Identification and characterization of a calcium oxalate crystal growth protein inhibitor from human renal stone matrix

by

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DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the supervision of Dr. Chanderdeep Tandon, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan-173215, Himachal Pradesh, and has not been submitted for any degree or diploma to any other university. All assistance and help received during the course of the investigation has been duly acknowledged.

Priyadarshini

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Place: Priyadarshini

Date:

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LIST OF ABBREVIATIONS

BM	Basolateral Membrane
Bru	Brushite
CaOx	Calcium Oxalate
CD	Collecting Duct
CL	Cardiolipin
COD	Calcium Oxalate Dihydrate
COM	Calcium Oxalate Monohydrate
EDTA	Ethylenediaminetetraacetic Acid
ESWL	Extracorporeal Shockwave Lithotrpsy
FTIR	Fourier Transform Infrared Spectroscopy
GAG	Glycosaminoglycan
Gla	γ-carboxyglutamic acid
HA	Hyaluronic Acid
HAP	Hydroxyapatite
HS	Heparan sulphate

IEF	Isoelectric Focussing
IH	Idiopathic Hypercalciuria
i.v.	intravenous
MOE	Molecular Operation Environment
NC	Nephrocalcin
OPN	Osteopontin
PC	phosphatidyl choline
PE	Phosphatidyl Ethanolamine
PH	Primary Hyperoxaluria
PI	Phosphatidyl Inositol
PMSF	*P*henylmethanesulphonylfluoride or Phenylmethylsulphonyl Fluoride
PNL	Percutaneous Nephrolithotomy
PolyD	Polyaspartic Acids
PS	Phosphatidylserine
SF	Stone Formers
SM	Sphingomyelin
SP	Solubility Product
SS	Supersaturation
THP	Tamm–Horsfall protein
UA	Uric Acid
UPFT-1	Prothrombin Fragment-1 (),

ABSTRACT

Kidney stone formation is a complex process involving multiple factors. Although saturation product for CaOx is frequently exceeded in normal urine, most humans do not form stones. It has been assumed that inhibitors of urolithiasis have protective effect, although their precise role has not been defined. Kidney stones invariably comprise a combination of inorganic crystals and organic macromolecules consisting principally of proteins. Many proteins occur in stones, but their role in urolithiasis remains unknown. Calculi contain some proteins normally present in urine, in addition to others arising from injury inflicted by the stones themselves, making it impossible to discriminate between those that bind to the stone as it grows, but play no role in its development, and those that may be involved in regulating the formation of stone crystals. The inhibition is generally understood to arise mainly from the non-dialyzable molecules of urine, particularly acidic glycoproteins and glycosaminoglycans. Some inhibitor molecules have been identified, including Tamm–Horsfall protein, uropontin , calgranulin, bikunin, and prothrombin F1 fragment. There are several hypotheses regarding kidney stone formation. According to one hypothesis, it is related to intratubular crystal nucleation, growth, and aggregation. While other hypothesis

explains that the locale of crystal deposition is at a renal interstitium near or at the tip of renal papillae.

Insoluble nature of kidney stone poses many challenges in extraction of proteins present in its organic matrix; therefore, a comparison of different methods of extraction from human calcium oxalate containing stones was done to examine the soluble matrix proteins involved in their biomineralization process. It was observed that EGTA extract exhibited highest inhibitory activity (98%) towards CaOx crystal growth followed by acetic acid (6.47%) and SDS extract (2.64%). Therefore, EGTA extraction method was selected for the renal stone extraction to carry out the further studies.

Proteins were isolated from the matrix of kidney stones containing CaOx as the major constituent using EGTA as a demineralizing agent. EGTA fraction (more than 10 kDa) exhibiting highest activity on CaOx crystal growth assay was subjected to bioactivity guided protein purification.

To purify the most potent antilithiatic protein different chromatographic techniques like anion exchange, molecular sieve and high performance liquid chromatography followed by SDS-PAGE were employed. Then, the effect of purified protein was studied on calcium oxalate crystal growth system followed by SDS-PAGE analysis. Purified protein was further characterized by MALDI-TOF-MS. Human phosphate cytidylyltransferase 1, choline, beta (mol. wt. ~42kDa and pI 5.99) was identified as a purified protein by peptide mass finger-print obtained from MALDI-TOF-MS followed by database search on MASCOT search engine. 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.

Interaction of identified protein with CaOx was further studied using bioinformatics tools. By *in silico* studies, 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.

Often calcium oxalate stones are mixed with various percentages of apatite or brushite, and some studies have shown that apatite is the principal component of Randall's plaque and the primary nidus at which calcium oxalate stones grow. Romberg *et al*. have reported that macromolecular modifiers of calcium oxalate crystallization are also active in the corresponding steps of calcium phosphate crystallization. The heterogeneous nucleation theory also underlines the importance of calcium phosphate crystals in calcium oxalate urolithiasis. All these evidence suggests that there is a close relationship between calcium phosphate and calcium oxalate.

Activity of calcium oxalate extract was studied on mineralization of calcium phosphate and it was observed that high molecular as well as low molecular weight biomolecules are capable of influencing initiation, growth and demineralization of calcium phosphate.

Lipids represent approximately 10.15% of stone matrix obtained by sonication and demineralization with EDTA. Presence of lipids in stone matrix indicates their role in kidney stone formation. Therefore, activity of lipid was also studied on calcium oxalate crystal growth and it was observed that lipids isolated from the matrix of calcium oxalate renal stones had an ability to inhibit growth of CaOx crystals.

Thus, the identification of human phosphate cytidylyltransferase 1, choline, beta as a novel CaOx crystal growth inhibitor involved in the biosynthesis of phosphatidylcholine which happens to be an important constituent of human renal stones throws light on its potential pathophysiological role in nephrolithiasis.

INTRODUCTION

1.1 Introduction

Urolithiasis, the deposition of stones in urinary tract has plagued man since long and continues to pose a universal health problem even today. Histological and archeological studies have clearly revealed that ancient man suffered from urinary tract stone disease. The earliest evidence dates back to around 4,800 B. C. when a bladder stone was found among the pelvic bones of a young predynastic Egyptian. The incidence of urolithiasis appears to have been generally increasing over the last 100 years, particularly in countries which have either hot climate or which have moved from an agriculture economy to one based on industrial and technological development (Zilberman et al. 2009).

Mineralization can occur both under physiological and pathological conditions. Physiological mineralization in bone and teeth seems to be a purposeful phenomenon as it offers structural integrity and maintains homeostasis by creating mineral sinks. However, the pathological mineralization in soft tissues, e.g. tendons, cartilage, aorta and salivary, biliary or urinary system do not offer any satisfactory explanation with respect to its significance.

Urinary stones have attracted greater scientific attention not only due to their high frequency of occurrence but also due to the serious functional implications associated with

formation of such stones. Although the urinary calculi can be lodged in any part of urinary tract yet during the last two decades or so, the frequency as well as the site of occurrence of stones has shown gradual shift from the lower urinary tract (bladder and ureters) to the upper urinary tract (kidneys).

The epidemiological studies have demonstrated that there are various stone belts or pockets in many developed as well as developing countries. As far as India is concerned two distinct stone belts having very high incidence of urinary calculi have been identified in Northern and Central India (Coblabawala 1971). Interestingly, the incidence of this disease has been reported to be very low in the southern and Eastern coastal parts of India. Dietary patterns have been thought to be primarily responsible for the low incidence of urinary calculi these regions.

During the process of water conservation, kidneys supersaturate urine (Carvalho and Nakagawa 1999; Khan and Canales 2009). Supersaturation (SS) in relation to calcium oxalate and phosphate salts is the driving force for crystallization in solutions like urine, which means that it will contain crystals that are formed spontaneously. If inhibitors of crystal formation were not able to act and control their size, the final result will be nephrolithiasis (Carvalho et al. 2002; Kurutz 2003).

Human renal stones are composed of crystalline and non-crystalline phases; 80% of stones are composed of calcium oxalate (CaOx) and the supporting structure (the organic matrix) accounts for 2–5% of the total stone weight (Boyce 1968), and is distributed throughout the architecture of all stones (Boyce and Garvey 1956). Proteins constitute a major portion of the matrix and the organic matrix is considered to be important in stone

formation and growth (Warpoheski et al. 1981). Macromolecules are suggested to modulate the course of crystallization by inducing crystal nucleation, growth and aggregation of crystals as well as their attachment to renal epithelial cells. 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).

Non-obstructing stones produce no symptoms or signs apart from hematuria. Stones less than 5 mm in diameter have a high chance of passage, those of 5–7 mm have a modest chance (50%) of passage, and those greater than 7 mm almost always require urological intervention (Andrew 2009). Ideally, stone analysis is performed by infrared spectroscopy or x-ray diffraction. Renal stone burden is best gauged using CT radiographs taken with 5-mm cuts, without infusion of contrast agents. The radiographic appearance and density of stones as measured by CT is a guide to their composition (Zarse et al. 2004). Extracorporeal shock wave lithotripsy (ESWL), in which sound waves are used to break the stone into small pieces that can more easily pass into the bladder, is widely used and valuable for small stones (Lingeman et al. 2003). Modern instruments facilitate passage of endoscopes up the ureter into the kidney pelvis and permit local stone disruption with high-powered lasers (Bagley 2002). Percutaneous stone removal via instruments introduced into the kidney through a small flank incision permits disruption and removal of even very large stones (Clayman 2005).

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. Thus, determination of 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 mechanisms involved in kidney stone formation by these inhibitory molecules have been recently performed (Nakagawa et al. 1985; Nakagawa et al. 1987) the majority of previous studies have been concerned with the overall kinetics of crystallization rather than molecular mechanisms which remain poorly defined. Thus, the aim of present study is to fractionate renal stone matrix proteins, to purify and characterize most potent antilithiatic protein from organic matrix of calcium oxalate renal stones and study their interaction with calcium oxalate *in silico* with following objectives:

OBJECTIVES

1) To prepare the renal calculi extract from surgically removed stones and study its effect on *in vitro* mineralization by the assay system involving calcium phosphate precipitation and calcium oxalate crystal growth.

2) To isolate, purify and characterize proteins from the renal calculi extract which are capable of influencing *in vitro* mineralization.

3) Assessment of molecular interactions between the identified protein and calcium oxalate *in silico*.

REVIEW OF LITERATURE

2.1. Kidney stone disease

It is estimated that at least 10-15 per cent of the population in the industrialized part of the world is afflicted by urinary tract stone disease. In some geographical areas the prevalence is even greater, for instance, in the Arabian countries. Therefore, it appears to be a changing pattern in as much as stone disease now is becoming more common in young women (Robertson et al. 2000). The formation of urolithiasis occurs in both men and women but the risk is generally higher in men (Davidson et al. 2009). With its multifactor etiology and high rate of recurrences, urinary tract stone disease provides a medical challenge. It is well established that calculi in the urinary tract occur more frequently in the natives of certain geographic regions of the world (e.g. Southern China, Northern India with some pockets in Assam and Manipur, certain regions of Turkey and Egypt, the Volga Valley in U.S.S.R., East coast of U.K. and South-Eastern States of U.S.A.) than others (Straffon and Higgins 1970). Since considerable variations exist in these so-called stone belts regarding the mineral content of the water and soil, dietary habits of their natives, and environmental conditions, it is safe to conclude that factors other than these may be important in the etiology of urolithiasis. For a group of first time calcium stone formers, the

expected risk of recurrent stone formation during a 10-year period was estimated to 30 per cent and in those who had formed at least two stones at the start of follow-up the corresponding figure was as high as 70 percent (Tiselius 2000). For patients with certain other types of stone the recurrence risk might be even more.

2.2. Aspects on the process of stone formation

During the process of water conservation, kidneys supersaturate urine (Carvalho et al. 1999). Supersaturation (SS) is the driving force for crystallization in solutions like urine, which means that it will contain crystals that are formed spontaneously. If inhibitors of crystal formation were not able to act and control their size, the final result will be nephrolithiasis and/or nephrocalcinosis (Carvalho et al. 2002). The understanding of crystalluria requires some knowledge of crystal nucleation, growth and aggregation, all of which depend greatly on solution concentration. Both the monohydrate and dihydrate species of calcium oxalate (CaOx) crystals are present in kidney stones (Wesson et al. 1998). It has been proposed that crystalluria may be predictive of a nephrolithogenic tendency (Rushton and Spector 1982). Also, crystalluria with oxalate crystal volume measurement is a non-invasive, easily performed investigation, and can give feedback on the efficacy of urolithiasis therapy (Jouvet et al. 1998). In terms of calcium oxalate crystallization the situation has proved to be much more complex and the various parts of this process are schematically summarized in figure 2.1 the fundamental steps in stone formation are

a) *Crystal nucleation*

b) *crystal growth*

c) *crystal aggregation*

d) *crystal retention*

The last step in the process is poorly understood, but most certainly an *assembling* of crystalline material is necessary to build up a stone. Nucleation of calcium oxalate is assumed to be induced by one or several promoters. Growth and aggregation of calcium oxalate crystals can proceed as long as the ion-activity product of calcium oxalate exceeds the solubility product (SP).

All these processes are counteracted by inhibitors. The inhibition is accomplished by either large or small molecules. Whether the initial crystallization takes place as free or fixed particles has been a matter of debate over the years and it has generally been assumed that the precipitation of

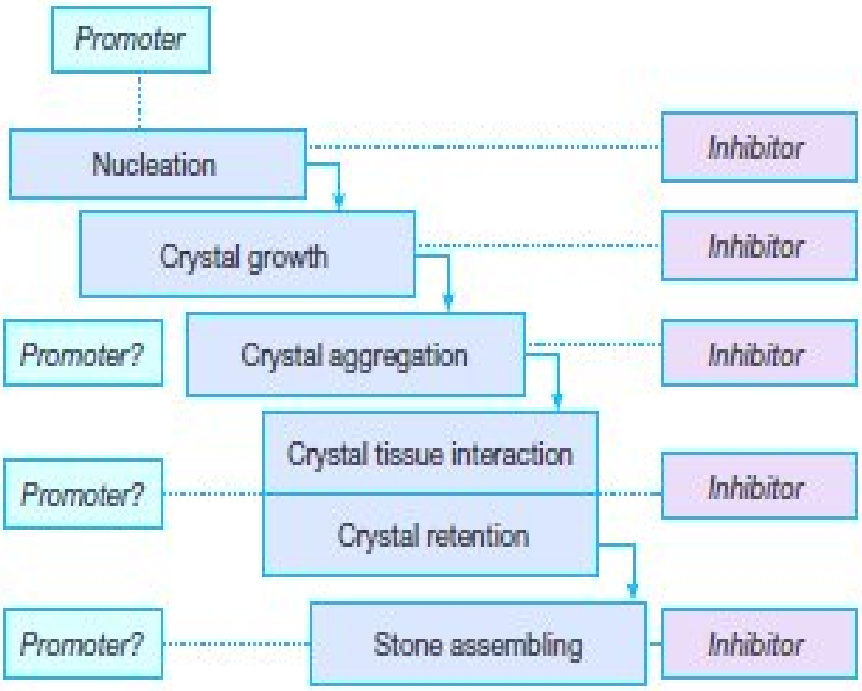


Figure 2.1. Major steps in calcium salt crystallization.

oxalate is too slow to give crystals of sufficient size to be trapped in the tubular system unless there is some kind of fixation of the crystalline material (Finlayson and Reis 1978). Recalculation of old data has, however, indicated that free particles of calcium phosphate as well as of calcium oxalate might form at the levels of supersaturation that occasionally are built up in nephron urine (Kok and Khan 1994). In this way crystals might become large enough to be trapped intra-tubularly. Irrespective of whether the initial crystallization is the result of free or fixed particles, stone formation cannot occur unless crystal material is retained in the renal collecting system. Retention of crystal material can, however, also be the result of interaction between the crystals and the cells and such a mechanism is assumed to play an important role (Mandel 1994; Khan 1996; Verkoelen et al. 1995, Verkoelen et al. 1997; Lieske and Deganello 1999). It needs to be emphasized again that if the urine is not

sufficiently supersaturated, there will be no salt precipitation and accordingly, under such conditions, the fundamental prerequisite for stone formation is lacking. This important fact is the theoretical basis for most of the recurrence preventive treatment regimens that are used today clinically in patients with urinary tract stone disease. In as much as recurrence preventive treatment ideally should be as selective as possible, there are two steps that are necessary to enable such an action. First, to decide whether the patient has a high supersaturation level or may be at risk of forming critically supersaturated urine. Thereby the supersaturation or the ion-activity product is the effective or secondary risk factor. Second, it is necessary to identify those factors that in an important way contribute to the supersaturation and the risk of crystallization. These latter variables can thus be considered as primary risk factors.

There is evidence that the process of calcium stone formation starts as a precipitation of calcium phosphate either in the loop of Henle or in the distal part of the distal tubule (Lupták et al. 1994; Tiselius 1996; Asplin et al. 1996; Kok 1997; Höjgaard and Tiselius 1999). Although the urine at these levels of the nephron might be critically supersaturated with calcium oxalate in patients with hyperoxaluria and in experimental animals following administration of ethylene glycol, the ion-activity product of calcium oxalate is usually too low to result in calcium oxalate crystal formation (Lupták et al. 1994). Any crystallization that occurs in this part of the nephron most certainly is facilitated by promoters (Figure 2.2) and it has been suggested that lipoprotein membranes from the brush border of proximal tubular cells might serve this purpose (Khan et al. 2000). Experimental research has shown that the brush border membrane might be injured by free radicals formed as the result of toxic effects on the cell. This might lead to lipid peroxidation and cell death (Thamilselvan

and Khan 1998). The released membrane fragments that are transported down the nephron thereby can supply a suitable surface for deposition of both calcium oxalate and calcium phosphate. There are also other less specific constituents of nephron urine that have the capacity to induce nucleation of calcium salts, such as, for instance, blood cells, crystals of sodium urate, cholesterol, or other intratubular particles. Crystals of calcium phosphate that form at a high nephron level (Tiselius 1997) (Figure 2.2) might be dissolved when they are exposed to the acid urine in the collecting duct (Höjgaard 1999).

Dissolution of calcium phosphate causes a high level of supersaturation with calcium oxalate by increased urine concentration of calcium. Nucleation of calcium oxalate can thus take place either by epitaxis on the surface of the dissolving calcium phosphate crystal or by nucleation, with or without the contribution of a promoter, or by nucleation in the macromolecular environment that surrounds the calcium phosphate crystals. The latter process might take place either freely in the tubular lumen or more likely at tubular wall.

Under normal conditions, the crystals of calcium oxalate and calcium phosphate that form are small and well protected from crystal growth and crystal aggregation by a cover of inhibitory macromolecules. The negatively charged macromolecules have a high affinity to the positively charged surface of calcium salt crystals. The aggregation inhibiting properties of small as well as of large molecules are related to their electronegativity which establishes repulsive forces between adjacent crystals and between the crystals and the similarly negatively charged macromolecular layer on the surface of tubular cells (Figure 2.3).

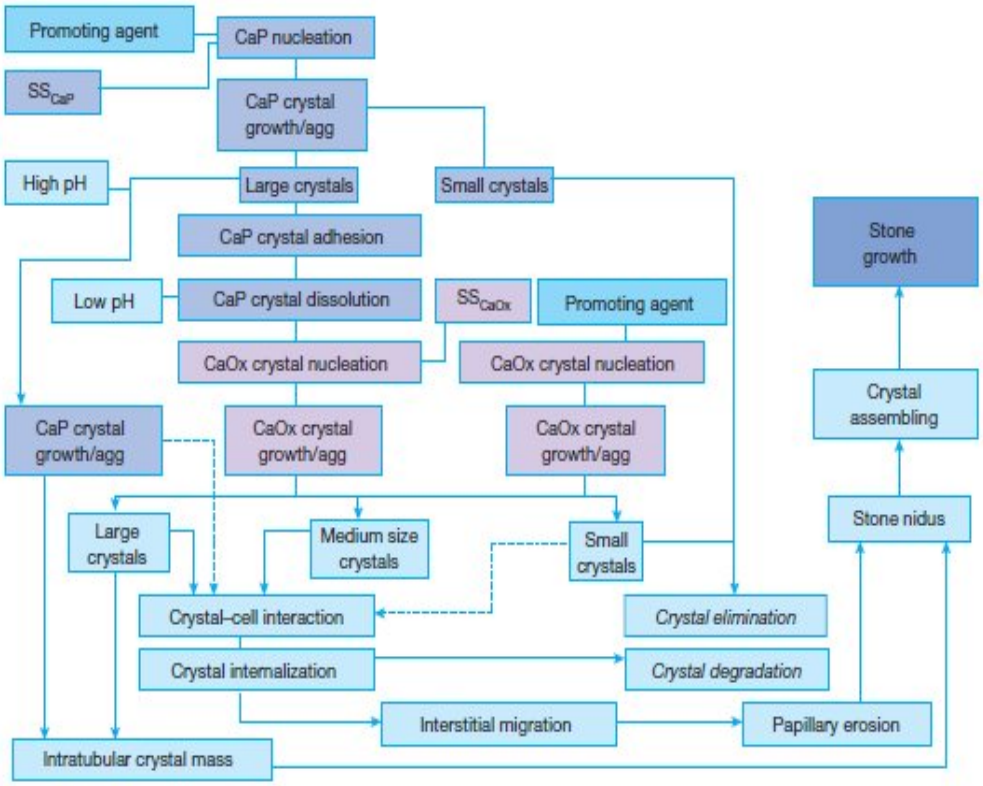


Figure 2.2. Overview of the various possible steps in calcium stone formation. (SS=supersaturation)

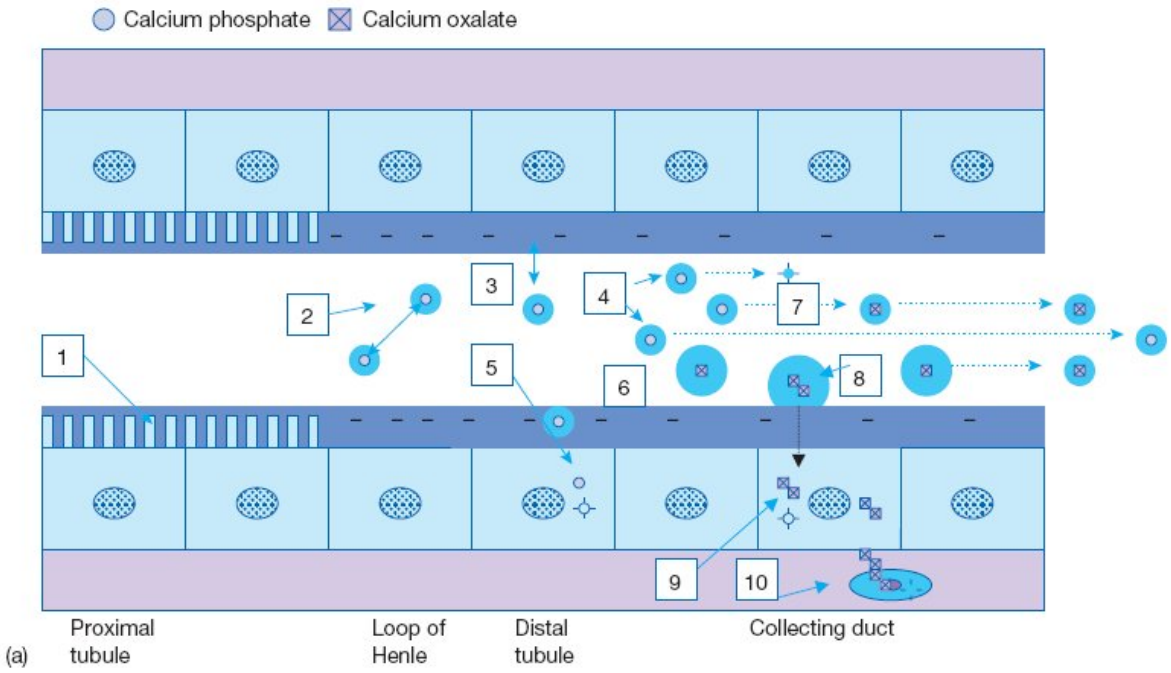


Figure 2.3. (a) Hypothetical interpretation of the possible series of events of the *normal crystallization* in urine: (1) brush-border membrane of proximal tubular cells; (2) repulsion between small calcium phosphate crystals; (3) between calcium phosphate crystals and tubular cells; (4) elimination of small calcium phosphate crystals by dissolution or passage with urine; (5) internalization and intracellular dissolution of calcium phosphate crystals; (6) primary nucleation of calcium oxalate; (7) calcium oxalate nucleation induced by calcium phosphate; (8) attachment of small calcium oxalate crystals to the tubular cell; (9) internalization and dissolution; (10) macrophage destruction of calcium oxalate crystals in the interstitial tissue, small intraluminal crystals of calcium oxalate are excreted with urine.

