DISCUSSION
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Protein inhibitors of CaOx crystal growth from different sources have been proposed to play an important role in kidney stone disease for several decades (Aggarwal et al. 2000; Aggarwal et al. 2005; Kaur et al. 2009; Bijarnia et al. 2009). Identification of additional stone inhibitory proteins was hampered in the past by limitations in protein identification methods thereby making the identification of novel proteins or other low abundance molecules quite difficult without prior knowledge of their involvement in this process. Recent advances in the technologies are therefore mandatory to study CaOx crystal growth inhibitors for better understanding of the pathophysiology and pathogenesis of kidney stone disease. Many proteins present in the organic matrix play an important role in kidney stone formation (Aggarwal et al. 2000), but only few of them are identified and fully characterized. The purpose of the present study was to use biochemical purification methods and recent advances in mass spectrometric protein identification to characterize and identify a novel calcium oxalate crystal growth inhibitor from the organic matrix of human renal stones.

A comparison of different methods of protein extraction from COM stones was done to examine the soluble matrix proteins involved in the calcium oxalate monohydrate (COM) stone biomineralization process. The protein concentration of the supernatants obtained using three different extraction methods: 2% SDS, 0.05M EGTA and 10% acetic acid was measured by Bradford method. Maximum amount of proteins from human renal stone matrix was obtained from SDS extraction method followed by EGTA and acetic acid extraction methods respectively. The above observations were corroborated by SDS-PAGE analysis of the proteins extracted by three different extraction methods viz. 2% SDS, 0.05M
EGTA and 10% acetic acid respectively. A negligible inhibitory activity on CaOx crystal growth system was observed in the proteins extracted by acetic acid method. Proteins extracted by SDS method did not show any activity at all. It may be due to the denaturing property of SDS as a result of which extracted proteins might have lost their activity regardless of the high yield of protein yielded by SDS extract. Based on these observations EGTA fraction (more than 10 kDa) exhibiting highest inhibitory activity on CaOx crystal growth assay was subjected to chromatography using a strong anion exchanger. The purification was performed systematically using anionic and molecular-sieve chromatography followed by SDS-PAGE analysis after each purification step. It was found that fraction P6 obtained after anion exchange chromatography exhibited highest inhibitory activity against CaOx crystal growth and its SDS-PAGE analysis showed the presence of few bands. However, when this fraction P6 was further purified by molecular-sieve chromatography, the fractions (P1', P2', P3' and P4') so obtained all showed inhibitory activity. Purified potent protein fraction P2', which showed a single band of ~ 42 kDa was found to possess highest inhibitory activity against CaOx crystal growth. Analysis of fraction P2' by MALDI-TOF-MS resulted in peptide mass finger print which when followed by database search (SwissProt) on a MASCOT server matched significantly with human phosphate cytidylyltransferase 1, choline, beta. This protein is present in CDP-choline pathway and is used in catalyzing the condensation of CTP and phosphorylcholine to form CDP-choline as the rate limiting and regulatory step in the CDP-choline pathway. In this pathway, choline is phosphorylated to form phosphorylcholine, which reacts with cytidine triphosphate to form cytidine 5'-diphosphocholine (CDP-choline), the immediate precursor of phosphatidylcholine. Phosphatidylcholine (lecithin) is the predominant phospholipid
(>50%) in most mammalian membranes. Phosphatidylcholine also happens to be the major phospholipid of renal membranes (Leah and Toback 1980). Incidentally, the molecular weight of fraction P2' (~42 kDa) after its SDS-PAGE analysis and homogeneity confirmation by RP-HPLC was found to be same as that of human phosphate cytidylyltransferase 1, choline, beta. In this study, human phosphate cytidylyltransferase 1, choline, beta which is identified from human renal stone matrix as a novel CaOx crystal growth inhibitor holds a direct relevance since it is involved in the formation of phosphatidylcholine which is a constituent of calcium oxalate stones (Priyadarshini et al. 2009). Khan et al. have reported that calcium oxalate stones contain cardiolipids, sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol in lipid extract (Khan et al. 1988). It was found that lipid extract of renal stone exhibits inhibitory activity against CaOx crystal growth (Priyadarshini et al. 2008). CDP-choline leads to the formation of phosphatidylcholine and finally choline is formed. Choline is a dietary component essential for the normal function of all cells. It, or its metabolites, assures the structural integrity and signaling functions of cell membranes. Most choline in the body is found in phospholipids such as phosphatidylcholine and sphingomyelin. In most mammals, prolonged ingestion of a diet deficient in choline (and adequate though limited in methionine and folate content) has consequences that include hepatic, renal, pancreatic, memory, and growth disorders (Zeisel and Blusztajn 1994). Interestingly, Lipostabil (a drug having phosphatidylcholine and deoxycholate) is also reported to improve enzymic indices of renal tubular damaging enzymes viz. alkaline phosphatase, gammaglutamyl transferase, alpha-glucosidase and
lactate which promote nephrolithiasis (Neimark et al. 1998). This is also in conformity with the inhibitory role of this novel protein which is reported here, in urolithiasis.

