CHARACTERIZATION OF THE PHYSIOCHEMICAL AND BIOCHEMICAL PROPERTIES OF THE URINARY PROTEIN BIKUNIN IN SOUTH AFRICAN BLACK AND WHITE SUBJECTS WITH RESPECT TO CALCIUM OXALATE KIDNEY STONE FORMATION

Nontobeko F. Mabizela

Thesis Presented for the Degree of
Doctor of Philosophy
In the Department of Chemistry
University of Cape Town
September 2006

UNIVERSITY OF CAPE TOWN

I dedicate this thesis to my parents, Nompumelelo Doreen and Richard Sabelo Mabizela, my sister Nontokozo Magasela; her husband Hilton Magasela and my brother Lwazi Mabizela. These are the people that urged me on by way of their untiring support and seemingly unlimited belief in me. Thank you for the love and encouragement.

ACKNOWLEDGEMENTS

The process of putting together this thesis was both a challenge and a wonderful opportunity for my personal growth, an experience I will not trade for anything, for this I give honour and glory to God Almighty. I believe it's only appropriate to acknowledge all of the persons who during the time of preparing this thesis provided me with useful and helpful assistance. Without their support and consideration, this thesis would likely not have matured.

Firstly, I would like to thank my supervisors, **Prof. A. L. Rodgers** who first stimulated my interest in kidney stone research and for mentoring in my project and in the writing of this thesis and **Prof. E. D. Sturrock** for his guidance throughout the bikunin isolation process which constituted a rather difficult and challenging act.

My deepest thanks also goes to **Dr Dawn Webber** for her dedication and interest in helping put together this thesis and interpreting the data.

Ms Sylva Schwager for her assistance in teaching a chemist the biochemistry techniques and most of all with the isolation and characterization of bikunin.

I would also like to thank **Ms Diane Pinnock** for her assistance with the chemical composition analysis of the urine and for encouragement. **Ms Gretchen Baretta** for her encouragement and support.

Ms Ntsapokazi Deppa for her assistance in the lab, moral support and friendship. **Mr Takalani Theka** and **Dr Shameez Allie-Hamdulay** for helping me proof read my thesis. **Ms Madhu Chauhan**, Department of Molecular and Cell Biology, for allowing me to use the department's centrifuges and cold rooms. **Ms Amandeep Kaur** and **Lara Donaldson** for their technical assistance with MALDI-TOF MS and **Dr David Harvey** of Oxford University for Nano-ESI MS.

Thanks are also due to colleagues I worked with in the laboratory, **Ms Ntombovuyo Bungane and Mr Abel Mathopa** for their friendship and support and also to **Dr Sonja Lewandowski, Ms Dalielah Jappie** and **Ayesha Parker**.

Finally, I would also like to thank all volunteers who donated urine samples and the National Research Foundation (NRF) and University of Cape Town for financial support.

CONFERENCE PROCEEDINGS

Publications

Mabizela N. F., Rodgers A. L., Schwager S. L. U. and Sturrock ED. 2004. Analysis of urinary bikunin in calcium oxalate crystals from the black and white population groups in South Africa. Urological Research (2004), **32**(1): 140

Mabizela N. F., Rodgers A. L., Webber D., Schwager S. L. U. and Sturrock ED. 2005. Isolation of bikunin from urine and inhibitory effect of protein precipitate in the urine of black and white subjects. Urological Research (2005), **32** (2): 161

Conference presentations

- Poster: 10th International Symposium on Urolithiasis, Hong Kong, 2004
- Poster and oral: 11th European Symposium on Urolithiasis, Germany, 2005.

ABSTRACT

Approximately 12 % of males in the Western world are likely to suffer at least one kidney stone in their life. The incidence of kidney stone disease in South Africa is similar in white males; however stone occurrence in the South African black population is extremely rare. The difference between the incidences of kidney stones in the black and white populations in South Africa is unexplained. In general, the role of several urinary proteins in the pathogenesis of this disease has been established. Bikunin is an example. The primary aim of this study was to isolate bikunin from the urine of healthy black and white male subjects and to investigate whether the protein from the black group is a more powerful inhibitor of CaOx crystallization than that from the stone prone white group.

A wide range of crystallization experiments were used. These included [^{14}C]-oxalate deposition, CaOx crystal aggregation and nucleation as well as determination of Zeta potential and particle number-volume-size distribution. Four different forms of bikunin (commercial bikunin, protein precipitate containing bikunin, purified bikunin and deglycosylated bikunin) were studied. Bikunin was isolated and purified from the proteins precipitated from the urine of black and white subjects using immunoaffinity chromatography. Its purity was confirmed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF MS). Commercial bikunin was deglycosylated using chondroitinase ABC and its inhibitory effect was studied in both synthetic urine and the urine obtained from black and white subjects in order to gain some insight into the role played by bikunin glycans in inhibiting calcium oxalate crystallisation.

The crystallization experiments performed on the commercial bikunin provided convincing evidence that bikunin is an inhibitor of CaOx crystal growth and aggregation rather than nucleation. Further evidence on the possible role played by bikunin in retarding *in vivo* CaOx crystallization was provided by the experiments conducted on the protein precipitate containing bikunin.

These experiments demonstrated that the proteins precipitated from the urine of the two race groups retard CaOx crystallization irrespective of the urine from which it was derived. Structural differences were observed in the bikunin purified from the urine of the two race groups using immunoaffinity chromatography. MALDI-TOF MS demonstrated that bikunin isolated from the white subjects had less coverage of the bikunin amino acid, while the coverage by bikunin isolated from black subjects was larger. The concentration for urinary bikunin was determined to be 0.46 mg/L in blacks and 0.32 mg/L in white subjects but this difference was not statistically significant. Crystallization experiments demonstrated that purified bikunin, irrespective of the race group from which it was derived, is a strong inhibitor of CaOx growth and aggregation and a weak inhibitor of CaOx crystal nucleation. Importantly, aggregation inhibition by bikunin from black subjects was superior. A study comparing the inhibitory activity of bikunin after the removal of the glycans provided convincing evidence that bikunin glycans are responsible for this protein's ability to retard CaOx crystallization. Differences in the morphology of the crystal precipitated from the urine of black and white subjects were also noted using SEM. A predominantly COM crystal morphology was observed in the urine of black subjects compared to mainly COD in the white subjects urine.

The results of this PhD project have provided meaningful insights into the inhibitory role played by urinary bikunin in the pathogenesis of CaOx urolithiasis in general and have also provided evidence that this protein may be a key role player in protecting the South African black population against this disease.

ABBREVIATIONS AND SYMBOLS

α_1-m	α_1-microglobulin
Amps	ammonium persulphate
ANOVA	analysis of variance
BB	bikunin isolated from urine of black subjects
BP	proteins isolated from urine of black subjects
BPP	protein precipitate isolated from urine of black subjects
BUF	black subjects' ultrafiltered urine
BSA	bovine serum albumin
CaOx	calcium oxalate
CB	commercial bikunin
COM	calcium oxalate monohydrate crystals
COD	calcium oxalate dihydrate crystals
COT	calcium oxalate trihydrate crystals
cpm	counts per minute
CS	chondroitin sulphate
DB	deglycosylated bikunin
GAG	glycosaminoglycan
H1	heavy chain 1
H2	heavy chain 2
HPLC	high performance liquid chromatography
I_A	inhibition of aggregation
I_n	inhibition of nucleation
IgG	immunoglobulin
kDa	kilo dalton
MALDI -TOF	Matrix-assisted laser desorption/ionisation time-of-flight
MS	mass spectrometry
MSL	metastable limit
Na_2Ox	sodium oxalate
PMSF	phenylmethylsulphonylfluoride
RS	relative supersaturation
SE	standard error
SEM	scanning electron microscope
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
WB	bikunin isolated from urine of white subjects
WP	proteins isolated from urine of white subjects
WPP	protein precipitate isolated from urine of white subjects
WUF	white subjects' ultrafiltered urine
TEMED	N,N,N'N'-tetramethylethylendiamine
Tris	Tris (hydroxylmethyl)methylamine
XRD	x-ray powder diffraction
Zp	zeta potential

TABLE OF CONTENTS

Chapter 1: Introduction

Chapter 2: General methods and results

Chapter 3: Inhibitory activity of commercial bikunin

Chapter 4: Protein Precipitates and their Effects on CaOx Crystallization Processes

Chapter 5: Isolation, purification and characterization of bikunin

Chapter 6: Crystallization studies of purified bikunin in urine and inorganic solution

Chapter 7: Deglycosylation of bikunin and crystallization experiments in ultrafiltered urine from black and white subjects

Chapter 8: Conclusion, future outlook and references

Chapter 1

Introduction

1.1 General

Kidney stones, renal stones, and renal calculi are terms used to refer to the condition in which crystallized substances in the urine combine to form hard, solid and non-metallic concretions. These substances originate as microscopic particles and develop into a stone which can be located anywhere in the urinary tract (Figure 1.1) where they cause irritation and secondary infection. The medical term for this condition is urolithiasis.

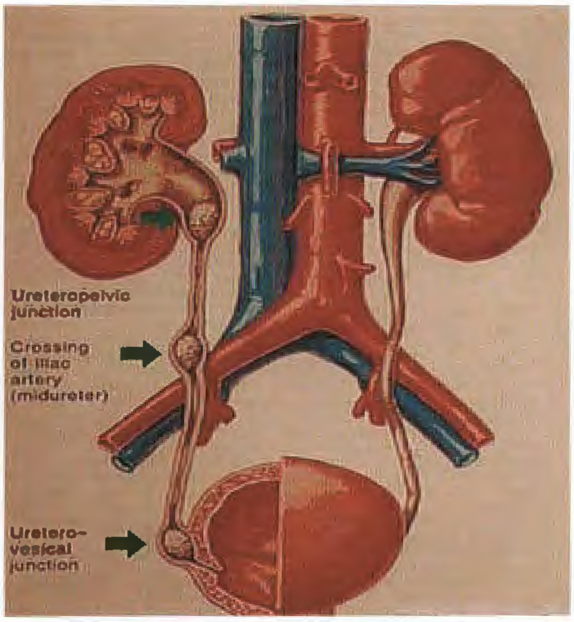


Figure 1.1 *Urinary stones can be found anywhere in the urinary tract (http://www.ocurology.com/stones.html)*

The disease was reported by Sir Grafton Elliot Smith in 1923 when stones were found in the pelvic bone of an Egyptian mummy from a 4400 BC tomb in Egypt (Clarke 1968).

The formation of kidney stones is a relatively common condition that develops in 12 % of men and 5 % of women in industrialized countries (Blacklock 1982). It is evident from the literature that urolithiasis is a multi-factorial disease dependent on a wide range of epidemiological variables such as climate, geography, race, occupation, diet, drinking water as well as clinical conditions (these will be discussed later). When waste material in the urine does not dissolve completely, crystals are likely to form. Small stones can pass out of the body on their own, although this can be painful. Large stones may require surgery, or they may be broken into smaller pieces with sound waves in a procedure called ultrasonic lithotripsy. The chemical composition of stones depends on the chemical imbalance in the urine. The discovery of an organic component in kidney stones dates back to 1684 (Butt 1956).

There are four general categories of urinary stones according to their chemical composition (Elliot 1973; Gill *et al.* 1984; Ito *et al.* 1992; Coe *et al.* 2005).

Calcium stones: Approximately 85 % of stones are composed predominately of calcium compounds (Elliot 1973; Gill *et al.* 1984; Ito *et al.* 1992). Calcium and oxalate are the most components of urinary calculi. Stones of this type are caused by an imbalance of these components in the urine. Another common stone type is calcium phosphate (Elliot 1973; Gill *et al.* 1984; Ito *at al.* 1992). The most common urinary risk factors for calcium stone production are hypercalciuria (excess calcium in the urine) (Broadus *et al.* 1984; Brutus *et al.* 1994), low level of citrate (Rudman *et al.* 1982; Hosting *et al.* 1985; Cupisti *et al.* 1992), high levels of oxalate (Merhoff 2001) and uric acid (Robertson *et al.* 1978). The work described in this thesis focuses on calcium oxalate (CaOx) urolithiasis.

Magnesium ammonium phosphate stones (struvite): Struvite stones are also called infection stones because they form due to the presence of long standing urinary infection with certain bacteria that are able to split the urea into ammonium. This then allows ammonium to complex with magnesium and phosphate in the urine to form crystals and stones (Coe *et al.* 2005). These stones represent only 2.8 % of urinary calculi (Ramello *et al.* 2000; Coe *et al.* 2005).

Uric acid stones: This type of stone occurs when the acid level in the urine is high as a result of dehydration and low urinary pH (Ramello *et al.* 2000; Coe *et al.* 2005). Approximately 9-10% of patients with kidney stone disease develop this type of stone (Ramello *et al.* 2000; Coe *et al.* 2005).

Cystine stones: These stones are caused by an inherited kidney disorder that involves the excretion of amino acid of which cystine is a member. These stones represent only a small percentage (1-2%) of urinary calculi (Ramello *et al.* 2000; Coe *et al.* 2005).

1.2 EPIDEMIOLOGICAL FACTORS

Various epidemiological factors have been reported to affect the risk of kidney stone formation. These include climate and geography, ethnicity, age, gender profession and diet. Each of these is discussed briefly below. The probability of forming stones varies in different parts of the world, the highest risk being reported in Saudi Arabia (20 %) (Robertson and Hughes 1994). The risk has been reported to be lower in Asia (1-5 %) than in Europe (5-9 %), North America (5-9 %), Canada (12 %) and the USA 13 % (Ramello *et al.* 2000). Urinary stone occurrence is higher in countries with warm or hot climates. It is likely that stone formation under these circumstances is provoked by dehydration with resultant reduction of urine flow rate and increase in the concentration of solutes in the urine (Blacklock 1982).

Ethnicity may be an important epidemiological factor in urolithiasis as there are reports that it occurs more frequently in whites than blacks, irrespective of the geographic area concerned (Ramello *et al.* 2000). For example, it has been reported that kidney stone disease in the USA is more prevalent in whites than in blacks (Finlayson 1978; Soucie *et al.* 1994). Several other groups have also been reported to show stone rarity, namely the Indians of Mexico, Peru, Ecuador and Bolivia (Finlayson 1978), Eskimos and Australia's aboriginal population (Scott 1977). In South Africa, stones occur in 10 -15 % of the white population but it is extremely rare (<1 %) in the black race group (Modlin 1968). Indeed, it is this latter phenomenon that forms the primary motivation for undertaking the research described in this thesis.

Stone formation is influenced by gender and age. Stones are more frequent in men than in women (i.e. male-to-female ratio is 4:1), although in children, the boy-to-girl ratio is 3:2 (Scott *et al.* 1977; Hesse *et al.* 1986). Peak presentation for adults is middle age. Children can present with stones at any age (e.g. premature, newborn to teenager) (Ljunghall *et al.* 1977).

1.3 DIETARY FACTORS

Diet is believed to be an important contributory factor in the aetiology of urolithiasis since several components of a normal diet can be lithogenic (Andersen 1973; Robertson *et al.* 1987, 2000, Trinchieri *et al.* 1991; Curhan *et al.* 1993; Hesse *et al.* 1993; Massey *et al.* 1993; Goldfarb *et al.* 1994). Fluid therapy is a front-line approach for all stone formers because it is safe, cheap, and effective. A low fluid intake leads to concentrated urine and increases the risk of stone formation. Therefore increasing water intake to dilute urine and increase frequency of urination is an important part of treatment of the disease (Robertson *et al.* 1987; Ryall *et al.* 1989; McCormack *et al.* 1991; Borghi *et al.* 1996; Parivar *et al.* 1996; Rodgers 1997).

Other dietary interventions can reduce the risk of stone formation. Restricting sodium and animal-protein consumption produces changes in the urinary environment that should benefit the majority of stone formers, including a decrease in calcium and increase in citrate excretion (Blacklock 1982, Robertson and Peacock 1983). In the past, calcium oxalate stone patients were advised to restrict their intake of dietary calcium, as there was a belief that a high calcium intake increased the risk of stone formation (Curhan *et al.* 1993, 1997). However, indiscriminant calcium restriction should be avoided, because it restricts the gastrointestinal binding of oxalate, leading to its increased absorption (Curhan *et al.* 1993, 1997). Indeed, higher dietary intake of calcium may actually decrease the risk due to increased binding of calcium to oxalate in the gastro-intestinal tract, leading to a decrease in urinary oxalate excretion (Curhan *et al.* 1993, 1997). On the other hand, a high oxalate intake may contribute to calcium oxalate stone production. Oxalate restriction should be considered for calcium oxalate stone formers, especially those with hyperoxaluria (Coe *et al.* 2005; Garse *et al.* 2000).

Bran is a rich source of insoluble fiber that has been reported to reduce the absorption of calcium, which in turn causes a decrease in urinary calcium when included in the diet (Shah 1980; Ebisuno *et al.* 1986). Oats, wheat bran and rice-bran are a good source of fiber foods that have been reported to have an effect in the kidney stone formation (Ebisuno *et al.* 1986). They all contain phytate. This dietary agent has been found to be important in decreasing the rate of urolothiasis (Grases and March 1989; Conte *et al.* 1999; Grases *et al.* 1999). Phytates (IP6) are salts of phytic acid formed with calcium, magnesium, iron, zinc and copper (Conte *et al.* 1999; Garses and Llobera 1999). Phytate, a substance present in blood, urine, interstitial and intracellular fluids has been reported to inhibit crystallization of calcium salts (oxalate and phosphate) by reducing the level of calcium in the urine and therefore reducing the risk of forming kidney stones (Grases and March 1989; Conte *et al.* 1999; Grases 2000). It is a powerful agent for preventing calcifications in biological fluids and has been used in urolithiasis treatment (Grases and Costa-Bauza 1999). Moreover, it has been reported that phytate is significantly lower in stone formers (0.5 mg/L) than in healthy individuals (2.5 mg/L) (March *et al.* 1998; Grasse *et al.* 1999).

On the other hand, carbohydrates have been reported to increase urinary oxalate (Li *et al.* 1986; Nguyen *et al.* 1989, 1994; Reddy *et al.* 2002) and urinary calcium (Lemann *et al.* 1969; Thom *et al.* 1981; Rofe *et al.* 1986; Nguyen *et al.* 1995), such that some clinicians have recommended that stone patients must avoid sugary products (Gaby 1996; Piesse 1985; Nguyen *et al.* 1986; 1994).

1.4 PHYSICO-CHEMICAL FACTORS

1.4.1 Mechanism of stone formation

Kidney stone formation is a process that involves nucleation, growth and aggregation of crystals and their retention within the kidney (Gill 1984; Hess and Kok 1996). It is the end result of several physicochemical processes that commence with nucleation of crystals from a supersaturated solution. Many hypotheses have been advanced to account for these processes (Howard and Thomas 1958; Lonsdale 1968; Robertson and Nordin 1982; Kok and Khan 1994; Hess and Kok 1996; Ryall 1989; Hess *et al.* 2001; Coe *et al.* 2005).

Supersaturation

Supersaturation is a prerequisite for the occurrence of crystallization in urine (Finlayson *et al.* 1978). The level of saturation of the urine with respect to each of the stone-forming compounds in urine determines whether crystals will form and whether they will aggregate or grow. Supersaturation permits the formation of crystals that can be retained in the nephron and form the nidus of a stone (Kavanagh and Rao 1994). The continual passage of the urine excessively supersaturated with one of the stone-forming salts may lead to spontaneous precipitation of that salt independently of inhibitors of crystallization. Urine is predominantly supersaturated with calcium oxalate monohydrate (COM) and dihydrate (COD) (Hess and Kok 1996). However, the occurrence of the trihydrate (COT) in urine and stones is rare (Hess and Kok 1996).

Crystal nucleation

The initial phase transformation that leads to the formation of urinary stones is called crystal nucleation (Finlayson *et al.* 1978; Finlayson *et al.* 1984). This process begins with the clustering of stone salts in solution into loose conglomerates which can grow due to the decrease in free energy that is associated with the change from liquid to solid phase (Finlayson *et al.* 1978; Finlayson *et al.* 1984; Hess and Kok 1996). The crystal nucleation step requires urine to be supersaturated with respect to crystallizing salts such as CaOx. For all solutions (including urine) there are three zone of relative supersaturation (RS) as demonstrated in Figure 1.2. (Hess and Kok 1996). The latter provides a summary of three scenarios that might be a driving force in the crystallization of salts. Briefly, scenario 1 describes the formation of crystals in a solution without any crystals. In this case relative supersaturation is increased by addition of Ca^{2+} and Ox^{2+} until RS reaches values in the range of 80-100. The solution will then become unstable as it passes the metastable limit (formation product) and the crystals will start to form spontaneously. This process is termed homogeneous nucleation (Finlayson 1978; Nordin *et al.* 1993; Hess and Kok 1996).

Scenario 2 describes a solution containing CaOx crystals and the metastable limit is lower. The presence of CaOx crystals increases the concentration of ions beyond the supersaturation value. Thus the RS of a solution is continuously increased and the formation product is exceeded culminating in spontaneous nucleation. This process is called secondary nucleation. Heterogeneous/secondary nucleation in scenario 3 is a process that is unlikely to occur in the urine due to the presence of many foreign contaminants, such as crystals and macromolecules which may serve as nuclei and cause heterogeneous nucleation. Epitaxy is an extension of the theory of heterogeneous nucleation (Lonsdale 1968; Thorne *et al.* 1983; Mandel *et al.* 1991). In this process, the material of one crystal precipitates onto another crystal and the lattice dimension of the two matches and induces the crystallization in urine.