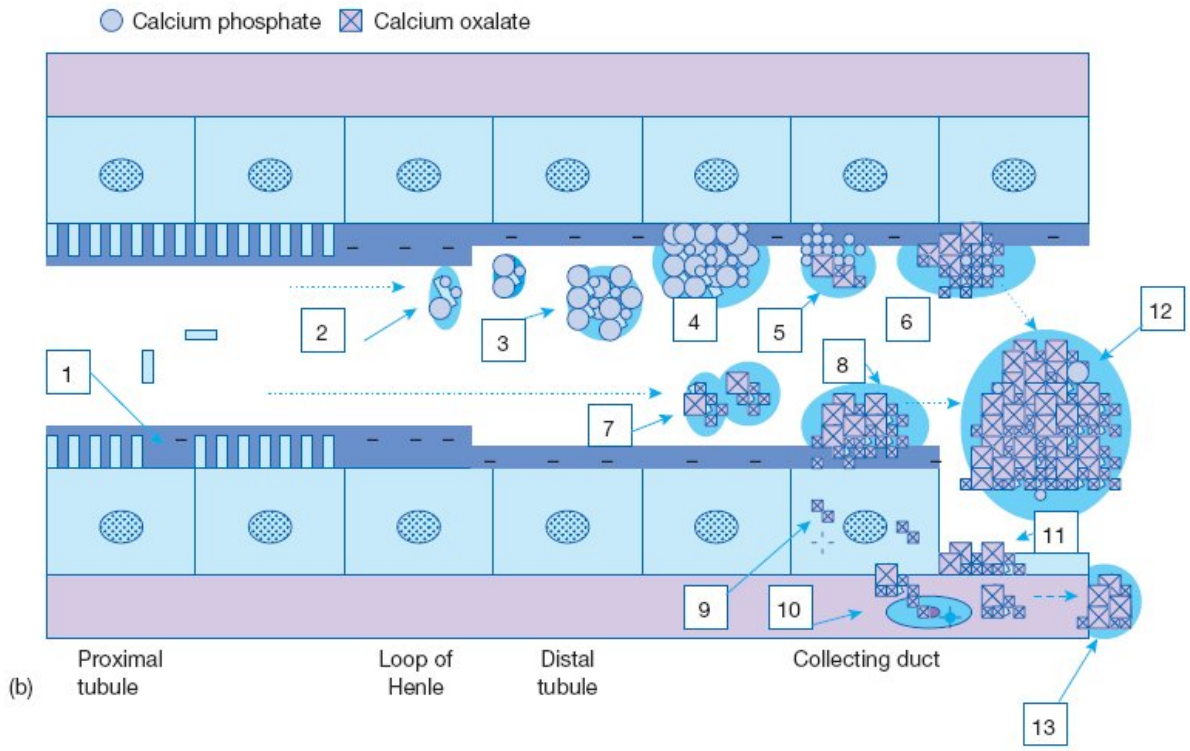


Figure 2.3. (b) Hypothetical interpretation of the possible series of events of the *pathological crystallization* in urine: (1) destruction of the brush-border membrane of proximal tubular cells; (2) nucleation of calcium phosphate crystals promoted by membrane fragments; (3) formation of large masses of calcium phosphate crystals by growth and aggregation; (4) adherence of crystal aggregates to the tubular surface; (5) dissolution of calcium phosphate in acid urine and nucleation of calcium oxalate; (6) formation of a large mass of calcium oxalate and calcium phosphate crystals attached to the tubular wall; (7) primary nucleation of calcium oxalate induced by membrane fragments with or without participation of urinary macromolecules; (8) attachment to the tubular cell of large calcium oxalate aggregates; (9) partial dissolution of internalized calcium oxalate crystal material; (10) migration of crystals to the interstitial tissue where the capacity of macrophages is insufficient to cope with the large crystal masses; (11) destruction of tubular cells with binding of crystals to the basolateral membrane; (12) formation of an intratubular stone nidus by an assembling and trapping of crystal aggregates; (13) interstitial migration of crystalline material to the papillary tip.

In this way, it is likely that small crystals are fast and can easily move through the tubular system and be excreted with urine. It is possible that small calcium phosphate crystals are completely dissolved during their transport through the collecting duct. Under

appropriate conditions primary nucleation of calcium oxalate might occur in collecting duct urine. As long as these crystals remain small and are well protected from growth and aggregation they leave the tubular system without problems. The interaction between the tubular cells and crystals are modified by several macromolecules excreted with urine or secreted by the tubular cells (Verkoelen et al. 1997; Lieske et al. 1997c)

Experimental studies with cell cultures have shown that calcium oxalate monohydrate crystals adhere to tubular cells in a specific and rapid way (Lieske and Coe 1996). The cellular affinity for brushite (Bru) crystals was much less pronounced. It has moreover, been shown that a number of polyanions might prevent the adherence of crystals to cells. Such an effect accordingly was recorded for glycosaminoglycans (heparin, heparan sulfate, hyaluronic acid, and chondroitin sulfate), citrate, nephrocalcin, and uropontin. Tamm–Horsfall protein (THP) on the other hand, did not counteract the adherence of crystals to the cells, but inhibited crystal endocytosis. It was concluded that crystals of calcium oxalate binds to sialic acid residues on cell surface glycoproteins. Also, the lipids of the plasma membrane appear to be of great importance for the adherence of crystals (Bigelow et al. 1997).

Normally, cells lining the tubular system are well protected from crystal adherence, but the situation alters dramatically following cell injury. The same principle for crystal attachment as for calcium oxalate is applicable to crystals of hydroxyapatite (HAP) (Lieske et al. 1997b). Crystals of calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD), and HAP that have adhered to the cell surface might be internalized by endocytosis (Lieske et al.1992; Lieske et al. 1999). Some of these crystals might be dissolved by the action of lysosomal enzymes within the tubular cell, whereas others might be transported to

the basolateral membrane and expelled into the interstitial tissue where macrophages and other inflammatory cells can take care of the crystals and destroy them (Khan 1996). In this way a substantial amount of crystalline material might be removed from the tubular system and it is likely that this is one of the defence systems that the kidney has developed to protect from intratubular crystallization and obstruction. In response to high concentrations of oxalate or calcium oxalate crystals tubular cells might proliferate and thus increase the capacity to eliminate crystals. The risk of crystal adherence is certainly greatest for calcium phosphate and calcium oxalate crystals that are large and thus move slowly through the nephron. For very large crystals and crystal masses the repulsive forces described above are probably insufficient to counteract both crystal aggregation and crystal–cell adherence. It is also well recognized that patients with calcium stone disease excrete in their urine larger crystals and crystal aggregates than normal subjects. Given these principles for the normal crystallization in the nephron, a pathological crystallization leading to stone formation might be the net result of one or several abnormalities or defects in the control of this process (Figure 2.3). Low concentrations or structural abnormalities of crystallization modifying macromolecules or small molecules will cause increased growth and aggregation of crystals so that large crystal masses form either of calcium phosphate or of calcium oxalate (Coe and Parks 1990). Large crystal masses with or without insufficient protection by macromolecules will adhere to the tubular cell. The crystals might alter the plasma membrane so that endocytosis occurs whereas crystals of reasonable size can be taken care of and destroyed by the cell, larger crystal agglomerates might cause cell death (Khan et al. 2000; Koul et al. 1996). When the crystals that are bound to the basolateral membrane or deposited interstitially are too large, the capacity of macrophages and inflammatory cells

will probably be insufficient to cope with the crystals. Such crystal material might accordingly be transported within the interstitial tissue down to the papilla where it can provide a basis for crystal deposition and growth following erosion of the epithelial surface. It is understood that the insufficient or defect control of the crystallization process also will result in development of large crystals or crystal agglomerates that remain within the tubular lumen. Under such conditions crystals of calcium oxalate or calcium phosphate might be trapped at the lower and narrow end of the collecting duct and thereby serve as a nidus for further crystal deposition in the supersaturated urine. Figure 2.4 summarizes the various possibilities of calcium salt crystalluria and calcium stone formation. Small crystals of calcium oxalate or calcium phosphate that might have formed in the nephron can disappear either by intraluminal dissolution or by cellular action. These crystals can also be excreted with urine as microscopic crystalluria, which is a common finding in both stone formers and normal subjects.

A primary precipitation of calcium phosphate and subsequent dissolution in acid collecting duct urine can give rise to crystal aggregates containing calcium oxalate and calcium phosphate or, in case of complete dissolution, pure calcium oxalate.

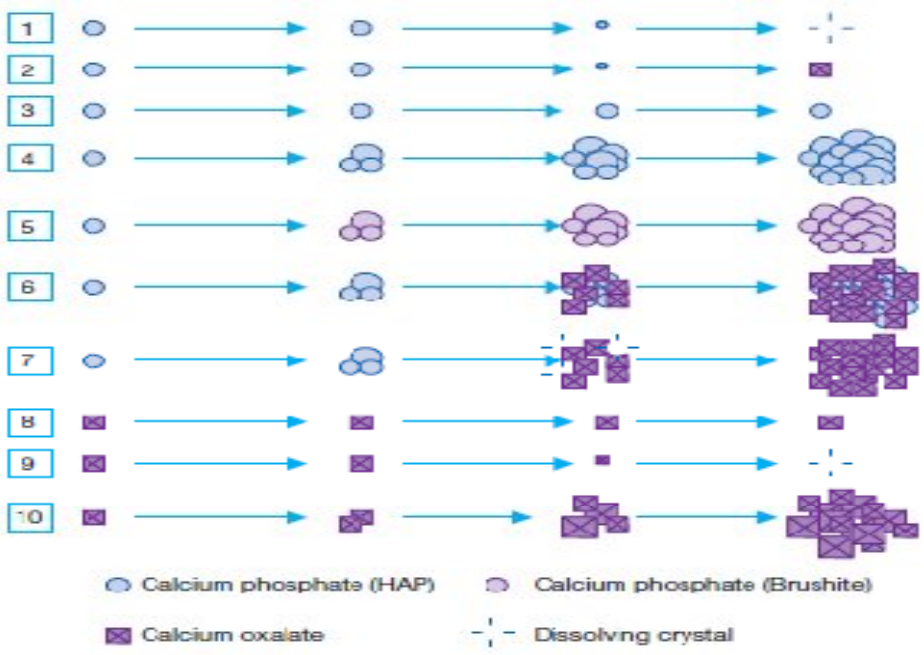


Figure 2.4. Possible outcomes of calcium salt crystallization in the urinary tract. Small crystals of calcium phosphate or calcium oxalate can disappear by dissolution (1, 9) or remain small (3, 8). Nucleation of calcium oxalate might be induced by calcium phosphate (2, 6, 7). Following complete dissolution of calcium phosphate pure calcium oxalate aggregates can form (7). In constantly alkaline urine there is no precipitation of calcium oxalate and the result will be pure calcium phosphate aggregates, either in the form of hydroxyapatite (4) or brushite (5). A primary nucleation of calcium oxalate results in small crystals (8) or crystal aggregates (10).

Pure calcium oxalate aggregates also might form when calcium oxalate is primarily precipitated in the nephron unrelated to a calcium phosphate crystal phase (Lieske et al. 1998). In patients with a constantly high pH, calcium phosphate crystals will not dissolve

and inasmuch as calcium phosphate is the favoured crystal phase in alkaline urine, pure calcium phosphate crystals and stones will be the result. Such crystals most commonly consist of HAP, but might occasionally be composed of Bru, particularly at a lower pH.

2.3. Epidemiology of renal stones

Urolithiasis is a clinical disorder which is known to be caused by multiple etiologic factors. These could be preurinary and urinary risk factors leading to metabolic disorders. The preurinary factors could be both intrinsic and extrinsic.

2.3.1. Preurinary risk factors

A. Intrinsic factors

a) Heredity – Several workers have given evidence to support the concept that the familial incidence of urinary calculi is related to heredity. Now it is known to be a polygenic defect influencing various enzymes of oxalate, uric acid metabolism (Khan and Canales 2009). Ethnicity has also been shown to be an important factor for calculosis. For example, a low incidence of calculi has been found in Negroes of Africa and America, North American Indians, natives of Israel, South Indians and South Americans.

b) Age - The maximum incidence of idiopathic calcium stone disease has been shown to occur between the third and the fifth decade of life. It has been found to be quite uncommon in children and elderly people (Soucie et al. 1994; Hiatt and Friedman 1982).

c) Sex – Males are generally known to be more prone to calcium stone disease as compared to females. This could be due to the fact that as compared to females, males are known to excrete more calcium, oxalate, uric acid in their urine leading to its higher saturation. The

anatomical structure of the female urogenital tract also facilitates easy passage of initially formed crystalloid. Moreover, effect of estrogens in increasing the urinary citrate excretion which have a solubilising effect on calcium could be responsible for the low incidence of urinary calculi in females. The incidence of renal calculi is four times higher in males as compared to females (Davidson et al. 2009; Hiatt and Friedman 1982).

B. Extrinsic factors

a) Geographical distribution- The incidence pattern of renal stones in India has been determined and two high incidence stone belts have been found to occur (Coblabawala 1971). The first belt starts from Amritsar in North and while passing through Delhi and Agra ends up in U. P. The other belt which starts from Jamnagar in the West Coast extends inward towards in Jabalpur in Central India. Very low incidence has been found in West Bengal and coastal areas of Maharashtra, Karnataka, Kerala, Tamilnadu and Andhra Pradesh.

The Northern part of the United States (except in Negroes) has also been shown to have a relatively high incidence of urinary calculi. Other geographic areas surveyed and reported to have high incidence include Central Europe, Japan, North India, Pakistan, Northern Australia, parts of Malaysia and China (Zerwekh et al. 1983).

b) Climatic factors – The high summer temperatures in South Eastern United States have been shown to be responsible for high incidence of urinary calculi. Reasons for higher incidence in summers could be an increased conversion of vitamin D to its active metabolites resulting in increased calcium absorption from intestines. Decrease in urine production due to greater loss of water from sweat causes an increase in the concentration of

stone constituents in urine and hence supersaturation of urine with stone forming constituents leading to calculosis (Milliner 1995).

c) Dietary factors – The dietary content of animal proteins and fats has been found to be approximately 5 times higher and that of sugar 10 times greater in developed countries (Milliner 1995) than in Africa (South of Sahara) where occurrence of Ca-stones is very rare. Use of refined carbohydrates and animal proteins has further been shown to increase urinary calcium and oxalate excretion which could be responsible for high incidence of renal calculi.

Sucrose has been observed to cause a significant increase in intestinal calcium absorption in idiopathic stone formers as well as in normal subjects. The effect of nutrient rich and fiber depleted diets on absorptive and excretory mechanism is likely to increase urinary supersaturation with respect to CaOx (Taylor et al. 2009).

Vitamin K deficiency is known to be associated with stones of renal origin. Vitamin K is known to promote the formation of gamma-carboxy glutamic acid which has high affinity for Calcium. If the renal carboxy glutamic proteins were to play a role in tubular handling then a decrease in their formation as possibly reflected by the decrease in urinary gamma-carboxy glutamic acid excretion in vitamin K depleted animals should lead to an altered tubular handling at calcium (Angayarkanni and Selvam 1998). Thiamine deficiency has also been reported to increase the risk of calcium lithiasis by causing hyperoxaluria and/or decreasing the citrate excretion in urine.

d) Occupation- It has been found that urinary calculi are more likely to be found in individuals having sedentary occupations as compared to individuals doing manual work

(Taylor et al. 2009). The possibility exists that the physically strenuous nature of the manual work leads to the disruption of the crystal aggregation phenomenon in the urinary tract.

e) Water intake – A number of investigators have shown that increased water intake and increased urinary output decreases the incidence of urinary calculi (Miggiano and Migneco 2007). A small urinary volume, whether caused by low fluid intake or increased fluid loss through other routes has been shown to result in increased stone formation.

2.3.2. Urinary factors

a) Effect of pH- Uric acid stones occur in patients with very low urine pH (below pH 5) and in those with hyperuricosuria (Sakhee et al. 2002). In some patients low urine pH is caused by a defect in renal ammonia secretion that results in less buffering of secreted hydrogen ion and lower urine pH.

b) Concentration of salts in urine - The key process in the development of kidney stones is supersaturation. Salts such as calcium oxalate, uric acid, cystine, or xanthine can become extremely concentrated under certain circumstances. If the volume of the urine is significantly reduced; or if abnormally high amounts of crystal forming salts are present, they precipitate out and form crystals.

c) Volume of urine – Increasing urinary volume is an important tool in the prevention of calcium renal stones. Urine dilution considerably reduces crystallization phenomena induced *in vitro* by an oxalate load in both calcium stone-formers and normal subjects (Guerra et al. 2005).

d) Enzymes – The initial step in the pathogenesis of urolithiasis must be the precipitation of an organic matrix of mucoproteins. An important factor in this process may be the activity of and/ or concentration of the urinary enzyme, urokinase, which would affect the level of urinary mucoproteins. A decrease has been observed in urinary urokinase concentration of renal stone patients which, once again, underlines the possible involvement of urokinase in renal stone formation (du Toit et al. 1997).

2.4. STONE & STONE MATRIX

Like other products of crystallization in biological systems (Lowenstam and Weiner 1989), stones are a composite of crystals and organic material, often referred to as matrix (Boyce 1972; Iwata et al. 1988; Khan and Hackett 1993). A variety of crystals including CaOx, CaP, uric acid, struvite, and cystine can be present in stone (Finlayson 1978; Finlayson 1977; Khan and Hackett 1987). CaP crystals appear most frequently in both the urine and stones however, CaOx is the major crystal in most stones. Stones, particularly those containing CaOx or uric acid have a compact structure. Their outer surfaces appear smooth at low magnification but reveal the presence of individual tabular or plate-like CaOx monohydrate (COM) crystals at higher magnifications. Crystal habits are generally not evident on surfaces exposed by cutting or fragmenting the stone. Such surfaces are typically stratified with radial striations and concentric laminations or layers, with radial striations being the predominant feature. Some of the striations run through many laminations while others are limited to only one. Many converge to a point at the base of a lamination

mimicking the arrangement of petals in a flower. These points are suggested to be the nucleation sites of crystals.

The laminations are approximately 50-60 μm thick and in many stones can be easily separated from each other exposing the underlying surfaces. The latter show the same structure as the outer stone surface, with protruding tips of the tabular COM crystals frequently covered with amorphous to flaky matrix material. Overall it is a highly ordered structure. Many stones have a well-defined nucleus that is less ordered, with a granular and non-stratified appearance. It is generally occupied by spherulitic or amorphous CaP and/or aggregates of dumbbell shaped twinned COM crystals. CaP frequently fills the space between CaOx crystals as well as that in the concentric laminations.

The organic matrix of most urinary stones accounts for 2-3% of their total dry weight. Matrix consists of macromolecules generally present in the urine. These molecules play a significant role in the development of kidney stones. Some of them promote crystal formation, growth, aggregation and retention, while others inhibit these processes. Their activity is often complex and depends on the urine conditions prevailing at the time of crystallization or retention. The same macromolecule can both promote and inhibit a process. For example macromolecules behave differently in solution than when they are attached or adsorbed to a surface. It may well be, that compounds free in solution cover a crystal surface and inhibit its growth or ability to aggregate while the same compound bound to a surface acts to accumulate salt ions and forms a template for the first nucleus. The latter will play a role when stone formation involves processes at cell surfaces and in the sub-epithelial space (Kok and Schell-Feith 1999).

