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Isolation and partial characterization of crystal matrix protein as a potent inhibitor of calcium oxalate crystal aggregation: evidence of activation peptide of human prothrombin

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Abstract In order to clarify the characteristics of crystal matrix protein (CMP), which exhibits a remarkable affinity for calcium oxalate crystals and may be important in stone pathogenesis, we have isolated CMP from macromolecular matrix substances of newly-formed calcium oxalate crystals. Purification of CMP consisted of calcium oxalate crystal formation, dissolution of crystals, electrodialysis, anion exchange chromatography and high-performance liquid chromatography. CMP showed the protein band of 31 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The N-terminal amino acid sequence of CMP was identical to that of human prothrombin. Both anti-CMP polyclonal antibody and anti-human prothrombin antibody cross-reacted well with human prothrombin and CMP in Western blotting. Its amino acid composition and its molecular weight of 31 kDa strongly suggest that CMP is the activation peptide of human prothrombin.

Key words Calcium oxalate crystal · Crystal matrix protein · Crystal aggregation · Aggregation inhibitor · Human prothrombin · Activation peptide (fragment 1 + 2)

The organic matrix of a kidney stone accounts for 2–3% of its total weight. Mucoprotein and glycosaminoglycans (GAGs) are found in the matrix. These macromolecules were initially thought to act as promoters of stone formation and growth because they easily bind to calcium. Later, however, such macromolecules were shown to act as inhibitors of the growth and aggregation of calcium oxalate crystals [5, 7, 8, 16, 19], and many efforts have been focused on the inhibitory role of the matrix of the stone. The question of whether each macromolecule, such as GAGs and glycoprotein, acts as a promoter or an inhibitor is still unresolved. Some macromolecules have been identified from urine or stone as inhibitors, for example nephrocalcin [11–13] and uropontin [18] from human urine as a potent inhibitor of calcium oxalate stone or crystals.

Many investigators have tried to find GAGs as well as other macromolecules in urinary stones, not calcium oxalate crystals. When stones that have been in existence for many years are used as starting materials, additional effects of blood, serum or epithelial cells on the surface must be considered. Khan and Hackett reported that the structure of calcium oxalate crystals was similar to that of the stones and that organic material was present on the surface of crystals, as well as inside them, by electron microscopy [6]. Therefore, newly-formed calcium oxalate crystals should be the best materials to study macromolecules in crystals. Doyle et al. [4] isolated crystal matrix protein (CMP) from fresh calcium oxalate crystals. Their CMP, however, still seemed to be crude because GAG was included in the macromolecular substances in the crystals.

This paper reports further purification of the intracrystalline macromolecular substances to obtain pure CMP and describes studies of amino acid composition and sequence as well as immunochemical investigations.

Materials and methods

Collection and preparation of urine samples

Twenty-four-hour urine specimens were collected from five healthy men between 30 and 43 years old, then NaCl was added at the final concentration of 580 mM and the urine was kept at 4°C for 48 h to remove Tamm-Horsfall mucoprotein. No specimen showed any sign of blood. Urine specimens were then centrifuged at 6000 *g* for 60 min and passed through 5-μm Millipore GV filters, followed by 0.22-μm filters.

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Crystal preparation

The method of inducing calcium oxalate crystallization in undiluted urine has been described [15, 21]. Shortly, minimum amount of sodium oxalate required to produce crystals in 200 µl urine was determined as the metastable limit using microplate and inverted microscope. Once the metastable limit was determined, the amount of oxalate which gave the final concentration of 0.3 mM excess of the limit was added to urine specimens dropwisely. A Coulter counter (Model TAII, coupled to an NEC personal computer) was used to monitor the crystal particle size. The same amounts of oxalate were added after 1 h and 2 h to promote the formation of calcium oxalate crystals. The crystals were washed on a 0.22-µm Millipore filter six times with 0.1 M sodium hydroxide and then with deionized and doubly distilled water (d.d. water) to wash out the surface substances completely, then lyophilized, weighed and demineralized by the addition of 100 ml 0.25 M EDTA (pH 8.0) to every 1 g crystals at 4°C in the presence of protease inhibitors (0.1 M 6-aminohexanoic acid, 1 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride and 10 mM *N*-ethylmaleimide).