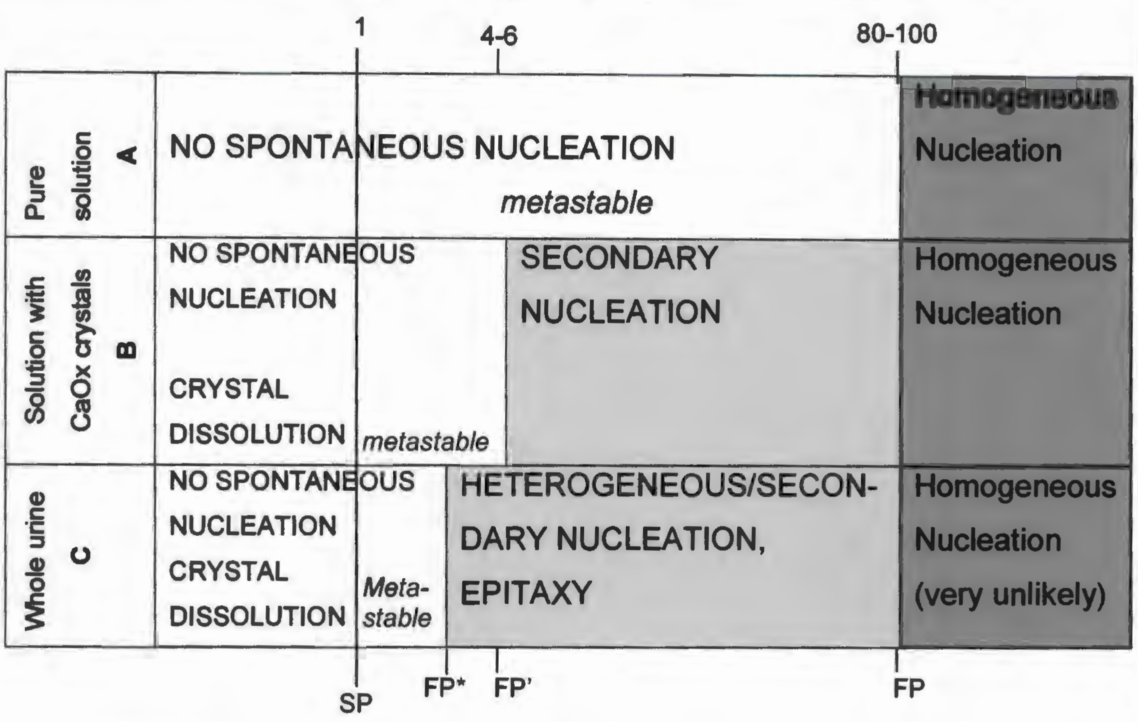


Figure 1.2: Relative supersaturation and CaOx crystal nucleation. A: Aqueous solution of calcium and oxalate. B: Aqueous solution of calcium oxalate crystals. C: Whole urine containing foreign surfaces and crystals (Hess and Kok 1996). SP= solubility product, FP= formation product

Crystal growth

The addition of new crystal components onto a crystal nucleus is called crystal growth. Crystal growth occurs mainly due to the net difference between the energy needed for the formation of new crystalline components in solution and the crystal lattice itself (Hess and Kok 1996). The incorporation of new crystals components into a crystal lattice is a complicated mechanism (Hess and Kok 1996). Crystal growth occurs due to processes called speciation, bulk diffusion and adsorption. Speciation refers to the binding of monomers to form complexes and these monomers migrate through the solution by a process called bulk diffusion. Bulk diffusion refers to the process in which the newly formed crystals travel through the solution and attach to the crystal by adsorption (Hess and Kok, 1996).

Supersaturation is usually approximated for undissolved salts by the ratio of their concentration in the urine to their solubilities and calculated by computer algorithms using eq.1 below (Gill 1984; Coe *et al.* 2005).

Equation 1 $-dS/dt = dP/dt = \kappa AS^{\eta}$

where S= supersaturation; t= time; P= precipitate; κ = reaction rate constant; A= surface area for crystallization; η = order of the reaction.

At supersaturation values less than 1, crystals of a substance will dissolve and crystal can form and grow at values greater than 1.

Crystal aggregation

The term aggregation refers to the process whereby crystals combine to form a large particle under all states of saturation (Hess and Kok 1996). Primary aggregation of particles is a result of malgrowth of crystals resulting in the formation of a polycrystalline particle that expands wide mutually without any predominant orientation from the surface of other crystals (Söhnel and Grases 1995). Secondary aggregation proceeds by collision of particles suspended in the liquid induced by shear forces acting in the liquid (Söhnel and Grases 1995).

Compared to nucleation and growth, crystal aggregation is more important in the process of stone formation because calcium oxalate growth is too slow to produce clinically significant particles within the transit time in the urinary tract, whereas aggregation occurs within seconds and is thus more dangerous for the formation of large crystalline particles in renal tubules (Kok *et al.* 1988; Hess and Kok 1996).

1.5 INHIBITORS

Inhibitors are chemical substances which occur naturally in urine and which retard or prevent the crystallization of salts. They have been a consistent theme of urinary stone research and fall into two classes depending on their molecular weight, namely "micromolecules", such as citrate, pyrophosphate and magnesium, and "macromolecules", which include proteins and glycosaminoglycans. Each of these will be discussed briefly below.

1.5.1 MICROMOLECULES

Several small molecules, such as citrate; pyrophosphate and magnesium have been identified to as inhibitors of urinary crystallization salts (Pak 1987; Ryall *et al.* 1988; Pak 1991; 1994; Ryall 1997; Khan and Kok 2004).

Citrate: Citrate has been associated with calcium stone disease since 1934 (Hastings *et al.* 1934; Ryall 1997). A number of studies have demonstrated that citrate has an inhibitory effect on crystal growth (Welshman *et al.* 1972; Meyer *et al.* 1975), nucleation (Doremus *et al.* 1978; Hallson *et al.* 1983; Schwille *et al.* 1999) and aggregation (Ryall *et al.* 1981; Kok *et al.* 1988) in urine. It is also known to bind to the CaOx crystal surface (Ryall *et al.* 1981, Kok *et al.* 1988) thereby influencing both crystal growth and aggregation. As a result it is regarded as a chelator of calcium ions, which efficiently lowers supersaturation, the driving force for crystallization. Citrate reduces supersaturation by binding to calcium (Khan and Kok 2004). A decrease in citrate excretion (hypocitraturia) has been reported in the urine of stone formers, thereby providing convincing evidence in support of it being a critical risk factor in kidney stone disease (Hodgkinson 1962; Rudman *et al.* 1982; Menon and Mahle 1983; Nicar 1983; Hosking *et al.* 1985; Pak 1987; Cupisti *et al.* 1992; Pak 1994; Ryall 1997; Khan and Kok 2004). The role of citrate in urolithiasis in the clinical treatment has been extensively studied by Pak (Pak 1987; Pak 1991; Pak 1994) and supplementation has become an accepted for stone therapy (Ryall 1997). Amongst these supplements are potassium citrate (Schwille *et al.* 1987; Berg *et al.* 1990; Ogawa 1994), calcium citrate (Levine *et al.* 1994; Sakhaee *et al.* 1994), calcium-sodium citrate (Schwille *et al.* 1997) and potassium-magnesium citrate (Ettinger *et al.* 1997). In a recent study evidence was provided that the use of sodium citrate, in the form of CitroSoda, favourably altered many risk factors of calcium oxalate urolithiasis and proved to be an effective preparation for the prophylactic and therapeutic management of calcium oxalate urolithiasis (Allie-Hamdulay and Rodgers 2005).

Pyrophosphate: Pyrophosphate was the first inhibitory component of urine to be isolated and identified (Fleish and Bisaz 1962; Ryall 1997). It is present in the urine at concentrations of 15-100 µM (Khan *et al.* 2004) and has been found to inhibit COM crystal growth by 50 % at 16-20 µM (Ryall *et al.* 1988). It has also been reported to inhibit CaOx crystal nucleation (Doremus *et al.* 1978), growth (Meyer *et al.* 1975, Welshman *et al.* 1972) and crystal aggregation (Ryall *et al.* 1981) in inorganic solutions. Pyrophosphate excretion has been reported to be significantly reduced in stone formers as compared with healthy individuals (Conte *et al.* 1989). The excretion of pyrophosphate can be increased by oral orthophosphate therapy (Russel *et al.* 1976), potassium phosphate (Breslau *et al.* 1995) and diclofenan sodium (Sharma *et al.* 1992); however therapy has not proven effective in preventing stone formation (Khan and Kok 2004).

Magnesium: Magnesium has long been associated with kidney stone disease. It has been reported that it can reduce the crystallization of CaOx by complexing with the oxalate to form soluble magnesium oxalate, thus reducing the amount of oxalate ions in the urine (Desmars and Dermas 1973, Ryall 1997). Although magnesium has been reported to inhibit CaOx nucleation (Doremus *et al.* 1978), growth (Li *et al.* 1985) and aggregation (Ryall *et al.* 1981), other studies have also reported that it has no effect on nucleation (Li *et al.* 1985), growth (Meyer and Smith 1975; Welshman *et al.* 1972) and aggregation (Robertson and Peackock 1972). A similar confusing array has also been seen on reports on the levels of urinary magnesium in stone formers and healthy subjects (Ryall 1997). In one study, urinary magnesium in stone formers was found to be equal to or higher than healthy individuals (Johansson *et al.* 1980), whereas in other study it was found to be lower in stone formers (Trinchieri *et al.* 1991). Other conflicting results have been reported in the literature regarding the use of magnesium supplementation in the treatment of urolithiasis. For example, it was found in one study that magnesium unfavourably increased urinary calcium excretion and urinary pH (Fetner *et al.* 1978). On the other hand other studies have reported prophlytic effects (Moore and Brunce 1964; Johansson *et al.* 1980; Parivar *et al.* 1996) and found a significant decrease in oxalate excretion after magnesium supplementation (Zimmerman *et al.* 2003).

1.5.2 MACROMOLECULES

Of the two classes of inhibitors described, macromolecules are the most widely investigated today. In the context of urolithiasis, “macromolecules” refer to urinary glycosaminoglycans and proteins. Numerous studies have shown that normal urine contains macromolecular substances that are capable of preventing and reducing CaOx crystal formation and aggregation (Boyce 1968; Morse and Resnick *et al.* 1989; Jones *and* Resnick 1990; Ryall *et al.* 2000; Khan and Kok 2004).

Glycosaminoglycans (GAGs)

GAGs (chondroitin sulphate (ChS), dermatan sulphate, hyaluronic acid (HA), heparin/heparan sulphate and keratin) are polysaccharides, composed of repeating disaccharide units (Roberts and Resnick 1986). ChS is the most abundant urine GAG (Roberts and Resnick 1986). The organic matrix of the stone accounts for 2-3 % of the total dry weight and heparan sulphate (HS) and HA have been the only GAGs that have been shown to be present in both the matrix of stones, and the CaOx crystals formed in the urine (Roberts and Resnick 1986; Ryall *et al.* 1997; Khan *et. al.* 2004). GAGs have a varying molecular weight of 18 - 40 kDa and account for 20 % of the matrix of kidney

stones. ***In vitro*** **studies have demonstrated that they inhibit growth and aggregation of calcium oxalate crystals** (Roberts and Resnick 1986; Ryall *et al.* 1997; Khan and Kok 2004). **Studies have shown that HS, HA and ChS affects the precipitation of CaOx** (Khan and Kok 2004). **ChS has been reported to inhibit crystal growth, aggregation, nucleation mass deposition and agglomeration** (Shum *et al.* 1999; Erturk *et al.* 2002; Schepers *et al.* 2002). **On the other hand GAGs have been reported to promote nucleation** (Robertson *et al.* 1989) **and growth rate** (Kohri *et al.*1989). **GAGs contain post-translation modifications such as phosphorylation or glycosylation, and these may be important for their inhibitory activity. There are no significant urinary excretion differences of between stone formers and healthy subjects** (Harangi *et al.* 1996).

Urinary Proteins

The organic matrix of kidney stones constitutes 2.5 % of dry weight containing lipids, carbohydrates and proteins. The latter comprise 64 % of the stone matrix itself (Boyce and Garvey 1956). **Albumin, α and γ-globulin and Tamm-Horsfall Protein (THP) were the first proteins to be identified in the stone matrix** (Boyce *et al.* 1968). **Several proteins have been identified as having a role in the inhibition and promotion of CaOx crystallization. These include nephrocalcin** (Nakagawa *et al.* 1983; Coe *et al.* 1991); **uropontin (osteopontin)** (Worcester 1992; Worcester *et al.* 1995; Worcester *et al.* 1996; Khan *et al.* 2004); **THP** (Grant *et al.* 1973; Rose *et al.* 1982; Hess 1992; Hess *et al.* 1993); **Albumin** (Boyce 1968; Atmani *et al.* 1998; Ryall *et al.* 1991; Grover *et al.* 1998); **urinary prothrombin fragment 1 (UPTF1)** (Doyle *et al.* 1991; Grover *et al.* 1999; Durrbaum *et al.* 2001; Webber *et al.* 2002; Webber *et al.* 2003); **inter-α-(trypsin) inhibitor** (Sorenson *et al.* 1990; Atmani *et al.* 1993; Dawson *et al.* 1998; Moriyama *et al.* 2001) **and bikunin** (Shulman 1955; Balduyck *et al.* 1982; Sugiki *et al.* 1989; Fries *et al.* 2000; Médétognon-Bonissan *et al.* 1999; Yang *et al.* 2005). **Bikunin and inter-α-(trypsin) (I-α-I) inhibitor will be discussed in detail later.**

Nephrocalcin (NC): This is a glycoprotein with a molecular weight of ~14 kDa. It has been reported to self-aggregate into larger molecules that range from 23-68 kDa (Worcester *et al.* 1987; Nakagawa *et al.* 1990). NC is composed of 110 amino acid residues of which 25 % are glutamic and aspartic acid (Khan *et al.* 2004). It has been reported that it contains 2 cystein and 2 or 3 γ-carboxyglutamic acid residues which play a significant part in its ability to inhibit CaOx aggregation (Hess *et al.* 1989; Coe *et al.* 1991; Nakagawa *et al.* 1991), nucleation and growth (Hess *et al.* 1989; Coe *et al.* 1991; Khan and Kok 2004). Besides inhibition of the physical processes of crystal formation and aggregation, NC is also able to prevent attachment of crystals to the surface of renal epithelial cells. NC isolated from the urine of stone formers has been found to be structurally abnormal and to lack Gla residues (Nakagawa *et al.* 1983). The identity of NC remains to be authenticated because its amino acid sequence has not been described (Khan *et al.* 2004).

Uropontin (Osteopontin): Urinary UPN was originally isolated as a glycoprotein with a molecular weight of 50 kDa (Khan *et al.* 2004) and has a high asparagine content accounting for 16 - 20 % of the amino acids. UPN is a member of the aspartic acid-rich protein super family, a family characterised by a structure containing the Arg-Gly-Asp (RGD) sequence (Kohri *et al.* 1990; Konya *et al.* 2003). It is found in urine, human CaOx stones and CaOx and calcium phosphate (CaP) crystals grown in human urine (Elliot and Rabinowits 1980; Wesson et al. 1998; Worcester 1996). UPN was originally isolated from mineral bone matrix and thereafter isolated from both human urine and primary cultures of mouse kidney cortical cells, by virtue of its ability to inhibit calcium oxalate crystal growth (Butler 1989; Malyankar *et al.* 1997).

UPN can modulate calcium oxalate crystallization by inhibiting crystal nucleation, growth and aggregation (Elliot and Rabinowits 1980; Shigara *et al.* 1992; Hoyer 1994; Lieske *et al.* 1995; Asplin *et al.* 1997; Wesson *et al.* 1998; Worcester 1996). Like NC, UPN can inhibit binding of COM to BSC-1 cells (Wesson *et al.* 1998). It has not been tested for an effect on crystal endocytosis, its RGD peptides are able to block crystal uptake by these cells in vitro (Konya *et al.* 2003). Stone formers have been reported to excrete less UPN than non-stone formers, presumably because of the incorporation of it in crystal of the growing stone (Khan and Kok 2004)

Tamm-Horsfall protein (THP): THP is the most abundant protein in human urine with a molecular weight of 80 -100 kDa (Tamm and Horsfall 1950; Ryall 1997). It is produced in the kidney and specially localized in the epithelial cells of the loop of Henle. THP, also called uromucoid is another urinary glycoprotein, which has long been believed to play a role in stone formation (Grant *et al.* 1973; Hess 1992).

Although THP has been the most intensely studied protein associated with urolithiasis, varying results from different studies have been controversial and thus its exact contribution to urolithiasis remains unclear (Khan and Kok 2004). Some studies report that THP inhibits and promotes CaOx crystallization depending on the experimental conditions (Grover *et al.* 1998; Hess 1994) while others have reported that THP can inhibit CaOx and CaP crystallization (Rose *et al.* 1982). Several reports have indicated that THP inhibits nucleation and growth and aggregation (Finlyason 1978; Hess *et al.* 1991; Hess *et al.* 1993). Other studies have reported that THP has no effect on nucleation and a minor effect on crystal growth (Yoshioka *et al.* 1989; Hess 1992; 1994). There appears to be no differences in THP excretion of healthy subjects compared to stone formers (Grant 1973; Bichler *et al.* 1976; Erwin et al. 1994) however structural differences have been reported in THP isolated from healthy individuals and stone formers. It was demonstrated that THP isolated from the urine of stone formers contained less carbohydrates (mainly sialic acid) than the THP obtained from healthy subjects (Hess *et al.* 1991; Hess *et al.* 1995).

Albumin: After THP, albumin is the most abundant protein in the urine (Hess *et al.* 1989; Grover *et al.* 1998). This protein is manufactured in the liver and has been detected as the major component in the matrix of all urinary stones (Boyce 1968) as well as in urinary crystals (Atmani *et al.* 1998). Albumin has a molecular weight of 66 kDa. The effect of albumin on CaOx crystallization has been extensively studied both in inorganic solution and whole urine (Hess 1989; Edyvane 1987; Ryall et al. 1991; Mensah 2004). It has been reported to have a strong affinity for COM crystals but does not inhibit its growth (Edyvane *et al.* 1987; Ryall *et al.* 1991). It has also been shown that albumin inhibits CaOx crystal aggregation in a concentration dependant manner (Ryall *et al.* 1991; Worcester 1996; Grover *et al.* 1998) and promotes CaOx nucleation (Grover *et al.* 1994; Khan *et al.* 2004). Albumin has the ability to bind other urinary proteins and it exists either in monomeric or polymeric form when dissolved in solution.

Prothrombin Fragment 1 (UPTF1): Before being called UPTF1 this protein was originally referred to as crystal matrix protein (CMP) because of its selective inclusion into CaOx crystals (Doyle *et al.* 1991). The amino acid sequence analysis showed an identity to prothrombin and was therefore called UPTF1. UPTF1 is a protein with a molecular weight of 31 kDa. This protein is found in calcium stones and is also located in greater quantities in the human kidney of the stone formers than in the healthy controls (Doyle *et al.* 1991; 1995).

UPTF1 was found to be a potent inhibitor of CaOx growth and aggregation in ultrafilted urine (Ryall *et al.* 1995; Doyle *et al.* 1991). In this experiment UPTF1 decreased crystal aggregation and the amount of CaOx deposition at 1.25 -10mg/L concentrations. UPTF1 was also demonstrated to inhibit crystal growth and aggregation by 25 % at a concentration of 1.25 mg/L. In an experiment comparing inhibitory activity of different proteins, UPTF1 was found to be a better aggregation inhibitor than THP, prothrombin, α-1m or albumin and it was the only protein that reduced crystal growth (Grover *et al.* 1999). Studies on UPTF1 isolated from the urine of black and white South African subjects will be described in Section 1.6 which follows.

1.6 CaOx UROLITHIASIS IN SOUTH AFRICA

As stated earlier, the occurrence of kidney stones in the black South African population is extremely rare (Modlin 1968; Whalley *et al.* 1998). However, kidney stones in the white population occur with no less frequency than in other Western communities. The first recorded case of a kidney stone in a black South African was in 1927, when Vermooten reviewed the Johannesburg General Hospital records from 1922-1935 (Vermooten 1937). One black patient was treated for renal calculi during that period compared to 273 white patients. Urinary tract CaOx stones have been reported to be the most common stone type found in black South Africans on those rare occasions when such a condition arises (Beukes *et al.* 1987).

In an attempt to identify factors which may play a role in the exceptionally rare incidence of stone formation in the black population, studies involving urine chemistry, diet, solute handling and the presence and absence of promoters and inhibitors in this ethnic group have been undertaken in the Kidney Stone Research Laboratory at the University of Cape Town during the past several years (Craig *et al.* 2000, 2001; Durrbaum *et al.* 2001; Lewandowski *et al.* 2001; Rodgers and Lewandowski 2002; Webber et *al.* 2002; Webber *et al.* 2003; Lewandowski and Rodgers 2004 (a); Lewandowski and Rodgers 2004 (b); Webber *et al.* 2005; Rodgers 2006).

Differences in comparing the 24 hr urinary composition in normal black and white male subjects have been reported by other workers. These have shown that blacks have higher urinary volumes, osmolality, ionized calcium and sodium levels, while their total calcium, phosphate and citric acid levels and urinary pH are lower than the white population (Modlin 1968; Whalley *et al.* 1998). Results from our own urine studies have confirmed that citrate is lower in black groups (Lewandowski *et al.* 2001). This is counter-intuitive because urinary citrate is one of the most powerful natural inhibitors of CaOx crystal formation. Furthermore, this same study showed that urinary oxalate was higher in a small group of black subjects when compared to a matched group of white subjects. Thus urine composition alone does not appear to adequately account for the rarity of stones amongst South Africa's black population.

Other studies from our research group and others have indicated that dietary intake of calcium, oxalate and protein is significantly lower in black subjects (Vermooten 1937; Wise *et al.* 1961; Modlin 1968; Whalley *et al.* 1998; Lewandowski *et al.* 2001). Furthermore, in a study in which a combined low calcium-high oxalate diet was administered to black and white subjects, oxaluria increased significantly in the white group (as expected) but not in the black group (Lewandowski *et al.* 2002). This observation prompted the authors to suggest that blacks possibly have a lower oxalate absorbance rate (Whalley *et al.* 1998; Lewandowski *et al.* 2002) and possibly a different renal handling mechanism with respect to calcium and oxalate (Lewandowski *et al.* 2002).

Since routine urinary components cannot explain the rarity of stones in black South Africans, our research group has also undertaken an ongoing investigation of the role of urinary proteins in this context. Since urinary proteins have been shown to have inhibitory properties in other studies elsewhere in the world, it was decided to continue probing this area in the South African context. These included UPTF1, THP and albumin. These studies have yielded interesting results.

THP was isolated from the urine of black and white male subjects and various techniques including matrix-assisted laser desorption / ionization time of flight mass spectrometry (MALDI-TOF MS), amino acid analysis and tryptic finger printing digestion were used to characterize the protein (Craig *et al.* 2000). Structural differences were also observed in the THP isolated from the two race groups. For example, THP from the black subjects had a higher molecular weight, more cystein and glycine, and less alanine, valine, leucine and phenylalanine (Craig *et al.* 2004; Rodgers 2006). The inhibitory effect of THP on CaOx crystallization was tested in various crystallization experiments. THP isolated from black subjects was demonstrated to be a more potent inhibitor of COM crystal aggregation than that from whites (Craig *et al.* 2001; Rodgers

2006). It was therefore concluded that the stone rarity in the black population might be linked to the more potent activity of this race group's THP in inhibiting aggregation (Craig *et al.* 2001).

