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Characterization of protein components of human urinary crystal surface binding substance

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Abstract We previously extracted crystal surface binding substance (CSBS) from human urine and showed that it appeared to constitute a substantial proportion of urinary macromolecular inhibitors of calcium oxalate crystallization. CSBS was isolated from human urine and fractionated by three consecutive chromatography procedures in order to characterize protein inhibitors of calcium oxalate crystallization. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and NH_2-terminal amino acid sequencing revealed that inhibitory fractions eluted from a final, hydroxyapatite column contained prothrombin and osteopontin. Hydroxyapatite column fractions also contained other, unidentified protein inhibitors of calcium oxalate crystallization. CSBS contained also human serum albumin, α_1-acid glycoprotein, α_1-microglobulin, α_2-HS glycoprotein, retinol-binding protein, transferrin, and Tamm-Horsfall protein, but these proteins seemed to play no direct role in inhibitory activity.

Key words Calcium oxalate · Crystallization · Protein inhibitor · Crystal surface binding substance · Prothrombin · Osteopontin

Introduction

There have been many investigations about a relationship between urinary stone formation and urinary substances. Urinary macromolecules are currently thought to play a more important role in stone formation than small molecular substances because of their strong inhibitory activity on calcium oxalate crystal growth or aggregation [8, 11]. Among urinary macromolecules, several proteins, including nephrocalcin [15], uropontin [17], crystal matrix protein [18], uronic-acid-rich protein [1], and osteopontin [10] have been identified and mentioned as the most important substances affecting stone formation. Recently crystal matrix protein has been shown to be a urinary prothrombin activation peptide F1 [5] and uronic-acid-rich protein to be an inter-α-trypsin inhibitor [2]. However, we think that it is quite difficult to explain a stone-forming process with only one protein and it is more reasonable to consider several proteins each acting on stone formation. The aim of our present study was to characterize the inhibitory proteins which absorbed on to calcium oxalate crystals. It is very important to analyze these proteins because, at the least, they have a function in binding to crystals.

Materials and methods

Preparation of crystal surface binding substance (CSBS)

We collected 20 l of urine from healthy men; $NaNO_3$ was added to the urine at a concentration of 0.02% and stored at −20°C until enough samples had been obtained. After incubation at 37°C with shaking, the urine was passed first through a Whatman no. 1 paper filter and then through a 0.22 μm Millipore filter (Millipore, Bedford, Massarusetts). Spontaneous crystallization of calcium oxalate was induced by mixing the urine with solutions containing 1 M $CaCl_2$ and 0.1 M sodium oxalate in a volume ratio of 1000:32:320 and incubating for 6 h with shaking at 37°C in a water bath. The mixture was then centrifuged, the supernatant decanted, and the crystal pellet washed with a saturated solution of calcium oxalate to remove urinary contaminants. The crystals were then suspended in 10% (w/v) EDTA (tetrasodium salt) (pH 8.0), in which they dissolved within 30 min. The solution was concentrated and EDTA removed by ultrafiltration with a Labomodule (Asahi Kasei, Tokyo, Japan) filtration cell with a molecular size cut off of 6 kDa. Finally, the concentrated and desalinated CSBS solution was freeze-dried.

Ion-exchange chromatography and hydroxyapatite chromatography

Two steps of ion-exchange chromatography and hydroxyapatite chromatography were performed at 4°C in the presence of protease

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inhibitors (0.1 M 6-aminohexanoic acid, 1 mM benzamidine hydrochloride, 1 mM phenylmethysulfonyl fluoride and 10 mM *N*-ethylmaleimide) to isolate proteins from CSBS. CSBS was first dialyzed through a solution containing 10 mM TRIS-HCl (pH 6.8) and 7 M urea, and then applied to a column (1.6 × 12 cm) of diethylaminoethyl (DEAE). Sepharose CL-6B (Pharmacia Biotech, N.J.) that had been equilibrated with the same buffer. CSBS constituents were eluted with a gradient of 0 to 2 M NaCl in the same buffer. Fractions were dialyzed through distilled water to eliminate urea, freeze-dried, and assayed for protein, glycosaminoglycans (GAGs), and crystal growth inhibitory activity. Protein concentration was measured by the method of Lowry et al. [13] and uronic acid contained in GAGs were measured with the 1,9-dimethylmethylene blue (DMB) assay [7]. Crystal growth inhibitory activity was measured as described below.