Boyce *et al.* defined and established the importance of stone matrix in urolithiasis, proposing that the matrix actively participates in the assembly of kidney stones (Xie et al. 2001). In their view, the matrix acts as a template and controls crystallization within its bounds. An opposite hypothesis was advanced by Vermeulen *et al.* who viewed the matrix and its ubiquitous presence as merely coincidental because stones form by crystallization in urine in the presence of large macromolecules (Vermeulen and Lyon 1956; Finlayson et al. 1961). According to them the matrix is adventitiously acquired, primarily by physical adsorption of urinary mucoproteins on crystal surfaces. Another hypothesis proposed by Khan et al explains that the role of matrix compounds is different in the formation of the stone center and in the subsequent build-up of the stone. The first is a short-term event involving crystal formation and retention. The second is a long term event occurring after a stone nidus has been formed and retained. Both events do not necessarily take place at the same site. Solution depletion (Leal and Finlayson 1977) and examination of crystals incubated in protein solutions by transmission electron microscopy (Khan et al. 1983) tested the theory of physical adsorption of urine proteins on surfaces of CaOx crystals. Results showed proteins have a strong affinity for CaOx crystals. Adsorption of anionic proteins was sensitive to calcium ion concentration, whereas cationic protein adsorption depended upon the oxalate ion concentration with temperature and pH playing only a minor role. Proteins formed a discontinuous coat around the crystals ranging in thickness from 10 to 20nm. It has been suggested that newly formed crystals with a macromolecular coat are less likely to dissolve during the routine urinary ionic and pH changes and therein may lie the importance of matrix in stone formation (Khan and Hackett 1993).

2.4.1. Morphology of renal stone

Morphological examination of decalcified and intact stones shows that matrix is pervasive, distributed throughout the stones, and has amorphous and fibrous components (Boyce 1972; Iwata et al. 1988; Khan et al. 1988). The crystal matrix association is so intimate that the dipyramidal habit of CaOx dihydrate [COD], the monoclinic or plate-like habit of COM and spherulitic habit of CaP stay intact after total removal of the mineral content (Khan and Hackett 1986). Scanning electron microscopic examination of decalcified COM stones showed the matrix to be organized in concentric layers of 2-5 μm thick. The thickness was uniform throughout the circumference and smaller than the laminations seen in an intact stone. Successive layers appeared as rings of an onion bulb with little or no space in between. They consisted of loosely or tightly matted fibers and contained empty columns representing crystal ghosts presumably formed by dissolution of tabular COM crystals. Crystal ghosts were often arranged radially in relation to the stone centre, reminiscent of the radially arranged crystals in the intact stones.

Examination of the concentric layers by transmission electron microscopy confirmed that they contained radially arranged columnar crystal ghosts surrounded by an amorphous electron dense coat and embedded in a fibrous matrix. Electron dense material was also found inside crystal ghosts. Cellular degradation products including degenerating nuclei, mitochondria, endoplasmic reticulum and membrane fragments as well as vesicles occupied the intercrystalline spaces (Khan and Hackett 1993). The layered matrix was sudanophilic (Khan et al. 1988) and stained positive with periodic acid Schiff (PAS) and colloidal iron (Khan and Hackett 1986), indicating the presence of lipids, glycosaminoglycans (GAG) and proteins. Examination of decalcified stones using antibodies against osteopontin (OPN) and

calprotectin showed them to stain both the stone center and concentric laminations (Tawada et al. 1999). OPN was detected both inside the crystals as well as on their surfaces. Ultra structural examination of decalcified stones also showed the crystal associated matrix to stain positive with malachite green indicating the presence of phospholipids (Khan et al. 1996).

2.4.2. Chemical Composition

The organic matrix of urinary stones contains lipids, GAG's (20%), carbohydrates and proteins. Proteins comprise approximately 64% of the matrix. Table 2.1 lists the compounds, which have been identified in matrices of urinary stones. Most of them are proteinaceous in nature. A number of other proteins have also been detected but not identified. Initially lipids were not recognized as constituent of stone matrix (Boyce 1972) even though detected as an osmiophilic substance during histochemical examination of decalcified stones. All urine macromolecules can become part of stone matrix but only some are there because they have participated in crystallization and stone formation. This appreciation led investigators to study crystallization *in vitro;* using freshly collected urine to determine the macromolecules that become a part of the crystal matrix (Morse and Resnick 1989).

2.4.2.1. Glycosaminoglycans (GAGs)

Heparan sulphate (HS) and hyaluronic acid (HA) are the two major GAGs in the matrix of both stones and CaOx crystals formed in urine (Roberts and Resnick 1986; Yamaguchi et al. 1993). GAGs can account for up to 20% of the stone matrix (Nishio et al.

1985). The most abundant urine GAG, chondroitin sulphate (CS) was not found in these matrices indicating selective incorporation (Suzuki et al.1994a). Keratan sulphate and dermatan sulphates are present in trace amounts.

2.4.2.2. Proteins

More than twenty individual proteins have been detected in the matrix of various types of stones. While most of them have been identified (Table 2.1), few are fully characterized and some still remain nameless (Binette et al. 1996) and a few await confirmation of their identity (Tang et al. 1995). There are Human serum albumin (HAS), α and γ-globulin and Tamm-Horsfall Protein (THP) were the first proteins identified in stone matrix (Boyce et al. 1962).

Albumin is a major component of the matrix of all stone types including CaOx, uric acid, struvite and cystine. It is also found in the matrix of CaOx and CaP crystals precipitated from human urine and it is more pronounced in crystals induced in stone formers urine (Atmani et al. 1998; Atmani and Khan 2002).

Both CaOx and CaP crystals are known to adsorb HAS. THP is not always detected in stones and even then in only minor quantities, 0.002-1.04 mg/g (w/w) of stone (Grant et al. 1973).

Table 2.1. Proteins, GAGs, lipids and small molecule detected in CaOx/CaP stones and/or crystals.

	Proteins	15	Renal lithostatin
1	Albumin	16	Retinol binding protein
2	α-1-microglobulin	17	Superoxide dismutase

3	α-1-acid glycoprotein	18	Tamm-Horsfall Protein
4	α and γ globulins	19	Transferin
5	α-1-antitrypsin		**Glycosaminoglycans**
6	Apolipoprotein A1	20	Heparan sulfate
7	β-2-microglobulin	21	Hyaluronic acid
8	Calprotectin (Calgranulin)		**Lipids**
9	Haemoglobin	23	Phospholipids
10	Inter-α-Inhibitor	24	Cholesterol
11	Nephrocalcin	25	Glycolipids
12	Neutrophil elastase		**Small molecules**
13	Osteopontin (Uropontin)	26	Pyrophosphate
14	Porin		

THP associated with CaOx crystals is easily removed by washing the crystals with sodium hydroxide solution (Maslamani et al. 2000; Gokhale et al. 1996a) indicating THP's loose interaction with the CaOx crystal surfaces. Ultrastructural investigations of human CaOx urinary stones and CaOx nephroliths induced in an animal model supported the hypothesis that THP is not included in the crystals (Gokhale et al. 1996a Gokhale et al.1996b). This may explain THP's scanty presence in the stone matrix. Of the other proteins listed in Table 2.1, osteopontin (OPN), α-1- microglobulin, urinary prothrombin fragment-1 (UPFT-1), and light and heavy chains of inter-α-inhibitor have been identified in the matrix of CaOx and CaP crystals precipitated from the urine of normal and stone forming individuals (Atmani and Khan 2002). Ultrastructural examination reveals OPN to be pervasive in the crystals and stones and a key component of the matrix of CaOx stones (Mckee et al. 1994; Hoyer 1994).

More OPN is present in CaOx monohydrate stones (800 µg/100mg stone) than in COD stones (10 µg/100mg stone).

2.5. MODULATORS OF CRYSTAL FORMATION AND RETENTION

In urine, three classes of modulators are recognized; low MW compounds like citrate and pyrophosphate, glyco-proteins, high MW non-protein compounds like acid mucopolysaccharides, glycosaminoglycans and various types of lipids. They modulate crystal formation and retention in the urinary tract either directly by interacting with the crystal or indirectly by influencing the urinary environment. From crystallization experiments with urine, it appears that in non-stone formers the concerted actions of these compounds ensure that:

1. The crystals formed remain unaggregated and small enough to be excreted (Kok et al. 1986a; Kok et al. 1990);

2. The crystals have a reduced affinity for epithelial cells (Verkoelen et al. 1996; Schepers et al. 2002; Lieske et al. 1995) and;

3. The crystals if needed are easily recognized and removed by macrophages (de Water et al. 2001).

Crystallization in confined spaces, e.g. simulating Randall's plaque formation at the basal membrane below the tubular epithelium has been less studied but even here, inhibitors can decrease crystal growth rates (Achilles et al 1991; Achilles et al. 1995). Which inhibitors are the most effective? The first approach to answering this question has been to identify individual urine compounds and test their "inhibitory" potency in crystallization and

cell-culture systems. The next problem has been to translate these data to the whole urine situation where singular inhibitors may also co-operate or compete with each other and where restrictions posed by the kidney and urinary tract itself (flow-rates, residence time and changing urine composition) affect their inhibitory and stimulatory powers. Undiluted whole urine strongly affects calcium salt nucleation, crystal growth and crystal aggregation. When preformed CaOx crystals were added to supersaturated whole undiluted urine their growth was almost completely stopped. Crystal growth only occurred when the supersaturation was drastically increased by adding extra oxalate (Kok 1996). Urine has an overabundance of inhibitors. Tested *in vitro* as single compounds some are clearly more effective than the others, however, experimental data suggest that when the most efficient compounds are lacking, others readily take over. For instance, the low MW compound citrate can inhibit crystal growth very effectively at concentrations between 0.1mM and 1 mM. When citrate was added at these concentrations to urine, however, it did not change the growth inhibitory action of that urine (Kok et al.1986b). In studies of large groups of stone formers and healthy controls where urine was tested in a 1:5 dilution, approximating the degree of dilution existing in the collecting ducts, both urine from stone formers and normal subjects strongly inhibited CaOx crystal growth (Kok et al.1986a; Kok et al. 1990; Erwin et al.1994). When all macromolecules were removed by ultrafiltration, the degree of crystal growth inhibition was only slightly reduced (Drach et al. 1979). *In vitro* tests have confirmed that macromolecules are the most effective inhibitors of crystal growth. Apparently the low MW compounds take over the inhibitory function when the high MW compounds are gone. An additional effect of crystal growth inhibition may be that supersaturation will persist longer and the process of nucleation will have more time to proceed (Erwin et al.1994). How

relevant this is, in view of the short transit times of urine through the nephron (a few minutes) (Kok and Khan 1994), is not clear. Normal urine can also strongly inhibit crystal aggregation. This function is reduced in single stone former urine and severely reduced in recurrent stone former urine (Kok et al. 1986; Kok et al. 1990). Aggregation is important as it can lead to particle retention, just like crystal cell interactions and disturbed flow conditions (Kok and Khan 1994). The inhibition of aggregation in urine is correlated to the citrate concentration (Kok et al. 1990). However, in ultrafiltered urine this relationship is gone (Koide et al 1981). Apparently citrate modulates the effect that high MW compounds have on crystal aggregation. In addition, it was found that the urinary macromolecular fraction (>10.000 D MW) of single stone formers inhibited crystal aggregation less than that of normals and even less by those from recurrent stone formers (Erwin et al. 1994). In this study 70-90% of the inhibitory activity was destroyed by proteinase treatment. Citrate has been shown to improve the inhibitory effect of THP on crystal aggregation (Hess et al. 2000). Overall it appears that urine contains numerous components, both small and large that competes and cooperates in modulating crystallization and inhibiting stone formation.

2.5.1. Low Molecular Weight Compounds

2.5.1.1. Pyrophosphate and Bisphosphonate

Pyrophosphate is present in urine at concentrations of 15-100μM. In a seeded crystal growth system, it inhibits COM crystal growth by 50% at 16-20 μM (Schwille et al. 1988; Kok et al. 1988; Ryall et al. 1988; Sidhu et al. 1986). It can also inhibit COM crystal growth

inside a gel matrix (Achilles et al. 1989) and effectively inhibits the growth of CaPs (Grases et al. 2000; Robertson 1973). If it is equally efficient in urine it can contribute 50% crystal COM growth inhibition in the collecting ducts (5 times dilution) and up to 80% in the urine. This efficacy prompted interest in therapies that raise the urine output of pyrophosphate and in non-biodegradable pyrophosphate analogues, bisphosphonates. These inhibit COM crystal growth at least as good as pyrophosphate, 50% inhibition at 1-20 μM concentrations (Kok 1995). Another feature of pyrophosphate, 2.0 10-4 mol/l, which it shares with citrate, 1.0 10-3 mol/l, is that COD is preferentially formed in its presence. The critical pyrophosphate concentration above which COD formation is prevented may be lowered to the physiological range by adding citrate (Yuzawa 1998). This is of interest as COD is the major crystal phase in normal crystalluria (Cerini et al. 1999) while there is more COM in stone former crystalluria and COM predominates in stones. The effects of pyrophosphate and bisphosphonates on crystal aggregation are more complex. Pyrophosphate increasingly inhibits crystal aggregation at increasing concentrations (Kok et al. 1988). At its concentration range in the collecting ducts it could contribute to the whole urine effect on aggregation. While some bisphosphonates have a comparable effect, others show no effect, a stimulatory effect on aggregation or even a biphasic effect, inhibiting aggregation at low concentrations and stimulating it at higher concentrations (Kok 1995). From experiments with a series of bisphosphonates where slight variations were made in their structure it was concluded that bisphosphonates bind to the crystal surface by a combined action of the two phosphonate groups and side chains in close proximity. Increasing the affinity for calcium of these side groups increased the capacity to inhibit crystal growth. The presence of two calcium binding phosphonate groups makes bisphosphonates to likely form large

polynuclear complexes with calcium ions acting as a bridge (Bone et al. 1979). These complexes act as one macromolecular structure and inhibition of aggregation is reversed to stimulation (Kok 1995). The complexes bind to more than one crystal at the same time and act as a bridge (viscous binding). Viscous binding can also explain why some macromolecules may at the same time strongly inhibit crystal growth and strongly stimulate crystal aggregation (Kok et al. 1986). Bisphosphonates with a large side chain (steric hindrance) do not form such large complexes and do not show stimulation of crystal aggregation. Growth inhibition by bisphosphonates also depends on their protonation state, thus on the pH and its pKa-values. The triply deprotonated form, present when the pH surpasses the pKa3-value, is the most effective in inhibiting crystal growth. A pH-dependency is also found for pyrophosphate and citrate (Caudarella and Vescini 2009). In the urine pH range ionic species of pyrophosphate are PP^{4-}, HPP^{3-} and H_2PP^{2-}. The first two adsorb onto COM crystals (Wagner and Finlayson 1978) and will predominate at higher pH values. Variation of the pKa3 value of a bisposphonate increases its activity at low urine pH values and might reduce its anti bone resorptive capacity. It may be possible to construct a bisphosphonate that strongly inhibits CaOx crystal growth and crystal aggregation at the urine pH levels and does not interfere with bone resorption activity at the low pH levels existing under active osteoclasts. Since pyrophosphate is an effective inhibitor under non-urine conditions, several groups have investigated if stone formers have a low urine pyrophosphate excretion. Pyrophosphate enters the urine in the glomerular filtrate. The plasma concentration is 2-3 μM, of which 70-80% is ultrafiltrable. The urine excretion rate is variable. In male non-stone formers the concentration averages 20-40 μM, the 24hr excretion rate is 30-60 μmoles (range 15-98 μmoles). It is nevertheless possible that

increasing pyrophosphate excretion raises the growth inhibitory power of urine and as such is beneficial.

2.5.1.2. Citrate

Citrate inhibits COM crystal growth at concentrations above 0.1 mM (Hess et al. 2000), which is in the range of its concentration in the loop of Henle (Kok 1996). It is also inhibits crystal growth in a gel matrix (Achilles et al. 1989). Citrate may contribute to crystal growth inhibition at sites where other, macromolecular, modulators have not entered the fluid yet (Caudarella and Vescini 2009). Citrate also affects crystal aggregation, both in solution (Kok et al. 1988) and in a matrix situation (Achilles et al. 1997). Tested as single modulator present, citrate inhibits crystal aggregation at concentrations above 0.1mM (Kok et al. 1988), and thus could be active up to the loop of Henle. However, this data cannot be directly extrapolated to the whole urine situation. When whole urine is tested in 1:5 dilution (the Dilution State in the collecting duct) urine is found to strongly inhibit crystal aggregation, and there is a strong correlation with the urine citrate concentration (Kok et al. 1986; Kok et al. 1990). But when all macromolecules are removed and citrate remains, the urine loses most of its capacity to inhibit crystal aggregation and the relationship between crystal aggregation inhibition and citrate concentration is lost.

2.5.2. High MW Compounds

2.5.2.1. Glycosaminoglycans (GAGS)

In 1684 Anton von Heyde discovered the presence of a mucoprotein matrix in stone (Gruber 1934). Later, urine was found to contain many different anionic proteins and non-protein anions like (GAGS), RNA and acid mucopolysaccharides. Most prominent are the

GAGS, polyanionic compounds with varying MW of usually18-40 kDa but up to 106 Da. GAGS can enter the urine by filtration, by release from the glomerular basement membrane (Pitcock et al. 1988) from the surface of the tubular epithelial lining and the urothelium further down the urinary tract, including the bladder (Edyvane et al 1983). Well-known GAGS include heparin (not present in urine) and the urinary GAGS heparan sulfate (HS), chondroitin sulfate A B and C (CS-A, CS-B, CS-C) dermatan sulfate (DS), keratan sulfate (KS) and the non-sulfated hyaluronic acid (HA). Some, but not all urinary GAGS are found in crystals and stones (Morse and Resnick 1989; Nishio et al. 1985; Roberts and Resnick 1986; Yamaguchi et al 1993; Suzuki et al. 1994a).

Although quantity does not appear to play a role, some data indicate that the quality of GAGS may vary. Urinary macromolecules and urine from children inhibit crystal aggregation better than urine of adults. The macromolecule fraction of pediatric urine contained more GAGs (Teller et al. 1962). GAGs from stone formers had an increased nucleation promoting activity but similar crystal growth inhibitory activity (Erturk et al. 2002). The first appeared related to a changed action of HA in stone formers (Gohel et al. 1992). However, CS of healthy individuals also showed a basal crystallization-promoting property (Shum et al. 1999). Under inorganic solutions and urine conditions the non-urine GAG heparin is the most effective on a molar basis. Of the GAGs present in urine HS is most effective followed at a distance by CS and HA. The heparin analog pentosan polysulfate has an efficacy between heparin and CS. With respect to crystal-cell interactions, coating of crystals by GAGs decreased the binding of crystals to renal epithelial cells in culture, but did not completely abolish it (Schepers et al. 2002; Lieske et al. 1995; de Water et al. 2001).

2.5.2.2. Proteins

2.5.2.2.1. Tamm-Horsfall Protein

THP was first isolated from the urine by Tamm and Horsfall and characterised as a glycoprotein that inhibits viral hemagglutination (Tamm and Horsfall 1950). It is one of the most abundant proteins in normal human urine and the major constituent of urinary casts. Muchmore and Decker isolated a protein called uromodulin from the urine of pregnant women (Muchmore and Decker 1985). Based on amino acid and carbohydrate analysis THP and uromodulin were shown to be identical (Kumar and Muchmore 1990). THP has a molecular weight of approximately 80 kDa with a tendency to aggregate to the polymeric form. Polymerisation is increased in the presence of free calcium ions, at high ionic strength, osmolality, and at low pH.

THP has been the subject of extensive research for its implication in stone formation. However, its exact contribution to urolithiasis remains unclear and the results of various studies have been controversial (Hess 1992). Some studies indicated that THP promoted CaOx and CaP crystallization (Hallson and Rose 1979; Yoshioka et al. 1989). Other studies demonstrated that the macromolecule does not support CaOx crystallization and has no effect on spontaneous precipitation (Yoshioka et al. 1989; Weaver et al. 2009).

Still other studies indicated that THP has no effect on nucleation or growth, but is a potent inhibitor of CaOx crystal aggregation (Hess et al. 1991; Hess et al. 1993). Hess *et al.* found that the addition of citrate reduced CaOx crystal aggregation by reducing the self aggregation of THP isolated from stone formers urine. It is important to point out that low citrate or hypocitraturia is common in stone formers and can contribute to crystal

aggregation and stone formation in this fashion. THP activity is controlled by its concentration, urinary osmolality and physicochemical environment of the urine (Scurr and Robertson 1986). For example, at low concentrations, THP has a minor effect on CaOx crystallization yet promotes it at higher concentrations. Also, when ionic strength was increased or the pH lowered the inhibition of CaOx monohydrate crystal aggregation by THP was decreased (Hess et al. 1991). Apparently, at high ionic strength, high THP concentration and low pH, the viscosity of THP increases due to its polymerisation. Several studies have shown that there is no significant difference in the daily urinary excretion of THP between normal subjects and CaOx stone formers (Bichler et al. 1976). This fact led Hess *et al.* to hypothesize that THP of stone formers is structurally different from that of the healthy subjects (Hess et al. 1991). They showed that THP isolated from the urine of stone formers contained less carbohydrate (mainly sialic acid) than the THP obtained from control subjects (Hess et al. 1995). It has been suggested that the abnormality may be inherited, but sufficient evidence to support this concept is not available at this time.

Studies have also shown differences in sialic acid contents and surface charge between THP from stone formers and normal individuals. Isoelectric focussing (IEF) studies have shown that THP from healthy individuals has a pI value of approximately 3.5, while THP from recurrent stone formers has pI values between 4.5 and 6 and the two exhibit completely different IEF patterns (Schnierle 1995). THP is exclusively produced in the kidneys. Based primarily on studies in rat kidneys, it is agreed that THP is specifically localized in epithelial cells of the thick ascending limbs of the loops of Henle (Hoyer et al. 1979; Bachmann et al. 1990) and is generally not seen in the papillary tubules. When CaOx crystal deposits, the nephroliths, are experimentally induced in rat kidneys, THP is seen in

close association with the crystals, both in the renal cortex as well as papillae (Gokhale et al. 1996a; Gokhale et al. 1996b). However, THP is not seen occluded inside the crystals nor produced by cells other than those lining the limbs of the Henle's loop (Gokhale et al. 2001). There is no significant biochemical differences in the THP between one secreted by normal rats or rats with CaOx nephroliths. They have similar amino acid composition, carbohydrate contents, molecular weights and rates of urinary excretion. However, THP from nephrolithic rats has slightly less sialic acid contents, 20% of the total carbohydrate in nephrolithic rats vs. 26% in normal rats. In an aggregation assay, both the normal rat THP and nephrolithic rat THP reduced CaOx crystal aggregation *in vitro* by approximately 47%. Results of these rat model studies led to the conclusions that THP is most likely involved in controlling aggregation and that the major difference between normal and stone formers THP may be their sialic acid contents. However animal studies cannot rule out THP's role in modulating crystal nucleation or growth. Another rat model study has shown increased expression of THP in kidneys following unilateral ureteric ligation, which caused tubular dilatation (Miyake et al. 1998). The results indicated that THP expression in kidneys may be increased without crystal deposition and that increased expression in nephrolithic kidneys may be a result of crystal associated injury to the renal epithelial cells. Even rat model studies have provided controversial results for THP. One study shows decreased renal expression of THP during CaOx crystal deposition (Marengo et al. 2002) while results of another study show upregulation of the THP gene (Katsuma et al. 2002).