Electrodialysis

The solution of demineralized crystal extracts was electrodialysed in the dialysis tube (Sigma, 10-kDa cut-off) against 25 mM Tris, 192 mM glycine buffer (pH 8.3) with four changes of the buffer at 80 V initially, with gradual increase to 120 V finally, then against d.d. water overnight at 4°C, changing the water three times, and lyophilized. Crystal matrix extract was dissolved in d.d. water and desalted with an Econo-Pac 10DG column (BioRad, 6-kDa cut-off).

Anion exchange chromatography

The desalted material was adjusted to 20 mM Tris-HCl (pH 7.2) and applied to an Econopac Q cartridge (BioRad) previously equilibrated in the same buffer. After washing with 20 ml of the same buffer, the column was eluted with a linear gradient of NaCl (0–0.8 M) in Econo System B (BioRad). The concentration of protein was monitored by the ultraviolet absorption at 280 nm (A_{280nm}). Fractions of 4 ml were collected. The concentration of GAGs in each fraction was measured by the Brumenkranz and Asboe-Hansen method [2]. Inhibition of calcium oxalate crystal aggregation and growth was measured by the seed crystal method [14].

Amino acid analysis and sequence determination

Proteins in fractions with high inhibitory activity from anion exchange chromatography were further separated from nonproteinaceous substances by reverse-phase high-performance liquid chromatography (HPLC) in a Brownlee RP-300 column (2.1 mm i.d. × 30 mm, C8, 300 Å) with a linear gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid. Amino acid composition was determined with a Hitachi model L-8500 amino acid analyzer after hydrolysis in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 24 h. Automated Edman degradation was performed with a model 477A Applied Biosystems Gas-phase Sequencer equipped with a 120A 3-phenyl-2-tiohydantoin (PTH) analyzer according to the standard analytical procedure from the manufacturer.

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

Materials were dissolved in 100 µl SDS sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.025% bromophenol blue) and heated for 6 min at 100°C. After cooling, 10-µl samples were electrophoresed in a 0.75-mm-thick 16% gel in a BioRad Mini-Protean II using Laemmli buffer system at 200 V for 45 min, then stained with Coomassie Brilliant Blue R-250 (CBB) and destained. Molecular weights of samples were compared with that of protein standard (BioRad).

Western blotting and immunochemical staining

After electrophoresis, gel was immersed in transfer buffer [25 mM Tris, 192 mM glycine and 20% (w/w) methanol, pH 8.3] and the protein was transferred onto a nitrocellulose membrane for 1 h at 100 V in a Mini TransBlot (BioRad) at 4°C. The nitrocellulose membrane was blocked with 3% gelatin in 10 mM Tris and 150 mM NaCl (Tris-buffered saline, TBS) for 24 h. After washing with TBS plus 0.05% Tween 20 (TBST), the membrane was soaked in the primary antibody diluted 200-fold in 1% gelatin in TBST for 2 h. After twice washing with TBST, the membrane was incubated in the second antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 3000-fold in 1% gelatin in TBST for 1 h. Finally, the membrane was stained with Immuno-Blot Assay (BioRad). The primary antibodies used were goat anti-human prothrombin polyclonal antibody (Nordic Immunological Laboratories, The Netherlands) and anti-CMP polyclonal antibody (kindly donated by Dr. Rosemary L. Ryall, Flinders Medical Centre, South Australia).

Immunoelectrophoresis

Normal human serum, 100-fold concentrated male urine, CMP and human prothrombin were electrophoresed in thin-layer 1% agarose gel, then reacted with antibody solutions (goat anti-human serum and goat anti-human prothrombin) for 24 h. The gel was stained with CBB for 20 min and destained.

Results

Anion exchange chromatography

The result of anion exchange chromatography of crystal matrix extract is shown in Fig. 1. Inhibitory activity of calcium oxalate crystal aggregation and A_{280nm} showed two peaks respectively. Only the second A_{280nm} peak, fraction *b*, had high inhibitory activity. The other inhibitory peak, fraction *c*, had a high concentration of GAG, which was identified as heparan sulfate by electrophoresis and heparitinase digestion (data not shown). Fraction *a* of the higher A_{280nm} peak and fractions *b* and *c* with high inhibitory activities were collected, lyophilized and used for electrophoretic study.