In another study, UPTF1 was isolated from the urine of black and white male subjects and characterized using *N*-terminal sequencing, amino acid analysis and MALDI-TOF MS (Webber *et al.* 2002). Structural differences were observed in the proteins isolated from the two race groups. For example, the amino acid analysis conducted under basic conditions demonstrated a greater number of Gla residues in UPTF1 from black subjects (Webber *et al.* 2003). The inhibitory activity of UPTF1 was compared using various crystallization experiments. UPTF1 from both race groups was found to be a potent inhibitor of CaOx growth and aggregation in ultrafiltered urine (Webber *et al.* 2002, 2003). It was also demonstrated that UPTF1 isolated from black male subjects is a stronger inhibitor of CaOx crystal growth and aggregation in synthetic urine and ultrafiltered urine than that isolated from whites (Webber *et al.* 2002).

Albumin is another protein that was selected for investigation on the basis of its reported inhibition on CaOx crystallization. Urinary albumin was isolated in order to study its role in the context of the urolithiasis anomaly amongst the South African population groups. Results from sedimentation and zeta-potential experiments demonstrated that albumin isolated from black male subjects inhibited crystal aggregation to a greater extent than that isolated from white subjects, and thereby confirmed its possible role in the protection of the black population (Mensah 2004; Rodgers *et al.* 2006)

The above-mentioned studies on urinary proteins have encouraged us to continue probing this area in our effort to identify factors which might afford this population group unique protection against kidney stone formation. The protein selected for further study in the present project was bikunin.

1.7 BIKUNIN

The set of plasma proteins comprising inter-α-(trypsin) Inhibitor (IαI) and related molecules is collectively referred to as the IαI family (Shulman 1955; Balduyck *et al.* 1982; Sugiki *et al.* 1989; Fries *et al.* 2000; Médétognon-Bonissan *et al.* 1999; Yang *et al.* 2005). The family is characterized by the unique covalent linkage between the heavy chains and the chondroitin sulphate chain of bikunin. Figure 1.3 is a schematic representation of IαI family. IαI proteins belong to the super-family of Kunitz-type protease inhibitors. IαI itself is a macromolecule with a molecular weight of 180 - 220 kDa (Gerbhard *et al.* 1990; Höchstrasser *et al.* 1979; Kobayasha *et al.* 1988; Atmani *et al.* 1999; Dean *et al.* 2000; Médétognon-Benissan *et al.* 1999; Suzuki *et al.* 2001). It is mainly synthesized in the liver and excreted in plasma and urine (Gerbhard *et al.* 1990).

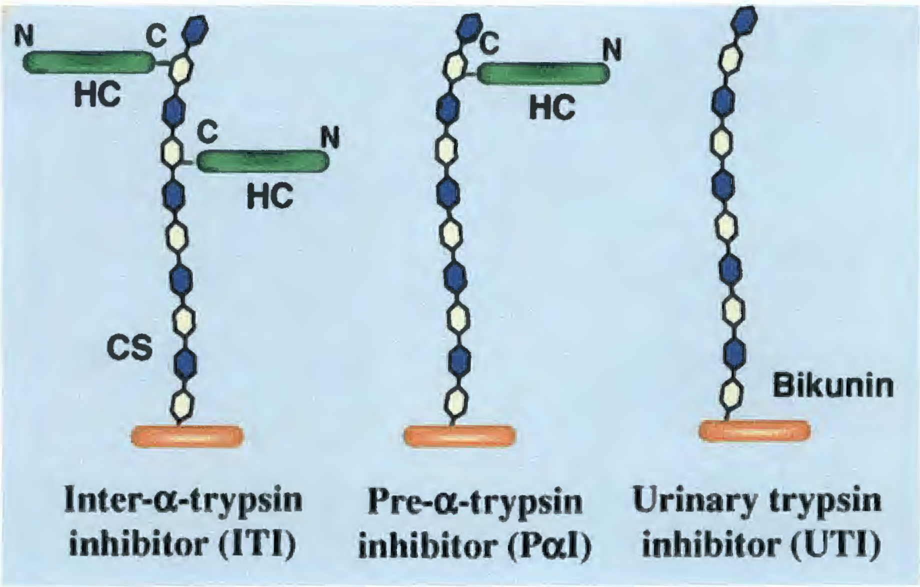


Figure 1.3: IαI family; HC- Heavy chain, CS- chondroitin sulphate chain (Picture taken from http://www.glycoforum.gr.jp/science/hyaluronan/HA22/HA22E.html)

It is comprised of three distinct polypeptide chains. Two heavy chains, H1 and H2, have molecular weights in the ranges of 65 -101 kDa and 70 -106 kDa, respectively (Médétognon-Benissan *et al.* 1999; Dean *et al.* 2000; Suzuki *et al.* 2001). These are covalently linked via a chondroitin sulphate moiety to a light chain known as bikunin. The covalent chondroitin sulphate inter-link between these three peptides is termed a protein-glycosaminoglycan-protein cross link (Kobayasha *et al.* 1988).

The connection between IαI and stone disease was established in 1909, when it was demonstrated that the proteolytic activity of trypsin was inhibited by urine (Gebhard and Hochstrasser 1986). The physiological role of IαI remains unknown (Atmani *et al* 2004).

Bikunin is referred to by several other names in the literature, such as urinary trypsin inhibitor (UTI), ulistatin, urinastatin, acid- stable trypsin inhibitor (ASTI), uronic-acid-rich protein (UAP) and HI-30 (Shulman 1955; Sugiki *et al.* 1989; Faarvagn *et al.* 1965; Gerbhard *et al.* 1990; Fries and Blom 2000). Bikunin was first isolated in 1955 (Shulman 1955) by researchers working on mucopolysaccharide, leukemia and urolithiasis, and it was originally referred to as UTI (Shulman 1955; Balduyck *et al.* 1982; Fries and Blom 2000). UTI was found to remain soluble and active after treatment with strong acids and was therefore also called ASTI (Sugiki *et al.* 1989).

The name UAP was given in view of its composition. It was later named HI-30 because of its molecular weight and after a polypeptide identical to UTI was released when it was digested with trypsin. Other names for bikunin such as mingin (Astrup and Nissen 1964; Faarvagn *et al.* 1965), ulistatin, urinastatin have also been used (Gerbhard *et al.* 1990; Fries and Blom 2000). In 1990, it was proposed that the name bikunin be adopted (Gerbhard *et al.* 1990). This name is now commonly used to refer to this protein. Bikunin is a structure-based name since the amino acid sequencing has shown that the protein contains two proteinase inhibitor domains (Gerbhard *et al.* 1990).

Bikunin is synthesized in the liver and originates from common mRNA that also codes for another protein, α1-microglobulin (α1-m) (Atmani 2001; Fries and Blom 2000). After bikunin is synthesized, cleavage occurs in the Golgi apparatus of the cell and it is released and eliminated in urine or serum either in its free form or bound to the two heavy chains of IαI (Fries and Blom 2000). Immunohistochemical staining using renal tissue samples and reverse transcriptase polymerase chain reaction experiments have demonstrated that bikunin is produced in the kidneys (Xu *et al.* 1998). It is localized in the cells of the proximal tubular cells of normal human beings and it is highly glycosylated (Fries and Blom 2000; Médétognon-Bonissan *et al.* 1999; Yang *et al.* 2005). Bikunin is composed of a 15-16 kDa polypeptide chain, and ~7-8 kDa chondroitin sulphate side chain and an *N*-linked oligosaccharide of about 1-2 kDa (Médétognon-Bonissan *et al.* 1999; Yang *et al.* 2005). The bikunin glycans account for approximately one third of the molecular mass including the important chondroitin sulphate chain, which links bikunin to the heavy chains. In 1981, the bikunin carbohydrates were identified as : a) an *O*-linked (GalNAc-rich chain) attached to serine residue at position 10 of the core protein linked via the chondroitin sulphate that contains 15 disaccharide repeats; and b) an *N*-linked oligosaccharide at position 45 (Hochstrasse *et al.* 1981) Figure 1.4. The CS linkage to bikunin is a typical *O*-xylosyl-serine (Balduyck *at al.* 1986).

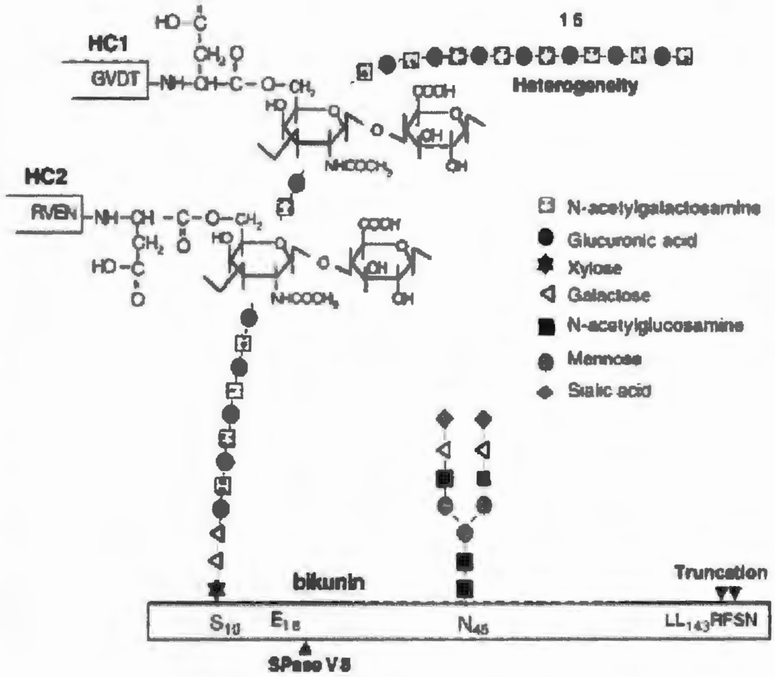


Figure 1.4: Bikunin glycosylation (Balduyck *at al.* 1986).

This light chain is responsible for the antitryptic activity of IαI in the inhibition of various enzymes such as trypsin and chymotrypsin (Atmani *et al.* 1996). Bikunin fragment is the N-terminal portion of IαI responsible for the protease inhibitory activity (Morri *et al.* 1985).

Varying molecular weights of bikunin isolated from urine have been reported: 70 kDa (Boyce *et al.* 1956); 68 kDa (Prat *et al.* 1989); 60-70 kDa by gel filtration (Muramatsu *et al.* 1980; Slota *et al.* 1994); 45 kDa (Bourguignon *et al.* 1989; Odum 1990; Malki *et al.* 1992; Route *et al.* 1992); 40 kDa (Sorensen *et al.* 1990; Kobayasha *et al.* 1998); 35 kDa (Atmani *et al.* 1993a; Tang *et al.* 1995; Ebisuno *et al.* 1999; Okuyama *et al.* 2003); 30 kDa (Enghild *et al.* 1989, Morikawa *et al.* 1994); 29 kDa; 20-25 kDa (Mizon *et al.* 2002); 17 and 24-28 kDa (Médétognon-Benissan *et al.* 1999; Yang *et al.*

2005). It has also been reported that bikunin degrades to lower molecular weights (14 and 8 kDa) after isolation (Höchstrasser *et al.* 1979; Kobayasha *et al.* 1988; Atmani *et al.* 1999). The many isomers appear to be a result of variations in the GAGs and polysaccharide side chains, and the amount of sulphation (Médétognon-Bonissan *et al.* 1999; Yang *et al.* 2005).

Difficulties have been encountered in isolating bikunin, especially when using immunoaffinity chromatography since commercially available antibodies bind to both bikunin and the heavy chains of IαI (Atmani *et al.* 1999). Trypsin-affinity chromatography has therefore been employed because bikunin binds trypsin and inhibits its activity (Atmani *et al.* 1999). Other methods, like ion exchange-trypsin affinity chromatography have also been exploited (Médétognon-Benissan *et al.* 1999). Bikunin isolated from human urine has been reported to have concentrations ranging from 6.25 nmol/L to 500 nmol/L in normal men and women (Dean *et al.* 2000; Kobayashi et al. 1998). However the 500 nmol/L urinary bikunin concentration reported by Kobayashi *et al.* (1998) has been seen as an overestimate, since it will translate to the equivalent of 17.5 mg/day, which is unlikely considering that albumin, which is the second most abundant urinary protein has an excretion concentration of less than 20 mg/L (Dean *et al.* 2000). These authors have proposed that the true physiological concentration range of bikunin is 6.25 - 18 nmol/L. Reports on urinary bikunin excretion in stone formers and healthy subjects are contradictory. The mean urinary bikunin concentration in one study was found to be higher in stone formers (5.2 µg/ml) than in healthy subjects (2.8 µg/ml) (Suzuki *et al.* 2001). However, it was found to be higher in healthy controls than in stone formers in another study (5 mg/L and 2.54 mg/L respectively) (Médétognon-Benissan *et al.* 1999).

The role of bikunin as an inhibitor of CaOx crystallization was first proposed by Nakagawa *et al.* (1985). The effect of bikunin in its various urinary forms on CaOx crystallization has been tested *in vitro*. It has been reported that bikunin isolated from human urine is a strong inhibitor of CaOx crystallization (Höchstrasser *et al.* 1984; Atmani *et al.* 1993a; Atmani *et al.* 1993b; Atmani *et al.* 1995; Sorensen *et al.* 1990). Many reports have demonstrated that bikunin inhibits CaOx nucleation (Höchstrasser *et. al.* 1984; Atmani *et al.*1999a; Atmani *et al.* 1999b; Okuyama *et al.* 1999); crystal growth (Atmani *et al.* 1993; Atmani *et al.* 1993b; Atmani *et al.* 1999; Okuyama *et al.* 2003) and crystal aggregation (Atmani *et al.* 1999). Bikunin isolated from the urine of stone formers has been found to be less glycosylated and showed less inhibitory effect than that purified from the urine of healthy subjects (Atmani *et al.* 1994; Ebisuno *et al.* 1999).

Regarding concentration, Atmani *et al.* (1999) found that urinary bikunin retarded nucleation by 58 to 67 % at concentrations of 2.0 to 2.5 µg/ml and inhibited crystal aggregation by 59 to 80 % at similar levels. In another study, it was demonstrated that bikunin fragments with molecular weights of 17 and 24 kDa inhibited CaOx crystallization in a concentration dependant manner, reaching 80 % at a concentration of 8 µg/ml (Médétognon-Benissan *et al.* 1999). Inhibition activity of 70 % was found in similar experiments with the intact 35 kDa protein at a concentration of 10 µg/ml (Atmani *et al.*1993). Bikunin can inhibit CaOx crystallization and alter the ratio of CaOx monohydrate (COM) to dihydrate crystallization (Wesson *et al.* 1998). Kobayashi *et al.* (1996) demonstrated that bikunin inhibits CaOx growth with increasing protein concentration when tested with a tracer of [^{14}C]-oxalate.

Bikunin has been implicated in the prevention of COM crystal adhesion to the renal ephithelial cells (Ebisuno *et al.* 1999; Khan and Kok 2004; Yang *et al.* 2005). In both studies Madine-Darby canine kidney (MDCK) cells were cultured and incubated with COM crystals in the presence and absence of various protein fractions isolated from human urine. They demonstrated that a protein with a molecular weight of 35 kDa and an *N*-terminal sequence homologous to bikunin inhibits crystal adhesion at minimum concentrations of 10 ng/ml and blocked adhesion completely at 200 ng/ml. Bikunin is negatively charged and is thought to be a calcium-binding protein. Its inhibitory action against crystal adhesion depends on the binding of bikunin to the crystal surface (Ebisuno *et al.* 1999).

1.8 OBJECTIVES

As described earlier in this chapter, bikunin has been found to inhibit CaOx crystal nucleation, growth and aggregation. The present study was undertaken to compare the inhibitory effect of the urinary bikunin protein from black and white South African subjects on CaOx crystallization

The objectives of this thesis were therefore:

- To compare the relative baseline inhibitory activity of whole urine from healthy white and black South African subjects
- To measure the relative inhibitory activity of commercially available bikunin in the urine of healthy subjects from both groups
- To isolate COM and COD from the urines of healthy black and white subjects
- To isolate protein precipitates (using ammonium sulphate) from the urines of both groups
- To measure the inhibitory activity of the protein precipitate in the urine of healthy subjects from both groups
- To analyze for the presence of bikunin in the protein precipitate, COM and COD matrix isolated from the urine of both groups
- To isolate bikunin from the protein precipitated from the urines of both subjects
- To characterize bikunin samples from both groups using SDS-PAGE, Western blot and MALDI-TOF MS
- To measure the inhibitory activity of bikunin in the urine of healthy subjects from both groups using a cross-over design
- To deglycosylate bikunin samples derived from the urine of both groups
- To measure the relative inhibitory activity of the deglycosylated protein fractions

Chapter 2

General Methods and Results

2.1 Introduction

This chapter describes the methods that were used in the different studies undertaken for this thesis.

The urine collection and its treatment and CaOx metastable limit (MSL) determination methods are described. The inhibitory effects of proteins on CaOx crystallization *in vitro* were investigated using different crystallization experiments. These experiments measured [^{14}C]-oxalate deposition and particle formation kinetics (particle number, particle volume and particle volume-size distribution). A scanning electron microscope (SEM) was also used to view deposited crystals.

Also described in this chapter are the experiments used to determine the inhibitory activity of proteins towards CaOx crystal aggregation and nucleation in inorganic solutions.

Finally, the general methods used for the protein characterization are described, namely sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration determination using the Bradford assay.

2.2 Methods

2.2.1 Urine collection, treatment and determination of CaOx metastable limits

For each of the studies described in this thesis, 24 hr urines were collected in plastic bottles without preservative from five black and five white healthy male subjects (age range 18-30 years) on their free unrestricted diets and with no history of kidney stones. The urines were tested for haematuria and nitrite using urinalysis test strips (Medi Test Combi 5N, Macherey-Nagel). The urines testing positive were excluded. Equal volumes (1 L) of the whole urines were pooled (Jappie and Rodgers 2000) according to race and 5 ml aliquots of each pooled sample were removed for compositional analyses.

The urinary pH and volume were measured routinely and the urinary calcium, magnesium, potassium and sodium were determined using a Varian 1275 Model (Australia) flame atomic absorption spectrometer (Willis *et al.* 1967, Trudeau and Freier 1967, Fernandez and Kahn 1971). Urinary oxalate was determined using oxalate decarboxylase (Chiriboga 1963), citrate by citrate lyase conversions to oxaloacetate (Gruber and Möllering 1966), and creatinine using picric acid (Rock *et al.* 1986). Phosphate was determined using ammonium molybdate (Dryer and Routh 1963) and uric acid using uricase (Fossati *et al.* 1980).

The supersaturation of urine with respect to CaOx, brushite and uric acid is a thermodynamic parameter which is most often expressed as the relative supersaturation (RS). The relative supersaturation values were calculated using EQUIL2, a speciation programme which computes the equilibrium concentrations of complexes of primary cations and anions commonly found in urine (Werness *et al.* 1985). The urine composition of the two race groups was compared using analysis of variance (ANOVA) and the results were considered statistically significant if $p \leq 0.05$.

For crystallization experiments the pooled urines collected from the same population group were pre-filtered through 0.75 µm (Macherey-Nagel, Germany) followed by 0.45 µm (Sartorius AG, Germany) nitrocellulose to remove debris and flocculents. The filtered pooled urine was then ultrafiltered (UF) through a 10 kDa cut-off membrane to remove all macromolecules greater than 10 kDa. The urine was incubated in a shaking water bath (100 rpm; Labcon Marketing Services, Johannesburg) at 37 °C and the metastable limit of the UF was measured according to the method described below.

The CaOx metastable limit (MSL) was determined according to the method described by Ryall *et al.* (1985). Briefly, 10 ml UF aliquots were pipetted into Coulter cups and incubated at 37 °C in a shaking waterbath (100 rpm) for 10 min. Thereafter, 100 µL of solutions of increasing concentrations of Na_2Ox (15 mmol/L to 195 mmol/L) were used to dose the urines and these were incubated at 37 °C and 100 rpm for 30 min. A Coulter Multisizer II (Coulter Electronic LTD; England) was used to measure the number of particles in each urine sample.

A typical plot of particle number versus Na_2Ox concentration is shown in Figure 2.1. The Na_2Ox concentration that induces crystallization (shown by a sudden increase in the particle number) is regarded as a measure of the CaOx metastable limit of the urine.

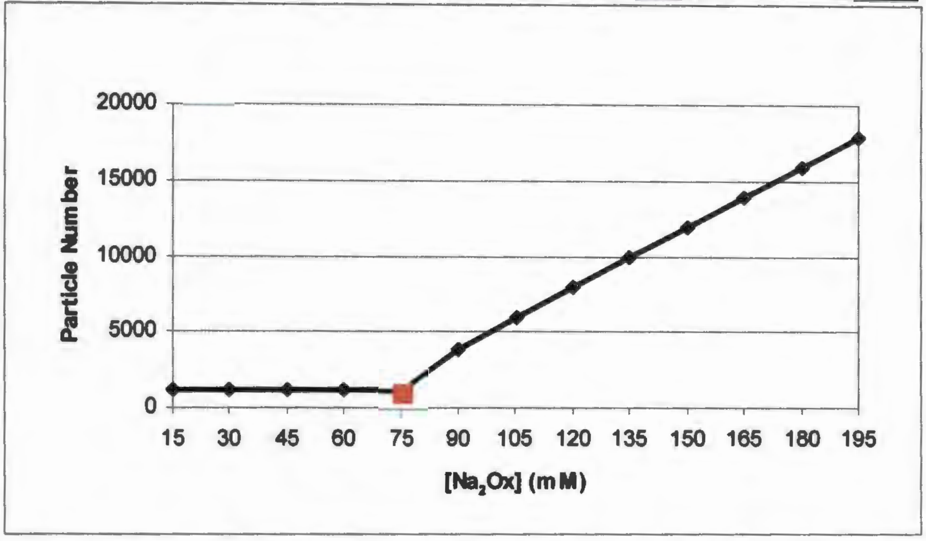


Figure 2.1: Typical example of the graph for a CaOx MSL determination, where the concentration of Na_2Ox corresponding to the red point indicated on the graph corresponds to a measure of the MSL.

2.2.2 Crystallization experiments

The following paragraphs describe experiments in which the inhibitory activity of "proteins" was investigated. This latter term is used generically to represent four different forms of bikunin, viz (i) commercial bikunin, (ii) protein precipitate containing bikunin (iii) purified bikunin and (iv) deglycosylated commercial bikunin. In all cases, a final protein concentration of 0.5 mg/L was used because it has been demonstrated that proteins extracted from urine inhibit CaOx crystallization by 80 % at this concentration (Doyle *et al.* 1995). The treatment, isolation and purification of each of these forms of bikunin are described respectively in Chapters 3, 4, 5 and 7 of this thesis.

Cross-over design

A cross over design experiment (Craig *et al.*1999) was used for the measurement of [^{14}C]-oxalate deposition and particle formation kinetics and is illustrated in Figure 2.2. Once the MSL of the UF was determined, equal volumes were pipetted into three flasks. The ultrafiltered urine sample without added proteins was used as a control in the [^{14}C]-crystal deposition and particle formation kinetics experiments. Proteins were added to the other two flasks as shown in the Figure 2.2.

