and size with the 'Improved Neubauer Chamber' haemocytometer under light microscope fitted with a high power objective (400×) and an ocular micrometer. Each sample was determined six times to obtain an average value. All crystal assessments were done in the urine ultrafiltrate in the presence of test material were expressed as ratios to that observed in the neat urine ultrafiltrate to minimize the effects due to micro-ions in the ultrafiltrate and thus demonstrate the effects due to the test GAG. *Crystal nucleation* is demonstrated when a ratio of >1 is indicated in the population density of the crystals and conversely *crystal suppression* (or crystal nucleating inhibition) is demonstrated when a ratio of <1 is indicated. *Crystal growth* is demonstrated when a ratio of >1 is indicated in the size of crystals and conversely crystal-growth inhibition is demonstrated when a ratio of <1 is indicated. The crystallization studies were repeated (see legend) to substantiate statistical comparisons.

3.6. Statistical analysis

All statistical analyses were performed using the Mann–Whitney *U*-test using Graphpad INSTAT (Ver. 3.06) and PRISM (Ver. 4.03) software (Graphpad Software Inc., San Diego, USA).

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References

1. Grover, P. K.; Ryall, R. L. *Clin. Sci.* **2002**, *102*, 425–434.
2. Drach, G. W.; Thorson, S.; Randolph, A. *J. Urol.* **1980**, *123*, 519–523.
3. Gohel, M. D. I.; Shum, D. K. Y.; Li, M. K. *Urol. Res.* **1992**, *20*, 13–17.
4. Koide, T.; Takemoto, M.; Itatani, H.; Takaha, M.; Sonoda, T. *Invest. Urol.* **1981**, *18*, 382–386.
5. Robertson, W. G.; Peacock, M.; Nordin, B. E. *Clin. Chim. Acta* **1973**, *43*, 31–37.
6. Doyle, I. R.; Ryall, R. L.; Marshall, V. R. *Clin. Chem.* **1991**, *37*, 1589–1594.
7. Nakagawa, Y.; Abram, V.; Kezdy, F. J.; Kaiser, E. T.; Coe, F. L. *J. Biol. Chem.* **1983**, *258*, 12594–12600.
8. Rose, G. A.; Sulaiman, S. *J. Urol.* **1982**, *127*, 177–179.
9. Ryall, R. L.; Grover, P. K.; Stapleton, A. M.; Barrell, D. K.; Tang, Y.; Moritz, R. L.; Simpson, R. J. *Clin. Sci. (Colch)* **1995**, *89*, 533–541.
10. Shiraga, H.; Min, W.; VanDusen, W. J.; Clayman, M. D.; Miner, D.; Terrell, C. H.; Sherbotie, J. R.; Foreman, J. W.; Przysiecki, C.; Neilson, E. G., et al. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 426–430.
11. Gill, W. B.; Karesh, J. W.; Garsin, L.; Roma, M. J. *Invest. Urol.* **1977**, *15*, 95–99.
12. Kitamura, T.; Zerwekh, J. E.; Pak, C. Y. *Kidney Int.* **1982**, *21*, 379–386.
13. Shirane, Y.; Hamao, T.; Kagawa, S. *Nippon Hinyokika Gakkai Zasshi* **1990**, *81*, 1065–1070.
14. Shum, D. K. Y.; Gohel, M. D. I. *Clin. Sci. (Colch)* **1993**, *85*, 33–39.
15. Khan, S. R.; Kok, D. J. *Front. Biosci.* **2004**, *9*, 1450–1482.
16. Rodgers, A. L. Promoters and Inhibitors—Introduction. In *Urolithiasis—Consensus and Controversies*; Rao, P. N., Kavanagh, J. P., Tiselius, H.-G., Eds.; South Manchester University Hospitals: Manchester, UK, 1995; pp 101–109.
17. Kok, D. J. Macromolecular Inhibitors and Promoters of Crystallization. In *Kidney Stones: Inside and Out*; Gohel, M. D. I., Au, W. T. D., Eds.; The Reprographic Unit, The Hong Kong Polytechnic University: Hong Kong, 2004; pp 45–48.
18. Boeve, E. R.; Cao, L. C.; Verkoelen, C. F.; Romijn, J. C.; de Bruijn, W. C.; Schroder, F. H. *World J. Urol.* **1994**, *12*, 43–48.
19. Fellstrom, B.; Danielson, B. G.; Ljunghall, S.; Wikstrom, B. *Clin. Chim. Acta* **1986**, *158*, 229–235.
20. Kohri, K.; Garside, J.; Blacklock, N. J. *Br. J. Urol.* **1989**, *63*, 584–590.
21. Robertson, W. G.; Scurr, D. S. *J. Urol.* **1986**, *135*, 1322–1326.
22. Wessler, E. *Biochem. J.* **1971**, *122*, 373–384.