Fractions enriched in both protein and inhibitory activity were pooled, dialyzed against 10 mM TRIS-HCl (pH 6.8) containing 7 M urea, and again applied to a column (1.6 × 12 cm) of DEAE-Sepharose CL-6B that had been equilibrated with the same buffer. Elution was performed with steps of 0, 125, 250, 500, and 1000 mM NaCl in the same buffer to separate proteins from GAGs. Fractions were dialyzed against distilled water, freeze-dried, and assayed for protein and GAGs. Fractions that contained only protein were pooled, dialyzed against 30 mM sodium phosphate buffer (pH 6.8), and applied to a column (0.6 × 3 cm) of hydroxyapatite (Pharmacia Biotech) that had been equilibrated with the same buffer. Proteins were eluted with steps of 30, 60, 125, 250, and 500 mM sodium phosphate buffer (pH 6.8). Fractions eluted at each step were pooled, dialyzed against distilled water, freeze-dried, and assayed for protein and inhibitory activity.

Assay of crystal growth inhibitory activity

Inhibition of calcium oxalate crystal growth was determined with the seed crystal system according to a modified version of the method of Robertson et al. [16]. Metastable synthetic urine was composed of 1 mM $CaCl_2$, 0.2 mM sodium oxalate, 150 mM NaCl, and 10 mM sodium cacodylate (pH 6.0), with a tracer dose of [^{14}C] oxalate. Seed crystals were prepared by adding 100 mg of commercially available calcium oxalate monohydrate crystals to 100 ml of distilled water, stirring with a magnetic stirrer for 2 h, and then 250 µl of seed crystals were added to 10 ml of synthetic urine containing the test sample, or distilled water as a control, and incubated for 4 h at 37°C with shaking in a water bath. The mixture was filtered through a 0.22 µm Millipore filter to remove crystals, the radioactivity in the filtrate was measured with a scintillation counter, and the amount of oxalate consumed for crystal growth was calculated. Inhibitory activity was determined (1−Cs/Cc) × 100%, where Cs is the amount of oxalate consumed with the test sample and Cc is the amount of oxalate consumed under control conditions.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were dissolved in loading buffer [62.5 mM TRIS-HCl (pH 6.8), 2% (w/v) SDS, 7% (v/v) glycerol, 0.005% (w/v) bromophenol blue] and heated for 5 min at 100°C. After cooling, samples were subjected to electrophoresis for 60 min at 40 mA in a 10–20% polyacrylamide gradient gel (Multi Gel, Daiichi, Tokyo, Japan) with a buffer containing 25 mM TRIS and 192 mM glycine, pH 8.4). Gels were stained with coomassie brilliant blue R-250, and the molecular size of sample proteins was determined by comparison with those of protein standards (Daiichi).

Amino acid sequence analysis

The NH_2-terminal amino acid sequences of proteins eluted from SDS-PAGE gels were determined with an Applied Biosystems gas-phase sequencer (model 476A), and analysis system (model 610A). Determined amino acid sequences were compared with known sequences using the GENETYX-MAC/CD database (v. 28 1995.2).