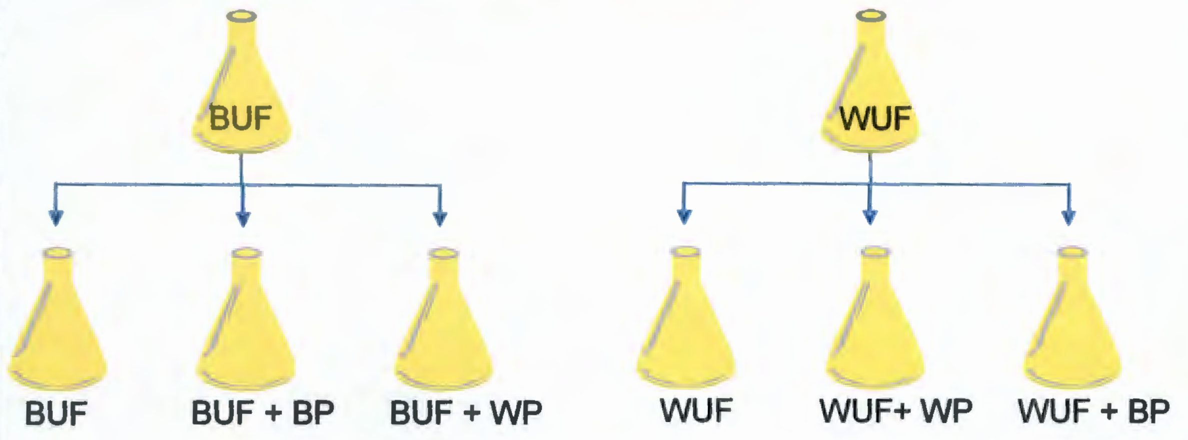


Figure 2.2: Cross-over design for crystallization experiments. BUF: pooled, ultrafiltered urine from black subjects; WUF: pooled, ultrafiltered urine from white subjects; BP: proteins from the urine of black subjects; WP: proteins from the urine of white subjects.

Effect of proteins on [^{14}C]-oxalate deposition kinetics

Crystal deposition experiments using radioactive [^{14}C]-oxalate were conducted according to the method of Ryall *et al.* (1995). These were used to determine the kinetics of CaOx crystallization as opposed to the kinetics of particle formation (described on page 38).

Ultrafiltered urine samples from black and white subjects, referred to as WUF and BUF respectively, were divided into three 30 ml aliquots and incubated in a shaking waterbath at 37 °C for 10 min. Proteins were added to two of the flasks to achieve a final concentration of 0.5 mg/L. The third flask contained ultrafiltrate without protein and was used as a control. 0.938 µCi of [^{14}C]-oxalate (NEN, Boston, USA) was then added to each 30 ml aliquot and CaOx crystallization was induced by the addition of a sodium oxalate (Na_2Ox) load 30 µM above the previously determined MSL. 2.5 ml samples were withdrawn from each flask at 30 min intervals and filtered through a 0.22 µm filter (Millipore) into 250 µL concentrated HCl to quench the reaction. Duplicate 1 ml aliquots of the acidic solutions were added to 10 ml scintillation fluid (Zinsser Analytic, Frankfurt) and counted in a liquid scintillation counter (LS 5000TD Scintillation Counter, Beckman Instruments) for 10 min each. The percentage of [^{14}C]-oxalate deposited was determined from the equation: % precipitated [^{14}C]-oxalate = 100 – [100 x (cpm at x min/ cpm at 0 min)]. Graphs of this variable, i.e. percentage of precipitated [^{14}C]-oxalate were constructed as a function of time. In order to facilitate interpretation of these graphs, trendlines corresponding to the gradient were computed. These are given on the right side of each curve.

Effect of proteins on CaOx crystal aggregation and nucleation

The aggregation assays (described in page on page 35) required slurries of COM crystals. The preparation of these crystals is described first.

Preparation of calcium oxalate monohydrate (COM) crystals

COM crystals were prepared by mixing equal volumes of 100 mM $CaCl_2$ and 100 mM Na_2Ox at a constant speed of 1 ml/min using a peristaltic pump (Pak *et al.* 1975). The mixture was stirred at 4 °C for 7 days. The precipitated crystals were separated by filtration through 0.22 µm filter paper, washed with distilled water followed by methanol and then dried at 95 °C for 1 hr.

XRD analysis

The crystals were analyzed by x-ray powder diffraction (XRD) (Phillips PW1050/25 goniometer) to ascertain whether they were COM or not. The interplanar *d*-spacing of the crystals were compared with the standard reference values for COM crystals (Sutor and Scheidt 1968). Figure 2.3 shows the XRD pattern of the crystals and the intensities. The observed peaks all corresponded to known COM reflections (Table 2.1).

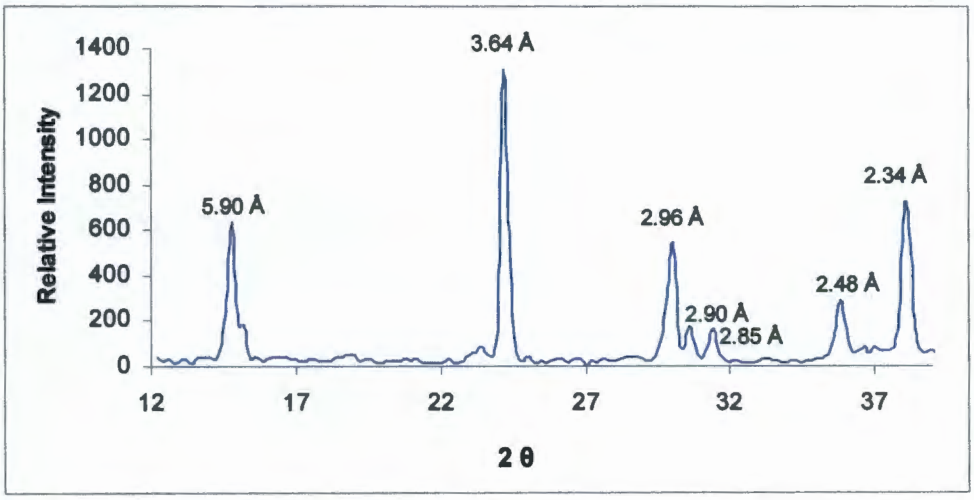


Figure 2.3: XRD pattern of CaOx crystals

Table 2.1: Reference XRD data of COM crystals together with the *d*-spacings (Å) of prominent peaks observed experimentally

Standard *d*-spacing and relative intensity data for COM		Present study
***d*-spacing (Å)**	**Relative intensity**	***d*-spacing (Å)**
5.93	100	5.90
5.79	25	
4.64	7	
4.52	6	
3.78 }	13	
3.76		
3.65	100	3.64
3.00	10	
2.97	46	2.96
2.91	12	2.90
2.89	10	
2.84	14	2.85
2.51	2	
2.48	30	2.48
2.41	5	
2.37	2	
2.34	90	2.34

Aggregation assay

Crystal aggregation was determined using the method developed by Hess *et al.* (1989). A crystal slurry (0.8 mg/ml) was prepared by adding COM crystals (as prepared above) to a solution of 10 mM tris(hydroxymethyl)-aminomethane (Tris)HCl buffer, pH 7.2, containing 90 mM NaCl. The slurry was equilibrated with constant stirring at 1100 rpm for 16 hr in a glass vial at 37 °C. After equilibration of the slurry, it was mixed with proteins from both race groups in a 10 mm glass cuvette and incubated at 37 °C for 2 hr with stirring at 1100 rpm. Using a spectrophotometer (Specord 40, Analytikjena, Germany) the absorbance was monitored at 620 nm and 37 °C for 15 min. The solution was stirred at 1000 rpm (corresponding to the maximum speed of the instrument) until equilibrium had been reached, which was demonstrated by a stable absorbance. Upon reaching equilibrium, stirring was stopped to induce aggregation. The sedimentation rate overtime (indicated by the "absorbance slope" in Figure 2.4) was measured which corresponded to the maximum rate of decrease in absorbance. A typical plot of such an experiment is shown in Figure 2.4. The percentage of crystal aggregation in the presence of proteins was calculated as $(T_s/T_{sc}) \times 100$ where T_{sc} is the turbidity slope of the control without proteins and T_S is the slope of the protein-dosed slurry. The inhibition of crystal aggregation (I_A) data was calculated as 100 % - percent aggregation (Hess *et al.* 1989).

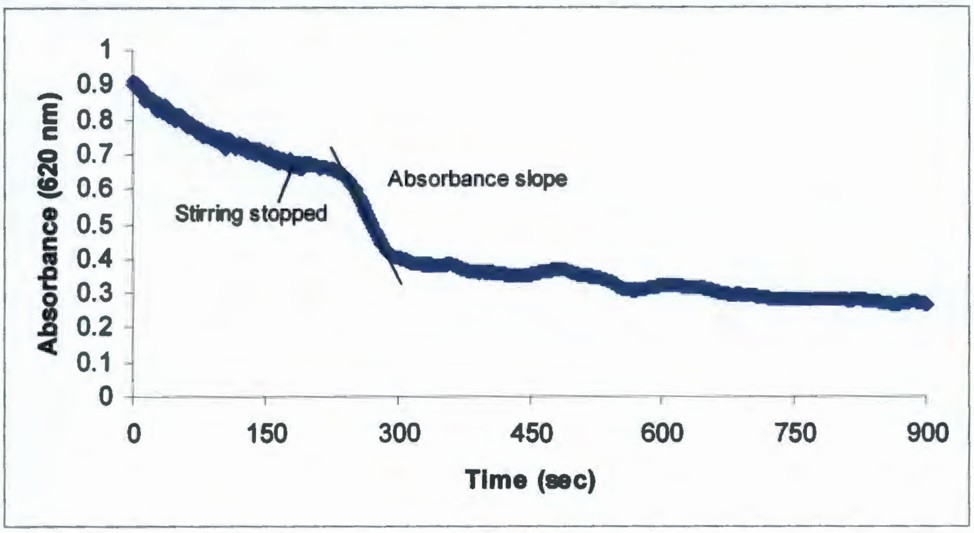


Figure 2.4: A typical plot of absorbance vs time from which sedimentation rate was determined

Nucleation assay

The method described by Hess *et al.* (1995) was used. Stock solutions of 8.5 mM $CaCl_2$ and 1 mM Na_2Ox, each containing 200 mM NaCl and 10 mM sodium acetate, pH 5.70, were prepared. The solutions were filtered (0.22 μm) before each experiment. Protein was added to solutions of calcium and oxalate in the volume ratio of 2:1:2 ($CaCl_2$: protein: Na_2Ox). The mixture was equilibrated at 37 °C with continuous stirring at 500 rpm. The absorbance at 620 nm was measured every 0.5 sec for 60 min at 37 °C using a spectrophotometer (Specord 40, Analytikjena, Germany). Figure 2.5 shows an example of a typical plot of absorbance versus time for this experiment. The percent nucleation inhibition induced by the proteins was determined from the equation:

% Nucleation inhibition (I_n) = $[1-T_{sc}/T_s] \times 100$ where T_{sc}= turbidity slope of the protein sample and T_s= slope of the control without the protein.

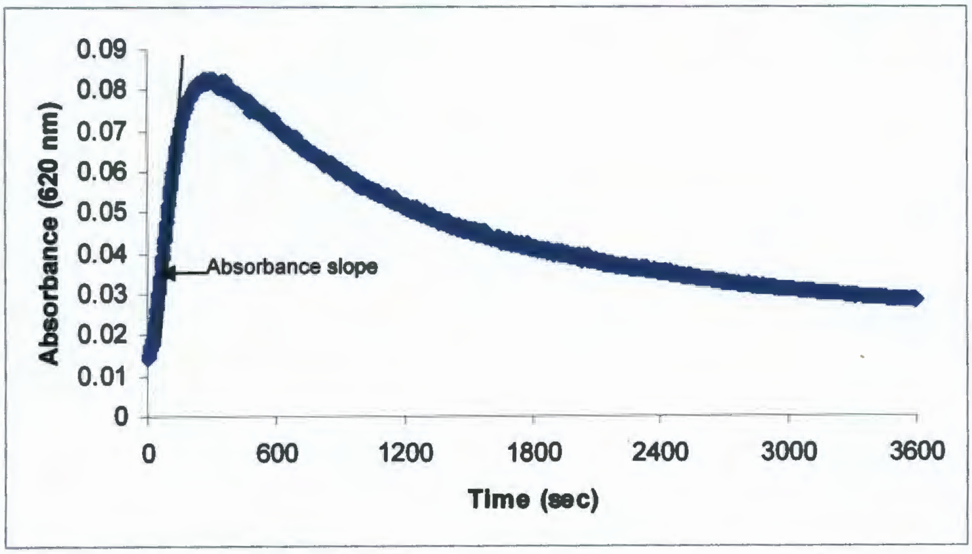


Figure 2.5: A typical plot of absorbance vs time for the nucleation experiment

Effect of proteins on particle formation kinetics

A Na_2Ox load, 30 µM in excess of the previously determined MSL, was added to each of the three flasks, each contained 50 ml WUF or BUF. Proteins from both race groups were added to two of these flasks as shown in the cross over design (Figure 2.2).

The urines were incubated at 37 °C in a shaking water bath (100 rpm) for 120 min and the effect of the proteins on the particle volume-size distribution, particle number and particle volume was measured at 30 min time intervals for 120 min using a Coulter Multisizer II (140 µm orifice, 2.8 - 90.0 µm particle size range, 256 channels). Graphs of particle number and volume were plotted as a function of time. In order to facilitate interpretation of these graphs, gradients corresponding to the first 30 min of the crystallization processes were computed. Modes are reported for the particle size distribution curves.

Scanning electron microscopy

At the completion of the kinetics experiments described above, 2 ml of urine from each flask was passed through a 0.22 µm filter to collect the deposited crystals. The filter paper was glued onto an aluminium stub for examination by scanning electronic microscopy. The crystals were then splutter-coated for 10 min with 3 - 5 nm of Au/Pd (Biorad, SEM coating system) and viewed using a Leica S440 scanning electron microscope (Leica Cambridge Ltd , England) operating at an accelerating voltage of 10 kV, at a working distance of 10 - 15 mm and under a probe current of 20 - 30 pA.

2.2.3 Characterization of proteins

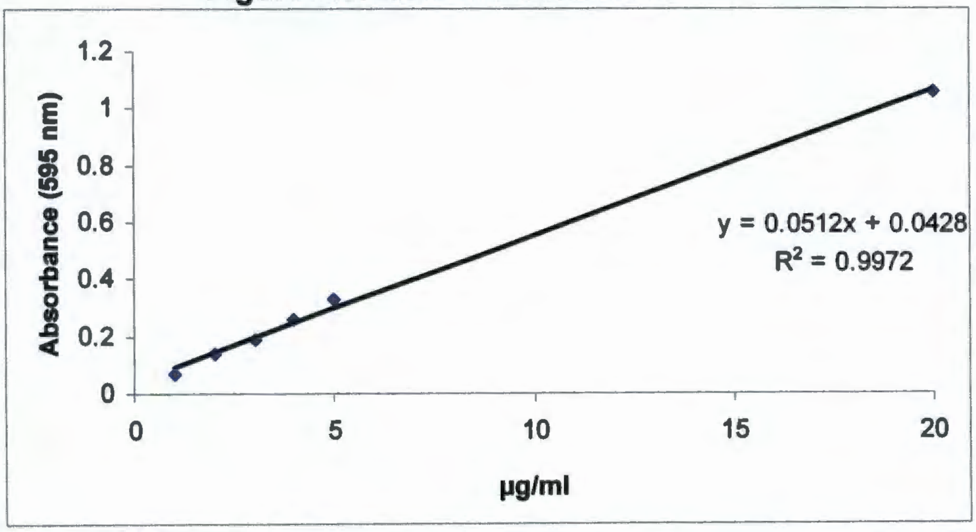
Determination of urinary protein concentration

In the experiments involving protein precipitates (Chapters 4 and 5). The Bradford assay method was used to measure the protein concentration (Bradford 1976). For the construction of a standard curve, duplicate samples of bovine serum albumin (BSA) were prepared in the concentration range 1 µg/ml - 20 µg/ml. Each sample contained 800 µL protein and 200 µL protein assay solution (BioRad). The mixtures were incubated for 5 min in eppendorf tubes. The absorbance was then measured at 595 nm (UV-vis Spectrophotometer, Anthelie) in disposable plastic cuvettes. The absorbance of BSA measured at different concentrations using the Bradford protein assay are presented in Table 2.2. The standard curve corresponding to these results is shown in Figure 2.6.

Table 2.2: BSA standard curve data

Concentration (µg/ml)	Average Absorbance (595 nm)	± SE
1	0.07	0.0032
2	0.14	0.0152
3	0.19	0.0021
4	0.26	0.0089
5	0.33	0.0059
20	1.06	0.0023

Figure 2.6: BSA standard curve



Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Using BioRad Mini-Protean II apparatus (BioRad Laboratories, Hercules, California, USA), the protein precipitate from each population group was analysed by 10 % SDS-PAGE (Methods in Appendix 1).

Samples containing 0.5 mg proteins were diluted in 15 µL distilled water and 1.5 µL 5 x SDS reduction buffer. The samples were boiled for five minutes and were allowed to cool down prior to electrophoresis. A low range molecular weight marker was included (Bio-Rad). Proteins were resolved on 10 % polyacrylamide mini gels by electrophoresing at 25 mA per gel until the dye-front had run off the gel. Protein bands were detected by staining with Coomassie Blue (0.05 % Coomassie Blue, 50 % methanol and 7 % acetic acid) for 1 hr, after which the background was destained (25 % ethanol and 7 % acetic acid) for approximately 30 min.

2.3 Results

Since urinary compositions and CaOx metastable limits were measured in all of the different studies in this thesis, results for these determinations are reported here. Results for specific studies are presented in the relevant chapters which follow.

2.3.1 Urine composition

Table 2.3 shows the average composition values and physicochemical parameters obtained for 20 pools of urine, each pool consisting of five urine samples from black and white subjects. The data for each pooled urine sample is shown in Appendix 1 [Black subjects: Tables A1.1, White subjects: Table A1.2 for commercial bikunin experiments], Appendix 2 [Black subjects: Tables A2.1, White subjects: Table A2.2 protein precipitate experiments], Appendix 3 [Black subjects: Tables A3.1, White subjects: Table A3.2 for isolated bikunin experiments] and Appendix 4 [Black subjects: Tables A4.1, White subjects: Table A4.2 for bikunin deglycosylation experiments].

Table 2.3: Average composition and physicochemical parameters of pooled urine samples from black (B) white (W) subjects

Variables	B	W	p-values
pH	6.10 ± 0.07	6.17 ± 0.07	0.484
Volume (ml/24hr)	5978 ± 448	5966 ± 448	0.984
Calcium (mmol/24hr)	9.09 ± 1.62	16.7 ± 1.62	0.003*
Citrate (mmol/24hr)	15.5 ± 1.9	16.7 ± 1.9	0.676
Chloride (mmol/24hr)	556 ± 35	580 ± 35	0.629
Creatinine (mmol/24hr)	59.1 ± 3.7	64.6 ± 3.7	0.304
Magnesium (mmol/24hr)	9.47 ± 1.20	12.38 ± 1.20	0.095
Oxalate (mmol/24hr)	1.20 ± 0.14	1.16 ± 0.14	0.821
Phosphate (mmol/24hr)	78.1 ± 6.64	101.7 ± 6.64	0.016*
Potassium (mmol/24hr)	117 ± 26	229 ± 26	0.004*
Sodium (mmol/24hr)	474 ± 126	617 ± 126	0.043*
Sulphate (mmol/24hr)	69.7 ± 5.7	77.8 ± 5.7	0.318
Uric acid (mmol/24hr)	12.4 ± 0.9	14.5 ± 0.9	0.093
RS Brushite	0.333 ±0.094	0.757 ± 0.094	0.0029*
RS CaOx	12.8 ± 14.2	44.1 ± 14.2	0.127
RS Uric acid	1.60 ± 0.30	1.52 ± 0.30	0.836

**Difference is statistically significant: $p \leq 0.05$. RS: relative supersaturation*

2.3.2 CaOx metastable limit

Figure 2.7 gives mean plots for the determination of the CaOx MSL in 20 pooled urine from black (Figure 2.7.1) and 18 pooled urine from white (Figure 2.7.2) subjects. These graphs show that the metastable limit in the urine of black subjects is higher than that in white subjects i.e. 0.075 vs 0.045 $mol.dm^{-3}$ respectively. The data and plots for each pooled urine sample are shown in Appendix 1 [Black subjects: Tables B1.1 and Figures A1.1, White subjects: Tables B1.2 and Figures A1.2 for commercial bikunin experiments], Appendix 2 [Black subjects: Tables B2.1 and Figures A2.1, White subjects: Tables B2.2 and Figures A2.2 for protein precipitate experiments], Appendix 3 [Black subjects: Tables B3.1 and Figures A3.1, White subjects: Tables B3.2 and Figures A3.2 for isolated bikunin experiments] and Appendix 4 [Black subjects: Tables D4.1 and Figures E4.1, White subjects: Tables D4.2 and Figures E4.2 for bikunin deglycosylation experiments].