SDS-PAGE

SDS-PAGE patterns of the molecular weight of crude crystal matrix extract, fractions *a*, *b* and *c* from anion exchange chromatography, concentrated male urine, serum and commercially available human prothrombin are shown in Fig. 2. Results showed that crystal matrix extract contained mainly 31-kDa protein which was

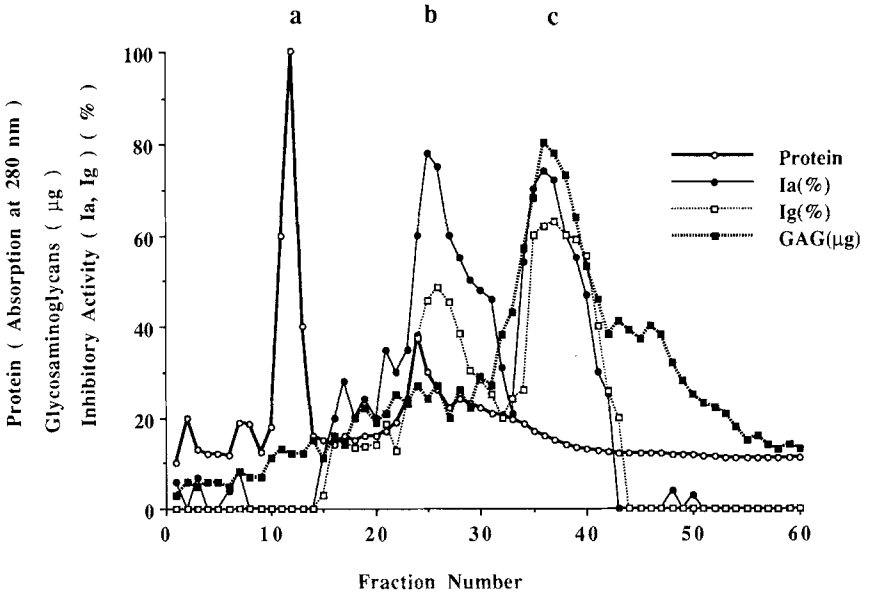


Fig. 1 Anion exchange chromatography of calcium oxalate crystal extract. Inhibitory activity of aggregation (*Ia*), growth (*Ig*) and the content of glycosaminoglycan (*GAG*) in each fraction were determined. Fractions *a* and *b* had high protein peak. Two fractions with high Ia were collected as fractions *b* and *c*

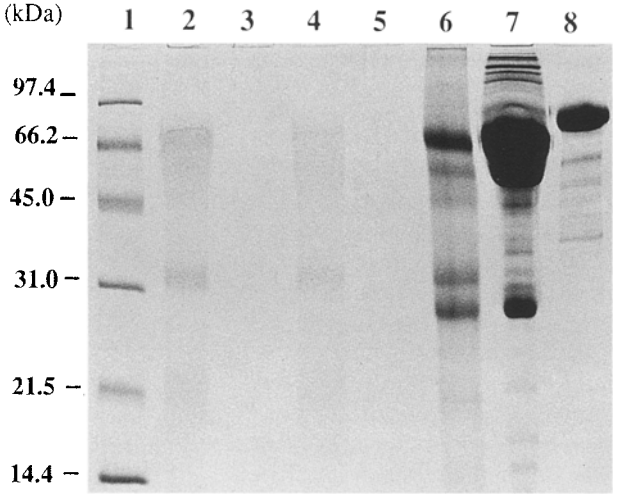


Fig. 2 Sodium dodecyl sulfate-polyacrylamide electrophoresis. *Lane 1,* protein standard; *lane 2,* crystal matrix extract; *lane 3,* fraction *a; lane 4,* fraction *b; lane 5,* fraction *c* obtained from anion exchange chromatography shown in Fig. 1; *lane 6,* normal male urine (100-fold concentrate); *lane 7,* normal human serum; *lane 8,* human prothrombin. In lanes 2 and 4, a, 31-kDa protein band (CMP) can be seen