Western blotting

To analyze the protein composition of CSBS, immunoblotting was carried out with antibodies to proteins which were reported to be contained in stone matrix [6] or to be involved in stone formation. Fractions that contained only protein were obtained from CSBS with above-mentioned ion-exchange chromatography on DEAE-Sepharose CL-6B with an NaCl gradient and stepwise elution, dialyzed through distilled water, and freeze-dried. After SDS-PAGE, proteins were transferred on to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) according to Burnette [3], and analyzed by immunoblotting with antibodies to ten proteins, human serum albumin, α_1-acid glycoprotein, α_1-microglobulin, α_1-antitrypsin, α_2-HS glycoprotein, β_2-microglobulin, retinol-binding protein, transferrin, Tamm-Horsfall mucoprotein, and prothrombin. They were all commercially available antibodies. Proteins were immunodetected with horseradish peroxidase-linked antimouse immunoglobulin (Ig) or antirabbit Ig using enhanced chemiluminescence system (ECL western blotting, Amersham, UK).

Results

CSBS was subjected to ion-exchange chromatography on DEAE-Sepharose CL-6B, and fractions enriched in protein and crystal growth inhibitory activity (38–100% inhibition), but also containing uronic acid, eluted between 0.2 and 0.8 M NaCl (Fig. 1). These fractions were pooled and subjected to DEAE-Sepharose CL-6B chromatography with stepwise rather than gradient elution. Fractions rich in protein were eluted by 250 mM NaCl, whereas those containing uronic acid were eluted by 500 mM NaCl (Fig. 2). The protein-rich fractions were then pooled and subjected to chromatography on a

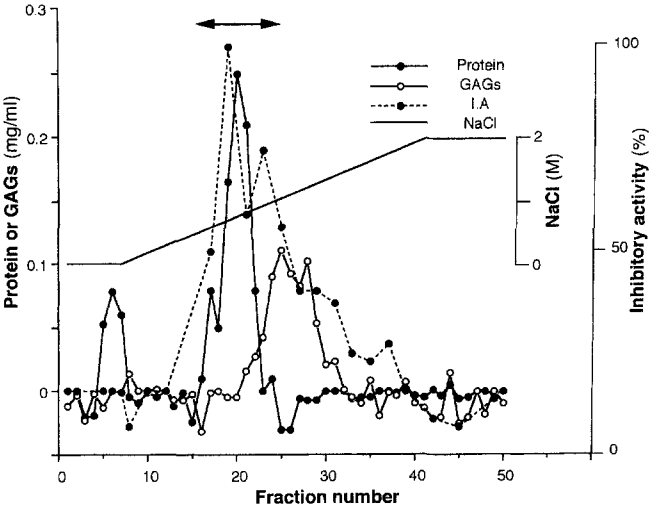

Fig. 1 Ion-exchange chromatography of crystal surface binding substance (CSBS) on diethylaminoethyl (DEAE)-Sepharose CL-6B with NaCl gradient elution. Protein, glycosaminoglycans (GAGs), and crystal growth inhibitory activity (*I.A*) were determined. Protein-rich fractions containing inhibitory activity (*arrow*) were pooled for further analysis

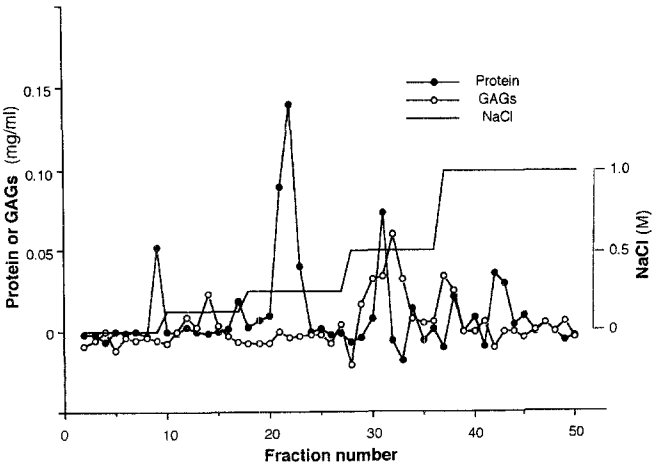

Fig. 2 Ion-exchange chromatography of pooled CSBS fractions on DEAE-Sepharose CL-6B with NaCl stepwise elution. Protein-rich fractions eluted by 250 mM NaCl were pooled for further analysis