2.5.2.2.2. Nephrocalcin (NC)

NC is a glycoprotein with a monomeric molecular weight of approximately 14 kDa and has a tendency to self-aggregate into a larger macromolecule and thus can exist as a

dimer, trimer or tetramer with molecular weights of 23-30, 45-48 or 60-68 kDa respectively (Worcester et al. 1992; Nakagawa et al. 1983; Nakagawa et al. 1985; Nakagawa et al. 1987; Nakagawa et al. 1981). NC can also bind to THP in the presence of calcium and magnesium ions. NC can be reversibly dissociated into its monomeric form with incubation in ethylenediaminetetraacetic acid (EDTA) for several days. NC is composed of 110 amino acid residues of which 25% are glutamic and aspartic acid. It contains 2 cysteine and 2 or 3 γ-carboxyglutamic acid (Gla) residues which are suggested to play a significant part in its ability to inhibit CaOx crystallization. Carbohydrate content represents about 10.3% of its weight, with no glucuronic acid and 0.4% sialic acid. Originally purified from human urine, NC has subsequently been isolated from human kidney tissue culture medium, human renal cell carcinoma, kidneys of many vertebrates, mouse renal proximal tubular cells in culture and rat kidney and urine (Coe et al. 1994; Nakagawa et al. 1989). Immunohistochemical techniques have localised NC in the renal epithelial cells of proximal tubules and thick ascending limb of Henle's loop (Nakagawa et al. 1990). The site of its synthesis has not yet been confirmed by localization of NC mRNA. Daily excretion of NC in human urine is about 5-16 mg (Worcester et al. 1992; Nakagawa et al. 1983). NC was originally regarded as the principal inhibitor of CaOx monohydrate (COM) crystallization in the urine, accounting for approximately 90% of the total urinary crystallization inhibitory activity (Worcester et al. 1992). According to the recent results however, the contribution of this inhibitor is suggested to be limited to only 16% (Worcester et al. 1993). NC is suggested to inhibit nucleation, growth, and aggregation of COM crystals. The fractional inhibition of nucleation due to the presence of NC was shown to be equal to that of urine, (Asplin et al. 1991) suggesting that this inhibitor accounts for the total nucleation inhibitory activity of urine.

However, the amino acid composition and carbohydrate contents of NCs from both the stone formers and normals appeared similar.

2.5.2.2.3. Inter-α-Inhibitor

Inter alpha inhibitor (IαI) are composed of a combination of heavy chains, H1 (60 kDa), H2 (70 kDa), H3 (90 kDa) covalently linked via a CS bridge to a light chain called bikunin (35- 45 kDa). The heavy and light chains also exist independently as single molecules. IαI (180-240 kDa) is a heterotrimer consisting of bikunin linked to heavy chains H1and H2. The macromolecule consisting of bikunin linked to heavy chain H2 is called IαI like inhibitor (IαLI). Bikunin is a broad-spectrum protease inhibitor and an acute-phase reactant. IαI and related proteins have been linked to various pathological conditions such as inflammatory diseases (Witte et al. 1982; Franck and Pederson 1983), cancer (Chawla et al. 1982; Yoshida et al. 1994; Thogersen and Enghild 1995), renal failure (Toki and Sumi 1982) and more recently the urinary stone disease.

Both heavy and light chains have been identified in the urine (Atmani et al. 1993a; Atmani et al. 1993b; Atmani and Khan 1995; Atmani et al. 1996a; Atmani et al. 1994; Suzuki et al. 2001). The average concentration of IαI in the plasma of healthy human subjects is approximately 450 mg/l. It was shown that bikunin isolated from the patients, contained less sialic acid and exhibited less crystallization inhibitory activity than that purified from the urine of healthy subject (Atmani et al. 1994). In a separate study mean urinary bikunin to creatinin ratio was found to be significantly higher in stone formers than in non-stone forming healthy male and female controls (Suzuki et al. 2001). Western analysis showed that a significantly higher proportion of stone patients had a 25kDa bikunin

in their urine in addition to the normal 40kDa species. 25kDa bikunin was similar to the deglycosylated bikunin and was less inhibitory.

With respect to kidney stone formation, Atmani *et al.* isolated a 35kDa urinary protein, which inhibited growth of CaOx crystals. They named the protein uronic acid rich protein (UAP), because of the high uronic acid content with D-glucuronic and L-iduronic acids being the major constituents (Atmani et al. 1993a). Amino acid composition revealed it to be rich in aspartic and glutamic acid residues, which account for 24% of the total amino acids. No Gla residues were detected. Basic and aromatic amino acids represented 10% and 13%. Carbohydrates accounted for 8.5% of its weight. N-terminal amino acid sequence analysis of human protein demonstrated the homology with IαI, specifically with bikunin (Atmani et al. 1993b). Later UAP was isolated the from the rat urine (Atmani and Khan 1995) and showed it to have characteristics similar to the human UAP in molecular weight, amino acid composition as well as the crystallization inhibitory activity. In addition, on Western blot analysis, both reacted with an inter-α-trypsin inhibitor antibody. Later, on the basis of bikunin antibody reaction with the UAP in western blot analysis and similarity of the sequence of first 25 N-terminal amino acid residues of UAP being identical to that of bikunin UAP was identified as bikunin (Atmani et al. 1996a). IαI proteins have been shown to inhibit CaOx crystallization *in vitro* (Atmani et al. 1993a; Atmani et al. 1993b; Atmani and Khan 1995; Atmani et al. 1996; Medetognon-Benissan et al. 1999; Kobayashi et al. 1998). The inhibitory activity is confined to the carboxy terminal of the bikunin fragment of IαI (Kobayashi et al. 1998).

2.5.2.2.4. Osteopontin

Its apparent molecular weight has been estimated from 44 to 75 kDa depending on the percentage of polyacrylamide gel used. This anomalous migration is assumed to be due to differences in glycosylation and phosphorylation. In addition to its existence as a monomeric form, the protein may also aggregate to form a higher molecular weight entity. Amino acid analysis of rat OP revealed that it contains 319 residues of which 36% are aspartic and glutamic acid (Denhardt and Guo 1993; Prince et al. 1992). It also contains 30 serine, 12 phosphoserine and one phosphothreonine residues.

Osteopontin from all species has high aspartate/asparagine contents accounting for as much as 16-20% of all amino acid residues in the molecule. In addition to bone cells, OPN is present in many epithelial tissues in kidneys, gastrointestinal tract, gall bladder, pancreas, lung, salivary gland and inner ear (Brown et al. 1992). It is also expressed in a variety of other cell types including macrophages (Pollack et al. 1994; Murry et al. 1994), activated T-cells, smooth muscle cells and endothelial cells.

The significantly higher incidence of a single base mutation in the OPN gene has been found in the patients with recurrent or familial nephrolithiasis (Yamate et al. 2000). OPN is intimately involved in both the physiological and pathological mineralisation processes including crystallization in the urine and development of calcific kidney stones support for the CaOx crystallization inhibitory actions of OPN (Langdon et al. 2009) is further strengthened by studies in OPN knockout mice (Wesson et al. 2003). When comparable hyperoxaluria is induced in OPN knockout and wild type mice, knockout mice developed significant intratubular deposition of CaOx crystals while wild type remained free of any crystals. In addition wild type hyperoxaluric mice showed significant increase in OPN expression in their kidneys, indicating a reno-protective role for OPN. Results of one

study show OPN favouring crystallization of COD over COM (Wesson et al. 1998), which may influence the development of kidney stones because renal epithelium is more likely to bind COM crystals than the COD crystals. It appears that structural defects and various post-translational modifications, such as glycosylation and phosphorylation may influence the effect of OPN on crystallization in urine.

2.5.2.2.5. Urinary Prothrombin Fragment –1(UPTF-1)

This protein is also known as crystal matrix protein (CMP) because it was found selectively associated with CaOx crystals experimentally induced in human urine (Doyle et al. 1991). Molecular weight of this protein was found to be 31 kDa. The amino acid sequence analysis of CMP showed an identity with prothrombin (Stapleton et al. 1993; Stapleton and Ryall 1994; Suzuki et al. 1994), a plasma protein involved in coagulation cascade. In the first 34 amino acid residues, 10 of the glutamic acids are γ-carboxylated. The carbohydrate contents represent 17% of its molecular weight. Suzuki *et al.* proposed that CMP is the activation peptide of human prothrombin (Suzuki et al. 1994b). By using specific antibodies for prothrombin and F1+2 fragment, Stapleton and Ryall demonstrated (Stapleton and Ryall 1994) that CMP is prothrombin fragment F1 (UPTF-1).

Recent studies have provided evidence that PT gene is expressed in both the human and rat kidneys indicating the possibility of PT biosynthesis in both human and rat kidneys (Grover et al. 2000; Suzuki et al. 1999; Grover et al. 1999). Recent studies using purified urinary proteins have confirmed earlier results and have demonstrated UPTF-1 to be an inhibitor of both crystal growth and aggregation (Ryall et al. 1989). Results of another study where a comparison was being made between the white and black South Africans with regard to urinary crystallization inhibition showed that UPTF-1 is a strong inhibitor of

crystal nucleation (Durrbaum et al. 2001). UPTF-1 from normal black males reduced crystal nucleation by 63.6% as compared to the protein from normal white males that reduced the nucleation by 23.4%.

2.5.2.2.6. Calgranulin (Calprotectin)

Calgranulin is a 28 kDa member of S100 family of calcium binding proteins, which are small, ubiquitous, and acidic proteins involved in normal developmental and structural activities (Zimmer et al. 1995). However, they are also implicated in a number of diseases (Kahn et al. 1982). The protein was recently isolated from human urine (Pillay et al. 1998) at a concentration of 3.5-10 nM. Purified urinary calgranulin inhibited both CaOx crystal growth (44%) and aggregation (50%) in nanomolar range. 28kDa calgranulin was cloned from the human kidney expression library. Western analysis of rat and human kidneys as well as renal epithelial cell lines, BSC-1 and MDCK confirmed its renal presence. Calgranulin is also known as leukocyte antigen L1 and has been identified in circulating neutrophils and 22 monocytes and has bacteriostatic antifungal activites (Steinback et al. 1990). It has also been identified in matrix of infectious or struvite stones (Bennett et al. 1994) and in CaP deposits formed by MDCK cells (Naito et al. 1997; Yasui et al.1997).

2.5.2.2.7. Albumin

Albumin is one of the most abundant proteins in the urine (Maslamani et al. 2000; Fraij 1989; Boyce and Garvey 1956) and has been detected in the matrix of both urinary stones (Fraij 1989; Boyce and Garvey 1956; Boyce 1968) as well as crystals (Atmani et al.

1998; Atmani and khan et al. 2002; Atmani et al. 1996a) made in the whole human urine. It is known to bind to CaOx as well as uric acid crystals (Worcester 1994; Dussol et al. 1995) but does not inhibit their growth (Worcester 1994). However, it has been shown to inhibit CaOx crystal aggregation in concentration dependent manner (Edyvane et al. 1986; Hess et al. 1995; Grover et al. 1998). When immobilized to surfaces and exposed to metastable solutions albumin promotes crystal nucleation (Cerini et al. 1999; Ebrahimpour et al. 1991). When dissolved in solution albumin exists either in monomeric or and polymeric form (Cerini et al. 1999). In metastable CaOx solutions both monomeric and polymeric forms promote nucleation of CaOx. In addition, nucleation by albumin leads exclusively to the formation of COD crystals. Urinary albumin purified from healthy subjects contained significantly more polymeric forms and was a stronger promoter of CaOx nucleation than albumin from idiopathic calcium stone formers. Promotion of CaOx nucleation and formation of large number of COD crystals might be protective. Nucleation of large number of small crystals would allow their easy elimination and decrease CaOx saturation preventing crystal growth and aggregation and subsequent stone formation. COD crystals are more common than COM crystals in non-stone formers urine and are generally found in lesser quantities in stones than COM crystals. In addition crystals present in the urine from non-stone formers are significantly smaller than those in stone formers urine. Albumin also exhibits the capacity to bind some of the urinary proteins. Interestingly, urinary proteins that show great affinity for albumin are also those that are included in the stone matrix. It is suggested that proteins become a part of stone matrix by binding to the albumin coating CaOx crystals. It is also suggested that unlike other calcium binding urinary proteins, albumin promotes nucleation by interacting with calcium through the carboxyl group.

Strong nucleation activity was observed at pH 7 but was totally eliminated at pH 4 when carboxyl groups are no longer ionized. In addition, morphological studies showed CaOx crystals to nucleate through calcium rich face (Cerini et al. 1999).

2.5.2.2.8. CD44

CD44 is a transmembrane protein and the main cell surface receptor for hyaluronan or hyaluronic acid (HA) as well as OPN (Weber et al. 1996). Both CD44 and HA are upregulated during injury and inflammtion and are involved in the formation of a cell coat or pericellular matrix on surfaces of proliferating and migrating cells. HA is restricted to the inner medullary interstitium of the normal kidneys. Distal collecting duct cells express both CD44 and HA on apical cell surfaces of the proliferating cells. At confluence however, CD44 is expressed at the basolateral membrane while HA is undetectable. Proliferating cells are receptive to adhesion of CaOx crystals, a property lost when cells become confluent. In addition removal of pericellular matrix by hyaluronidase treatment also results in loss of crystal adhesion property of the proliferating cells (Verhulst et al. 2003; Asselman et al. 2003). Based on theses observations it has been proposed that intact epithelium does not bind crystals because of the absence of a pericellular matrix and crystal attachment depends upon the expression of CD44, OPN and HA by the damaged renal epithelial cells (Verkoelen et al. 2000).

2.5.2.2.9. Trefoil Factor1

Chutipongtanate et al. have reported human trefoil factor 1 (TFF1) as CaOx crystal growth inhibitor (Chutipogtanate et al. 2005). It belongs to the trefoil factor family of proteins, is expressed predominately in gastric mucosa, and is synthesized by mucosal

epithelial cells. It has antiapoptotic and motogenic activities, and its main functions in the gastrointestinal tract are thought to involve maintenance of mucosal integrity and mucosal repair in response to inflammation or injury. TFF1 has been identified previously in human urine using radioimmunoassay and Western blotting. Urinary TFF1 at the concentration of 7 ng/ml inhibited CaOx crystal growth. The significant inhibitory effect was demonstrated after 10 minutes' incubation and remained significant through the end of the assay (1 hour).

2.5.2.2.10. Model Peptides

A number of studies have been carried out investigating the effect of model peptides on crystallization *in vitr*o. Polyaspartic acids (PolyD) with molecular weights of 8, 12, 15, 37.6 and ploy glutamic acids (PolyE) with molecular weight of 13 have been examined. A clear understanding of the crystallization inhibitory mechanisms of various glycoproteins has been the main purpose of these studies. Crystallization of CaOx was induced *in vitro* in a buffered salt solution containing calcium and oxalate in different ratios and at various supersaturations, in the absence or presence of the polypeptides with pH and ionic strength in the range of normal human urine (Wesson and Worcester 1996; Wesson et al. 2000). In the absence of proteins, CaOx monohydrate was the preferred crystalline form for all calcium to oxalate ratios (Wesson and Worcester 1996; Wesson et al. 2000). The number of CaOx monohydrate crystals increased with increasing oxalate concentrations. The presence of either the Poly D or E produced COD crystals. PolyE was less effective at producing COD than PolyD (Wesson et al. 2000). At a concentration of 800nM and equimolar Ca and Ox concentrations only 20% of the crystals were COD's. It did however have an effect on COM crystal morphology by producing dumbbell shaped crystals, a morphology common in

human and rat urine. Under similar conditions of supersaturation and Ca and Ox concentrations PolyD, however, favoured the formation of COD requiring very low concentrations <200nM. 12, 15 23 and 37.6 molecular weight PolyD were able to exclusively produce almost all COD's. Higher CaOx supersaturations required higher amounts of PolyD to cause COD formation. It is concluded that change from COM to COD is the result of inhibition of COM nucleation by protein adsorption onto nascent nuclei. COD is formed to relieve the chemical potential favouring crystallization. The importance of these results with regard to nephrolithiasis lies in the observations that COD crystals are less likely to adhere to the renal epithelium than COM crystals and thus, less likely to be retained in the kidneys and promote the formation of kidney stones(Wesson et al. 2000). Both PolyD and PolyE have also been tested for their effect upon COM crystal growth and adherence to renal epithelial cells in culture. Both proved potent inhibitors of the growth of COM crystals and also blocked the adhesion of COM to BSC-1 cells.

2.5.2.3. Lipids

Even though lipids account for a small proportion of the matrix; 7-14% in bone, 2-6% in dentin, 12-22% in newly mineralised enamel, 9.6% in submandibular salivary gland calculi and 10.2% in supragingival calculi (Wuthier 1981; Anderson 1983; Slomiany et al. 1982; Boskey et al.1983; Boskey et al. 1981), they play a significant role in calcification. They promote crystal nucleation, modulate growth and aggregation and become incorporated in growing calcifications. The matrix of all stones examined to date, including struvite, uric acid, CaOx and CaP contains lipids (Khan et al. 1988; Khan et al. 1996). The protein to lipid ratio is, however, higher in the matrix of struvite and uric acid stones than in

CaOx and CaP stone matrix. Even though there are no significant differences in types of lipid, the matrix of struvite stones contains more cholesterol, cholesterol ester and triglycerides than the other three stone types. One dimensional thin layer chromatography separated and identified various phospholipids and glycolipids including sphingomyelin (SM), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), cardiolipin (CL) and trace amounts of phosphatidylserine (PS) in all stone matrices. Occasionally, the stone matrix also contains phosphatidyl inositol (PI), lyso-PC, lyso-phosphatidic acid (PA) and lyso-PE. In all stones glycolipids include gangliosides, D-sphingnosine, and glucocerebrosides. In addition, the struvite stone matrix contains sulfatides and digalacto diglycerides while CaOx and CaP stone matrix contains cerebrosides 1 and 2 and digalacto-diglycerides. All stone matrices contain both complexed and non-complexed lipids. The amount of complexed lipids is highest in CaP and lowest in uric acid stones. Both complexed and noncomplexed lipids contain cholesterol, triglycerides, phospholipids and gangliosides. Both CaOx and CaP crystals induced in the urine contain lipids (Khan et al. 1996). There are no significant differences in either the nature of lipid constituents or the amounts of lipid per gram of crystal between the two types of calcific crystals. Glucocerebrosides are the most common glycolipids and SM the most common phospholipid. Gangliosides are the second most common glycolipid, PC and PE the most common phospholipids. Determinations of lipids in the urine before and after experimental induction of CaOx crystals show that the formation of crystals depletes the urine of its phospholipids indicating its incorporation in the crystal matrix. Almost all the urinary phospholipids become incorporated during the formation of crystals (Khan et al. 1996).

MATERIAL & METHOD

3.1. Collection of human renal stone sample and its extraction

Human renal calculi, surgically removed from the kidney stone patients were obtained from the Department of Urology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Stones were taken from those patients who were more than 25 years of age and were suffering from no other abnormality, and the stones were of a non-infectious nature. After FTIR analysis, the stones with calcium and oxalate as their major components were selected for further studies.

3.1.1. Protein extraction

3.1.1.1. EGTA extraction

Proteins were isolated from the matrix of kidney stones containing CaOx as the major constituent using EGTA as a demineralizing agent. Stones were washed in 0.15M NaCl with gentle stirring for 48 hours to remove the adhered blood, tissue etc. They were then dried and pulverized with a mortar and pestle. For extraction of the organic matrix of powdered stone; each gram of stone was suspended in 10mL of 0.05M EGTA, 1mM PMSF and 1% β-mercaptoethanol. The extraction was carried out for 4 days at 4ºC with constant stirring. The suspension was centrifuged for 30 minutes at 10,000g and at 4ºC. The supernatant of EGTA extract was filtered through Amicon ultra centrifugal filter device with a molecular weight cut off 10,000 daltons at 4ºC and concentrated to a known volume. Whole EGTA extract, greater than and less than 10,000 daltons fractions were stored at -20ºC for further studies (Aggarwal 2000).

3.1.1.2. SDS extraction

For SDS method, 1g stone powder was mixed with 4mL SDS reducing buffer (0.06M Tris HCl, pH6.8, 10% glycerol, 5% β-mercaptoethanol, and 2% SDS) and heated

(100ºC, 30min). Samples were centrifuged at 700g for 15 minutes at 4ºC and the supernatant collected. The supernatant was filtered through Amicon ultra centrifugal filter device with a molecular weight cut off 3,000 daltons at 4ºC to remove SDS and concentrated to a known volume (Williams et al. 2006).

3.1.1.3. Acetic acid extraction

For the acetic acid method, 1gm stone powder was vortexed with 5 mL 10% acetic acid and incubated for 30 min. Samples were centrifuged at 3,000g for 20 min at 4ºC and the supernatant was retained and filtered as described above (Williams et al. 2006).

3.1.2. Lipid extraction

Lipids were extracted from stone powder by the method of S R Khan et al. (Khan et al. 2002). 1 gm of stone was extracted in 30 mL of ice-cold chloroform: methanol: 0.05 mol/L Tris-HCl, pH 7.4 (2:1:1); using sonication at 4˚C. After sonication the sample was centrifuged at 10,000g to phase separate and pellet residue. The upper and lower phases were removed separately and pooled as aqueous and organic substances, respectively. Extraction solvent was added to the pellet and the process was repeated several times pooling the respective phases. Pooled organic phases were extracted with ethanol: ether (3:1) and then centrifuged. Non-complexed lipids were isolated from the supernatant. Complexed lipids were recovered from the pellet by dissolving it into DMSO by sonication. Complexed lipid in DMSO was preserved at -20˚C for further use.

3.1.3. Separation of biomolecules on the basis of their molecular weight

Renal stone extract obtained through EGTA extraction was centrifuged by using Amicon Ultra-4 centrifugal separating tubes (Millipore) of 10 kDa cut off molecular weight. Thus, two fractions >10kDa and <10 kDa were obtained. Whole EGTA extract, >10 kDa and <10kDa fraction were assayed on different assay system described below.

3.1.4. Protein concentration

Total protein concentration of the supernatant of renal stone matrix obtained by EGTA extraction method as mentioned above was determined using a commercial Bradford assay reagent (Bio-Rad Laboratories, Hercules CA).