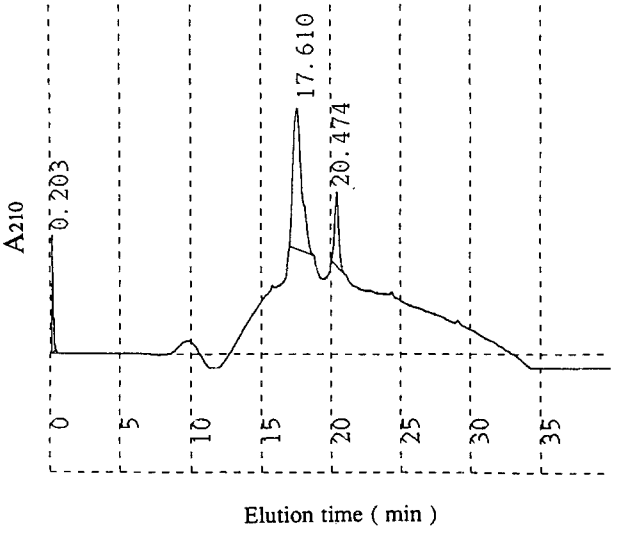


Fig. 3 Reverse-phase high-performance liquid chromatography. Two peaks appeared. There was nonproteinaceous impurity in the first peak. Only the second peak showed 31-kDa protein band in SDS-PAGE

eluted in fraction *b* and was thought to be CMP, as already reported [4]. In urine and serum mainly albumin band was visible. Fraction *b* still contained impurities, so it was further purified by reverse-phase HPLC (Fig. 3). Only the second peak contained a 31-kDa protein band in SDS-PAGE, while the first peak showed no protein band and presumably contained nonproteinaceous impurity substances (data not shown). Protein from the second peak was used for amino acid analysis and sequence determination.

Amino acid sequence and amino acid composition of CMP

The N-terminal amino acid sequence of CMP determined was

^{1} ^{5} ^{10}
Ala-Asn-Thr-Phe-Leu-Xxx-Xxx-Val-Arg-Lys-Gly-Asn-

^{15} ^{20}
Leu-Xxx-Arg-Xxx-Xxx-Val-Xxx-Xxx-Thr-,

Table 1 Amino acid composition of crystal matrix protein (CMP) and comparison with that of the activation peptide of human prothrombin (PAP) [16, 17]

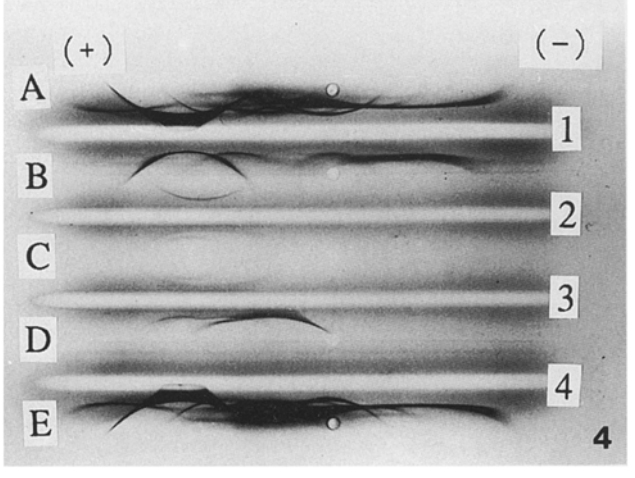
Amino acid	CMP	PAP
Asp	28.3	30
Thr	27.5	24
Ser	18.3	18
Glu	41.8	43
Pro	15.1	16
Gly	17.0	21
Ala	20.6	23
Val	12.8	16
1/2 Cys	16.8	16
Met	1.5	1
Ile	6.8	5
Leu	14.5	17
Tyr	8.9	10
Phe	6.6	9
Lys	7.6	7
His	5.1	5
Trp	2.1	5
Arg	19.2	18
Total	270.5	284
Molecular weight	31000	31514

which was identical to the N-terminal sequence of human prothrombin [3] by the homology search in the DDBJ database. Positions of Xxx which could not be identified as PTH amino acid completely correspond to γ-carboxy glutamic acid (Gla) positions. The amino acid composition of CMP is shown in Table 1. CMP has a relatively large number of acidic amino acids such as aspartic acid and glutamic acid. The number of amino acid residues calculated from the molecular weight of 31 kDa was 270.5. The composition of prothorombin activation peptide [1, 3] shows good agreement with that of CMP (Table 1). Gla residues in CMP were not measured in this experiment. The molecular weight of 31 kDa together with amino acid composition and N-terminal sequence strongly suggested that CMP was the activation peptide corresponding to residues 1–284 of human prothrombin (residues 1–579 are mature prothrombin with molecular weight of 71 kDa) as reported by Degen [3]. Although not shown here, CMP preparations from female urine and male recurrent stone former's urine showed completely the same molecular weight and N-terminal sequence as those of CMP from male urine shown here, suggesting that the prothrombin activation peptide is the common constituent of CMP.