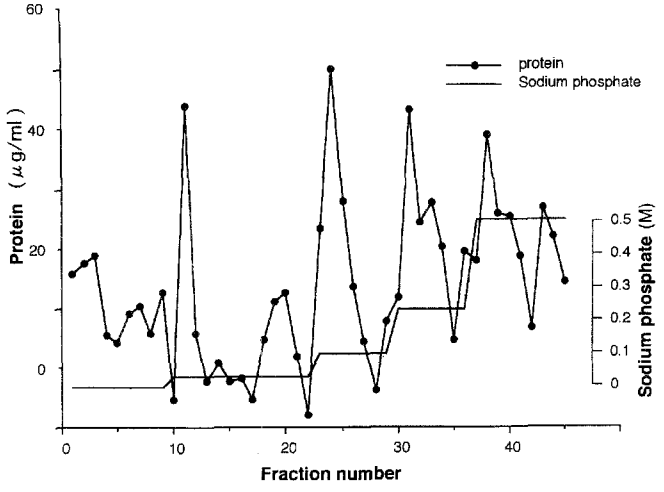

Fig. 3 Hydroxyapatite chromatography of pooled CSBS fractions with stepwise elution with sodium phosphate

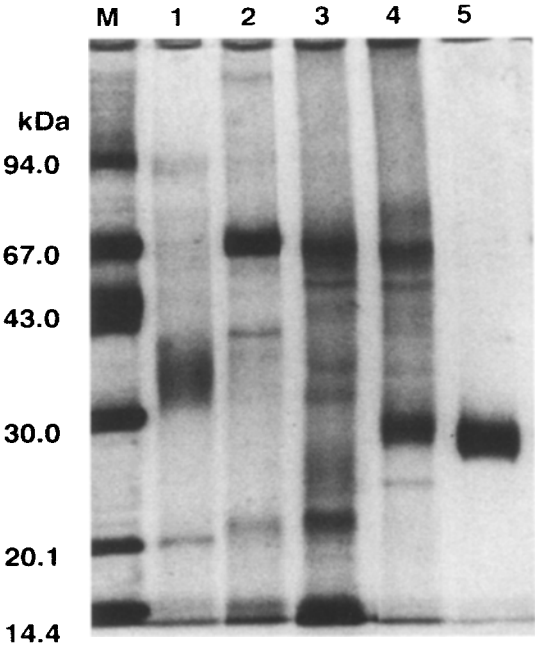

Fig. 4 SDS-PAGE analysis of fractions eluted from the hydroxyapatite column sodium phosphate at concentration of 30, 60, 125, 250, and 500 mM (lanes *1* to *5*, respectively). Lane *M* molecular size standards

column of hydroxyapatite. Protein-rich fractions were obtained with each concentration of sodium phosphate during stepwise elution as shown in Fig. 3. Fractions eluted from the hydroxyapatite column at each concentration of sodium phosphate were analyzed by SDS-PAGE. Only 30-kDa protein was shown in the fraction eluted at 500 mM sodium phosphate, and the fraction eluted at 60 mM sodium phosphate contained predominantly 67-kDa protein, whereas the fractions eluted at 125 and 250 mM sodium phosphate each contained several proteins as shown in Fig. 4. The NH_2-terminal amino acid sequence of the 30-kDa protein eluted by 500 mM sodium phosphate was determined to be Ala-Asn-Thr-Phe-Leu-X-X-Val-Arg-Lys-Gly-Asn-Leu-X-Arg-X-X-Val (where X is an unidentified residue), which shows 72% identity with the sequence of human prothrombin [4]. The NH_2-terminal amino acid sequence of the 67 kDa protein eluted by 60 mM sodium phosphate was determined to be Ala-Ile-Pro-Val-Ala-Gly-Asp-Leu-Asn-Ala-Pro-Ser-Asp-Trp-Asp-X-X-Gly-Lys,which shows an 89% identity with that of human osteopontin [10]. The crystal growth inhibitory activity of the fractions eluted from the hydroxyapatite column at the various concentrations of sodium phosphate were assayed. At a protein concentration of 5 μg/ml, the inhibitory activity of the fractions eluted at 125, 250, and 500 mM sodium phosphate was markedly greater than those of the fractions eluted at 30 and 60 mM sodium phosphate. The inhibitory activity of only the fraction eluted at 125 mM sodium phosphate remained high at a protein concentration of 0.5 μg/ml (Fig. 5).