Figure 2.7.1: Mean plot from black subjects

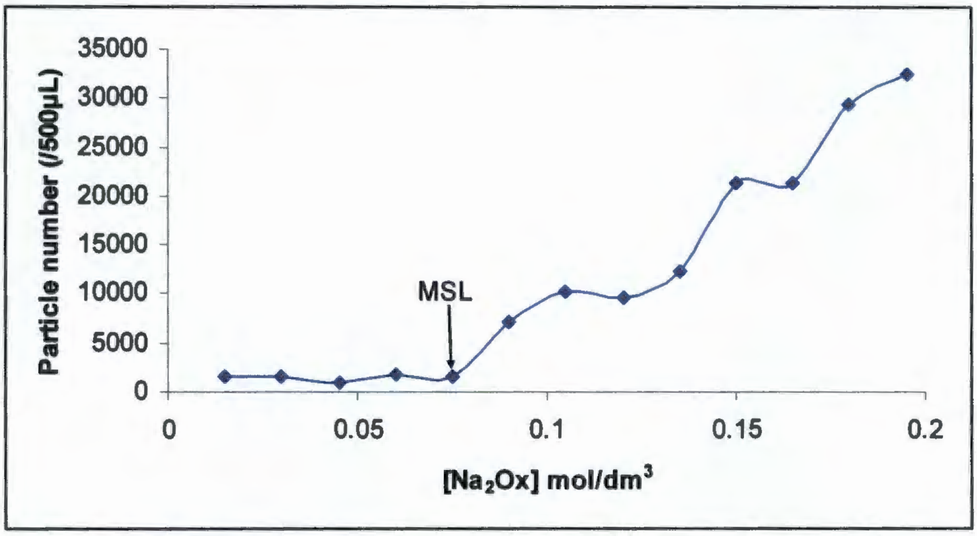
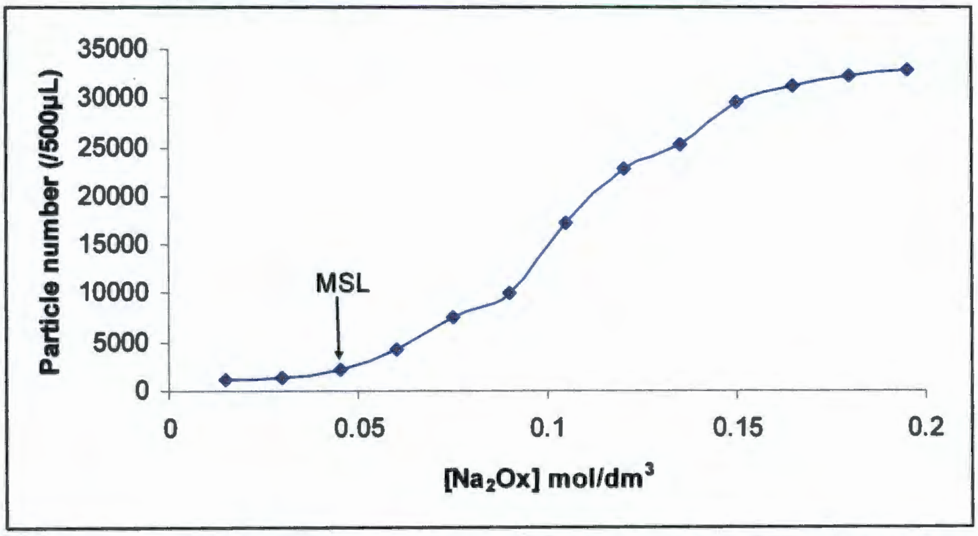


Figure 2.7.2: Mean plot from white subjects



2.3.3 Crystallization assays

Results for the various crystallization experiments involving the different forms of bikunin are presented in Chapters 3, 4, 5 and 7.

2.4 Discussion

The results for the 24 hr urine composition analysis (Table 2.3) showed that calcium is significantly lower in black subjects' urine. This confirms previous reports that the South African black race group has a significantly lower urinary calcium concentration than the white group (Whalley *et al.* 1998; Lewandowski *et al.* 2002). Other differences were also observed in the urine chemistry of black and white subjects in the present study. For example, phosphate and potassium were significantly lower in black subjects' urine. These differences were also observed in another study (Lewandowski *et al.* 2002). While the lower calcium concentration in black subjects is noteworthy, values nevertheless lie in the normal range. Thus the observed urinary differences do not explain the low occurrence of stones in the South African black population. Contrary to what has been reported in another study where it was demonstrated that citrate is significantly lower in black South African subjects (Lewandowski *et al.* 2002), the present study did not observe any differences between the urines of the two race groups with respect to this parameter.

Sodium was also found to be significantly lower in black subjects' urine in the present study. Contrary to this, Modlin (1968) reported a higher sodium excretion in blacks and suggested that sodium can act as an inhibitor of calcification by increasing the solubility of calcium.

Surprisingly, the relative supersaturation of CaOx in the two race groups was not statistically significant. As such, it might have been expected to be lower in the urine of black subjects because of the rarity of stones in this population group. Relative supersaturation is the prerequisite for the occurrence of crystallization in urine. Other studies have also not identified any differences in this important physicochemical property, thereby demonstrating that routine urine parameters alone are unlikely to provide an explanation for the rarity of stones in the black population (Lewandowski *et al.* 2002; Webber *et al.* 2003). Notwithstanding this point, the relative supersaturation of brushite in the present study was significantly lower in black subjects. This is interesting because brushite has been mooted as an initiating nidus in CaOx stone formation (Pak *et al.* 1984).

Several previous studies have indicated that proteins do not have an effect of the CaOx metastable limit (Grover *et al.* 1990, 1994; Ryall *et al.* 1995; Edyvane *et al.* 1997). As such, the present study simply compared the metastable limit of the two race groups without addition of proteins. These determinations showed that the MSL in black subjects in higher than in white subjects, thereby confirming that inducing CaOx crystallization in the urines of the former group is more difficult to achieve. These results are in agreement with the lower occurrence of urolithiasis in this group (Modlin 1968; Whalley *et al.* 1998; Rodgers *et al.* 2006).

Chapter 3

Inhibitory Activity of Commercial Bikunin

3.1 Introduction

As stated in Chapter 1, the primary objectives of the present project are to compare the relative inhibitory capacity of bikunin, isolated and purified from the urine of black and white South African subjects, in a host of different crystallization experiments and also to examine the effect of the urine environment on these capacities. As a strategic starting point, it was decided to develop, test and implement the various assays using commercially available bikunin (CB) rather than bikunin derived from both race groups. Besides enabling the establishment of the assays themselves, this approach allowed investigation of the protein's inhibitory performance in the urines of the respective groups. This chapter describes these experiments.

Objectives

The objective of this initial study was to conduct a variety of crystallization experiments in the urine of black and white South African subjects using commercially available bikunin in order to test the different assays and also to investigate whether the nature of the urine milieu has any influence on the capacity of this protein to inhibit CaOx crystallization processes.

3.2 Methods

3.2.1 Crystallization experiments

Urine collection and treatment

The urine that was used in these particular crystallization experiments was collected and pooled as previously described in Chapter 2.2.1, page 29. Five pools of urine were collected from black subjects and 3 pools from white subjects. The CaOx metastable limit of the urines was determined using a method previously described in Chapter 2.2.1, page 30. To assess the experiments' reproducibility, all the crystallization experiments presented in this chapter were performed in duplicate.

Effect of Commercial bikunin CB on [^{14}C]-oxalate deposition

The [^{14}C]-oxalate deposition experiments were carried out as described in Chapter 2.2.2, page 32. "CB1" and "CB2" refer to a duplicate experiments using commercially available bikunin.

Effect of CB on crystal aggregation

The spectrophotometer-based assay described in Chapter 2.2.2, page 35 was used to study the effect of the UF and CB on COM crystal aggregation in UF from the two race groups. The combinations of COM+CB, COM+BUF, COM+WUF, COM+BUF+CB and COM+WUF+CB were investigated.

Effect of CB on crystal nucleation

CaOx nucleation was studied in the absence and presence of CB as follows: C+CB, C+BUF, C+WUF, C+BUF+CB and C+WUF+CB where C is the control ($CaCl_2$ + Na_2Ox containing solutions). The method is explained in detail in Chapter 2.2.2, page 36.

Effect of CB on particle formation kinetics

Particle number-volume-size distribution

A Coulter Counter was used to determine the particle number-volume-size distributions in BUF and WUF before and after dosing with CB at a final concentration of 0.5 mg/L using the method previously described in Chapter 2.2.2, page 37. SEM was used to view the crystals precipitated as described in Chapter 2.2.2, page 37.

3.3 Results

3.3.1 Crystallization experiments

Effect of CB on [^{14}C]-oxalate deposition

Two unambiguous, reproducible results emerged form these experiments. Firstly, the total amount of CaOx crystals deposited after 90-120 min was significantly reduced when CB was added. In BUF, the reduction was 6 % while in WUF the reduction was 15 %. Secondly, the rate of CaOx deposition (as indicated by the gradient of the respective kinetics graphs) was also significantly reduced by the addition of CB. The magnitude of this effect was again greater in WUF. Typical plots are given Figure 3.1.1 and 3.1.2. The data for each of the five experiments is shown in Appendix 1 (Black subjects: Table C1.1 and Figure B1.1; White subjects: Table C1.2 and Figure B1.2). CB reduced [^{14}C]-oxalate deposition as demonstrated in Figure 3.1.

Figure 3.1.1

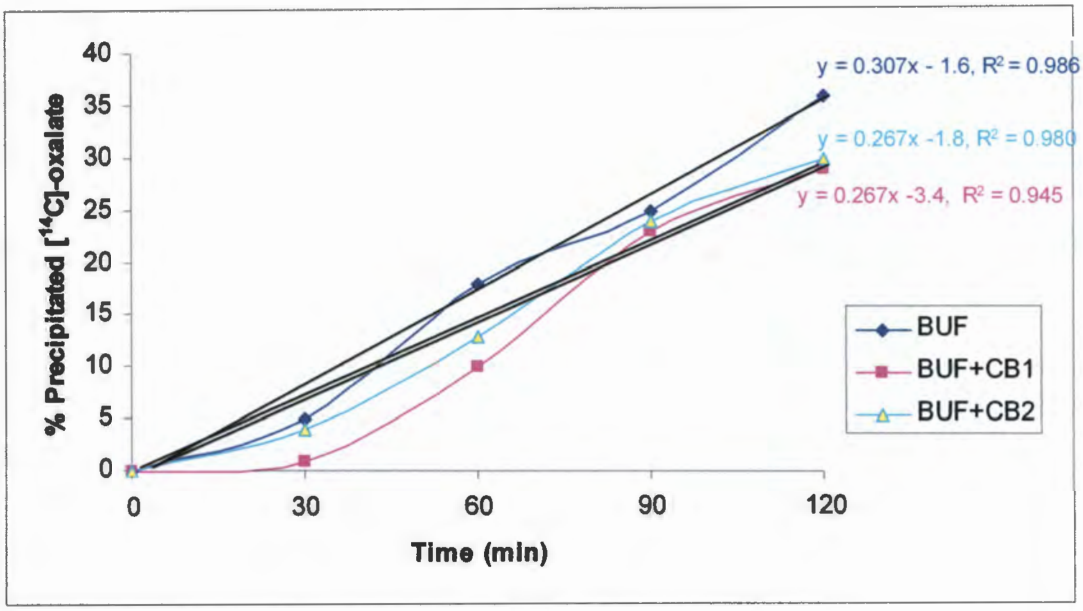


Figure 3.1.2

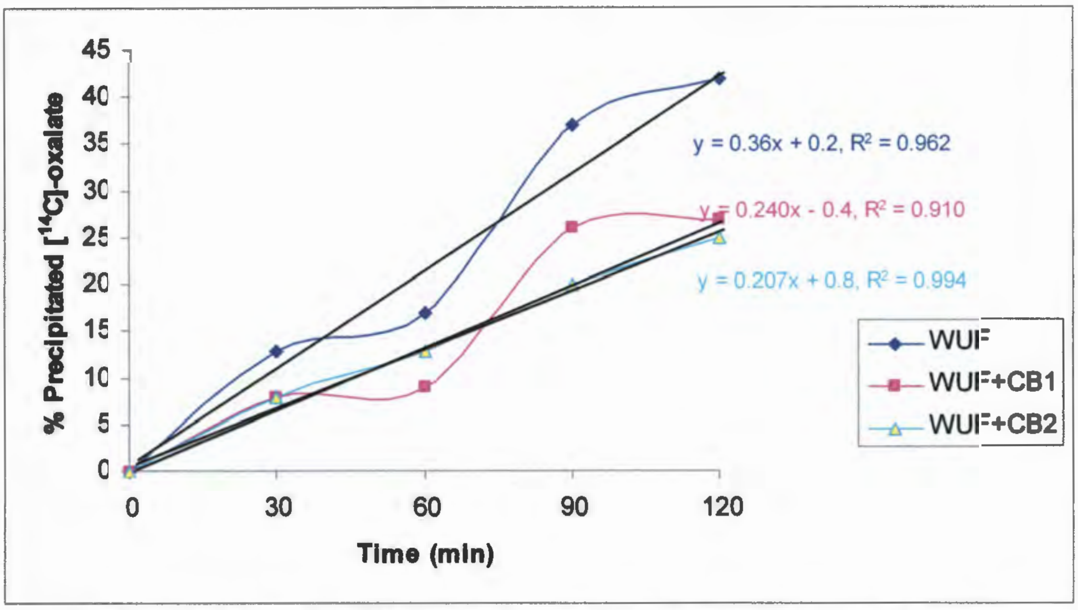


Figure 3.1: A typical plot of [^{14}C] - oxalate experiments of BUF (Figure 3.1.1) and WUF (Figure 3.1.2) urine before and after addition CB

Comments:

The results in these experiments show that CB inhibits the deposition of CaOx crystals in the urine of both race groups and that the decrease in WUF was greater than in BUF. Since differences in the chemical composition of WUF and BUF have already been highlighted, the [^{14}C]-deposition experiments have demonstrated that the nature of the urine milieu affects the capacity of CB to moderate the amount of CaOx deposition as well as its rate of formation.

Effect of CB on crystal aggregation

In all of these experiments, the addition of CB to a slurry of COM crystals inhibited aggregation significantly (range 85-97 %, mean 91 %) $p<0.05$. Table 3.1 gives the actual percentage inhibition values obtained in five experiments while the mean plot for these in shown in Figure 3.2. The plots for each experiment are shown in Appendix 1 (Figure C1.1).

Table 3.1: Absorbance slopes of COM crystal slurries before and after addition of CB and percent inhibition of aggregation (% I_A)

Sample	Absorbance slope ($\times10^{-5}$ s^{-1}) (R^2) ± SE	% I_A
COM (n=5)	-519 (0.998) ± 31.2	
COM+CB1	-76 (0.977)	85
COM+CB2	-61 (0.975)	88
COM+CB3	-30 (0.921)	94
COM+ CB4	-40 (0.881)	92
COM+ CB5	-14 (0.964)	97
Average: COM+CB	**-44 (0.943) ± 11.0**	**91**

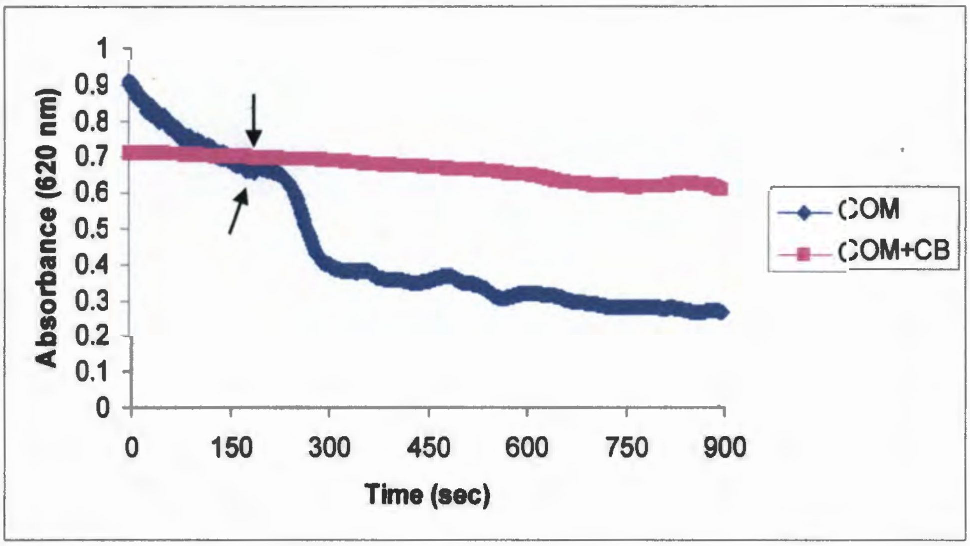


Figure 3.2: A typical plot of absorbance vs time of COM slurry before and after addition of CB. Arrows indicate the time at which stirring was stopped to induce crystal aggregation

Recognizing that urine is the chemical medium in which *in vivo* crystallization process occur, the effect on aggregation of CB in combination with UF was also tested. Average absorbance slopes and percent inhibition are shown in Table 3.2. The data and plot for each of these experiments are given in Appendix 1 (Black subjects: Table D1.1 and Figure D1.2; White subjects: Table D1.2 and Figure D1.3).

Table 3.2 shows that the mean percent inhibition when UF alone was added to the crystal slurry was 47 and 54 % for black and white subjects, respectively. These values increased significantly to 60 % in black subjects when CB was included in the mixture but insignificantly 56 % in white subjects. Figure 3.3 shows a typical plots for the change in absorbance in the presence and absence of CB and BUF (Figure 3.3.1) and WUF (Figure 3.3.2). These plots indicate that the addition of CB greatly reduced the sedimentation rate.

Table 3.2: Absorbance slopes and percent inhibition of aggregation (% I_A).of COM crystal slurries before and after addition of CB and UF

Sample	Absorbance slope ($\times 10^{-5}$ s^{-1}) (R^2)		% I_A		Average
		Duplicate		**Duplicate**	
COM (n=5)	-519 (0.998)				
COM+CB (n=5)	-44 (0.943)	-	**91**	-	**91**
COM+BUF(n=5)	-296 (0.987)	-298 (0.991)	**47**	**47**	**47**
COM+BUF+CB (n=5)	-196 (0.987)	-220 (0.987)	**62**	**58**	**60**
COM+WUF (n=3)	-238 (0.996)	-245 (0.925)	**54**	**53**	**54**
COM+WUF+CB (n=3)	-230 (0.996)	-229 (0.973)	**56**	**56**	**56**

Figure 3.3.1

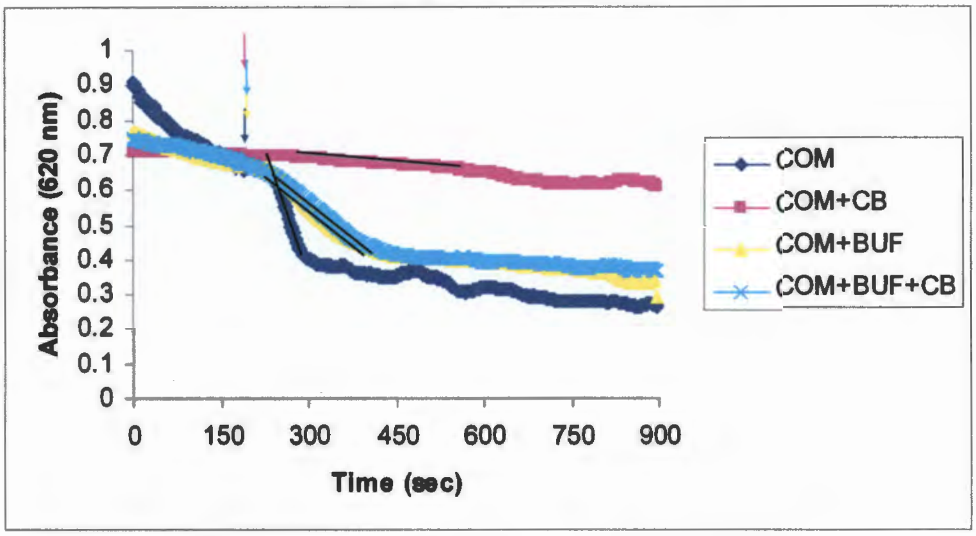


Figure 3.3.2

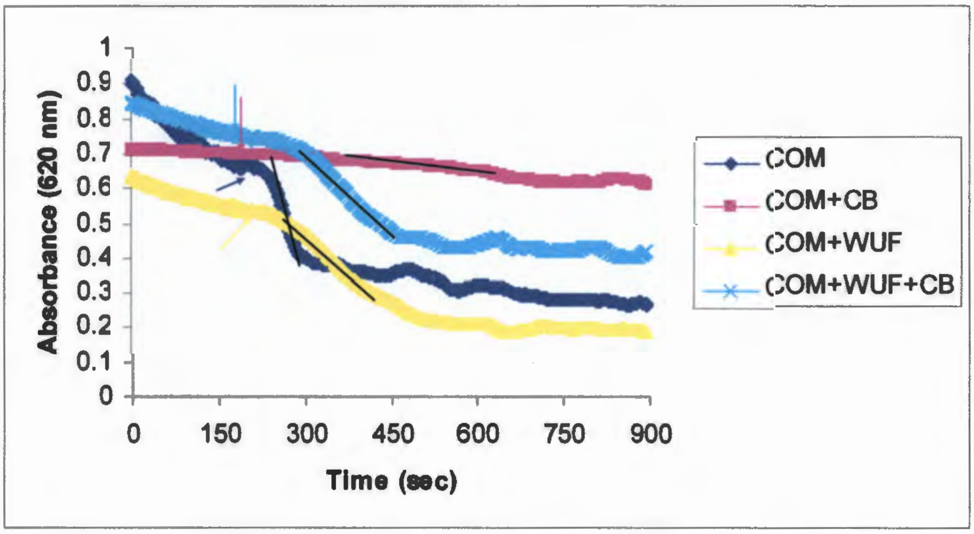


Figure 3.3: Plot of absorbance vs time of the sedimentation experiment before and after addition of CB and UF.

Comments:

It is evident from the aggregation experiments that CB is a strong inhibitor of crystal aggregation. This was demonstrated by a significant percent inhibition of 91 % when CB was added to COM crystals. When comparing the two race groups, superior aggregation inhibition was demonstrated in the urine of black subjects after addition of CB.

Effect of bikunin on nucleation

In these experiments, addition of CB to the control inhibited nucleation of CaOX crystals (range 36-60 %, mean 50 %). Individual values for each of the five experiments are shown in Table 3.3 while a typical plot of absorbance vs time for these experiments is given in Figure 3.4. The data and plots for each experiment are shown in Appendix 1 (Figure E1.1).