Principle

The assay (Bradford 1976) is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Reagents

1. Bradford reagent: 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL of 95% ethanol. To this solution 100 mL of 85% (w/v) phosphoric acid was added. Resultant solution was diluted to 1 liter and filtered through Whatman #1 paper just before use.
2. 1 M NaOH

Procedure

- 0.1mL of test solution was taken in a test tube.
- Equal volume of 1 M NaOH was added to each sample and was mixed.
- Finally, 5 mL of Bradford reagent was added and the solution was mixed and incubated at room temperature for 5 minutes.
- Similarly standard and blank tubes were prepared and absorbance of all samples was measured at 595 nm.

Calculation

The concentration of protein (µg/mL) was calculated using following formula

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ X concentration of standard}$$

3.1.5. Homogeneous assay system of initial mineral phase of calcium phosphate

Homogenous mineralization system was used to study the extent of *in vitro* mineral phase formation to determine the activity of calcium phosphate (CaP) precipitation (Kabra et al. 1978). The homogenous system consisted of 5mM $CaCl_2$ and 5mM KH_2PO_4. After incubating this system at 37^0C, precipitates obtained were centrifuged and the pellets were resuspended in 0.1N HCl. The calcium (Ca^{2+}) and phosphate ions (HPO_4^{2-}) concentration in the precipitate represented the extent of precipitation (crystallization) of these ions and the inhibitory biomolecule(s) will minimize the extent of their precipitation. The $Ca^{2+}$and HPO_4^{2-} ions were estimated by the methods of Trinder (Trinder 1960) and Gomori (Gomori 1941) respectively. Percentage inhibition or stimulation of mineral phase in the presence of renal stone extract (whole EGTA extract, >10kDa & <10kDa fraction) was calculated as:

%age Inhibition = [*(C-T)/C*] X 100, where *T* is the concentration of Ca^{2+}or HPO_4^{2-} ion of the precipitate formed in the assay system with the renal stone extract or lipid extract and *C* is the concentration of Ca^{2+}or HPO_4^{2-} ion of the precipitate formed in control system which had distilled water (Millipore).

3.1.5.1. Determination of calcium

Method of Trinder was used to determine the calcium ions in a sample.

Principle

Calcium ions get precipitated as napthyl hydroxamate by directly adding slight excess of calcium reagent. After centrifugation excess of reagent is removed by decantation and unwashed precipitates are dissolved in EDTA. Addition of ferric nitrate results in development of an orange red color, intensity of which is measured as the amount of calcium ions present in the sample.

Reagents

- Calcium reagent- This reagent was prepared by mixing two components and the volume was raised to 1000mL by adding distilled water, the resultant mixture was filtered and stored in dark reagent bottle. The two components were as follows:

  (a) 280 mg of Napthylhydroxamic acid in 100 mL of (95mL distilled water + 5mL ethanolamine + 2 gm of tartaric acid).

  (b) 9 gm of NaCl in 500 mL of distilled water.

- Color reagent- 60 gms $FeNO_3$ was dissolved in 500 mL of acidified distilled water (485 mL distil water with 15 mL of conc. HNO_3). Then, the volume was raised to 1000 mL with distilled water.
- EDTA- 2 gm EDTA was dissolved in 1000 mL of 0.1 N NaOH.
- Working standard- 2mM of calcium chloride.

Procedure

- 0.1mL of sample was dissolved in 2.5 mL of calcium reagent and incubated at room temperature for 30 minutes.
- The precipitate were obtained after centrifugation and then dissolved in 1.0 mL of 0.2% EDTA with boiling for 10 minutes.
- Finally, 3 mL coloring reagent was added and the absorbance was measured at 450 nm.

Calculations

The following formula was used to calculate the concentration of calcium ions (mM).

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ X concentration of standard}$$

3.1.5.2. Determination of phosphate

The phosphate ions concentration in the sample was determined by the method of Gomori.

Principle

Phosphate reacts with molybdic acid to form phosphomolybdic acid. Treatment of 2-methyl-4 aminosulfate causes reduction of phosphomolybdic acid to form deep blue colored complex which gives absorption maxima at 660 nm.

Reagents

- Molybdic acid- Prepared by mixing 2.5% ammonium molybdate dissolved in distilled water and 10 N H_2SO_4 in the ratio of 10:4.
- Metol reagent- Preparede by mixing 5% $NaHSO_3$ and 1% metol in distilled water.
- Working standard- 2mM of KH_2PO_4

Procedure

- 1.2 mL of molybdic acid was added to 0.2 mL of sample and incubated at room temperature for 10 minutes. The sample was diluted by 6.8 mL of distilled water.
- Then, 0.5 mL of metol reagent was added and the solutions were mixed properly.
- Finally the mixture was kept at room temperature for 20-30 minutes and absorbance was measured at 660 nm.

Calculations

The following formula was used to calculate the concentration of phosphate ions (mM).

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \quad X \quad \text{concentration of standard}$$

3.1.5.3. Determination of percentage inhibition of calcium phosphate mineral phase

The percentage inhibition of CaP mineralization was calculated using following formula.

$$\% \text{ age inhibition} = [(C-T)/C] \text{ X } 100$$

where, 'C' is the concentration of Ca^{2+} or HPO_4^{2-} ions of the precipitate formed in control system which had distilled water. 'T' is the concentration of Ca^{2+} or HPO_4^{2-} ions of the precipitate formed in assay system with the test sample.

3.1.6. Homogeneous assay system of growth and demineralization of calcium phosphate mineral phase

The growth and demineralization of preformed mineral phase consisting of calcium phosphate required initial precipitates of these minerals as obtained by the initiation of calcium phosphate mineral phase. To study the growth of the preformed mineral phase, the precipitates formed by the above method were resuspended in the same assay system having calcium and phosphate along with the three fractions of kidney stone extract. This assay system was incubated at 37°C for 30 minutes. Then Ca^{2+} and HPO_4^{2-} ions were estimated and the concentration of these ions represented the growth of precipitation of these ions over the previously formed mineral phase.

For demineralization, the preformed mineral phase was resuspended in the assay system with all the three fractions of kidney stone extract but without further addition of calcium and phosphate ions. This assay system was incubated at 37°C for 30 minutes. Ca^{2+}

and HPO_4^{2-} ions were estimated in supernatant to determine the demineralization of mineral phase by all the three fractions of kidney stone extract.

Different volumes of renal extract were added in the homogenous assay system compensated with water. Five test tubes were taken for every volume of stone extract. The concentration of calcium and phosphate ions of mineral phase in these test samples was determined. In case of growth of pre-formed mineral phase, concentration of Ca^{2+} and HPO_4^{2-} ions was deducted from the final concentration of Ca^{2+} and HPO_4^{2-} ions. The percentage inhibition or stimulation caused by different fractions of renal extract was calculated with respect to control system which had distilled water instead of kidney stone extract. In case of demineralization, the percentage inhibition of Ca^{2+} and HPO_4^{2-} ions demineralized, was calculated in supernatant.

3.1.7. Calcium oxalate crystal growth

Inhibitory activity against CaOx crystal growth was measured using the seeded, solution depletion assay (Chutipogtanate et al. 2005). COM crystal seed (from FTIR identified clinical kidney stones) was added to a solution containing 1mM calcium chloride ($CaCl_2$) and 1mM sodium oxalate ($Na_2C_2O_4$). The reaction of $CaCl_2$ and $Na_2C_2O_4$ with crystal seed would lead to deposition of CaOx on the crystal surfaces, thereby decreasing free oxalate that is detectable at λ 214 nm. When a protein is added into this solution, depletion of free oxalate ions will decrease if the protein inhibits CaOx crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30 second incubation with or without protein. The relative inhibitory activity was calculated as follows:

$$\% \text{ Relative inhibitory activity} = [(C–S)/C] \times 100$$

where, *C* is the rate of reduction of free oxalate without any protein and *S* is the rate of reduction of free oxalate with a test protein.

3.2. Purification of potent protein

3.2.1. Materials

Materials required were Macro Prep 25 Q strong anion exchanger (Bio-Rad laboratories), Bio gel P-100 gel (Medium), and column (50x1 cm).

3.2.2. Extraction

Only calcium oxalate renal stones confirmed after FTIR analysis were taken for the study. Protein extract was obtained through EGTA extraction method as described in section 3.1.1.1.

56.46 gms of calcium oxalate stones were collected. These stones were washed with 0.15 N NaCl. After drying at room temperature renal stones were powdered with the help of pestle and mortar. Renal powder was then extracted with EGTA extraction buffer (0.05M EGTA, 1mM PMSF and 1% β-mercaptoethanol). The extract thus obtained was centrifuged at 8000 rpm for 20 minutes at 4˚C. Supernatant was separated into >10 kDa and <10 kDa fractions by centrifugation with the help of Amicon Ultra-4 centrifugal separating tubes (Millipore) of 10 kDa cut off molecular weight.

3.2.3. Protein estimation

Protein concentration of whole EGTA extract, >10 kDa and <10 kDa fraction was estimated by Bradford assay (Bradford 1976).

3.2.4. Ion exchange chromatography

3.2.4.1. Sample

The fraction which exhibited highest activity against calcium oxalate and calcium phosphate assay system was filtered through Whatman paper. The sample obtained was lyophilized and was preserved at -80˚C.

3.2.4.2. Preparation of column

- **Washing of slurry**

Macro Prep 25 Q strong anion exchanger support was supplied hydrated in 20% (v/v) ethanol. 80 mL of exchanger was taken into a beaker and was allowed to settle for 2-3hrs. Ethanol solution was decanted. Matrix was washed with 2-3 bed volume of deionized water.

- **Equilibration of slurry**

After washing, matrix was mixed with starting buffer i. e. buffer A (20mM TrisCl +0.1M NaCl) having pH 7.4. After 2-3 wash with starting buffer pH of the slurry was checked with pH strips. When the color of pH strip matched with that of pH 7, equilibration of matrix was confirmed.

- **Column packing**

One third of column (50X1cm) was filled with equilibrating buffer. Supernatant of slurry was decanted and was mixed well. A funnel was kept at the top of column and slurry was poured over it slowly until the slurry reached the bottom of the column. After the column was half filled slurry was poured drop wise. For even packing bottom lid of the column was opened for the flow of packing buffer. When one third of the column was packed funnel was removed slowly and slurry was allowed to settle for 7-8 hours.

3.2.4.3. Sample loading & separation of biomolecules

Lyophilized sample was reconstituted with 2.75 mL of starting buffer and was loaded into the column. The bound protein was eluted with 0-90% of linear gradient of buffer A and B. Buffer A consists of 20mM Tris-Cl with 0.1M NaCl (pH 7.4) and Buffer B consists of 20mM Tris-Cl with 1.0 M NaCl (pH 7.4). Protein elution was done at flow rate of 1.0 mL/min using Automated Biologic LP system. Elution profile was made using LP data view software version 1.03. The absorbance of each fraction was read at 280 nm, simultaneously the conductance was also measured. The method used for anion exchange chromatography is illustrated in table 3.1

Fractions coming under the peak were pooled and dialyzed. Their activity was checked on calcium oxalate crystallization followed by SDS-PAGE run. Fraction which was showing highest activity and few bands was then lyophilized and was preserved for further purification.

Table 3.1 Method used for anion exchange chromatography.

Time (minutes)	Flow rate (mL/min)	Buffer
0.0-25	1.0	Buffer A
25-65	1.0	0-90% gradient of buffer A to buffer B
65-85	1.0	Buffer B
85-155	1.0	Buffer A

3.2.5. Molecular sieve chromatography

3.2.5.1. Preparation of the gel

10 gram of Bio-Gel P-100 Gel (Medium) was added to 20mM Tris HCl buffer in a beaker. It was allowed to hydrate for 12 hours at 20°C. After hydration was completed, half

of the supernatant was decanted and the hydrated gel was degassed for 5-10 minutes with occasional swirling. Two bed volume of degassed buffer was added to degassed gel.

3.2.5.2. Column packing

Column packing was done by using the same method as described in section 3.2.4.2 column packing for ion exchange chromatography.

3.2.5.3. Sample loading & separation of biomolecules

Fraction showing highest activity was concentrated and loaded on a Bio gel® P-100 gel molecular sieve column(50X1cm) equilibrated and eluted with the 20 mM Tris buffer (pH 7.4) at a flow rate of 0.1mL/min. The fractions which eluted out based on their molecular weights were pooled to study their activity on calcium oxalate crystal growth assay system. Potent fractions thus obtained were subjected to SDS-PAGE and reverse phase HPLC.

3.2.6. Electrophoresis

For SDS-PAGE, lyophilized samples were reconstituted in reducing sample buffer and analyzed by one-dimensional discontinuous SDS-PAGE using 1 mm thick, 12% separating and 4.4% stacking gels with a Mini-Protean III apparatus (Bio-Rad Laboratories). Protein bands were stained with silver using ProteoSilver™ Plus Silver Stain Kit (PROTSIL2,

Sigma-Aldrich Co.). Broad range molecular weight markers (catalog # 161-0317, Bio-Rad) were used as standards.

Table 3.2 Method used for molecular sieve chromatography

Time (minutes)	Flow rate (mL/min)	Buffer
0.00-1984	0.10	Buffer

3.2.7. RP-HPLC for homogeneity

Waters Spherisorb® C18 (5 μ, 4.6 X 250 mm) column with solvent A (0.1% TFA in water) and solvent B (100% acetonitrile containing 0.1% TFA) was used for determining the homogeneity of purified protein. Flow rate was maintained at 1 mL/min at the time of protein injection. The column was washed with solvent A and brought to 20% acetonitrile in 5 min. The bound protein was eluted with a linear gradient of acetonitrile (20 -70 %) over a period of 50 min. The detection was monitored at 280 nm using Waters 2996 photodiode array detector.

3.2.8. Tryptic in gel-digestion of purified protein

Single band detected after molecular sieve chromatography was excised from the gel and was destained with destainer provided in the ProteoSilver™ Plus Silver Stain Kit (PROTSIL2, Sigma-Aldrich Co.). Trypsin profile IGD kit (PPO100, Sigma-Aldrich Co.) was used for in gel digestion of purified protein. Destained gel piece was dried for approximately 15 to 30 minutes.

Trypsin solubilised in 1mM HCl and mixed with 40mM ammonium bicarbonate and 9% acetonitrile was added to the destained gel piece. Gel piece was fully covered by the addition of 40mM ammonium bicarbonate and 9% acetonitrile (pH 8.2) solution and was incubated for 5 hours at 37°C. After the incubation, liquid was removed from the gel piece

and transferred to a new labeled eppendorf tubes and was preserved for mass spectroscopic analysis.

3.2.9. Peptide mass fingerprinting by MALDI-TOF-MS

The proteolytic sample obtained after in gel digestion was premixed 1:2 with the matrix solution (α-Cyano-4-hydroxycinnamicacid) and spotted on the sample stage. It was dried at room temperature then washed with 0.1% TFA and was analyzed by Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Germany (Bremen)). The mass/charge spectra obtained were searched in MASCOT search engine (http://www.matrixscience.com). All the three databases (MSDB, SwissProt, NCBInr) available in the search engine was used. For search peptides were assumed monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Since we used human renal stones therefore, *Homo sapiens* taxonomy restriction was chosen, only one missed cleavage was allowed, and peptide mass tolerance of 1.2 kDa was used for peptide mass fingerprinting.

3.3. *In silico* study

3.3.1. Materials

Schrodinger software package was used for molecular modeling of identified protein. Calcium oxalate structure was drawn with the help of Molecular Operation Environment (MOE) software package (Chemical Computing Group, Montreal, Canada). After docking,

LIGPLOT program was used for describing the interactions between ligand and protein within a docked structure.

3.3.2. Homology modeling of identified protein

Homology modeling of the identified human phosphate cytidylyltransferase 1, choline, beta protein structure was modeled by using Prime module of Schrodinger (Prime version 1.5, Macromodel version 9.1, Schrodinger, LLC, New York, NY, 2005) software. The structure of protein was modeled on the basis of its structural similarity with the chain A CTP: glycerol-3 phosphate cytidylyltrnsferase of *Bacillus subtilis* (Protein Data Bank IC 1 COZ-A). The degree of identity between the template and the human CCT sequence was 31%, which enabled a preliminary model to be generated by Schrodinger. The sequence alignment was then improved manually and comparative homology method was used to build the structure.

3.3.3. Docking of Homology model of human CCT1 choline beta with calcium oxalate

Calcium oxalate structure was drawn with the help of molecular builder of MOE software developed by the chemical computing group inc. Montreal, Canada. Active site of the modeled protein was predicted by using active site finder tool of MOE software. Then docking of modeled protein with calcium oxalate was done in MOE–Dock. The docking energy calculation was carried out within a user-specified three-dimensional docking box (3D docking box) using the simulated annealing method under the MMFF94 X force field. The energy grids for docking were generated as *grid-based potential fields* by the MOE-Dock program, to reduce the calculation time.

Each docking energy value was calculated as the sum value of the electrostatic, Van der Waals, and flexibility energies. The interaction energy was calculated using the electrostatic and Van der Waals potential fields sampled on a grid overlaying the 3D docking box. The 3D docking box was interpolated at the atom positions by tri-linear interpolation. The Van der Waals parameters were taken from the currently active force field. The electrostatic field was calculated in the Coulombic manner using the current dielectric. MOE-Dock performed 25 independent docking runs, and wrote the resulting conformations and their energies to a molecular database file. The lowest docking energy conformation for each active site was chosen for LIGPLOT.

3.3.4. Point mutation of acidic amino acid of active site with neutral and positively charged amino acid

To further confirm the role of acidic amino acid in the inhibition process of kidney stone formation, they were point mutated with alanine, glycine, lysine, arginine and histidine in all the active site of the modeled protein. After mutation, protein was docked with calcium oxalate using MOE-Dock with same parameter of docking as was used for wild type.

3.3.5. Quality check of modeled protein

To check the quality of the modeled protein, Ramachandran Plot was drawn from the program Schrodinger, which checks the stereochemical quality of a protein structures, producing a number of postscript plots, analyzing its overall and residue-by-residue geometry, assured the reliability of the modeled protein.

RESULTS

4.1 Comparison of EGTA, SDS and acetic acid extraction methods for protein extraction from renal calculi

Three different extraction methods were used for extraction of proteins from the renal calculi. This study was done to compare different methods of protein extraction from COM stones to investigate the soluble matrix proteins involved in the formation of calcium oxalate monohydrate (COM) stone.

4.1.1. Protein concentration

Maximum amount of protein (0.64mg/mL) was extracted by using SDS method followed by 0.24mg/mL and 0.10mg/mL by EGTA and acetic acid extraction methods respectively. Figure 4.1 shows the concentration of proteins extracted by SDS, EGTA and acetic acid extracts respectively.

4.1.2. Effect of EGTA, SDS and Acetic extract on calcium oxalate (CaOx) crystal growth

Though the amount of protein extracted by SDS was high but its activity on CaOx crystal growth system was minimal. EGTA extract exhibited highest inhibitory activity on CaOx crystal growth out of the three extracts. Only 2.64% of inhibitory activity was shown by the SDS extract, while 6.47% by acetic acid which is insignificant when compared to the EGTA extraction process. % inhibition by different extraction methods is shown in table 4.1. Though, the amount of protein extracted by EGTA method was 0.24mg/mL but its activity on CaOx crystal growth was quite promising. Therefore, EGTA extraction method was selected for the renal stone extraction to carry out the further studies.

4.1.3. SDS-PAGE

SDS-PAGE analysis of three different extracts is shown in the Figure 4.2. Maximum numbers of protein bands were visible in SDS extract while few were visible in EGTA and acetic acid extracts which is in conformity with the amount of proteins extracted by SDS, EGTA and acetic acid extraction methods respectively (Figure 4.1).

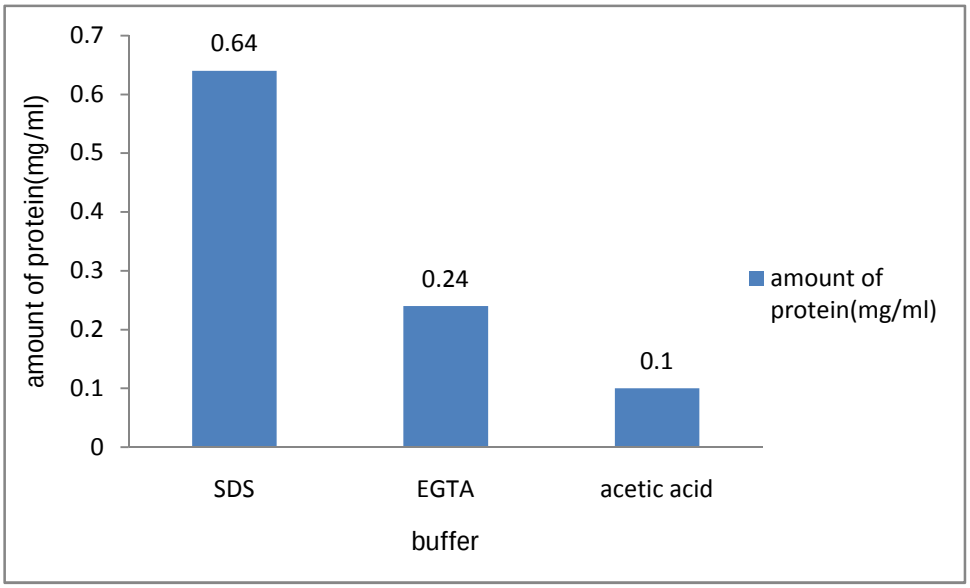


Figure 4.1. Amount of proteins extracted by SDS, EGTA and acetic acid. SDS extracted maximum protein followed by EGTA and acetic acid.

Table 4.1 Percentage inhibition exhibited by different extract on CaOx growth assay system. EGTA extract is exhibiting highest inhibitory activity among all extracting methods.