Eight of 10 proteins were found in CSBS by immunoblotting: human serum albumin, α_1-acid glycoprotein, α_1-microglobulin, α_2-HS glycoprotein, retinol-binding protein, transferrin, Tamm-Horsfall mucoprotein, and prothrombin as shown in Fig. 6. α_1-Antitrypsin and β_2-microglobulin were not detected in CSBS. To analyze the protein composition of fractions eluted from the hydroxyapatite column by the above-mentioned concentrations of sodium phosphate, immunoblotting was carried out with antibodies to eight proteins which were found in CSBS. Samples that contained 2 μg protein were subjected to SDS-PAGE, transferred on to PVDF membranes, and analyzed by immunoblotting. Transferrin, retinol-binding protein, and α_1-microglobulin were chiefly found in the fraction eluted from the hydroxyapatite column by sodium phosphate at a concentration of 30 mM. α_1-Acid glycoprotein was detected in fractions eluted by 60 mM and 125 mM sodium phosphate, and α_2-HS glycoprotein was found in frac-

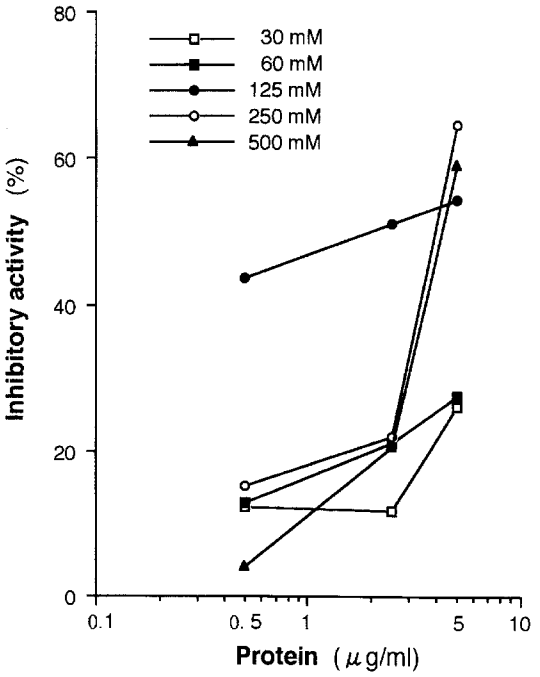

Fig. 5 Crystal growth inhibitory activity of fractions eluted from the hydroxyapatite column by the indicated concentrations of sodium phosphate.