Table 3.3: Absorbance vs time and percentage inhibition of nucleation (% I_n) slopes for the control solution ($CaCl_2$ + Na_2Ox) before and after addition of CB

Sample	Absorbance slope ($x10^{-5} s^{-1}$) (R^2) ± SE	% I_n
Control (C) (n=5)	47.1 (0.990) ± 2.75	
C+CB1	30.0 (0.990)	36
C+CB2	19.0 (0.984)	60
C+CB3	27.0 (0.978)	43
C+CB4	20.0 (0.980)	57
C+CB5	21.0 (0.965)	55
Average	**23.4 (0.961) ± 2.15**	**50**

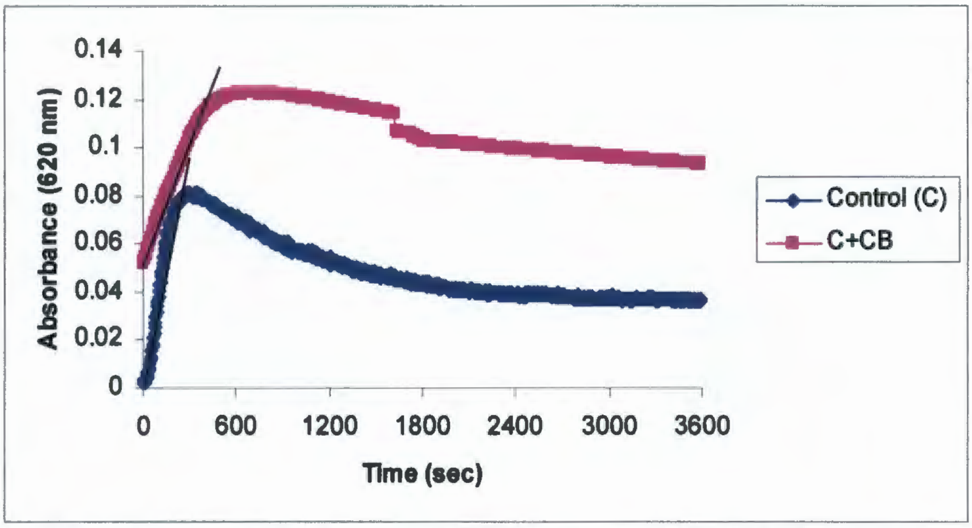


Figure 3.4: Plot of absorbance vs time of nucleation experiment before and after addition of CB

Table 3.4 shows that when ultrafiltered urine was added, inhibition of nucleation increased dramatically to values ≥ 90 %, irrespective of whether CB was included or not ($p<0.05$). The data and plots for each of these experiments are shown in Appendix 1 (Black subjects: Table E1.1 and Figure E1.2; White subjects: Table E1.2 and Figure E1.3). Typical plots of absorbance vs time for these experiments are shown in Figure 3.8.

Table 3.4: Absorbance vs time slopes and percentage inhibition of nucleation (% I_n) for the control solution ($CaCl_2$ + Na_2Ox) after addition of CB, BUF and WUF

Sample	Absorbance slope ($x10^{-5} s^{-1}$) (R^2)		% I_n		Average
		Duplicate		**Duplicate**	
Control (C) (n=5)	47.1 (0.990)				
C+CB (n=5)	23.4 (0.961)	-	**50**	-	**50**
C+BUF (n=5)	3.29 (0.972)	3.01 (0.901)	**93**	**94**	**94**
C+BUF+CB (n=5)	2.80 (0.971)	5.42 (0.988)	**92**	**89**	**90**
C+WUF (n=3)	3.01 (0.965)	2.80 (0.988)	**94**	**94**	**94**
C+WUF+CB (n=3)	2.70 (0.979)	2.00 (0.899)	**92**	**96**	**94**

Figure 3.5.1

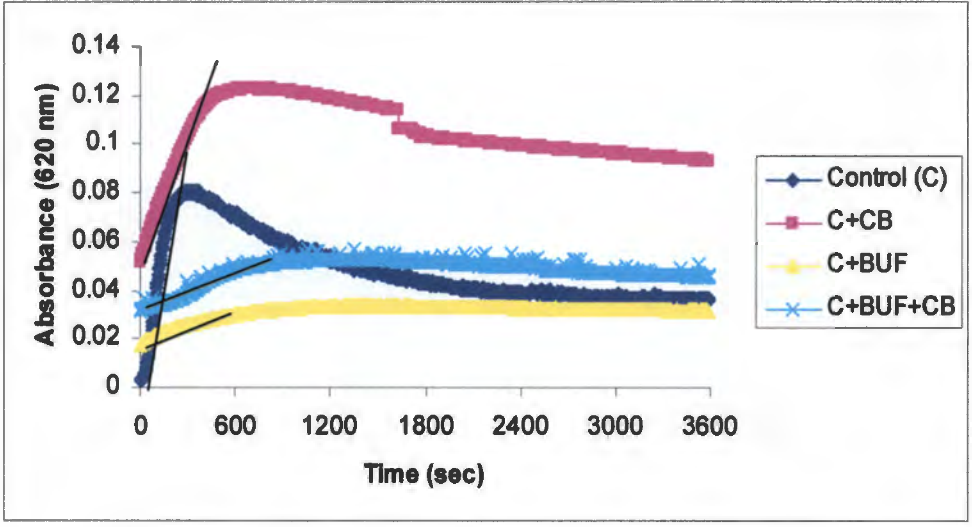


Figure 3.5.2

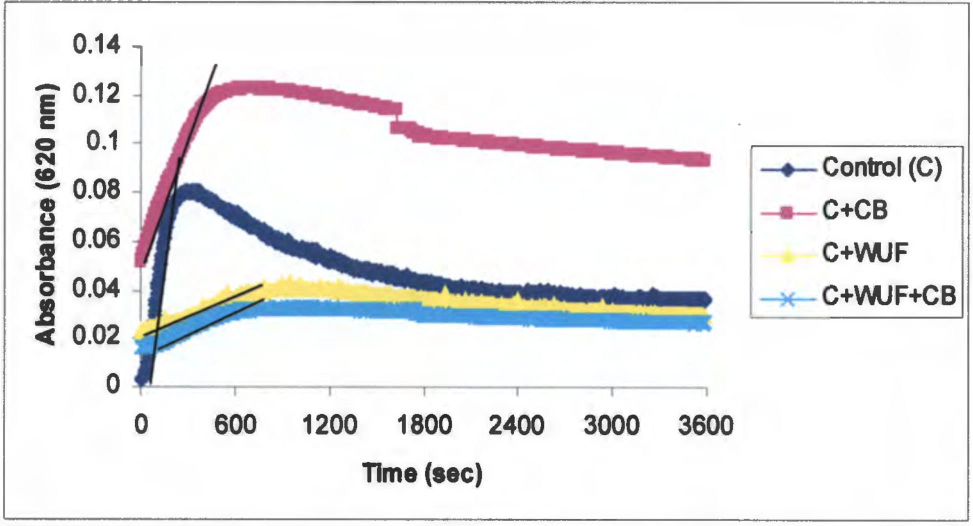


Figure 3.5: Average plot of absorbance vs time for the nucleation experiment before and after addition of CB , BUF (Figure 3.5.1) and WUF (Figure 3.5.2)

Comments:

The results of these experiments are highly interesting because, although CB was shown to be on inhibition of nucleation, ultrafiltered urine alone was far superior in this regard. Moreover, the addition of CB together with UF did not produce an additional effect. Thus it can be speculated that *in vivo*, CB has little effect, if any, on CaOx nucleation.

Effect of CB on particle formation kinetics

Particle size distribution

Particle size distribution curves were unimodal in four of five BUF samples and bimodal in all three of the WUF samples. Modes for these distributions are given in Table 3.5 while typical curves are given in Figure 3.6. Plots for each experiment at 30 min intervals are given in Appendix 1 (Blacks: Figure F1.1, Whites: Figure F1.2). Addition of CB caused a shift in the mean size distribution to smaller value in both BUF and WUF (Table 3.5). However, the bigger mode in WUF was not significantly affected.

Table 3.5: Particle size (µm) of ultrafiltered urine from black (BUF) and white (WUF) subjects before and after addition of CB at final concentrations of 0.5 mg/L

	BUF	BUF+CB1	BUF+CB2
Experiment 1	5.4 and 16.0	5.46 and 17.2	3.28 and 17.1
Experiment 2*	6.9	4.7	5.8
Experiment 3*	5.8	5.5	5.1
Experiment 4*	3.6	3.3	-
Experiment 5*	6.0	2.0	-
	WUF	**WUF+CB1**	**WUF+CB2**
Experiment 1*	4.4 and 12.4	3.3 and 16.1	4.1 and 16.7
Experiment 2*	3.6 and 26.3	3.3 and 26.2	2.9 and 24.8
Experiment 3	3.6 and 22.6	4.0 and 22.9	3.9 and 23.2

*Trend showing a decrease
Experiments 4 and 5 were not completed in duplicate

Figure 3.6.1

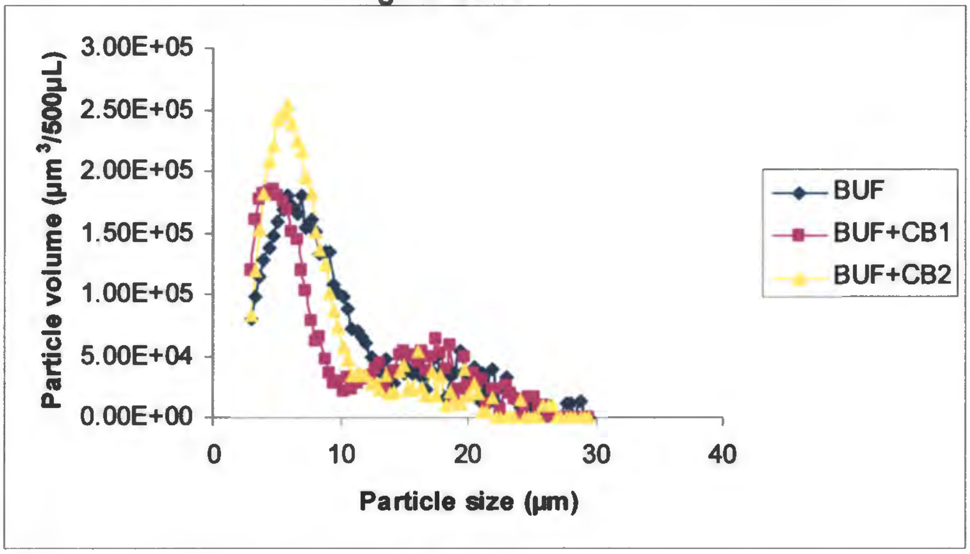


Figure 3.6.2

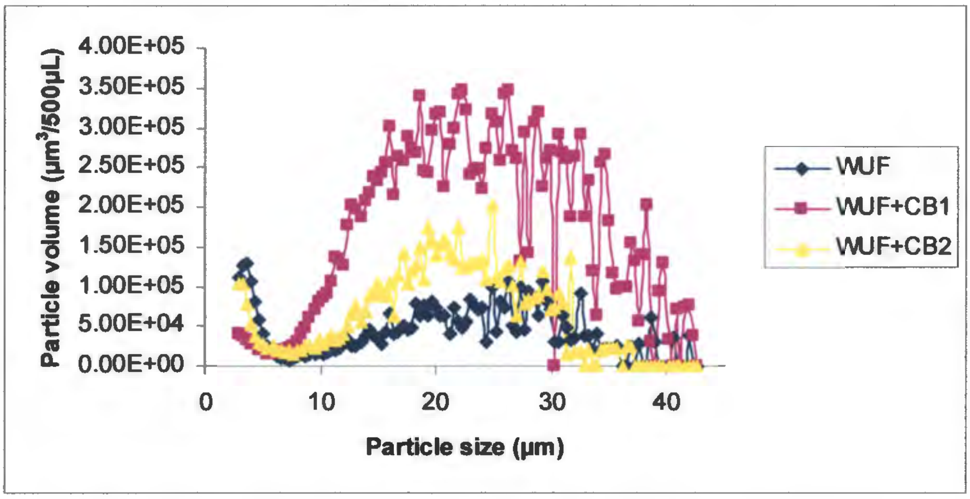


Figure 3.6: Typical particle size (µm) plot of ultrafiltered urine from black (Figure 3.6.1) and white (Figure 3.6.2) subjects before and after addition of CB.

Comments:

The observation of bimodal size distribution in WUF but not in BUF is interesting as it consisted with the notion that larger crystals are synonymous with stone disease. This in turn, is consisted with the higher incidence of stones in the white population. The shift to smaller sizes in the pressure of CB suggests that the protein has the capacity to be an inhibitor of CaOx growth. However, this cannot be conclusive since the shifts in particle sizes were not statistically significant.

SEM

Figure 3.7 shows the SEM micrographs of CaOx crystals precipitated from the BUF and WUF before and after addition of CB. The crystals were all viewed at 4K magnification. As depicted in Figure 3.7.1 crystals in BUF have COM morphology. The particle number reduced slightly after addition of CB and crystals appeared larger in relation to BUF (Figure 3.7.2). Crystallization in WUF demonstrated a predominantly COD morphology although COM crystals were also observed (Figure 3.7.3). Figure 3.4.4 shows that there was a slight decrease in the particle number and accompanied by an increase in the particle size after addition of CB. No morphology change was observed when CB was added to either BUF or WUF, nor was there any evidence of aggregation, both before and after addition of CB.

Figure 3.7.1: BUF

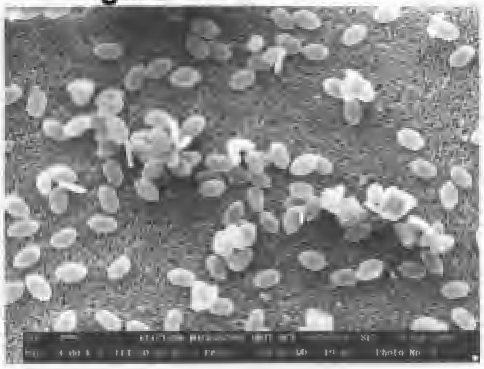


Figure 3.7.2: BUF+CB

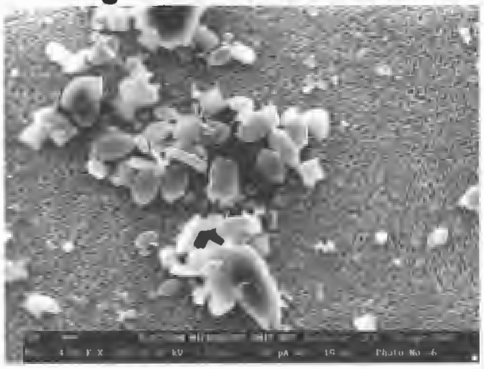


Figure 3.7.3: WUF

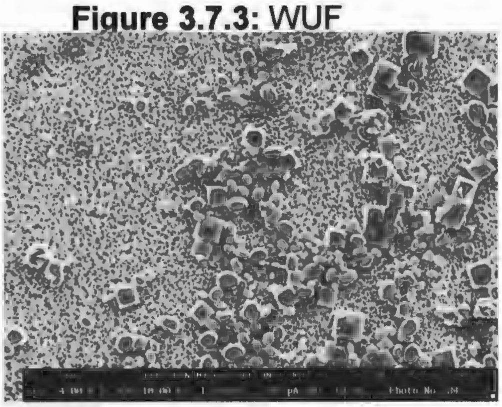


Figure 3.7.4: WUF+CB

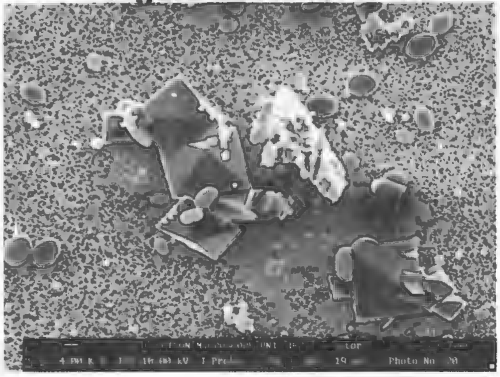


Figure 3.7: SEM of CaOx crystal deposition in BUF before (Figure 3.7.1) and after addition of CB (Figure 3.7.2) and WUF before (Figure 3.7.3) and after addition of CB (Figure 3.7.4).

Comments:

The observation of COM crystals in BUF and COD crystals in WUF is in agreement with previously reported studies (Webber *et al.* 2003). The observation itself is not surprising since COD crystals are known to form at higher calcium concentration (Cerini *et al.* 1999). Indeed, the present study (and others) has shown that white subjects have higher urinary calcium than their counterparts (Chapter 2, Table 2.3). The observation that particle size appeared to increase after addition of CB is in conflict with the (non significant) decrease in size suggested by the distribution curves in Figure 3.6, thereby ruling out the possibility of CB being an inhibitor of CaOx growth.

Particle number

Particle numbers at 120 min generally decreased in both BUF and WUF when CB was added, although increases were observed in some experiments. Initial particle formation kinetics, as determined by the gradients during the first 30 min, also showed decreases after addition of CB. Typical kinetic plots of particle number vs time are presented in Figure 3.8. Linear expressions for the gradients in the first 30 min are given on the right hand side of each curve. The data and plots for the each experiment are shown in Appendix 1 (Black subjects: Table F1.1; Figure G1.1, White subjects: Table F1.2; Figure G1.2).

Figure 3.8.1

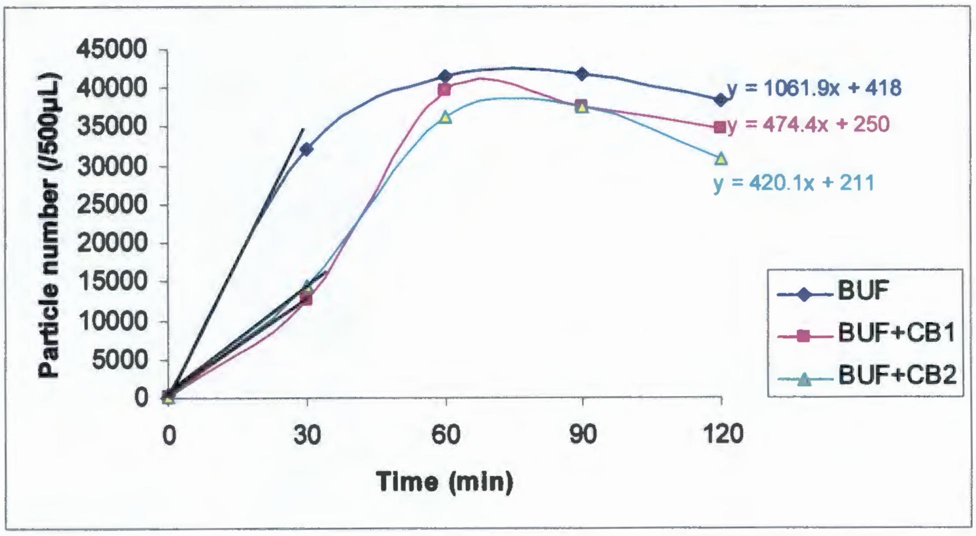


Figure 3.8.2

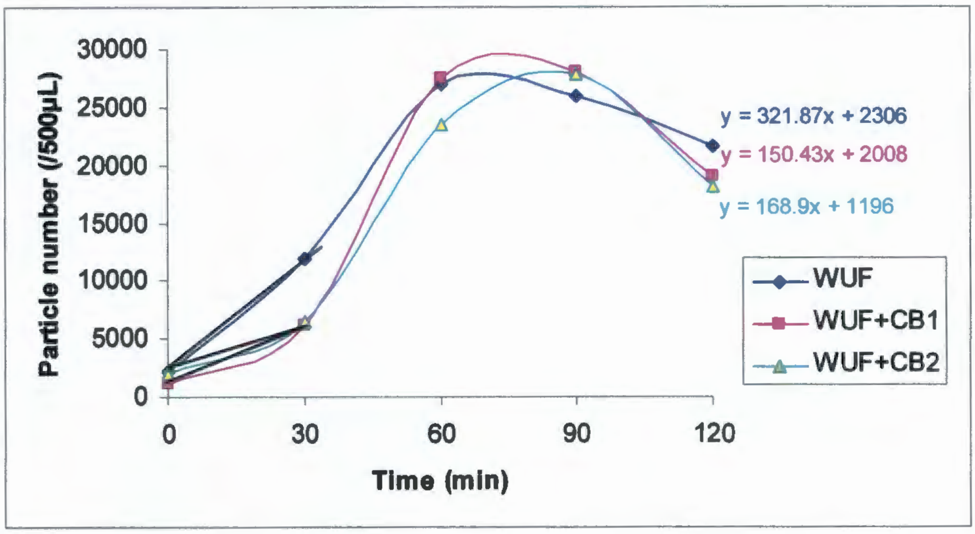


Figure 3.8: Plots of particle number in BUF (Figure 3.8.1) and WUF (Figure 3.8.2) before and after addition of CB at a final concentration of 0.5 mg/L. The linear expressions for the first 30 min (BUF) and 30 min (WUF) are given on the right hand side of each curve.

Comments:

During the particle counting process, the Coulter instrument is unable to distinguish between agglomerate of many small crystals and a single individual crystal. In both cases, a count of “one particle” will be registered. Thus, if agglomeration or disaggregation occurs during the crystallization experiments (as is likely) fluctuations in the particle count will be observed. Indeed, this did occur. As a result, interpretation of the data proved to be difficult. Nevertheless, general observations were possible. Thus, while identical trends for particle numbers and formation kinetics were not observed throughout, most of the experiments showed a decrease in particle number when CB was added to BUF and WUF. This suggests that CB has the capacity to either inhibit nucleation or promote aggregation. The experiments were not able to distinguish between BUF and WUF with regard to their respective roles in the crystallization process.

Particle volume

Three of five experiments showed a decrease in the particle volume when CB was added to BUF. When CB was added to WUF, two of three experiments demonstrated an increase in the particle volume. Figure 3.9 shows typical particle volume plots before and after addition of CB at a final concentration of 0.5 mg/L. The data and plots for each experiment at 30 min intervals are presented in Appendix 1 (Black subjects: Table G1.1, Figure H1.1, White subjects: Table G1.2, Figure H1.2). As with the particle number experiment the gradients were included in order to facilitate the interpretation.