Immunelectrophoresis and western blot analysis

Immunoelectrophoresis patterns of CMP, 100-fold concentrated urine and human male serum using anti-human prothrombin and anti-human serum are shown in Fig. 4. While anti-human serum reacted with serum and urine, not with CMP, anti-human prothrombin reacted with CMP and urine. In other words, substances which react with anti-human prothrombin exist in CMP and urine.

To confirm these results, western blotting was done for CMP, urine, serum and prothrombin using commercially available anti-human prothrombin antibody and anti-CMP polyclonal antibody (Fig. 5). CMP antibody reacted strongly with 31-kDa protein as well as prothrombin. Although other weak bands could be seen in lane 1, these are thought to represent multiple forms of the same peptide. Anti-human prothrombin antibody reacted well to both prothrombin and 31-kDa protein. The results



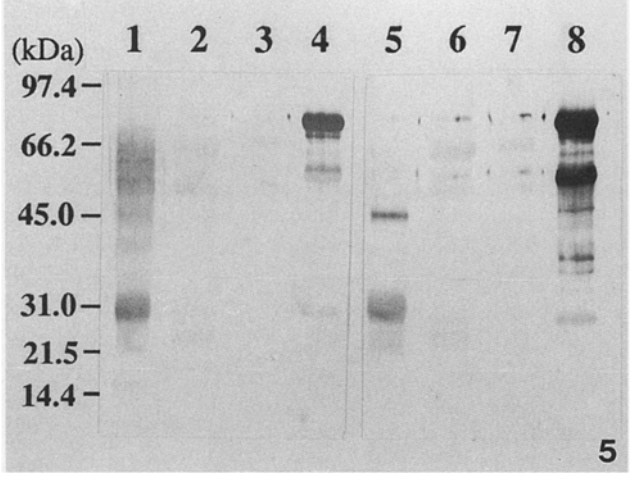


Fig. 4 Immunoelectrophoresis of CMP, normal male urine and normal serum using anti-human prothrombin antibody. *Wells A, E,* normal serum; *well B,* human urine (100 fold concentrated); *well C,* crystal matrix protein; *well D,* human prothrombin. *Troughs 1 and 4* contained antiserum to normal human serum, and *troughs 2 and 3* contained anti-human prothrombin. Anti-human prothrombin reacted with CMP and urine as well as prothrombin

Fig. 5 Western blotting of CMP (*lanes 1, 5*), human urine (*lanes 2, 6*), human serum (*lanes 3, 7*) and human prothrombin (*lanes 4, 8*). *Left* (lanes 1–4): antiserum to CMP was used; *right* (lanes 5–8): antiserum to human prothrombin was used. CMP antibody reacted strongly with 31-kDa protein as well as prothrombin. Prothrombin antibody reacted well to both prothrombin and 31-kDa protein

showed that CMP and human prothrombin share common epitopes and are therefore identical or closely related proteins.

Discussion

This study supports the conclusion of a previous investigation by Doyle et al. [4] that CMP found in newly formed calcium oxalate crystals exhibits a remarkable affinity for calcium oxalate crystals. CMP was named by the observation that major proteins such as Tamm-Horsfall glycoprotein, albumin, immunoglobulin, factor C_3, α_1- acid glycoprotein, α_1- microglobulin, apolipoprotein A-I and β_1-microglobulin were distinct from the most abundant protein in crystal matrix using the western blotting technique. Doyle et al. concluded that most urinary proteins play no direct role in calcium oxalate crystal formation and that CMP is included into calcium oxalate crystals as a highly selective phenomenon. Morse and Resnik found the protein component by almost the same approach, using two-dimensional gel electrophoresis [10], and suggested that the binding of urinary protein to calcium oxalate crystals formed in urine may be a selective phenomenon, because the amount and component of urinary protein did not reflect their relative concentration in the urine.