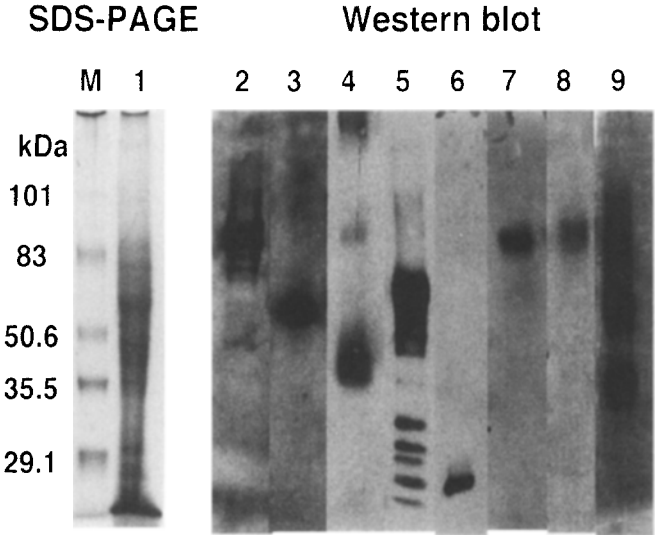

Fig. 6 SDS-PAGE and immunoblotting analysis of protein-rich fractions eluted from the DEAE-Sepharose CL-6B column by 250 mM NaCl. Lane *1* was the result of SDS-PAGE of protein-rich fraction. Lanes *2* to *9* were the results of immunoblotting. Lane *2* human serum albumin, *3* α_1-acid glycoprotein, *4* α_1-microglobulin, *5* α_2-HS glycoprotein, *6* retinol-binding protein, *7* transferrin, *8* Tamm-Horsfall mucoprotein, *9* prothrombin

tions eluted by 60 mM, 125 mM, and 250 mM sodium phosphate. Human prothrombin was detected in fractions eluted by 500 mM and 250 mM sodium phosphate. Tamm-Horsfall protein and human serum albumin were found in all fractions on hydroxyapatite column with sodium phosphate stepwise elution (Fig. 7).

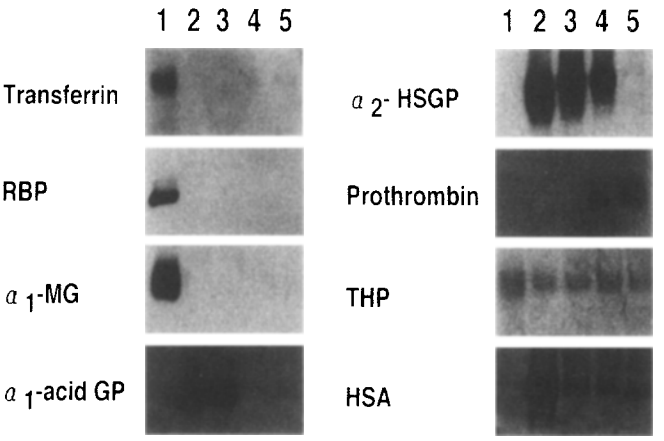

Fig. 7 Immunoblotting analysis of fractions eluted from the hydroxyapatite column by sodium phosphate at concentrations of 30, 60, 125, 250, and 500 mM (lanes *1* to *5*, respectively). *RBP* retinol-binding protein, α_1-*MG* α_1-microglobulin, α_1-*acid GP* α_1-acid glycoprotein, α_2-*HSGP* α_2-HS glycoprotein, *THP* Tamm-Horsfall protein, *HSA* human serum albumin

Discussion

Urinary macromolecules play an important inhibitory role in stone formation [9, 14] and proteins appear to be responsible for most of this inhibitory activity [11]. In the study of urinary stone formation, there have been three sources of proteins analyzed which are considered to affect stone formation. One is urine: nephrocalcin, uronic-acid-rich protein (UAP), uropontin and several other proteins are reported as important in the stone-forming process. Another source is a stone matrix: osteopontin is considered to play a most important role in stone formation. The other source is CSBS; crystal matrix extract [5] is included in this entity. We think that CSBS is related to stone matrix because analysis of GAGs showed that CSBS and stone matrix are quite similar [20]. Moreover, CSBS has a superiority to urine because of its substances binding calcium oxalate crystals and this fact reflects the functional interaction of CSBS.