Figure 3.9.1

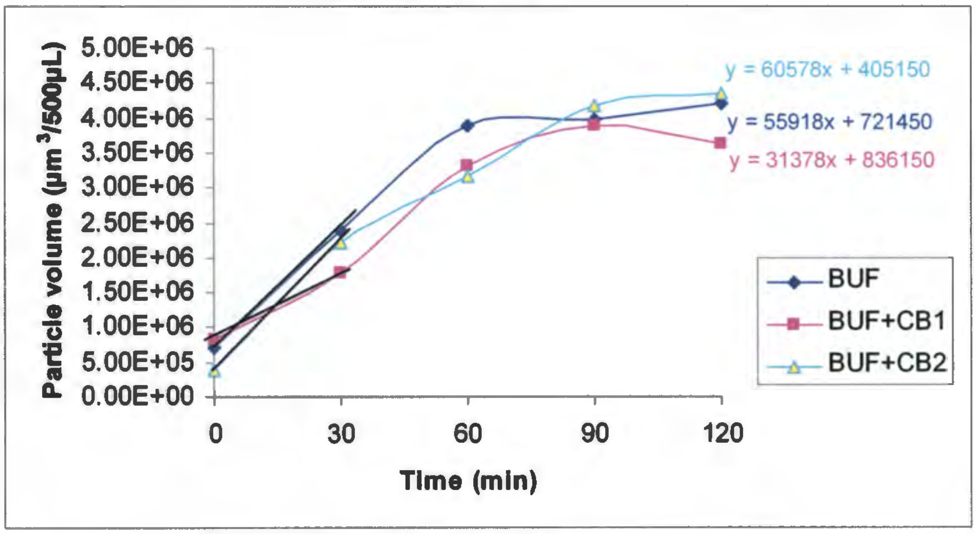


Figure 3.9.2

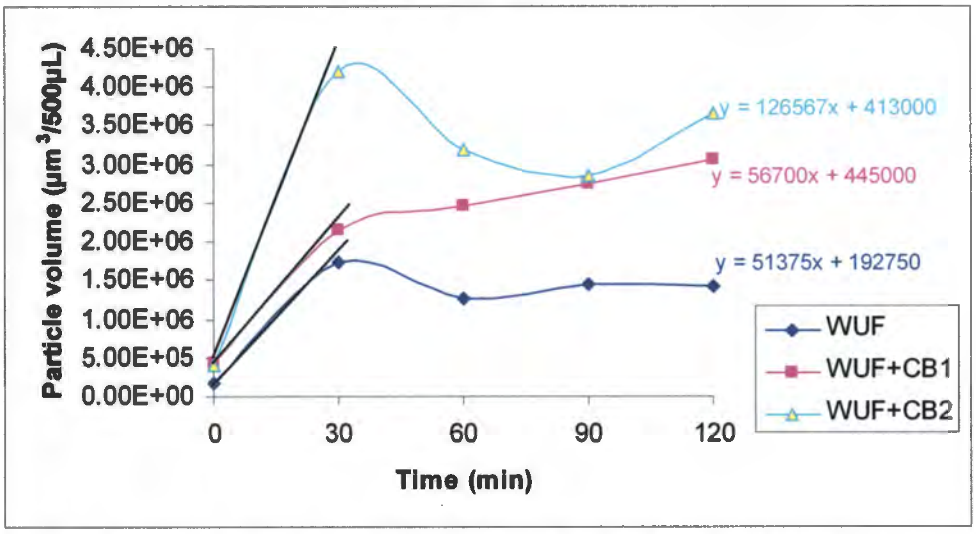


Figure 3.2: Typical plots of particle volume of ultrafiltered urine from BUF (Figure 3.9.1) and WUF (Figure 3.9.2) subjects before and after addition of CB

Comments:

When comparing the effect of CB on the particle volume in the ultrafiltered urines of the two race groups, it was observed that particle volume decreased when CB was added to BUF but increased when added to WUF. It is appropriate at this point to interpret the significance of an increase or decrease in particle volume. An increase in particle volume can occur as a consequence of the following processes.

i) an increase in nucleation
ii) disaggregation of existing clusters
iii) aggregation of existing particles with a concomitant inclusion of air pockets and urine debris.

Conversely, a decrease in particle volume can occur as a consequence of the following processes:

i) a decrease in nucleation
ii) aggregation of existing particles with 100 % efficiency, i.e without the entrapment of air and urine debris

Since the latter (i.e. aggregation with 100 % efficiency) is unlikely to happen, it can be reasonably concluded that a decrease in particle volume occurs as a result of a decrease in nucleation. Thus the effect of CB in BUF can be interpreted in this way. Since CB caused an increased in particle volume in WUF, one of the three processes mentioned above must be occurring. However in the Coulter experiment on particle number (pages 66-68), nucleation was observed to decrease indicating that either of the other two mechanisms is occurring i.e disaggregation of existing clusters or aggregation with entrapment. Since the earlier study described on pages 53-56 showed that CB inhibits aggregation, the volume increase can be attributed to this mechanism.

Regarding the kinetics of particle volume change, Figure 3.9.1 demonstrates that addition of CB to BUF decreased while Figure 3.9.2 showed an increase of the rate in WUF.

3.4 Discussion

As stated in the objectives for this particular set of experiments (page 47), it was decided to test various crystallization assays for their efficacy and practicability. These tests revealed that the [^{14}C]-oxalate deposition experiments, as well as those which investigated CaOx crystal sedimentation (to provide information about aggregation and nucleation mechanism) did indeed satisfy these criteria. In addition, scanning electron microscopy provided very useful visual data. However, as discussed earlier, Coulter Counter data proved to be difficult to interpret. In addition to the limitations of this technique which have already been mentioned, an additional short coming relates to its inability to distinguish between particles and crystals. As such, the data obtained using the Coulter Counter was interpreted as the effect of CB on the "particle" as opposed to "crystals". These particles or crystals could be single or aggregated and may or may not include organic debris. Thus an increase or decrease in the particle number and volume cannot be confidently attributed to the formation of the CaOx crystals. Therefore these experiments had to be supplemented with SEM and [^{14}C]-oxalate deposition experiments since they involve crystal formation. Nevertheless, despite the shortcomings in the Coulter technique, it was decided to retain this assay, recognizing that interpretation would present unique challenges.

Regarding the role of CB in modulating CaOx crystallization mechanisms, sedimentation experiments showed it to be an inhibitor of nucleation was supported by [^{14}C]-oxalate deposition experiments and by Coulter number data, albeit that the latter showed inhibition of particle formation rather than CaOx "crystal" formation. This is in agreement with other *in vitro* studies (Höchstrasser *et. al.* 1984; Atmani *et al.*1999a).

Sedimentation experiments in the present study showed that CB is an inhibitor of CaOx crystal aggregation and this is in agreement with literature *in vitro* studies where it was reported that bikunin is a strong inhibitor of CaOx aggregation (Atmani *et al.* 1999).

Although Coulter size distributions studies indicated that CB may be an inhibitor of CaOx crystal growth because it caused a shift in the mode to smaller sizes, this notion was not supported by SEM which showed an increase in particle size after addition of the protein.

A further objective in this particular study was to investigate whether the different urine milieus associated with BUF and WUF might influence the role of CB. However, prior to discussing the results pertaining to such an effect, attention is drawn to the marked differences between BUF and WUF independent of the presence of CB. Firstly, Coulter size distribution showed that after administration of sodium oxalate loads to initiate crystallization, two size modes occurred in WUF while only one such mode occurred in BUF. This itself is very interesting as Robertson (1968) in his pioneering study using a Coulter Counter reported two modes in stone formers' urine. However, irrespective of Robertson results, the observation of a second mode (in larger size range) in WUF but not in BUF is consistent with stone formation being common in the former group but rare in the latter group. Secondly, SEM demonstrated the formation of COD while COM formed in BUF, after addition of sodium oxalate. This too is interesting because COD crystals have been reported as having a lower capacity for attachment to renal cells (Wesson *et al.* 1998). Since the latter is a risk for stone formation, the observation of COM in BUF is consistent with the lower stone incidence in the black group. Thus fundamental differences exist between BUF and WUF. These differences indeed influence the activity of CB in modulating CaOx crystallization.

Compelling evidence in this regard was provided by the [^{14}C]-oxalate deposition measurements, the sedimentation experiments and the Coulter particle-volume data. The deposition experiments showed that the effect of CB in reducing CaOx formation was greater in BUF; finally Coulter particle volume studies showed that CB inhibits nucleation in BUF and aggregation in WUF. These results clearly show that the nature and chemical composition of the urine influences the performance (and role) of CB with respect to its capacity for modulating CaOx crystallization processes. This effect has not been previously reported for CB.

Chapter 4

Protein Precipitates and their Effects on CaOx Crystallization Processes

4.1 Introduction

As part of the strategic approach for the ultimate study of purified urinary bikunin from black and white subjects, the second series of experiments which were defined for this thesis involved the investigation of the effects of protein precipitates containing bikunin, rather than the protein itself. This chapter describes the preparation of these precipitates and their application in crystallization experiments similar to those in the previous chapter.

Objectives

- To prepare protein precipitates from the urines of healthy black (BPP) and white (WPP) subjects
- To characterize the protein precipitates using SDS-PAGE
- To test and compare the relative inhibitory activities of the protein precipitates in CaOx crystallization experiments as described in Chapter 3, but to include cross-over investigations

4.2 Methods

4.2.1 Urine collection and treatment

Twenty-four hour urine samples were collected tested and pooled as previously described in Chapter 2.2.1, page 30. In addition, for the preparation of protein precipitate, the pooled urine samples were extensively dialyzed against distilled water for 2 days using a 10 kDa cut-off membrane at 4 °C to remove urea, small glycosaminoglycans and peptides.

4.2.2 Protein precipitation

Protein precipitation using ammonium sulphate is a simple and easy method particularly when large volumes of urine are involved, and it is therefore widely used for preparative protein separation (Bell *et al.* 1983; Jiang *et al.* 2004; Yang *et al.* 2005). Proteins are soluble in aqueous solutions but at high salt concentrations most are insoluble. As such they will precipitate out of the salt solution by separating from the solution as a solid. Thus the strategy of protein precipitation using a high ammonium sulphate concentration was employed in the present study to obtain a mixture of proteins from urine.

Precipitation was induced by slow addition of ammonium sulphate (80 % saturation, 516 g/L) to the dialyzed urine with continuous stirring (Bell *et al.* 1983). The suspension was stirred for 1 hr and left to stand overnight at 4 °C. The suspension was then centrifuged for 30 min at 15 000 *g* (Beckman, JA-14). The pellet was washed with 90 % cold acetone, kept on ice for 15 min and centrifuged for another 30 min at 15 000 *g*. The supernatant was then discarded and the precipitate was dissolved in ~50 ml distilled water and dialyzed against distilled water overnight at 4 °C.

4.2.3 Crystallization experiments

Urine collection and CaOx metastable limit

CaOx metastable limits in the collected urines were determined as described in Chapter 2.2.1, page 30. Five pooled of urines were collected from both black and white subjects for the crystallization experiments presented in this chapter.

Effect of BPP and WPP on [^{14}C]-oxalate deposition

The [^{14}C]-oxalate deposition experiments before and after addition of BPP and WPP were carried out as described in Chapter 2.2.2, page 31. However, cross-over experiments, as described in Chapter 2.2.2, page 32 were included

Effect of BPP and WPP on crystal aggregation

The effect of BPP and WPP on the aggregation of COM crystals was determined using a method described in Chapter 2.2.2, page 35.

Effect of BPP and WPP on crystal nucleation

Nucleation assay was conducted in the absence and presence of BPP and WPP as described in Chapter 2.2.2, page 36.

Effect of BPP and WPP on particle formation kinetics

The effect of BPP and WPP on particle size, volume and number was tested as described in Chapter 2.2.2, page 37.

4.2.4 Characterization of protein precipitates

Protein concentration were determined using the Bradford assay as described in Chapter 2.2.3, page 38, while SDS-PAGE was used to identify the presence of bikunin (and other proteins) as described in Chapter 2.2.3, page 39.

4.3 Results

4.3.1 Isolation and characterization of protein precipitates

SDS-PAGE

The proteins precipitated from the urine using ammonium sulphate precipitation were analyzed by SDS-PAGE and stained with Coomassie Blue as shown in Figure 4.1. Figures 4.1.1 and 4.1.2 show the protein precipitates from both black (BPP) and white (WPP) subjects, respectively. The marker is included to estimate the molecular masses of the proteins. 1 mg protein (in 20µL) was loaded for both BPP and WPP. The protein precipitates from both race groups were qualitatively similar and both revealed a prominent band at ~60 kDa. Other protein bands were also observed at ~25, 30, 45 and 120 kDa.

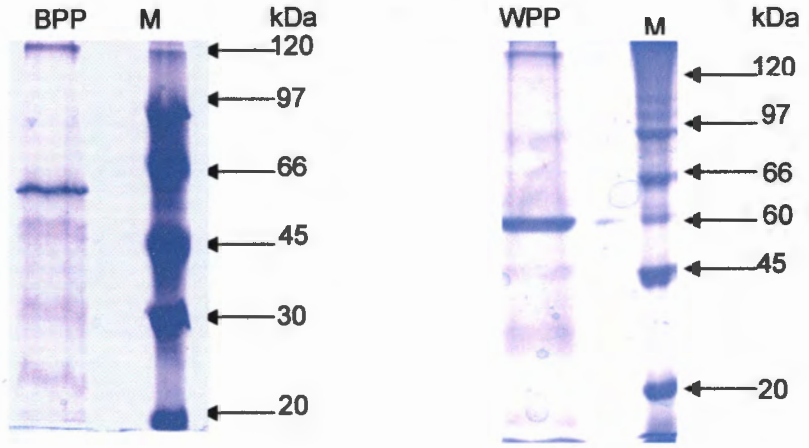


Figure 4.1.1

Figure 4.1.2

Figure 4.1: 10 % SDS-PAGE of proteins precipitated from the urines of black (Figure 4.1.1) and white subjects (Figure 4.1.2). Lane M is the low molecular weight marker.

Protein concentration determination

Table 4.1 shows the total concentration of proteins precipitated from 5 pooled urines from black and white subjects (each pool consisting of a 24 hr urine sample from 5 subjects). A large within-group range was observed in both population groups. The total protein concentration for black and white subjects was found to be 20.20 and 16.10 mg/L, respectively.

Table 4.1: Protein precipitate concentration (mg/L) from black and white subjects' urine

Sample	Protein concentration (mg/L)	Sample	Protein concentration (mg/L)
BPP1	10.00	WPP1	8.80
BPP2	23.25	WPP2	9.62
BPP3	11.60	WPP3	12.50
BPP4	19.20	WPP4	26.41
BPP5	26.02	WPP5	15.30
Average ± SE	**20.20 ± 3.16**	**Average ± SE**	**16.10 ± 3.16**

Comments:

The major band at ~60 kDa observed in the protein precipitate derived from the urine of both black and white subjects is likely to be due to albumin, the second most dominant protein in urine (Grover *et al.* 1998). There were no significant differences ($p>0.05$) observed in the total protein concentration derived from the two race groups. These concentrations all fell within the range reported in the literature (Ebisuno *et al.* 1999; Khan and Kok 2004).

4.3.2 Crystallization experiments

Effect of BPP and WPP on [^{14}C]-oxalate deposition

The results of these experiments showed that addition of the protein precipitate, irrespective of its origin, and irrespective of the urine in which it was tested decreased the percentage of [^{14}C]-oxalate deposition. This is shown in Figures 4.2.1 (BUF) and 4.2.2 (WUF). Of interest was the consistent observation that the protein precipitate performed best, i.e. induced the lowest percent deposition, when it was crossed-over into the ultrafiltered urine of the other race group. Thus WPP produced the lowest percentage deposition in BUF (Figure 4.2.1) while BPP has its most marked effect in WUF (Figure 4.2.2). The data and plots for all of the experiments are shown in Appendix 1 (Black subjects: Tables C2.1 and Figure B2.1; White subjects Tables C2.2 and Figure B2.2).

Figure 4.2.1

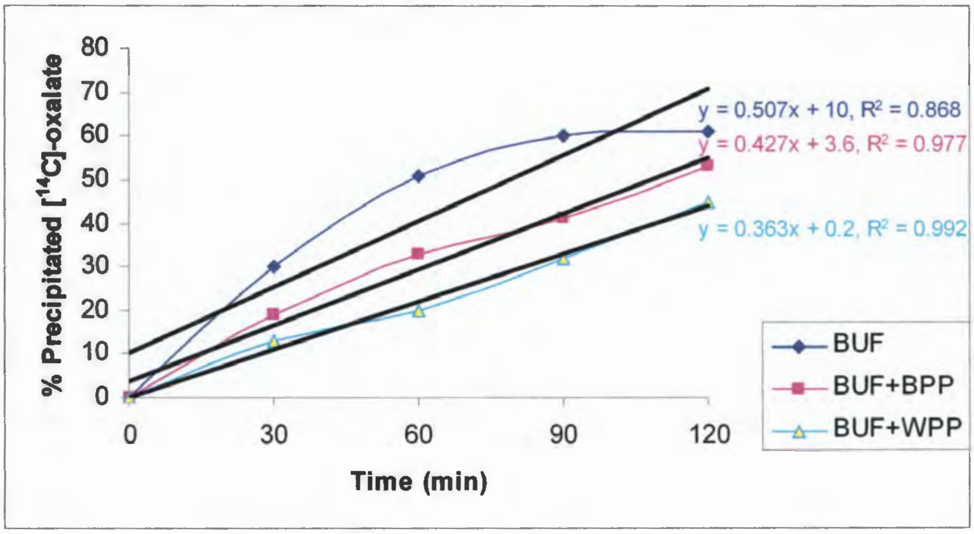


Figure 4.2.2

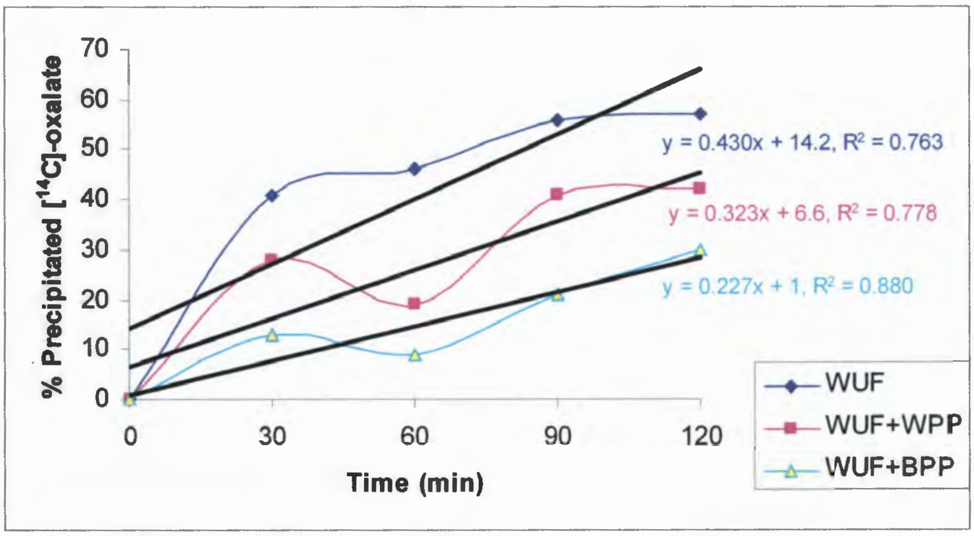


Figure 4.2: A typical plot of percent [^{14}C] - oxalate deposition in BUF (Figure 4.2.1) and WUF (Figure 4.2.2) urine before and after addition of protein precipitate from black (BPP) and white (WPP) subjects at final concentrations of 0.5 mg/L

Comments:

These results demonstrate that protein precipitates, irrespective of the ethnic origin, are inhibitors of CaOx deposition and that urine composition affects their performance in this regard. Surprisingly, crossed-over combinations were the most effective. This is contrary to previous results for THP (Craig *et al.* 2000) and UPTF1 (Webber *et al.* 2002) which showed a supportive synergistic relationship between the protein and its own urine. The results were unable to establish whether either of the protein precipitates was superior to the other. Nevertheless, the general principle that the urine milieu affects the inhibitory performance of urinary proteins is again apparent.

Effect of BPP and WPP on crystal aggregation

In these experiments, the addition of BPP and WPP to a slurry of COM crystals induced a significant inhibition of aggregation. The percentage inhibition by BPP (range 81-94 %, mean 88 %) was not significantly different to that of induced by WPP (range 81-90 %, mean 83 %). Figure 4.3 shows a typical plot of the absorbance vs time of a COM slurry before and after addition of BPP and WPP. This figure demonstrates that the sedimentation rate is reduced by the addition of protein precipitate, irrespective of its origin. The plots for each experiment are shown in Appendix 2 (Black subjects: Figure C2.1, White subjects: Figure C2.2).

Table 4.2: Gradients for the plots of absorbance vs time for COM crystal slurries before and after addition of BPP and WPP and corresponding inhibition of aggregation (% I_A).

Sample	Absorbance slope ($x10^{-5}s^{-1}$) (R^2) ± SE	% I_A
COM (n=5)	-430 (0.993) ± 16.2	
COM+BPP1	-70.0 (0.957)	84
COM+BPP2	-40.0 (0.964)	91
COM+BPP3	-80.0 (0.994)	81
COM+BPP4	-50.0 (0.965)	88
COM+BPP5	-23.0 (0.884)	94
Average	**-54.0 (0.998) ± 10.2**	**88**
COM+WPP1	-26.0 (0.982)	90
COM+WPP2	-40.0 (0.955)	84
COM+WPP3	-35.0 (0.752)	86
COM+WPP4	-62.0 (0.983)	75
COM+WPP5	-48.0 (0.890)	81
Average	**-42.0 (0.912) ± 6.10**	**83**

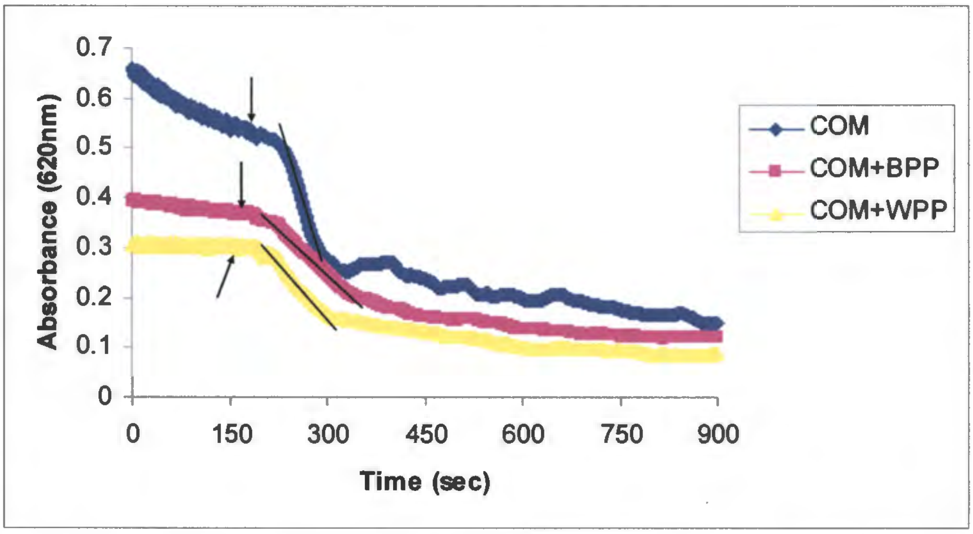


Figure 4.3: A typical plot of absorbance vs time for the sedimentation of COM crystal slurry before and after addition of BPP and WPP at 0.5 mg/L. Arrows indicate the time at which the stirring was stopped to induce crystal aggregation

Comments:

The protein precipitate from both race groups significantly inhibited COM aggregation to a similar extent. Aggregation is a process of crystals adhering together to form a large mass and allows the formation of larger particles and at a faster rate (Kok *et al.* 1993; 1994). Therefore the ability of BPP and WPP to retard this process highlights their protective role in kidney stone formation.

Effect of protein precipitate on crystal nucleation

These experiments showed strong inhibition of CaOx crystal nucleation by both BPP (range 80-90 %, mean 85 %) and WPP (range 47-93 %, mean 76 %). However, these values were not significantly different. A typical plot of the absorbance vs time of a decrease is shown in Figure 4.4. The individual plots for these experiments are shown in Appendix 2 (Black subjects: Figure D2.1: white subjects: Figure D2.2).