Buffer	% inhibition at 720 sec
EGTA	**98%**
SDS	2.64%

Acetic Acid	6.47%

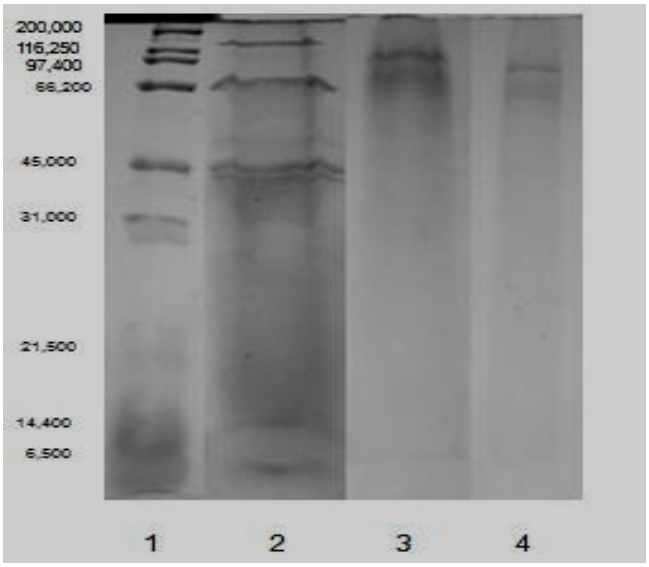


Figure 4.2. SDS-PAGE analysis of three different extract. First lane is showing the molecular weight marker while second, third and fourth is showing the bands of SDS, EGTA and acetic acid extract simultaneously. Maximum protein bands were visible in SDS extract while few were visible in EGTA and acetic acid extract.

4.2. Effect of lipid extract of renal stones on CaOx crystal growth

Different volumes of lipid extract were tested on CaOx crystal growth system. From Figure 4.3 it was observed that lipid extract did show an inhibition towards CaOx crystal growth however, with the increase in the volume of lipid extract inhibition decreases.

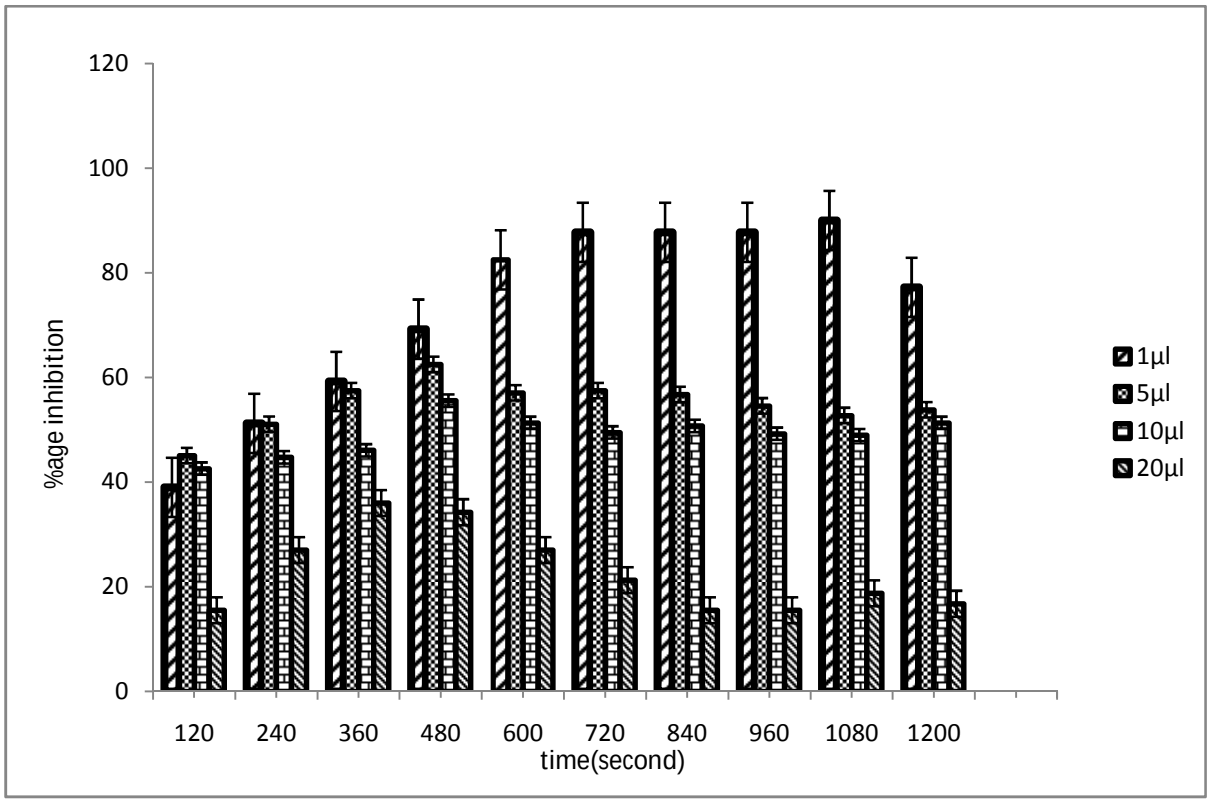


Figure 4.3. Effect of various volumes of lipid extract on CaOx crystal growth. Percentage inhibition of calcium oxalate growth by various volumes of lipid extract.

4.3. Effect of whole extract, >10 kDa and < 10kDa fractions on CaOx crystal growth system

Both high as well as low molecular weight biomolecules are reported to play role in kidney stone formation therefore, for this purpose EGTA extract was separated into >10kDa and <10kDa fractions. Since, EGTA extract was able to extract optimum amount of protein and also exhibited bioactivity therefore, it was selected for further investigations on calcium phosphate (CaP) mineralization system as well as on calcium oxalate crystal growth assay system.

Activities of all the three EGTA fractions obtained after filtering through Amicon ultra centrifugal filter device with a molecular weight cut off 10 kDa were studied on CaOx crystal growth assay system. Effect of various volumes of renal extract can be depicted from the graph shown in Figure 4.4. It was found that whole EGTA extract was showing highest percentage of inhibition. Whole EGTA extract (239.46 µg/mL protein) as well as its >10 kDa fraction (154.78 µg/mL protein) exhibited significant inhibitory activity as compared to the <10 kDa fraction (72.60 µg/mL protein).

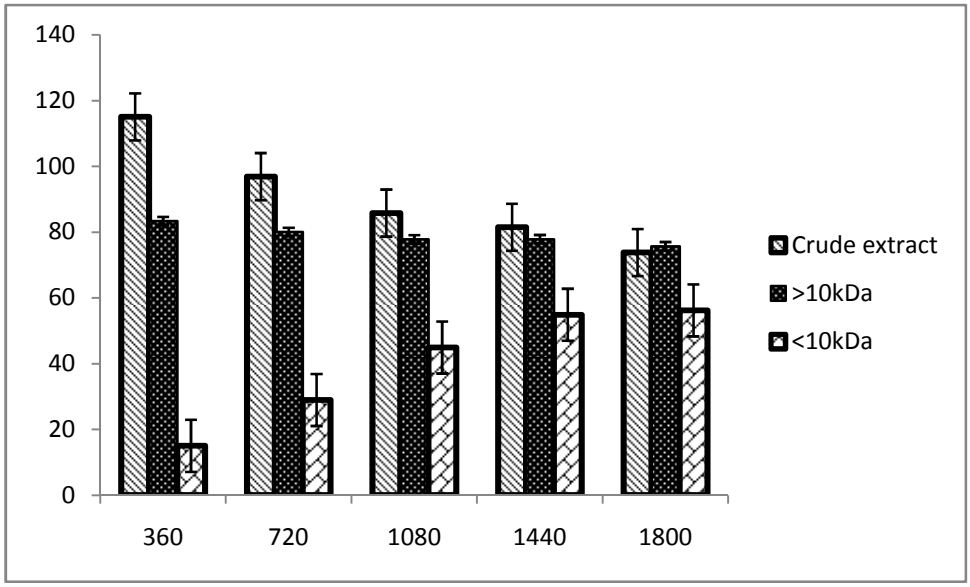


Figure 4.4 Percentage inhibition of whole EGTA extract, >10kDa and <10kDa fractions on CaOx crystal growth system.

4.4. Effect of whole extract, >10 kDa and < 10kDa fractions from human renal matrix of calcium oxalate monohydrate stones on *in vitro* calcium phosphate mineralization

Three phases of CaP mineralization viz. initiation, growth and demineralization were assessed by the whole EGTA extract, >10 kDa and < 10kDa fractions.

4.4.1. Effect of whole extract, >10 kDa and < 10kDa fractions on initial mineral phase of CaP assay system

Figure 4.5 depicts the effect of EGTA extract fractions on initial mineral phase formation. It is evident from the Figure 4.5a that 98.97% of phosphate ion inhibition was exhibited by whole renal stone extract, 85.9% by >10kDa and 92.09% by <10kDa fraction on *in vitro* homogenous assay system of calcium phosphate. However, Figure 4.5b shows that the whole extract showed maximum 81.64% of calcium ion inhibition. Interestingly, both type of activity stimulatory (maximum 25.2%) as well as inhibitory activity (maximum 25.01%) was shown by >10kDa fraction. <10kDa fraction showed 96.23% of maximum inhibition.

4.4.2. Effect of whole extract, >10 kDa and < 10kDa fractions on growth of preformed mineral phase

Figure 4.6 shows the percentage inhibition or stimulation of phosphate and calcium ions on growth of preformed mineral phase. From the figure, it could be inferred that inhibition of phosphate ions increased with the increase of whole extract volume. >10kDa fraction stimulated the growth of phosphate ions on preformed mineral phase. While <10kDa showed both types of activities (Figure 4.6a).

Inhibition of calcium ions was shown by whole extract and <10kDa fraction on the growth of preformed mineral phase. Stimulation was seen by various volumes of >10kDa fraction (Figure 4.6b). From the Figure, it is evident that whole EGTA extract is exhibiting maximum inhibitory potency towards phosphate ions but its activity is low towards calcium

ion inhibition. In addition, >10 kDa fraction showed maximum stimulatory activity against the growth of calcium ions but minimum stimulatory activity against phosphate ions growth on preformed mineral phase.

4.4.3. Effect of whole extract, >10 kDa and < 10kDa fractions on demineralization of preformed mineral phase

Release of phosphate ions increased with the increase of volume of different fractions. Figure 4.7a shows maximum amount of release of phosphate ions with whole extract. On the other hand, percentage release of calcium ions was decreased with the increase of different extract volumes. Low volume of whole extract exhibited the maximum ability to demineralize calcium ions from preformed mineral phase as is clearly depicted in Figure 4.7b. Among all the three fractions whole extract showed highest percentage of release of phosphate ions followed by <10kDa and >10kDa fractions.

It could be inferred that whole EGTA extract exhibited inhibitory activity in initial and growth mineral phase. Interestingly, stimulatory as well as inhibitory activity towards initial mineral phase was shown by >10 kDa fraction. Stimulatory activity was retained in growth mineral phase by this fraction. On the other hand, inhibitory activity was shown by <10kDa in initial mineral phase. Moreover, both type of activity was shown by <10kDa fraction in the case of phosphate ions but an inhibition was observed in the case of calcium ions on preformed 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 volume of all the three fractions.

Figure 4.5 a

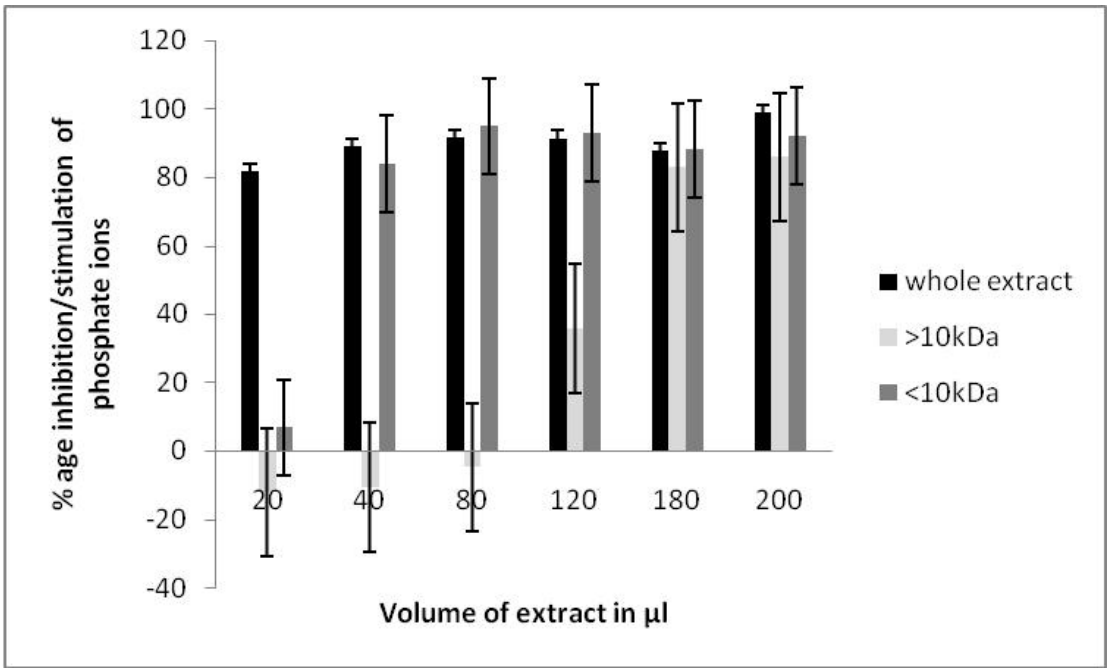


Figure 4.5 b

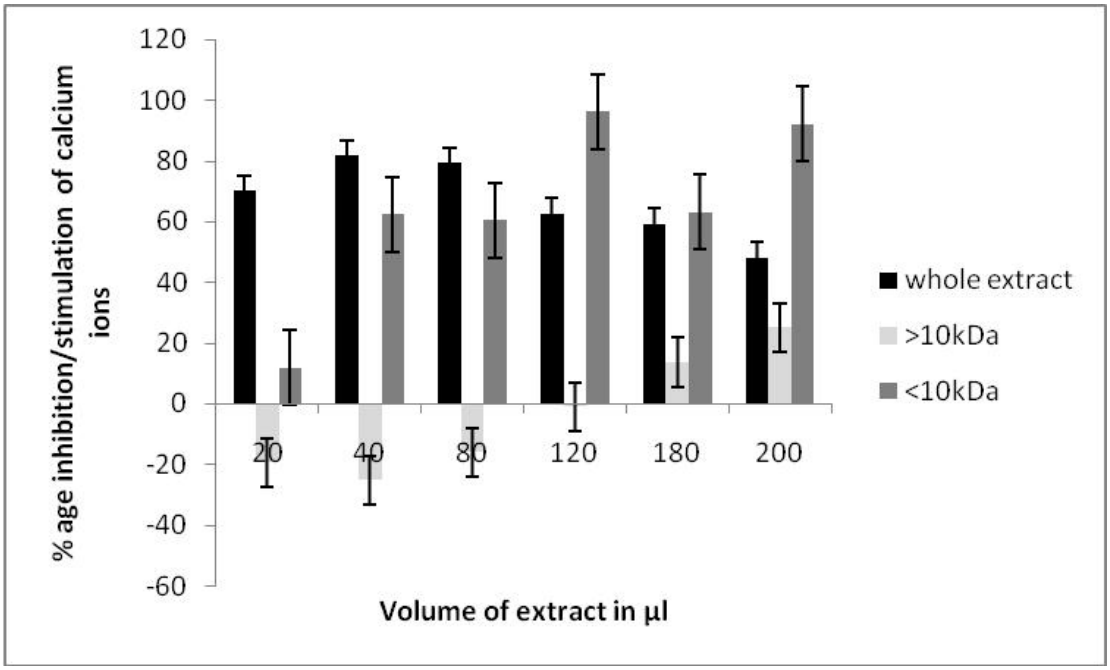


Figure 4.5 Effect of various volumes of renal stone extract(whole extract, >10kDa, <10kDa) on initial mineral phase. Figure 4.5 a. Percentage inhibition/stimulation of phosphate ions and calcium ions (Figure 4.5 b) by whole EGTA extract, >10kDa and <10kDa fractions different renal stone extracts.

Figure 4.6 a

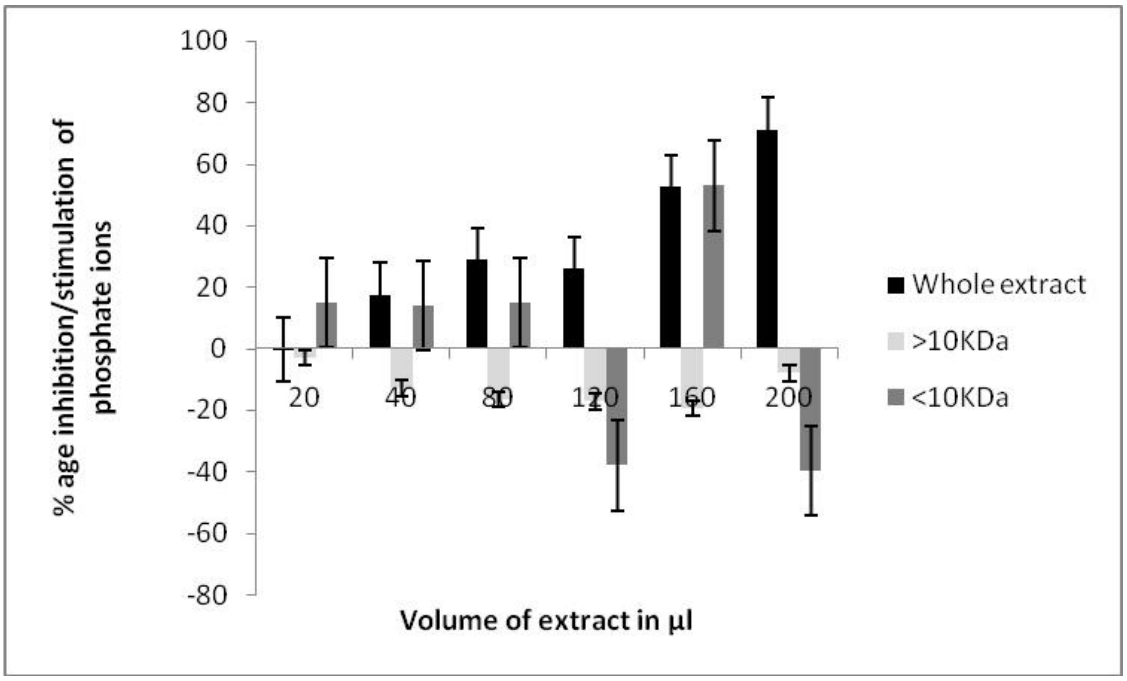


Figure 4.6 b

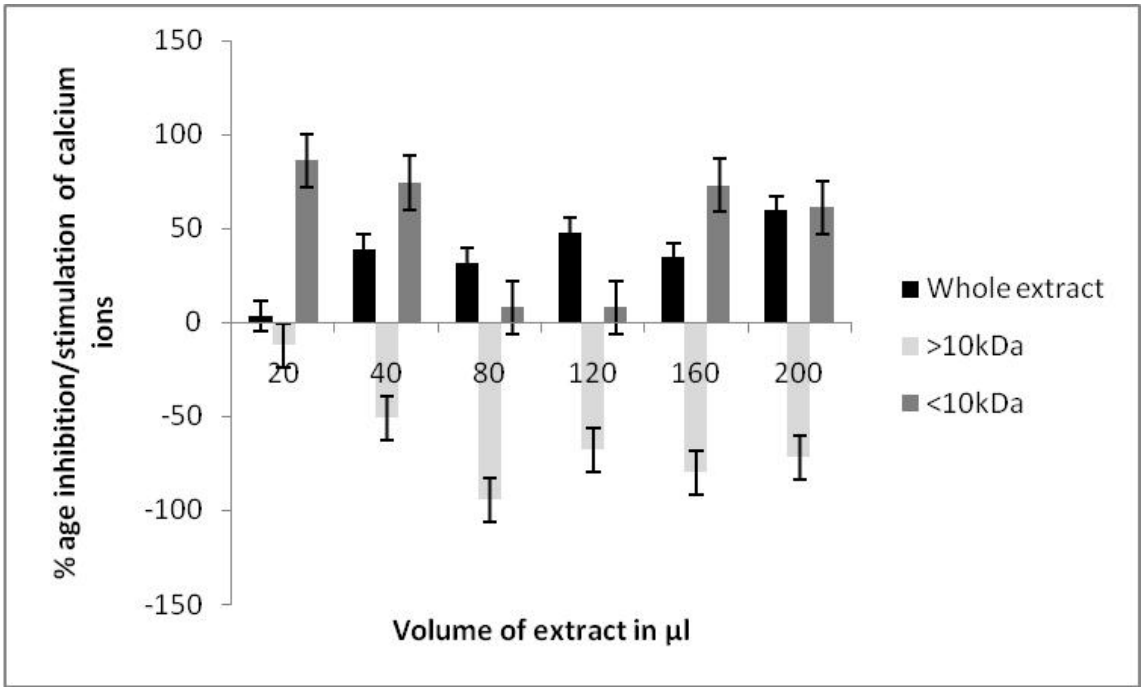


Figure 4.6 Percentage inhibition or stimulation of phosphate ions (a) and calcium ions (b) by different volumes of whole EGTA extract, >10kDa and <10kDa fractions of renal stone extract on the growth of preformed mineral phase.