Of all types of renal stones, calcium oxalate (CaOx) is the most common composition found by chemical analysis (Coe et al. 1992). CaOx crystal growth inhibitors (proteins, lipids, glycosaminoglycans, and inorganic compounds) have been proposed to play an important role in renal stone disease (Zerwekh et al. 1983; Coe et al. 1991). During the last few years, more and more research has been done at the cellular and molecular levels. In spite of these advances however, the clinical treatment of urolithiasis remain far from satisfactory. Stone recurrence in human beings can be predicted and is beyond the control of urologists, mainly because the mechanism of stone formation at molecular level is not yet fully understood (Aggarwal et al. 2000). Thus, determining the molecular mechanisms by which urinary constituents modulate calcium oxalate crystallization is crucial for understanding and controlling urolithiasis in humans. Although a few initial molecular-scale investigations of the controlling mechanisms of kidney stone formation by these inhibitory molecules have been recently performed (Shirane et al. 1999; Guo et al. 2002; Qiu et al. 2004; Jung et al. 2004), the majority of previous studies have been concerned with the overall kinetics of crystallization, rather than molecular mechanisms which remain poorly defined. Therefore, much attention was focused on the interaction of the purified human phosphate cytidylyltransferase1 (CCT) with calcium oxalate using bioinformatics tools. Cytidylyltransferases are critical enzymes involved in the biosynthetic pathways of lipids and complex carbohydrates. These enzymes catalyze a major step of energy input into biosynthesis by forming the activated intermediates, CDP-alcohols and CMP-sugars (Weber et al. 1999; Pattridge et al. 2003; Inatsugi et al. 2009).
In fact CCT is involved in the biosynthesis of phosphatidylcholine which happens to be an important constituent of human renal stones and is also reported to have an antilithiatic effect. Several cytidylyltransferases belong to a single family of structures, as defined by sequence similarities and signature sequence that occur in their catalytic domains. Among these enzymes is CTP: glycerol-3-phosphate cytidylyltransferase (GCT) from \textit{Bacillus subtilis}. Because GCT consists of only the catalytic domain, it is an excellent choice for initial structure function studies of the family (Weber et al. 1999; Pattridge et al. 2003; Park et al. 1997). The degree of identity between the template and the human CCT sequence was 31\%, which enabled a preliminary model to be generated by Schrodinger. Aspartic acid, glutamine, alanine, histidine, tyrosine were found to be interacting with calcium oxalate at site 1. More negative the docking score, stronger is the binding between ligand and protein’s active site (Bijarnia et al. 2008). The strong interaction between CCT’s active site and calcium oxalate predicts inhibition of the same. Highest docking score (-106.304) with site 3 indicates good binding of modeled protein with the ligand calcium oxalate. LIGPLOT of site 3 showed involvement of aspartic acid (Asp) at position 41 with calcium. Lysine, the basic amino acid at position 45, interacts with oxygen of oxalate group. Due to the large size of binding site 1, small size calcium oxalate was not able to interact with all amino acids. Small size of binding site 2 and site 3 was better for interaction with calcium oxalate. Whether a protein or other macromolecule acts as an inhibitor of growth and aggregation or a promoter of nucleation and aggregation implies that there must be some mechanism to explain the interaction with the mineral oxalate surfaces. The interaction between calcium and acidic Asp, Glu and Gla is certainly plausible, but it is equally conceivable that basic residues that are normally protonated at urinary pH and positively charged might experience an attraction