23. Asselman, M.; Verhulst, A.; Van Ballegooijen, E. S.; Bangma, C. H.; Verkoelen, C. F.; De Broe, M. E. *Kidney Int.* **2005**, *68*, 71–83.
24. Yamaguchi, S.; Yoshioka, T.; Utsunomiya, M.; Koide, T.; Osafune, M.; Okuyama, A.; Sonoda, T. *Urol. Res.* **1993**, *21*, 187–192.
25. Iwata, H.; Terado, T.; Kin, M.; Nishio, S.; Matsumoto, A.; Takeuchi, M. *Nippon Hinyokika Gakkai Zasshi* **1991**, *82*, 405–411.
26. Gohel, M. D. I.; Shum, D. K. Y.; Li, M. K. *Clin. Chim. Acta* **1994**, *231*, 11–22.
27. Shum, D. K. Y.; Gohel, M. D. I.; Tam, P. C. *J. Am. Soc. Nephrol.* **1999**, *10*, S397–S403.
28. Wood, K. M.; Curtis, C. G.; Powell, G. M.; Wusteman, F. S. *Biochem. J.* **1976**, *158*, 39–46.
29. Mitsuhashi, H.; Tsukada, Y.; Ono, K.; Yano, S.; Naruse, T. *Clin. Nephrol.* **1993**, *39*, 231–238.
30. Vernier, R. L.; Klein, D. J.; Sisson, S. P.; Mahan, J. D.; Oegema, T. R.; Brown, D. M. *N. Engl. J. Med.* **1983**, *309*, 1001–1009.
31. Kanwar, Y. S.; Rosenzweig, L. J.; Jakubowski, M. L. *Lab. Invest.* **1983**, *49*, 216–225.
32. Wasteson, A.; Wessler, E. *Biochim. Biophys. Acta* **1971**, *252*, 13–17.
33. Scott, J. E.; Newton, D. J. *Connect. Tissue Res.* **1975**, *3*, 157–164.
34. Ito, H.; Coe, F. L. *Am. J. Physiol.* **1977**, *233*, F455–F463.
35. Hess, B.; Nakagawa, Y.; Coe, F. L. *Am. J. Physiol.* **1989**, *257*, F99–F106.
36. Murata, K.; Harada, T.; Fujiwara, T.; Furuhashi, T. *Biochim. Biophys. Acta* **1971**, *230*, 583–594.
37. Shield, J. P.; Carradus, M.; Stone, J. E.; Hunt, L. P.; Baum, J. D.; Pennock, C. A. *Ann. Clin. Biochem.* **1995**, *32*, 557–560.
38. Hennessey, P. T.; Hurst, R. E.; Hemstreet, G. P., 3; Cutter, G. *Cancer Res.* **1981**, *41*, 3868–3873.
39. Longas, M. O.; Wisch, P.; Lebwohl, M. G.; Fleischmajer, R. *Clin. Chim. Acta* **1986**, *155*, 227–236.
40. Murata, K.; Takeda, M. *Clin. Chim. Acta* **1980**, *108*, 49–59.
41. Baggio, B.; Gambaro, G.; Cicerello, E.; Mastrosimone, S.; Marzaro, G.; Borsatti, A.; Pagano, F. *Clin. Biochem.* **1987**, *20*, 449–450.
42. Angell, A. H.; Resnick, M. I. *J. Urol.* **1989**, *141*, 1255–1258.
43. Hesse, A.; Wuzel, H.; Vahlensieck, W. *Am. J. Kidney Dis.* **1991**, *17*, 414–419.
44. Ebisuno, S.; Kohjimoto, Y.; Yoshida, T.; Ohkawa, T. *Urol. Res.* **1993**, *21*, 265–268.
45. Rodgers, A. L.; Ball, D.; Harper, W. *Scan. Microsc.* **1994**, *8*, 71–77.
46. Shum, D. K. Y.; Gohel, M. D. I.; Liong, E. Effects of Glycoproteins and Glycosaminoglycans on the Crystallization of Urinary Calcium Oxalate. In *Urolithiasis—Consensus and Controversies*; Rao, P. N., Kavanagh, J. P., Tiselius, H.-G., Eds.; South Manchester University Hospitals: Manchester, UK, 1995; pp 421–423.
47. Scott, J. E. *Methods of Biochemical Analysis*; Interscience: New York, 1960; pp 145–197.
48. Newton, D. J.; Scott, J. E.; Whiteman, P. *Anal. Biochem.* **1974**, *62*, 268–273.
49. Cornish, L. S.; Boyde, T. R. C.; Chung, Y. C.; Ng, C. S. *Lab. Prac.* **1987**, *36*, 35–37.
50. Bitter, T.; Muir, H. *Anal. Biochem.* **1962**, *4*, 330–334.
51. Saito, H.; Yamagata, T.; Suzuki, S. *J. Biol. Chem.* **1968**, *243*, 1536–1542.
52. Carey, D. J.; Todd, M. S. *J. Biol. Chem.* **1986**, *261*, 7518–7525.
53. Rice, K. G.; Rottink, M. K.; Linhardt, R. J. *Biochem. J.* **1987**, *244*, 515–522.
54. Knudson, W.; Gundlach, M. W.; Schmid, T. M.; Conrad, H. E. *Biochemistry* **1984**, *23*, 368–375.
55. Silbert, J. E.; Sugumaran, G.; Cogburn, J. N. *Biochem. J.* **1993**, *296*, 119–126.
56. Gohel, M. D. I. Ph.D. Thesis, The University of Hong Kong, 1997.
57. Gianotti, M.; Genestar, C.; Palou, A.; Pons, A.; Conte, A.; Grases, F. *Int. Urol. Nephrol.* **1989**, *21*, 281–288.
58. Trinchieri, A.; Mandressi, A.; Luongo, P.; Rovera, F.; Longo, G. *Scand. J. Urol. Nephrol.* **1992**, *26*, 379–386.