Figure 4.7 a

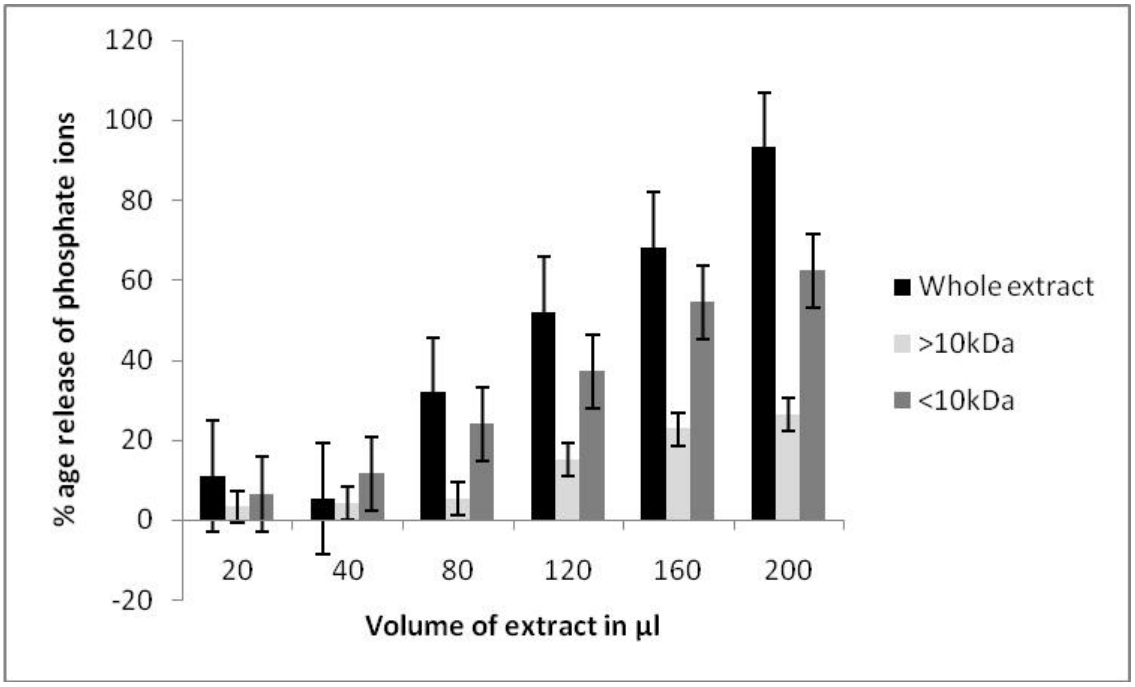


Figure 4.7 b

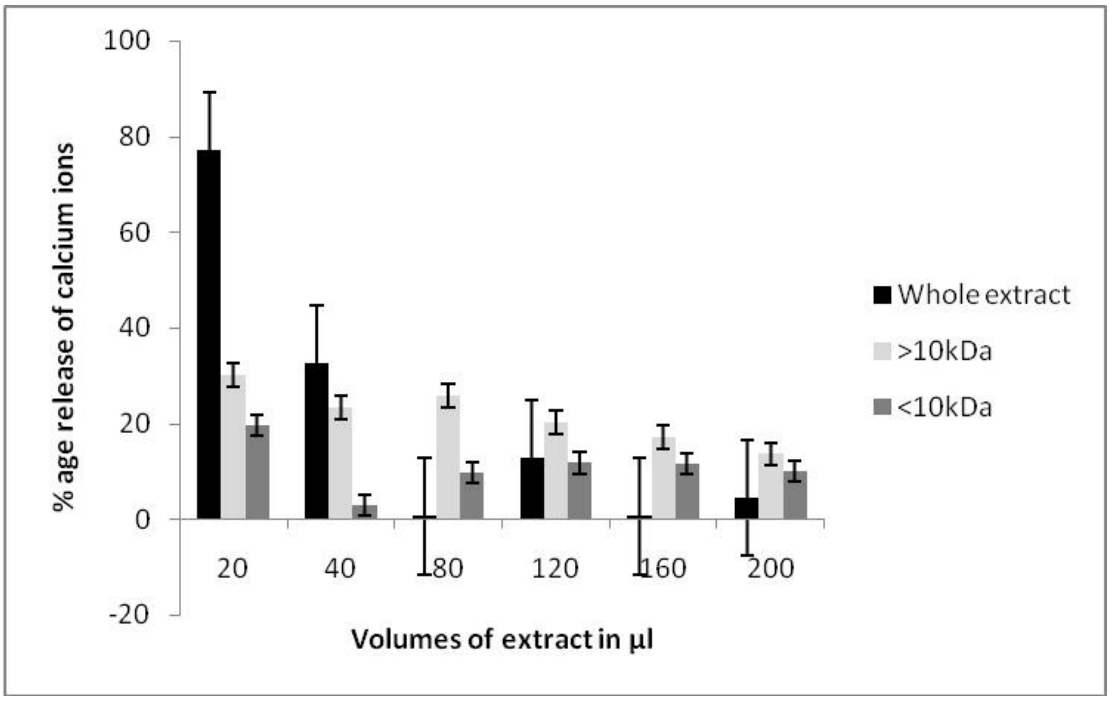


Figure 4.7 Effect of various volumes of renal stone extract (whole extract, >10kDa, <10kDa) on demineralization of preformed mineral phase. Percentage of phosphate ions (a) and calcium ions (b) demineralized by different fractions of renal stone extract.

4.5. Purification and identification of potent protein

More than 10kDa fraction exhibited maximum activity towards calcium oxalate crystal growth assay system and hence was loaded on a strong anion exchanger Macro Prep® 25 Q column. Consecutive fractions were collected with increasing gradient, pooled and were named fraction P1 to P7 (Figure 4.8). It was found that P6 fraction exhibited highest inhibitory activity against CaOx crystal growth and its SDS-PAGE analysis showed presence of few bands (Figure 4.9 & Figure 4.10).This fraction was further purified by molecular sieve chromatography on a Bio gel® P-100 gel molecular sieve column (50 X 1cm).

Proteins were eluted at pH 7.4 with 20 mM Tris buffer. Fractions were collected and pooled as P1', P2', P3', and P4' (Figure 4.11) and examined for inhibitory activity against CaOx crystal growth. It was found that fraction P2' had a relatively strong inhibitory activity (Figure 4.12). All the three fractions were analyzed further by 12% SDS-PAGE and pooled fraction P2' showed the presence of a single band having molecular weight ~ 42 kDa indicating that a novel CaOx crystal growth inhibitor from human renal stone matrix protein was purified (Figure 4.13).

The homogeneity of fraction P2' obtained after molecular-sieve chromatography was confirmed on RP-HPLC which showed a single peak at a retention time of ~9 minutes (Figure 4.14).

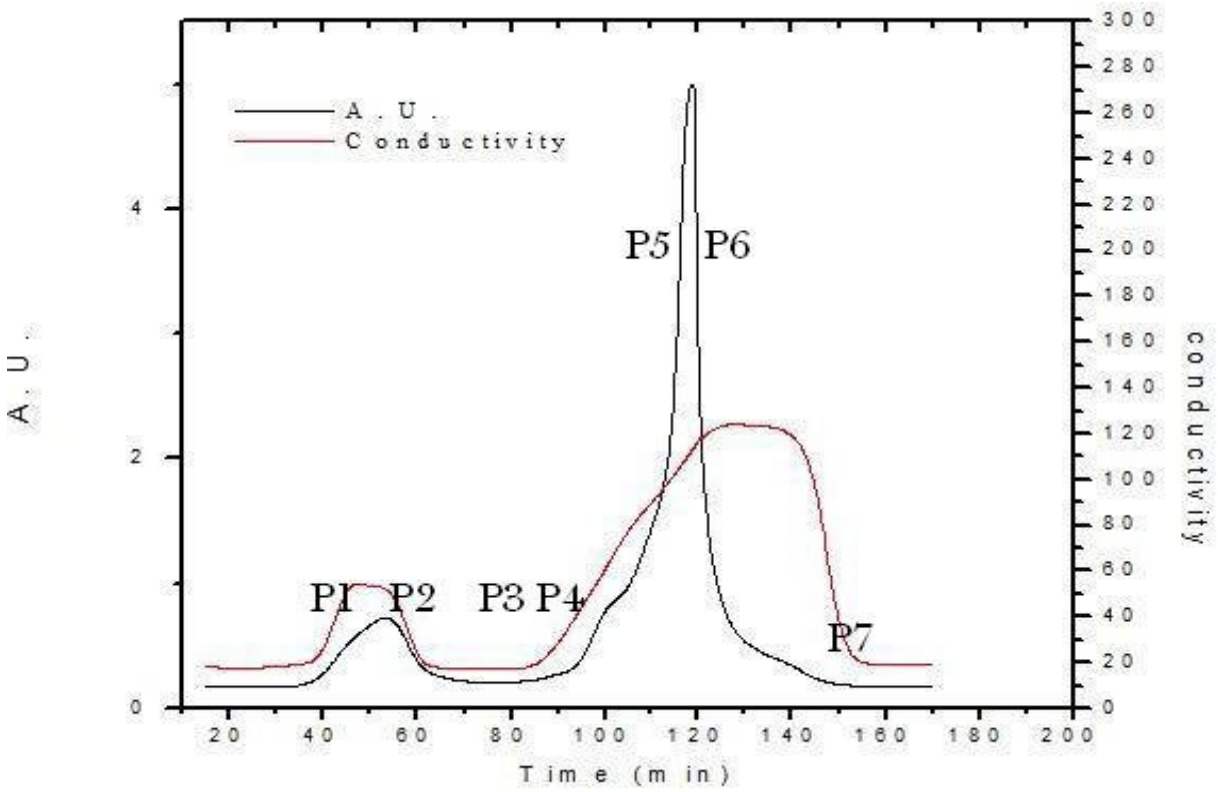


Figure 4.8 Elution profile of protein sample loaded on anion exchanger. Seven fractions (P1 to P7) were collected with a linear gradient of NaCl.

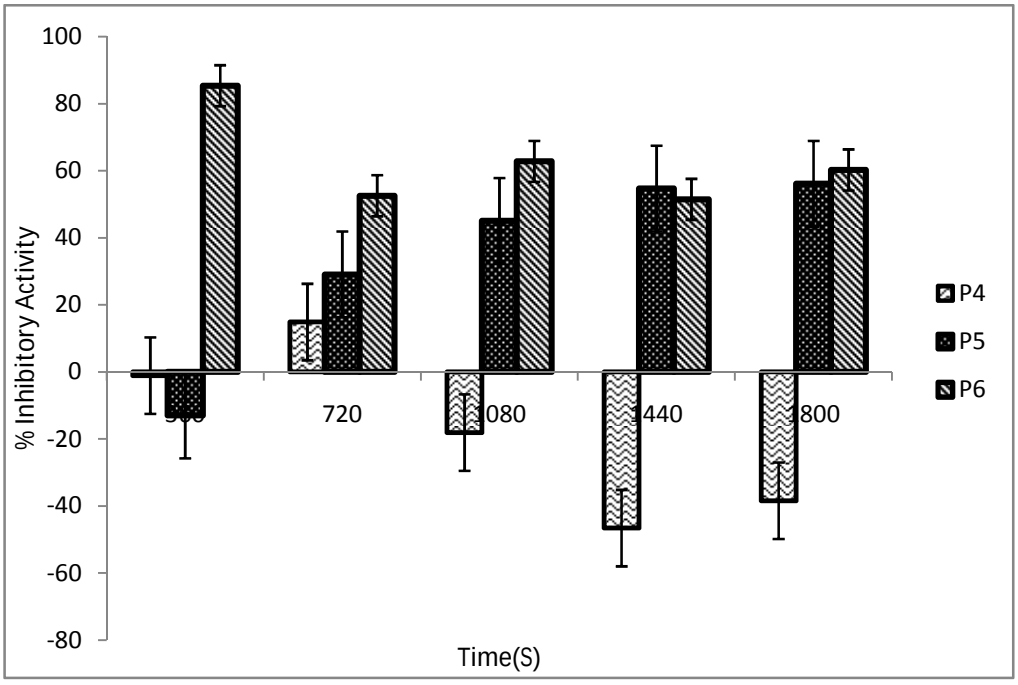


Figure 4.9 Characterization of inhibitory activity of various fractions after anion exchange chromatography. Fraction P6 showed highest inhibitory activity against CaOx crystal growth.

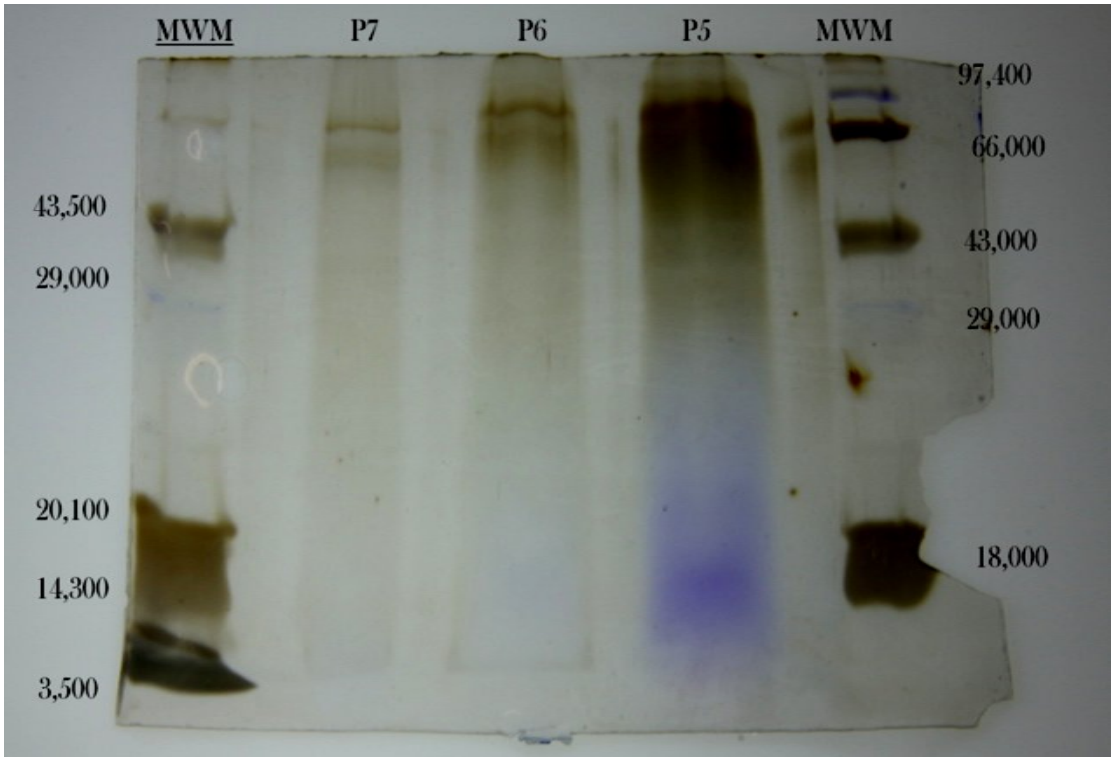


Figure 4.10 SDS-PAGE of pooled fractions after anion exchange chromatography

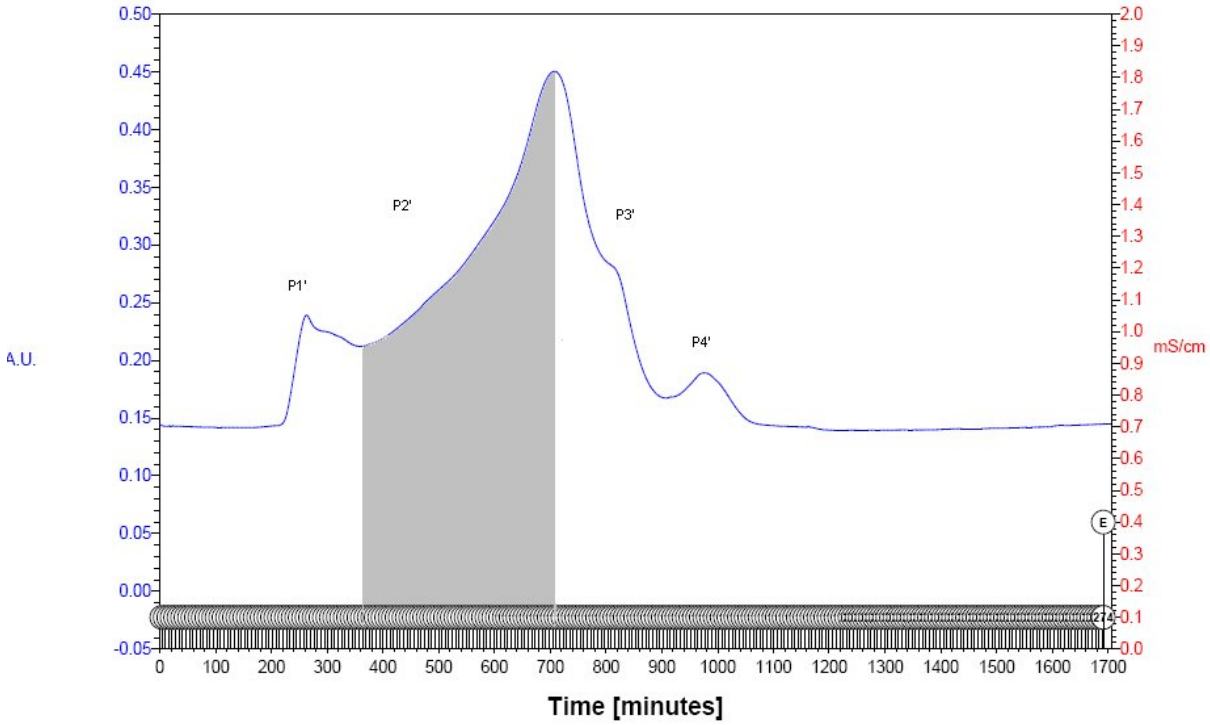


Figure 4.11 Elution profile of fraction P6 loaded on molecular sieve chromatography column after anion exchange chromatography. The eluting proteins were detected at 280 nm. Shaded portion (fraction P2') indicate elution of most active fraction having highest inhibitory activity.

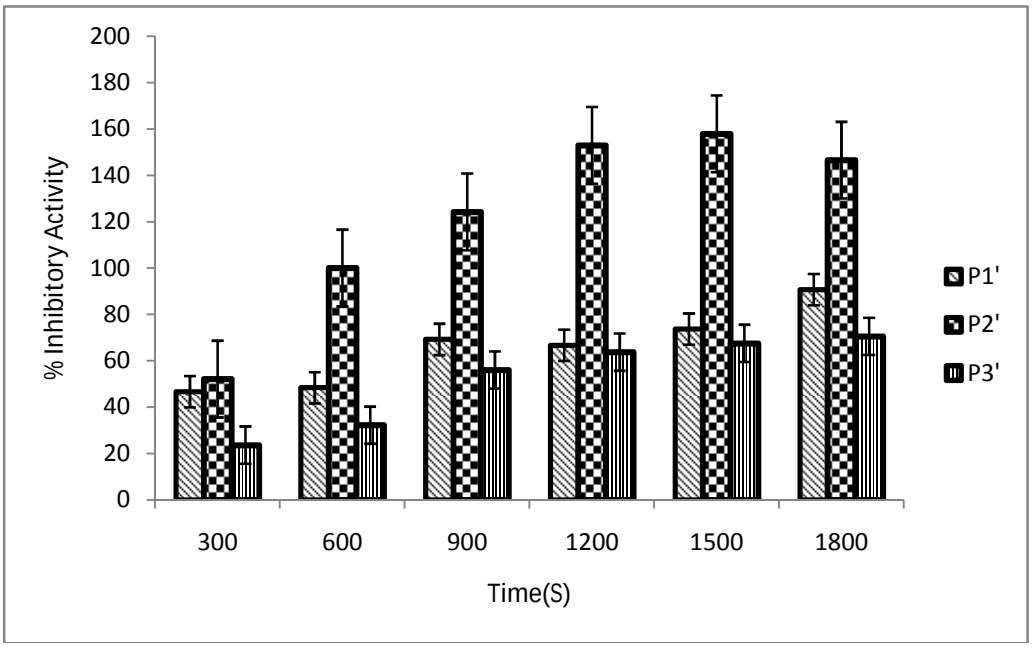


Figure 4.12 Percentage activity of pooled fractions of molecular sieve chromatography. P2' fraction is showing maximum inhibitory activity.

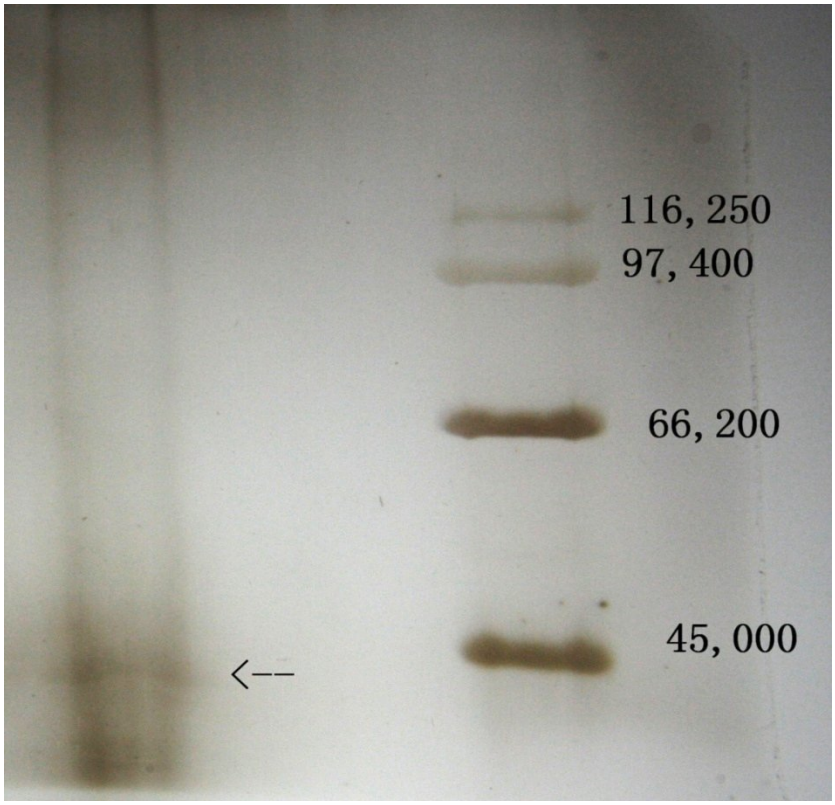


Figure 4.13 SDS-PAGE of fraction P2' after molecular sieve chromatography. A single band of MW~ 42kDa is shown by an arrow.

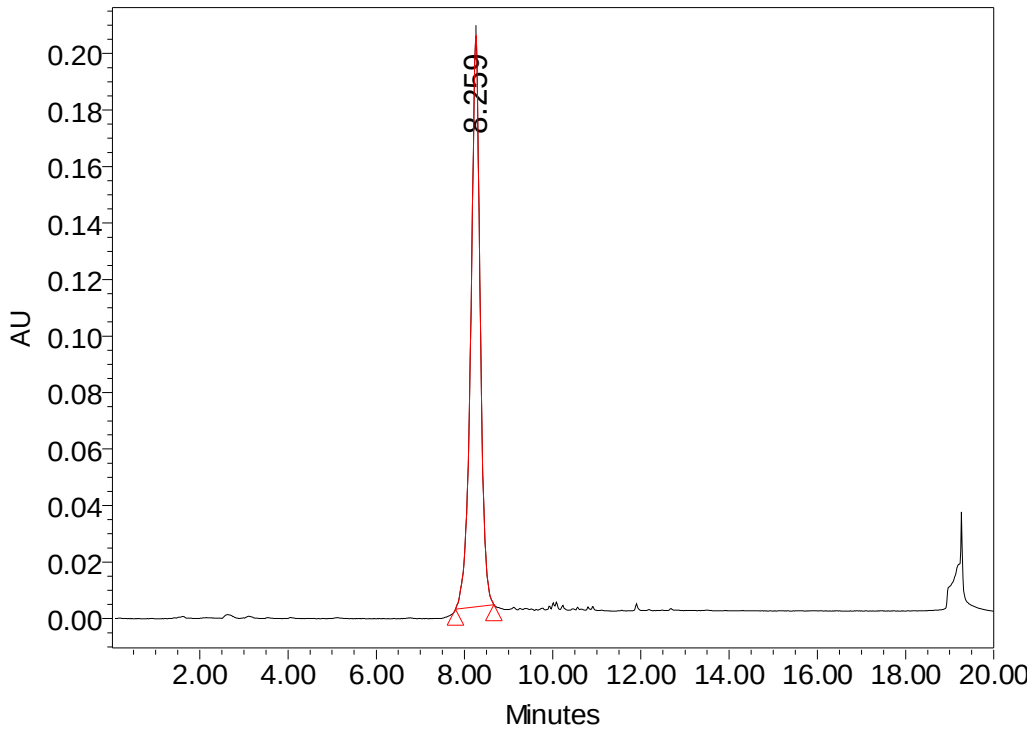


Figure 4.14 Homogeneity and molecular mass determination of protein from molecular sieve chromatography having highest CaOx inhibitory activity. RP-HPLC of fraction P2' for homogeneity, showing a single peak at 9 min.