toward negatively charged oxalate groups (Gul and Rez 2007). This is corroborated by the presence of basic amino acids too in the inhibitory proteins (Tandon et al. 1998). In either case, steric constraints from 3D conformation of the molecule might limit the number of these simple interactions (Gul and Rez 2007). Positive docking score with mutated binding sites confirms the inhibitory role of acidic amino acids (Nakagawa et al. 1987; Nakagawa et al. 1985; Shiraga et al. 1992). There are reports that members of cytidylyltransferase family have a number of residues that are conserved (Weber et al. 1999; Pattridge et al. 2003; Park et al. 1997). In present study, 40% sequence in binding site 1 and 20% sequence in site 2, in the conserved region of CCT was found. Interestingly, conserved amino acids are contained within the catalytic core of the CCTs (Kalmar et al. 1990). It was found that the acidic amino acids are interacting with the calcium while basic amino acids interact with oxygen of calcium oxalate. This was further substantiated when substitution of these acidic amino acids with alanine, glycine, lysine, arginine and histidine completely diminished the interaction with calcium oxalate. These findings are in conformity with the presence of acidic amino acids in the various inhibitors of calcification from human beings (Nakagawa et al. 1987; Nakagawa et al. 1985; Shiraga et al. 1992) as well as acidic nature of antilithiatic proteins from plants (Kaur et al. 2009; Bijarnia et al. 2009).

Among all types of kidney stones, the frequency of calcium stone is 70–80%, struvite stone 5–10%, uric acid stone 5–10%, and cystine stone 1% (Morton and Wooltorton 2002). Calcium oxalate is the primary component of 70–80% of calcium stones (Herring 1962; Mandel and Mandel 1989; Tefekli et al. 2003) with calcium phosphate being the predominant component in the rest of calcium stones. A recent study has reported that the occurrence of calcium phosphate containing stones has increased over time (Mandel et al.
Calcium phosphate occurs in stones in several different forms: amorphous calcium phosphate (ACP), hydroxyapatite (HAP), brushite (Bru), whitlockite, and carbonate apatite (CarbAp). The first product that precipitates is an ACP, which subsequently is converted to the crystal phases, octacalcium phosphate (OCP) and HAP or occasionally Bru. Hydroxyapatite is the thermodynamically most stable calcium phosphate crystal phase and it is also the major crystal phase in mixed calcium oxalate/calcium phosphate stones. Under certain conditions brushite (Bru; calcium hydrogen phosphate) is formed (Leusmann et al. 1995; Györy and Ashby 1999; Hesse and Heimbach 1999; Kalaiselvi et al. 1999). Since, as much as 80% of the stones studied were mixtures of calcium oxalate monohydrate (whewellite) and calcium phosphate (hydroxyapatite) in various proportions, (Fazil 2009) hence, in the present study, the effect of renal calculi organic matrix biomolecules from calcium oxalate monohydrate stones was also evaluated on in vitro calcium phosphate (CaP) crystallization. Whole EGTA extract exhibited inhibitory activity in initial and growth of CaP mineral phase. Stimulatory and inhibitory activity was shown by >10 kDa fraction in initial mineral phase. Stimulatory activity was retained in growth mineral phase by this fraction. <10kDa had inhibitory activity in initial CaP mineral phase. Both inhibitory and stimulatory activity was shown by <10kDa fraction in CaP growth mineral phase.