We previously showed that CSBS exhibited marked inhibition on calcium oxalate crystal growth and contained both proteins and GAGs [12]. In this study, we demonstrated that two protein components were included in CSBS by N-terminal amino acid sequence analysis. These two proteins appeared strongly in the electrophoresis gel and were thought to be the dominant proteins by volume in CSBS. A 30-kDa protein that was eluted from a hydroxyapatite column by 500 mM sodium phoshate possessed an NH_2-terminal amino acid sequence 72% identical to the sequence of human prothrombin. This observation is consistent with the results of an analysis of crystal matrix protein by Suzuki et al. [18]. They concluded that activation peptide of human prothrombin inhibited calcium oxalate crystal aggregation. In our study, the 30-kDa protein was also thought

to reveal calcium oxalate crystal growth inhibition. The NH_2-terminal amino acid sequence of a 67-kDa protein that was eluted from the hydroxyapatite column by 60 mM sodium phosphate was 89% identical to that of human osteopontin. Umekawa et al. [19] speculated that osteopontin had an inhibitory effect on calcium oxalate crystallization because it was abundant in acidic amino acids and bound to hydroxyapatite by high affinity. However, the crystal growth inhibitory activity of the 67-kDa protein fraction was markedly lower than that of the 30-kDa fraction. So it is speculated that the 67 kDa protein has high affinity to calcium oxalate crystal and lower inhibitory activity on calcium oxalate crystallization.

Doyle et al. [5] showed that crystal matrix protein was the most abundant protein in crystal matrix and that it was distinct from major urinary proteins such as Tamm-Horsfall glycoprotein, albumin, Ig, complement factor C3, α_1-acid glycoprotein, α_1-microglobulin, apolipoprotein A1, and β_1-microglobulin. They concluded that most urinary proteins played no direct role in calcium oxalate crystal formation and that the incorporation of crystal matrix protein constituents into calcium oxalate crystals was a highly selective process. Similarly, CSBS appeared to be adsorbed on to calcium oxalate crystals selectively during crystallization when analyzed for the GAGs component [12]. In this study, it was shown by immunoblotting analysis that CSBS contains human serum albumin, α_1-acid glycoprotein, α_1-microglobulin, α_2-HS glycoprotein, retinol-binding protein, transferrin, Tamm-Horsfall mucoprotein, and prothrombin. Proteins contained in CSBS were different from those in crystal matrix extract [5]. This could be caused by a difference in the procedure for preparation in that calcium and oxalate were mixed in CSBS, whereas only oxalate was loaded in crystal matrix extract. α_1-Antitrypsin and β_2-microglobulin were reported to be found in stone matrix, but could not be found in CSBS. These two proteins would seem to bind calcium oxalate stone not selectively but through a secondary process such as inflammation or tubular damage.

Hydroxyapatite chromatography revealed that CSBS appears to contain inhibitors of calcium oxalate crystal growth other than prothrombin and osteopontin. In particular, the fraction eluted by 125 mM sodium phosphate contained marked inhibitory activity that, unlike the activity of the other fractions, was maintained after tenfold sample dilution. Four proteins – α_1-acid glycoprotein, α_2-HS glycoprotein, Tamm-Horsfall protein, and human serum albumin – were contained in this fraction, but it was considered that these four proteins play no direct role in this marked inhibitory activity because they were also contained in other fractions with weak inhibitory activity. In this study, we could not clarify the role of several proteins contained in CSBS other than crystal matrix protein and osteopontin as inhibitors. Also the significance of the Tamm-Horsfall protein and human serum albumin existing in CSBS could not be ascertained. However, these proteins revealed binding ability with calcium oxalate crystals selectively.

The identities of the fraction eluted by 125 mM sodium phosphate remain to be determined. Neither crystal matrix protein nor osteopontin were contained in this fraction. It could not be decided in this study whether an unknown single strong inhibitory protein was contained in this fraction or several proteins were interacting as a strong inhibitor. However, other unknown proteins are included in this fraction and they are thought to be dominant inhibitory proteins in CSBS. Similar work on urine of recurrent calcium oxalate stone formers will be needed.

In conclusion, we found several proteins in CSBS, some of which played a part as an inhibitor while others did not, but more interesting is that proteins other than crystal matrix protein and osteopontin played a strong inhibitory role in CSBS. Not one but many proteins are thought to play important roles in the interactions of crystals and the stone-forming process.

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