4.5.1. Mass spectrometric identification of human phosphate cytidylyltransferase 1

The protein band detected in fraction P2' was excised, in-gel tryptic digested and identified by matrix assisted laser desorption/ionization–time of flight (MALDI-TOF) MS. The MALDI-TOF-MS spectrum of purified protein P2' is shown in Figure 4.15. Using the Mascot search engine (http://www.matrixscience.com), the MALDI-TOF data obtained from fraction P2' was matched significantly with human phosphate cytidylyltransferase 1, choline, beta [Homo sapiens]. The matching score (Figure 4.16) of mascot search was 38 and sequence coverage was 17%. Phosphate cytidylyltransferase 1 (gi|21361202) catalyzes the condensation of CTP and phosphocholine to form CDP-choline as the rate-limiting and regulatory step in the CDP-choline pathway.

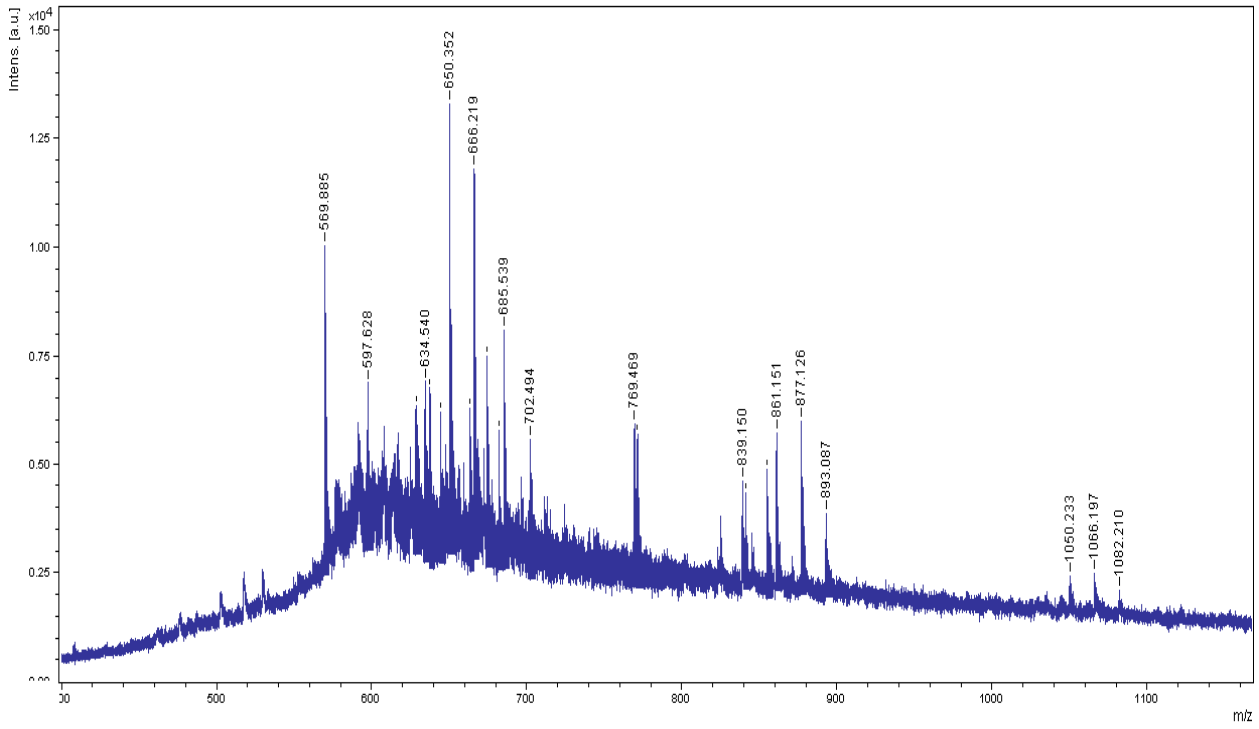


Figure 4.15 Identification of novel protein by MALDI-TOF MS. Peptide mass fingerprinting by MALDI-TOF MS obtained from trypsinized fraction P2'.

Mascot Search Results

Protein View

Match to: **AAD35088** Score: **38** Expect: **21**
AF148464 NID: - Homo sapiens

Nominal mass (M_r): **42199**; Calculated pI value: **5.99**
NCBI BLAST search of AAD35088 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
PCY1B_HUMAN from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **24**
Number of mass values matched: **11**
Sequence Coverage: **17%**

Matched peptides shown in **Bold Red**

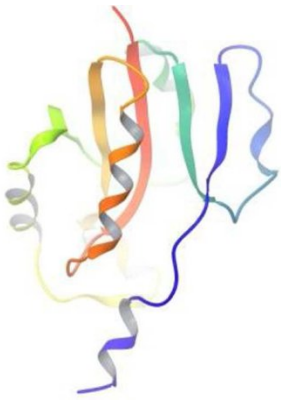
1 MPVVTTDAES ETGIPKSLSN EPPSETMEEI EHTCPQPRLT LTAPAPFADE
51 TNCQCQAPHE K**LTIAQARLG TPADRPVR**VY ADGIFDLFHS GHARALMQAK
101 TLFPNSYLLV GVCSDDLTHK FKGFTVMNEA ER**YEALR**HCR **YVDEVIR**DAP
151 WTLTPEFLEK HKIDFVAHDD IPYSSAGSDD VYKHIKEAGM FVPTQRTEGI
201 STSDIITRIV RDYDVYAR**RN LQRGYTAK**EL NVSFINEKRY RFQNQVDKMK
251 EKVK**NVEERS KEFVNR**VEEK **SHDLIQK**WEE KSREFIGNFL ELFGPDGAWK
301 **QMFQER**SSRM LQALSPKQSP VSSPTRSRSP SRSPSPTFSW LPLKTSPPSS
351 PKAASASISS MSEGDEDEK

Figure.4.16 Mascot search result showed that purified protein is similar to Phosphate cytidylyltransferase 1 choline beta [Homo sapiens]

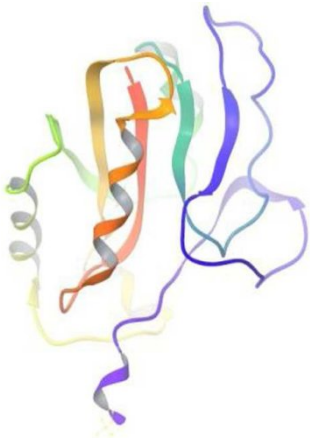
4.6. In silico study

4.6.1. Homology modeling of human CCT1 choline beta and docking with calcium oxalate

The protein phosphate cytidylyltransferase 1, choline, beta (CCT) was homology modeled based on sequence and structure alignments with the chain A CTP: glycerol-3 phosphate cytidylyltrnsferase (GCT) of *Bacillus subtilis* (Protein Data Bank IC 1 COZ-A) (Fig 4.17a and 4.17b). Three binding sites were predicted by the MOE site finder in the wild type CCT and were named site1, site2 and site3. Calcium oxalate was docked within the active site using the Monte Carlo docking procedure of MOE and repeated cycles of protein and substrate minimization.



a. X-ray crystallographic structure of GCT



b. Homology model of CCT used as template.

Figure. 4.17 X-ray crystallographic structure of GCT was used as a template (fig. a) for homology modelling. (Fig. b) showing homology model of CCT.

During the initial stage of the docking procedure, the side chains of the protein are fixed. The best-ranking docking modes of the ligands are identified and energy minimized in the protein, while allowing full side chain flexibility. Docking of calcium oxalate with all the three binding sites gave best docking score of -59.8251, -56.4486 and -106.304 with site1, site2 and site3 respectively (Table 4.2a).

LIGPLOT analysis of the binding site reveals involvement of different amino acids of protein with calcium oxalate. In site1 (first binding site) glycine, histidine, alanine, asparatic acid, glutamine, tyrosine and aspartic acid at 16, 17, 20, 94, 120, 138 and 139 positions were involved in interaction with calcium oxalate (Figure 4.18a). In the site 2, methionine at 22 and tyrosine at 66 positions were found to be participating in the interaction with calcium oxalate (Figure 4.18b). Aspartic acid, lysine and alanine at 41, 45 and 74 positions respectively in site3 interacted with calcium oxalate. In this binding site, most of the atoms of calcium oxalate are highly exposed. Aspartic acid at position 41 is covalently bound to the calcium of the ligand (calcium oxalate). Lysine at position 45 is bound to the oxygen, where it is acting as side chain receptor (Figure 4.18c).

In all of these active sites of the wild type modeled protein human CCT 1 choline beta, it was observed that acidic amino acids played a significant role in the interaction with calcium oxalate.

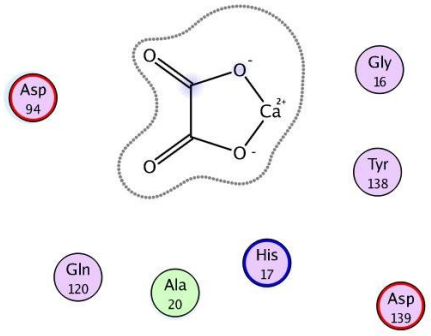


Figure a

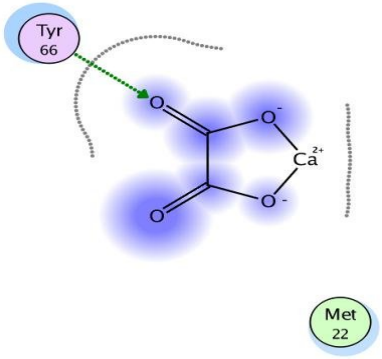


Figure b

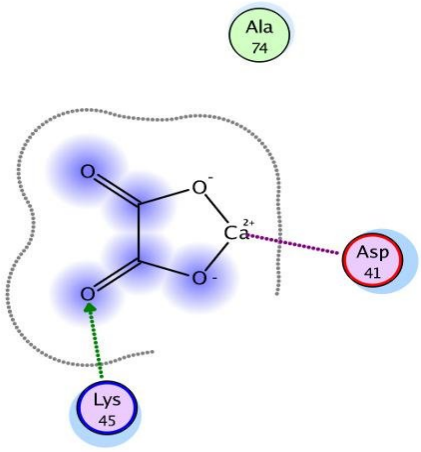


Figure c

Figure 4.18 Ligplot showing interaction of various amino acids present in binding site1 (Figure a), site 2 (Figure b) and site 3 (Figure c). Aspartate at position 94 & 139 is

interacting with calcium oxalate in binding site 1 while in binding site 3 it is binding with calcium. Amino acids tyrosine and lysine is binding with oxygen of calcium oxalate in binding site 2 and site 3 respectively.

From Ramachandran plot (Figure 4.19) it was found that out of 369 amino acids of CCT, 329 were in red region (89.15%) and 32 (8.67%) in yellow region and 8 (2.1%) was in outer region. This result indicates that the quality of the modeled protein was highly reliable.

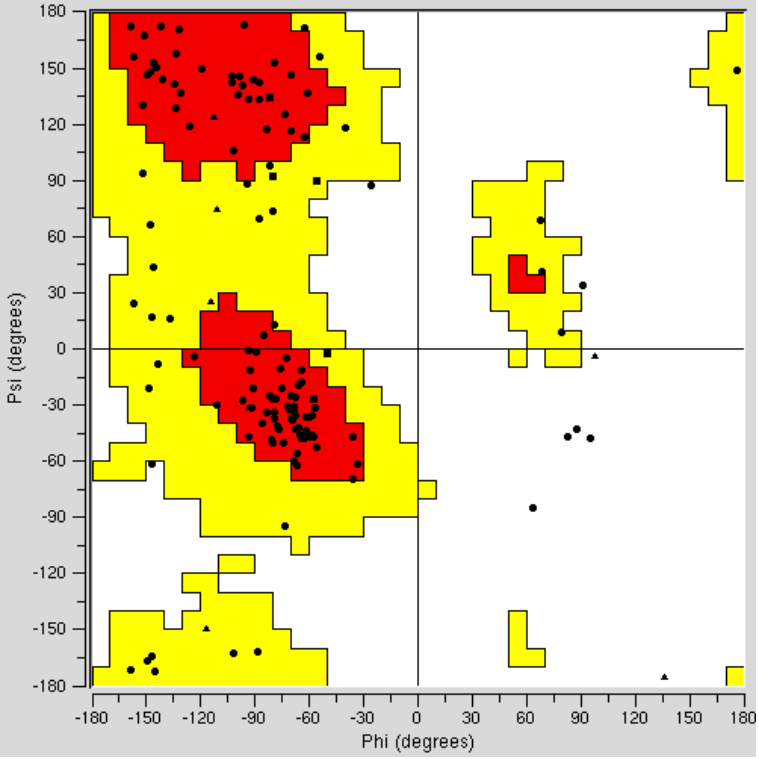


Figure 4.19 Ramachandran plot showing maximum amino acids in red region.

4.6.2. Point mutation of acidic amino acid of active site and docking with calcium oxalate

Upon substitution of acidic amino acids present in the active sites (site 1 and site 3) of the wild type modeled protein CCT with alanine, glycine, lysine, arginine and histidine, positive docking scores were obtained indicating a poor interaction with calcium oxalate. Since, no acidic amino acid was present in the binding site 2 therefore, it was not mutated. No docking score was found when acidic amino acid present in binding site1was point mutated with arginine and histidine (Table 4.2b). In all of these active sites of the wild type modeled protein CCT, it was observed that acidic amino acids played a significant role in the interaction with calcium oxalate.

Table 4.2 Table (a) showing docking score of wild type CCT and table (b) of mutated type. Negative value of wild type is indicating good binding with CaOx while positive value showing poor binding.

Table 4.2a

	Site1	Site2	Site3
Wild type human CCT	-59.8251	-56.4486	-106.3040

Table 4.2b

Point mutation of CCT with amino acids A,G,K,R,H	Site1	Site2	Site3
Alanine(A)	313.2496	_	7.3765
Glycine(G)	370.3134	_	257.1444
Lysine(K)	494.8806	_	7.8061
Arginine (R)	_	_	634.8440

Histidine (H)	_	_	343.5878

These studies clearly demonstrate that acidic amino acids present in the wild type CCT showed a good docking score, an indicator of interaction with calcium oxalate, while substitution of these acidic amino acids with alanine, glycine, lysine, arginine and histidine gave a poor docking score. (Table 4.2b)

DISCUSSION

Discussion

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, phosphatidylionositol, 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 (Neĭmark 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 *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 site1, small size calcium oxalate was not able to interact with all amino acids. Small size of binding site2 and site3 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.

2003). 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* (Devuyst et al. 2005; Kumar and Lieske 2006). OPN is a ubiquitously expressed phosphoglycoprotein that regulates bone biomineralization and ectopic calcification (Giachelli 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.

CONCLUSION

Kidney supersaturates urine with slightly soluble salts like calcium oxalates and calcium phosphates during the process of water conservation (Kok 2002). Except for special cases, the glomerular filtrate is not supersaturated. Supersaturation for calcium phosphate (CaP) is first achieved in the loop of Henle (Kok 1996), while that for calcium oxalate (CaOx) occurs in the distal tubules. These sites move along the nephron because of changes in the concentrations of calcium, phosphate or oxalate in the nephronic fluid, or variation in the nephron function (Kok 1996; Kok and Schell-Feith 1999). High concentrations present in the deep papillary nephron segments may permeate to the basement membranes and renal interstitium notwithstanding the low trans-membrane permeability of the thin loop membranes. When supersaturation is high enough and lasts long enough or promoters are present, crystals nucleate in the urine and are either excreted as crystalluria (Robertson and Peacock 1972) or deposit in renal tissue, appearing as so-called Randall's plaques (Randall 1940). Both types of nuclei may increase in size by growth and/ or aggregation (Coe and Parks 1988). In most people, crystals formed in the urine are discharged without any discomfort (Robertson and Peacock 1972; Hallson and Rose 1976; Finlayson 1978; Finlayson 1977; Finlayson et al. 1990). In hyperoxaluric animal models and hyperoxaluric patients crystal deposits remain in the urinary space, causing tubular epithelial damage and formation of large aggregates (Khan 1995; Kok and Khan 1994). In addition, in hypercalciuric stone formers subepithelial deposits are found in the loop of Henle (Evan et al. 2003). Randall's plaques remain just that in most people who never form stones (Low and Stoller 1999); but in some, they may grow through the interstitium towards the papillary surface and become a nidus for stone formation (Evan et al. 2003). The fact that the widespread occurrence of crystalluria and Randall's plaques leads to stone formation in

much fewer people and then usually only once or twice a lifetime suggests there are mechanisms that ensure crystals pass harmlessly and plaques stay as plaques. These mechanisms act at all levels of crystallization and stone formation: supersaturation, nucleation, crystal growth, aggregation, crystal structure and habit, crystal surface properties and crystal interactions with epithelial cells.

Many urinary compounds have a protective role, usually involving an affinity for the crystals or its constituents. The common occurrence of biomolecules in renal stones has prompted the present study on identification and characterization of a calcium oxalate crystal growth protein inhibitor from human renal stone matrix and *in silico* interaction of purified protein with calcium oxalate.

1) Three different extraction methods viz. EGTA, SDS and acetic acid were used for the extraction of renal stone matrix. Maximum amount of proteins from human renal stone matrix was obtained from SDS extraction method followed by EGTA and acetic acid extraction methods respectively. Though, the amount of protein extracted by EGTA extraction method was lesser compared to SDS extraction method but its activity on CaOx crystal growth was quite prominent in contrast to almost no activity observed in the case of SDS extract. EGTA extract exhibited highest inhibitory activity (98%) towards CaOx crystal growth followed by acetic acid (6.47%) and SDS extract (2.64%). Therefore, EGTA extraction method was selected for the renal stone extraction to carry out the further studies.

2) An anionic (MW ~ 42 kDa) CaOx crystal growth protein inhibitor was purified from human renal stone matrix by bioactivity guided purification using anion exchange chromatography and molecular sieve chromatography and SDS-PAGE analysis. Finally,

homogeneity of purified protein was confirmed by HPLC. Further, the protein was in gel tryptic digested and characterized by MALDI-TOF-MS.

3) Protein was identified as human phosphate cytidylyltransferase 1 (CCT), choline, beta when m/z data obtained after MALDI–TOF-MS of digested protein was searched in MASCOT search engine. Phosphate cytidylyltransferase 1, choline, beta is a novel CaOx crystal growth inhibitor and holds a direct relevance as it is involved in the formation of phosphatidylcholine which is a constituent of calcium oxalate stones.

4) *In silico* studies revealed that interaction between calcium oxalate and acidic amino acids present in the binding sites of wild type human phosphate cytidylyltransferase 1 (CCT) showed a good docking score, an indicator of excellent interaction with calcium oxalate. However, upon substitution of these acidic amino acids with alanine, glycine, lysine, arginine and histidine, a poor docking score was observed suggesting no binding at all with calcium oxalate.

5) Lipids isolated from calcium oxalate renal stones had an ability to inhibit growth of CaOx crystals.

6) 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.

Publications in Peer Reviewed Journal

National

- Priyadarshini**,** Bijarnia RK, Kaur T, Singh SK, Tandon C. Comparison of the inhibitory activity of isolated lipids and proteins from calcium oxalate stones. (Abstract) *Indian Journal of Urology*. 2008; 24 suppl. 2: S111. (indexed in PubMed & SCOPUS) (ISSN 0970-1591)
- Priyadarshini**,** Shrawan Kumar Singh, Chanderdeep Tandon. Exploring the role of lipids isolated from human renal stone matrix in urolithiasis. (Abstract) *Indian Journal of Clinical Biochemistry,* 2009;24 suppl: 145. (ISSN 0970-1915)

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- Priyadarshini**,** Singh SK, Tandon C. Mass spectrometric identification of human phosphate cytidylyltransferase 1 as a novel calcium oxalate crystal growth inhibitor purified from human renal stone matrix. *Clinica Chimica Acta.* 2009; 408: 34–38 (indexed in PubMed & SCOPUS) (ISSN: 0009-8981)
- Priyadarshini, Shrawan Kumar Singh, Chanderdeep Tandon Effect of biomolecules from human renal matrix of calcium oxalate monohydrate stones on *in vitro* calcium phosphate crystallization. *International Brazilian Journal of Urology* (Accepted) (indexed in PubMed) **(**ISSN: 1677-6119)

Communicated

- Priyadarshini, Singh SK, Tandon C. Studies on matrix protein(s) from human calcium oxalate monohydrate (COM) stones. *Scandinavian Journal of Urology*

- Priyadarshini, Pradeep Kumar Naik, Shrawan Kumar Singh, Chanderdeep Tandon. *In silico* interaction of calcium oxalate with human phosphate cytidylyltransferase 1, a novel calcium oxalate crystal growth inhibitor purified from human renal stone matrix. *Journal of Molecular Modeling.*

International conference

Priyadarshini, Bijarnia RK, Kaur T, Singh SK, Tandon C Comparison of the inhibitory activity of isolated lipids and proteins from calcium oxalate stones. *9th Asian Congress of Urology, 3-5th Oct. 2008 New Delhi* .

National conference

Priyadarshini, Shrawan Kumar Singh, Chanderdeep Tandon. Exploring the role of lipids isolated from human renal stone matrix in urolithiasis. *36th annual conference of association of clinical biochemists of India (ACBICON 2009), November 5-7, 2009, Kochi.*

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