We used almost the same procedure as Doyle et al. [4] to purify CMP and found that macromolecular substances in the crystals still contained impurities such as a small amount of albumin and small peptides as shown in SDS-PAGE, in agreement with Morse and Resnik [10]. Moreover, we found heparan sulfate in the crystal matrix extract by GAG measurement and electrophoresis. In anion exchange chromatography, heparan sulfate was found in fraction *c*. The crystal matrix extract was further purified by anion exchange chromatography and reverse-phase HPLC. CMP was eluted in the first inhibitory peak fractions, which overlapped the second A_{280nm} peak, fraction *b*, in anion exchange chromatography completely apart from heparan sulfate containing fraction *c*. Although a major protein band of 31 kDa with a trace 45-kDa band in SDS-PAGE with CBB staining was obtained in this step, the 31-kDa protein was further purified by reverse-phase HPLC to near homogeneity.

To our knowledge, this is the first report that the N-terminal of the main protein component of calcium oxalate crystals shows the same amino acid sequence as human prothrombin. As CMP showed the molecular weight of 31 kDa, a possible candidate is the activation peptide or fragment 1 + 2 of human prothrombin, which has 284 amino acid residues and a calculated molecular weight of 31514 (Table 1). Mature prothrombin has 579 amino acid residues and a molecular weight of 71 kDa with carbohydrate [3]. Interestingly, human prothrombin is well known to be a plasma glycoprotein- and vitamin K-dependent, Gla-containing blood-clotting protein. Ten residues of Gla are located in the activation peptide, not in the mature thrombin sequence. Gla is produced by posttranslational carboxylation of specific glutamic acid residues in the precursor protein mediated by vitamin K. The activation of prothrombin requires the binding of Ca^{2+}. Deficiency of vitamin K produced by the administration of the vitamin K antagonist dicoumarol results in the production of abnormal prothrombin due to the lack of activation of glutamic acid to Gla. Uropontin, a potent inhibitor of calcium oxalate crystal growth reported by Shiraga et al., has no Gla residues [18].

In calcium-containing renal stone, Lian et al. [9] found protein-bound Gla. They separated the Gla-containing protein (six Gla per 100 amino acid residues) with a molecular weight of 17 kDa and reported that the amino acid composition had no relation to Gla-containing bone protein or fragment 1 of human prothrombin. Nakagawa et al. isolated a Gla-containing protein, nephrocalcin, from human urine as a potent inhibitor of crystal growth [11–13]. They stated that the glycoprotein derived from stone formers lacked Gla residues, in contrast to that from normal individuals, which contained 2–3 Gla residues per mole, and that patient inhibitors were almost normal in amino acid and carbohydrate compositions and molecular weight despite the abnormal behavior and composition. Therefore they concluded that the molecular disorder might be responsible for some component of calcium oxalate nephrolithiasis.

As mentioned earlier, CMP derived from recurrent stone formers showed the same N-terminal sequence as that of normal males or females. Recently Ryall and colleagues [17] showed that CMP from female had higher affinity to calcium oxalate crystals than that from male. This might lead the sex differences of stone disease. There is also the possibility that CMP from stone formers might have low affinity to crystals. Further experiments are needed to clarify these possibilities.

In this study, CMP was not found in serum but in urine. As to the origin of CMP, Stapleton [20] reported that CMP was produced in the kidney and was associated with the cytoplasm of the thick ascending limb of the loop of Henle and the distal convoluted tubule as shown by an immunostaining technique with polyclonal antibody against CMP.

From calcium oxalate crystal matrix, two inhibitors have been isolated, CMP and heparan sulfate. While CMP shows potent inhibition of calcium oxalate crystals, heparan sulfate strongly inhibits crystal aggregation in Coulter counter analysis and scanning electron microscopy. Studies on interaction between inhibitors and crystals, as well as between CMP and heparan sulfate, are required. In addition, a sensitive method must be developed to investigate the normal concentration of CMP and its affinity to the crystals.

In conclusion, the present study demonstrates the very presence of an inhibitor of calcium oxalate crystal aggregation and its identity with the activation peptide of human prothrombin. CMP is quite distinct from nephrocalcin and uropontin with regard to molecular weight, amino acid composition and amino acid sequence.

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