High percentage of phosphate ion was released with high volume of all the three fractions. But the opposite trend was observed with calcium ion demineralization. It was found that high percentage of calcium ion was released with low volumes of all the three fractions. Romberg et al. have reported that macromolecular modifiers of calcium oxalate crystallization (Romberg 1986) are also active in the corresponding steps of calcium phosphate crystallization. There is, however, evidence that Mg, citrate, and pyrophosphate
are the most important inhibitors of calcium phosphate crystal growth. There are reports explaining the activity of uric acid binding protein (Kalaiselvi 1999) and calcium phosphate binding protein (Nishio 2001) on calcium oxalate crystallization. The predominant proteins found in organic matrices of CaOx crystals induced in the urine of healthy controls were prothrombin-related proteins followed by albumin and osteopontin. In matrices of CaP crystals, the principal proteins were Tamm-Horsfall protein followed by albumin, prothrombin-related proteins and osteopontin (Atmani and Khan 2002). Osteopontin (OPN) and Tamm-Horsfall protein (THP) are two major urinary macromolecules that exhibit various activities that can influence calcium crystallization in vitro (Devuyyst et al. 2005; Kumar and Ljeske 2006). OPN is a ubiquitously expressed phosphoglycoprotein that regulates bone biomineralization and ectopic calcification (Giaehelli and Steitz 2000; Lan et al. 2007). So, these studies suggest that both high and low molecular weight biomolecules extracted from human renal matrix of calcium oxalate (CaOx) stones have a significant influence on calcium and phosphate (CaP) crystallization.

In the present study, human phosphate cytidylyltransferase 1, choline, beta from human renal stone matrix is identified as a novel CaOx crystal growth inhibitor and it is involved in the formation of phosphatidylcholine which is a constituent of calcium oxalate stones (Khan et al. 2002). In the light of this observation, it was worthwhile to check the effect of lipid extract of renal stones on in vitro growth of CaOx crystals. Lipids have been isolated from matrices of all types of mineralized tissues and have been suggested to play an important role in the mineralization process. Of various matrices, lipids make up 7-14% of bone, 2-6% of dentin, and 12-22% of newly mineralized enamel (Boskey 1981). Thus, the percentage of lipid in calcium oxalate stone matrix at 10.15% is comparable to that of the
other mineralized systems (Khan et al. 2002). Since most of the lipids encountered in urinary stones are those that are present in cellular membranes (Vogel et al. 1976; Jackson and Gotto 1974) and cellular degradation products have been observed in human calcium oxalate urinary stones by transmission electron microscopy (Finlayson et al. 1984), presence of lipids may represent passive incorporation of sloughed epithelial cells in growing stones. Conversely, membranes and membrane associated lipids may play an active role in urolithiasis by either stimulating or inhibiting biomineralization process. The selective mineralization process could be attributed to certain biomolecules present in the body fluids which can control the mineralization process by acting as either inhibitors or promoters of mineralization (Tandon et al. 1999). As there are proteins biomolecules reported in kidney stone which are involved in inhibiting kidney stone formation as well as in stimulating the same (Aggarwal et al. 2000; Aggarwal et al. 2005). Prothrombin, uronic acid rich protein, urinary prothrombin fragment1 act as inhibitor in urolithiasis whereas uropontin act as stimulator, while Tamm Horsfall Glycoprotein act as stimulator as well as inhibitor. 66 kDa protein from kidney stone is also reported to act as promoter (Aggarwal et al. 2000). While biomolecules isolated from urine also exhibited inhibitory effect (Moghadam 2003). Lipids are present in stone matrices of all stones irrespective of the inorganic nature of their major crystalline components, be they calcium oxalate, calcium phosphate, struvite or uric acid. Altered membrane lipids might promote selective nucleation and retention of calcium oxalate crystals and the process become a part of the growing crystals and stones (Khan et al. 2002). It was observed that the lipids extracted from kidney stones had an ability to inhibit growth of COM crystals. Not only protein but lipids were also involved in the inhibition of kidney stone formation (Priyadarshini et al. 2008). There is a good deal of
evidence supporting the hypothesis that phospholipids are associated with the initial mineralization of bone (Irving 1973). Lipid matrix is a good nucleator of CaOx crystals from metastable solution (Khan et al. 1988). But it may be possible that some kind of phospholipids is involved in the inhibition of mineralization as is reported in the present work.