Table 4.3: Gradients for the plots of absorbance vs time of the control solution ($CaCl_2$ + Na_2Ox) before and after addition of BPP and WPP, and percentage inhibition of nucleation (% I_n)

Sample	Absorbance slope ($\times10^{-5}s^{-1}$) (R^2)	% I_n
Control (C) (n=5)	54.3 (0.989) ± 4.98	
C+BPP1	10.0 (0.959)	82
C+BPP2	11.0 (0.950)	80
C+BPP3	8.0 (0.957)	85
C+BPP4	7.3 (0.886)	86
C+BPP5	5.3 (0.990)	90
Average	**8.3 (0.948) ± 1.00**	**85**
C+WPP1	5.3 (0.986)	90
C+WPP2	15.0 (0.901)	72
C+WPP3	29.0 (0.765)	47
C+WPP4	13.0 (0.890)	76
C+WPP5	4.0 (0.992)	93
Average	**13.3 (0.908) ± 4.50**	**76**

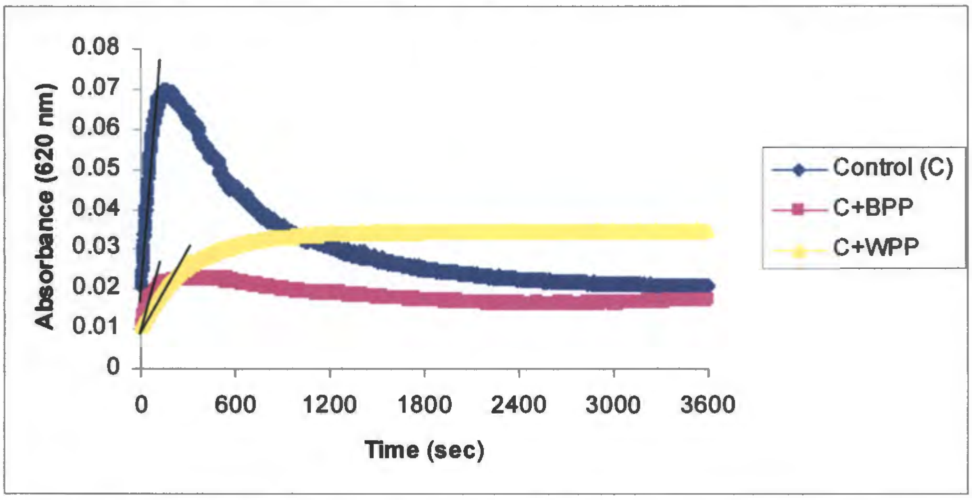


Figure 4.4: Plot of absorbance vs time for the nucleation assay before and after addition of BPP and WPP at 0.5 mg/L

Comments:

The results of these experiments have shown that urinary protein precipitates from both race groups are strong inhibitors of CaOx nucleation, and that there is no statistical difference between them in this regard.

Effect of BPP and WPP on particle formation kinetics

Particle size distribution

Bimodal particle size distributions were observed in two of five BUF samples, while a unimodal distribution were observed in four of five WUF samples. In general, there was a greater number of particles in WUF at baseline and these were of larger size (Figure 4.5). The latter figure shows the typical plot of the particle size distribution in ultrafiltered urine at 30 min intervals. In BUF, addition of BPP tended to produce smaller crystals but no clear evidence was evident when WPP was added (Table 4.4). In WUF, neither BPP nor WPP produced identifiable trends (Table 4.4). The plots from individual subjects are shown in Appendix 2 (Black subjects: Figures E2.1, White subjects: Figures E2.2).

Table 4.4: Particle size (µm) in ultrafiltered urine from black (BUF) and white (WUF) subjects before and after addition of BPP and WPP at final concentrations of 0.5 mg/L

	BUF	BUF+BPP	BUF+WPP
Experiment 1**	4.7 and 17.5	4.4 and 15.7	7.7 and 16.4
Experiment 2	5.5	4.7	5.8
Experiment 3	3.3	3.6	3.3
Experiment 4*	3.6 and 16.4	3.3 and 14.5	3.3 and 14.9
Experiment 5	4.3	3.3	5.4
	WUF	WUF+WPP	WUF+BPP
Experiment 1**	3.2 and 12.4	3.6 and 17.5	3.3 and 16.8
Experiment 2	10.6	16.8	8.0
Experiment 3**	7.3	9.5	10.6
Experiment 4*	8.0	7.7	4.7
Experiment 5*	8.3	4.4	5.1

* Trend showing a decrease
**Trend showing an increase

Figure 4.5.1

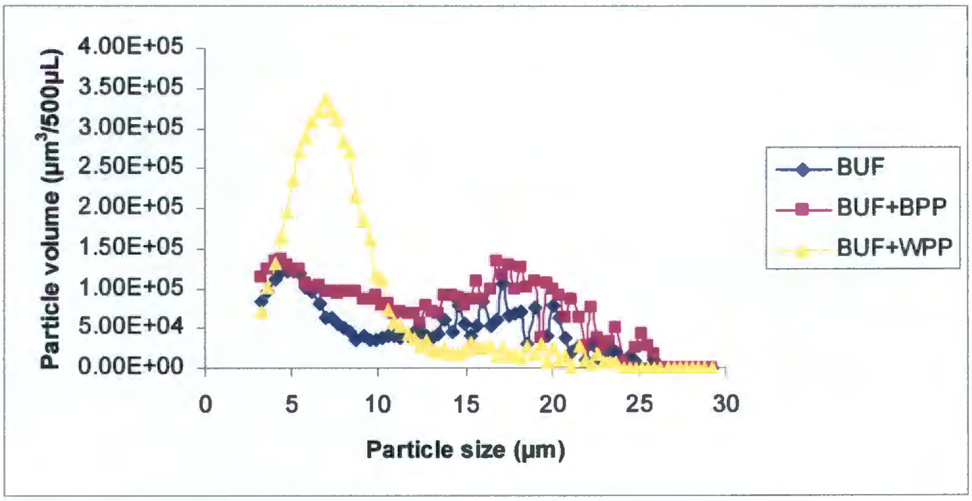


Figure 4.5.2

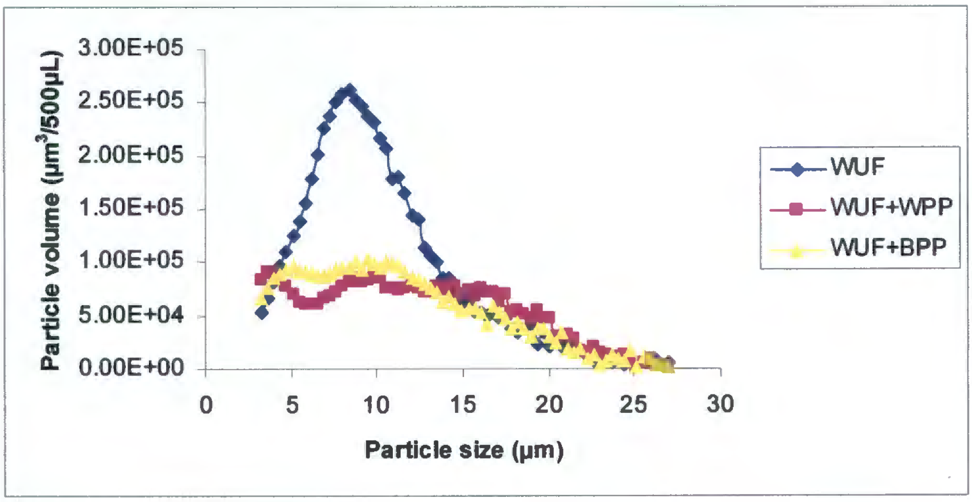


Figure 4.5: Particle size distribution in ultrafiltered urine from 5 black (Figure 4.5.1) and white males (Figure 4.5.2) before and after addition of BPP and WPP at final concentrations of 0.5 mg/L

Comments:

The observation of bimodal size distribution in BUF is somewhat surprising as it contradicts the general observation in the previous chapter that such distributions are rare. The importance of having many size distributions (as opposed to merely five) before drawing from conclusions is therefore apparent. The same point needs to be made for the other observed feature of the present set of distribution, namely that particle numbers and sizes were larger in WUF. While this is interesting as it is consistent with lower stone incidence in black subjects, the observation needs to be tested in a much larger series of particle number-size distribution.

Regarding the effects of the protein precipitated on particle size, no obvious trend was observed for WPP. However, a tendency for BPP to reduce size was observed, albeit that the trend was not convincing. Nevertheless, the possibility that BPP acts as inhibitory of CaOx crystal growth is noted here.

Particle number

In BUF, the general trend showed that particle number at 120 min decreased when protein precipitate was added to the test solution, irrespective of the protein precipitate's origin, although increases in particle number were also observed. A similar trend in WUF was not observed. Consistent reproducible differences between BPP and WPP were not evident. Typical plots of particle number vs. time are given in Table 4.6 while the data and plots for each of these experiments are shown in Appendix 2, Tables B (Black subjects: Tables D2.1 and Figures F2.1; White subjects: Tables D2.2 and Figure F2.2).

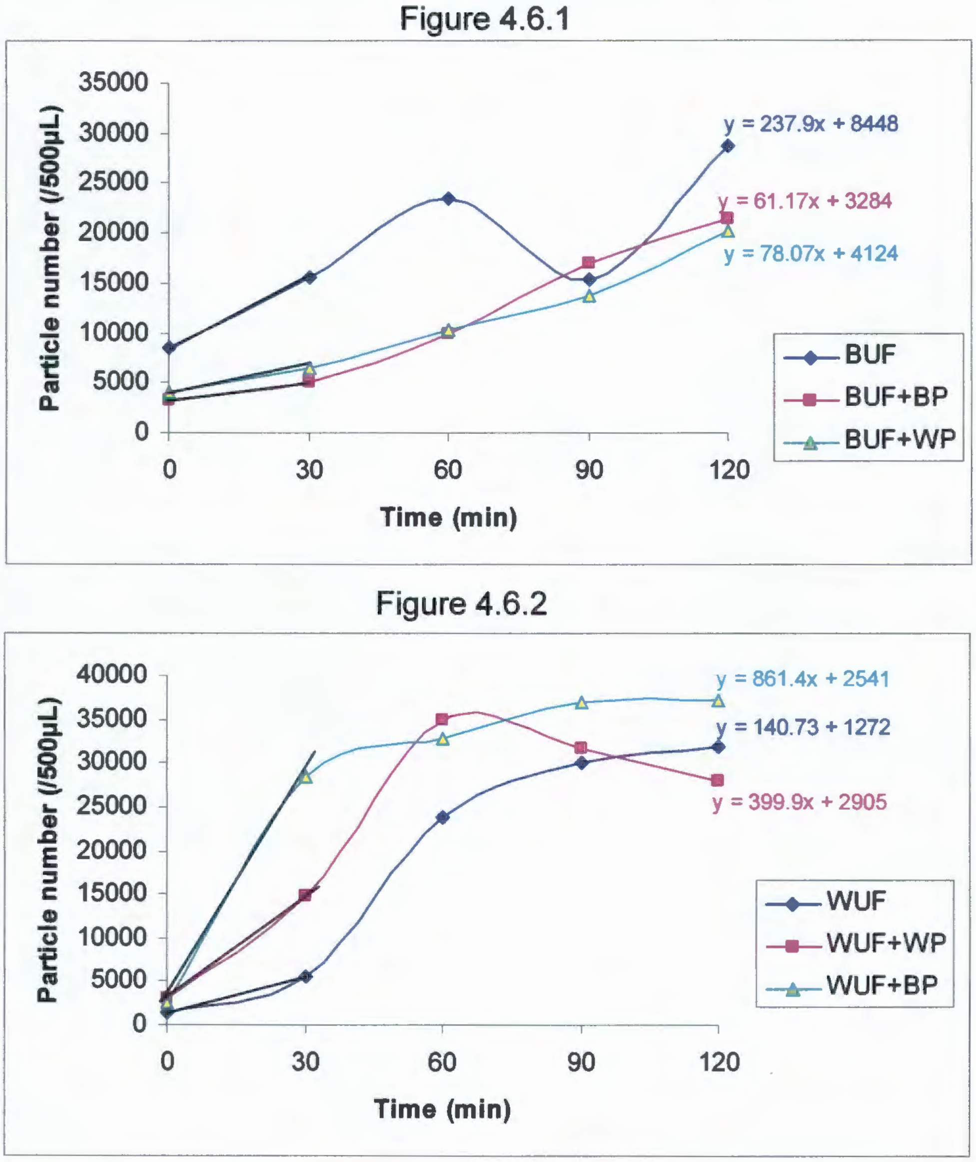


Figure 4.6.1

Figure 4.6.2

Figure 4.6: Typical kinetics plots for the particle formation in the ultrafiltered urine of black (Figure 4.6.1) and white subjects (Figure 4.6.2) before and after addition of BPP and WPP at final concentrations of 0.5 mg/L

Comments:

The data obtained in these experiments failed to show clearly defined trends, thereby highlighting again the difficulty of interpreting Coulter Counter data. The only trend which was weakly suggested was that protein precipitates may be inhibitors of CaOx crystal nucleation in BUF but not in WUF. This again draws attention to the influence that urine composition may have in directing crystallization processes.

Particle volume

The general trend in these experiments showed a decrease in particle volume when both BPP and WPP were added to BUF and WUF. It was not obvious whether either of these produces a consistently greater effect. Typical plots are given in Figure 4.7.1 and 4.7.2 and plots for each experiment are shown in Appendix 2, Tables (Black subjects: Table E2.1 and Figure G2.1; White subjects Table E2.2 and Figure G2.2).

Figure 4.7.1

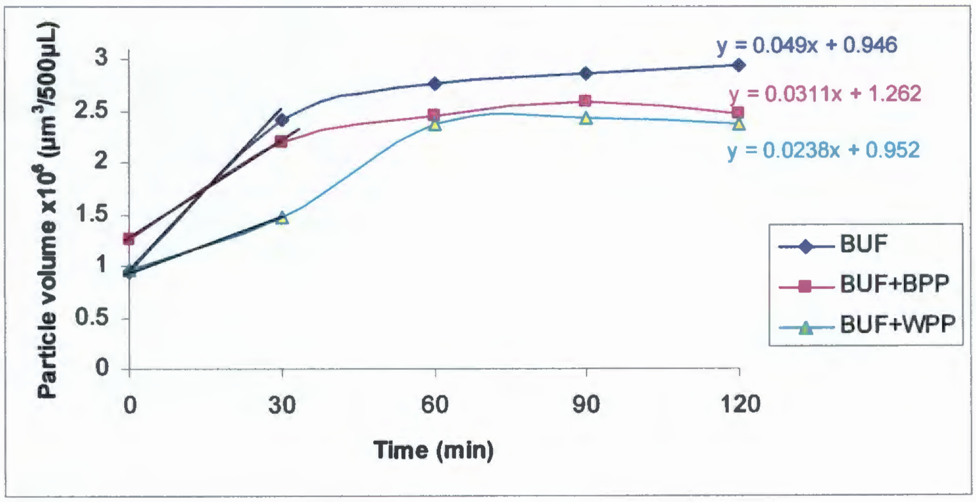


Figure 4.7.2

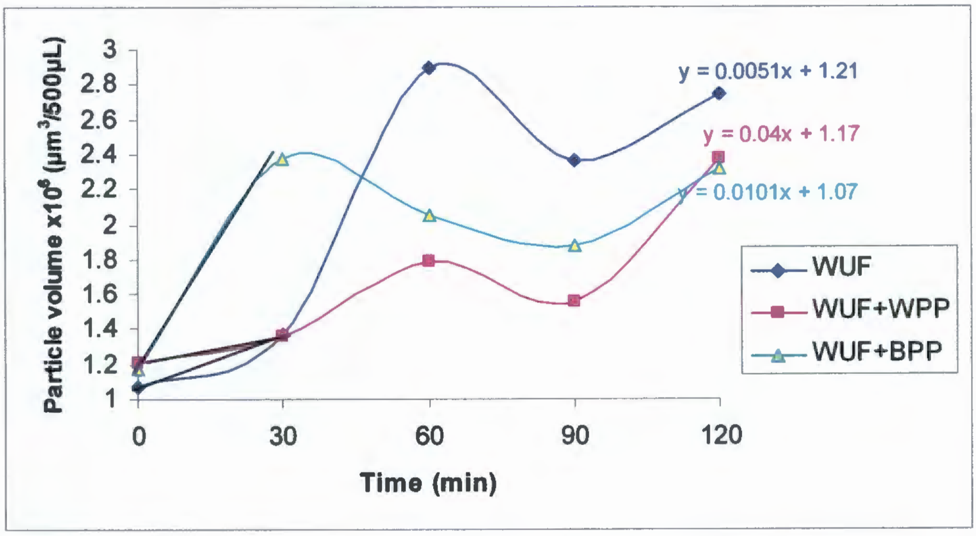


Figure 4.7: Plots of a typical graph of the particle volume in BUF (Figure 4.7.1) and WUF (Figure 4.7.2) before and after addition of BPP and WPP at final concentrations of 0.5 mg/L at 30 min intervals.

Comments:

The particle volume which showed a decreasing trend when BPP and WPP was added on either BUF or WUF is an indicating of a decrease in the total mass induced crystalline material. As explained earlier in Chapter 3, the final conclusion on the ability of BPP and WPP cannot be based on this experiment alone and therefore we can only speculated that these experiments indicate aggregation inhibition by the proteins included in the precipitate, irrespective of the race group from which it was derived.

As explained in Chapter 3, a decrease in particle volume is most likely indicative of inhibition of nucleation or growth. Unlike the other Coulter data, trends for the particle volume were consistent and reproducible in both BUF and WUF, thereby allowing a more confident interpretation. These results therefore suggestion that the protein precipitates acts as inhibitors of nucleation and growth.

4.4 Discussion

The results of the experiments describes in this chapter were disappointing because consistent, definitive trends were not detected, nor was it possible to identify differences in the behavior of the protein precipitates from the two race groups.

Evidence suggesting that protein precipitates are inhibitors of CaOx crystal nucleation was compellingly provided by the [^{14}C]-oxalate deposition and was weakly supported by the Coulter number and volume data. The sedimentation experiments also showed that the protein precipitates inhibit CaOx crystal aggregation. Weak evidence suggesting the possibility of growth, inhibition was provided by the Coulter size and volume data. The findings in this chapter are in agreement with previous studies which have shown that proteins precipitates from the urine inhibit CaOx nucleation, aggregation and growth (Boyce *et al.* 1968; Coe *et al.* 1991; Grover *et al.* 1998).

Chapter 5

Isolation, purification and characterization of bikunin

5.1 Introduction

In the previous chapter, the results demonstrated that urinary protein precipitates have inhibitory properties with respect to CaOx crystallization. While these studies are of interest and help us speculate generally on the role of urinary protein inhibition in providing the South African black population with protection against CaOx urolithiasis, they do not allow us to draw any firm conclusion about individual proteins. In particular, it is not possible to deduce the role of bikunin, which is the protein selected for study in this thesis. This chapter describes attempts to isolate, purify and characterize bikunin from the urine of both race groups so that their relative inhibitory capacities can be investigated.

Bikunin isolated from human urine has been reported to have a concentration of 6.25-18 nmol/L (Dean *et al.* 2003). The protein is composed of a 16 kDa polypeptide chain, a 7-8 kDa chondroitin sulphate side chain and an *N*-linked oligosaccharide of about 1-2 kDa (Médétognon-Benissan *et al.* 1999; Yang *et al.* 2005). Structural variations in the glycosaminoglycan moiety, the polysaccharide side chains and the amount of sulphation appear to result in the existence of several charged isoforms bikunin (Yuki *et al.* 1993).

Objectives

The objectives for this part of the study are as follows:

- To prepare precipitates of COM and COD crystals from the urine of healthy black and white male subjects to test the presence of bikunin
- To prepare the protein precipitate from the urine of both groups to test the presence of bikunin
- To isolate and purify urinary bikunin from the protein precipitates using immunoaffinity chromatography
- To characterize purified bikunin using the following techniques:
    1) SDS-PAGE
    2) Western blotting
    3) Matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI-TOF MS)

5.2 Methods

5.2.1 Precipitation of calcium oxalate monohydrate (COM) and dihydrate (COD) crystals from urine

24 hr urine samples from five black and five white healthy male subjects were collected and tested for haematuria and infection using urinalysis test strips (Medi Test Combi 5N, Macherey-Nagel). Urines testing positive for either were excluded. One liter of urine from each subject was then pooled according to race and filtered through a 0.75 µm pre-filter followed by a 0.45 µm nitrocellulose filter paper before use.

The urine was heated to 37 °C in a shaking water bath at 100 rpm. The Ca^{2+} concentration was adjusted to 0.5 mM by dilution with distilled water (Hess 1989, Webber *et al.* 2003) while it was adjusted to 12 mM (by addition of $CaCl_2$) to induce the formation of pure COD crystals (Webber *et al.* 2003). After the adjustment of urinary calcium, the CaOx metastable limit (MSL) of each portion was measured and crystallization was induced by the dropwise addition of 10 % Na_2Ox (v/v) at 30 µM above MSL. The dropwise addition of Na_2Ox was repeated at 0, 1 and 2 hrs. The precipitated crystals were collected after a total incubation period of 3 hrs, filtered though 0.22 µm filter paper and thoroughly washed with distilled (XRD) water and dried at 95 °C for 1 hr.

XRD

X-ray powder diffraction was used to identify the hydrates which had formed. The interplanar *d*-spacings of the CaOx crystals were compared with the standard reference values for COM and COD (Table 5.1). The XRD patterns of the crystals together with the d-spacings (Å) of prominent peaks correspond to those shown in Table 5.1. Figure 5.1 shows plots of the x-ray powder diffraction patterns and the *d*-spacing of COM

Table 5.1: Interplanar spacings and relative intensities of powder patterns of CaOx crystals

*Standard d-spacing and relative intensity data				Present Study	
COM		COD		COM	COD
d-spacing (Å)	Relative Intensity (I)	d-spacing (Å)	Relative Intensity (I)	d-spacing	d-spacing
5.93	100	8.70	12	5.93	
5.79		6.31			
	25		100		6.32
4.64	7	6.15			
4.52	6	4.40	45		4.48
3.78					
	13	3.89	14		3.93
3.76		3.67	8		3.70
3.65	100	3.58	1	5.64	
3.00	10	3.38	2		
2.97	46	3.15	3	2.96	3.15
2.91	12	3.09			
			18		
2.89	10	3.07		2.90	
2.84	14	2.81	20		2.79
2.51	2	2.77			
			85		
2.48	30	2.75			
2.41	5	2.41	14	2.49	2.41
2.37	2	2.39	14		2.37
2.34	90	2.33	10	2.34	

** Sutor and Scheidt 1968*

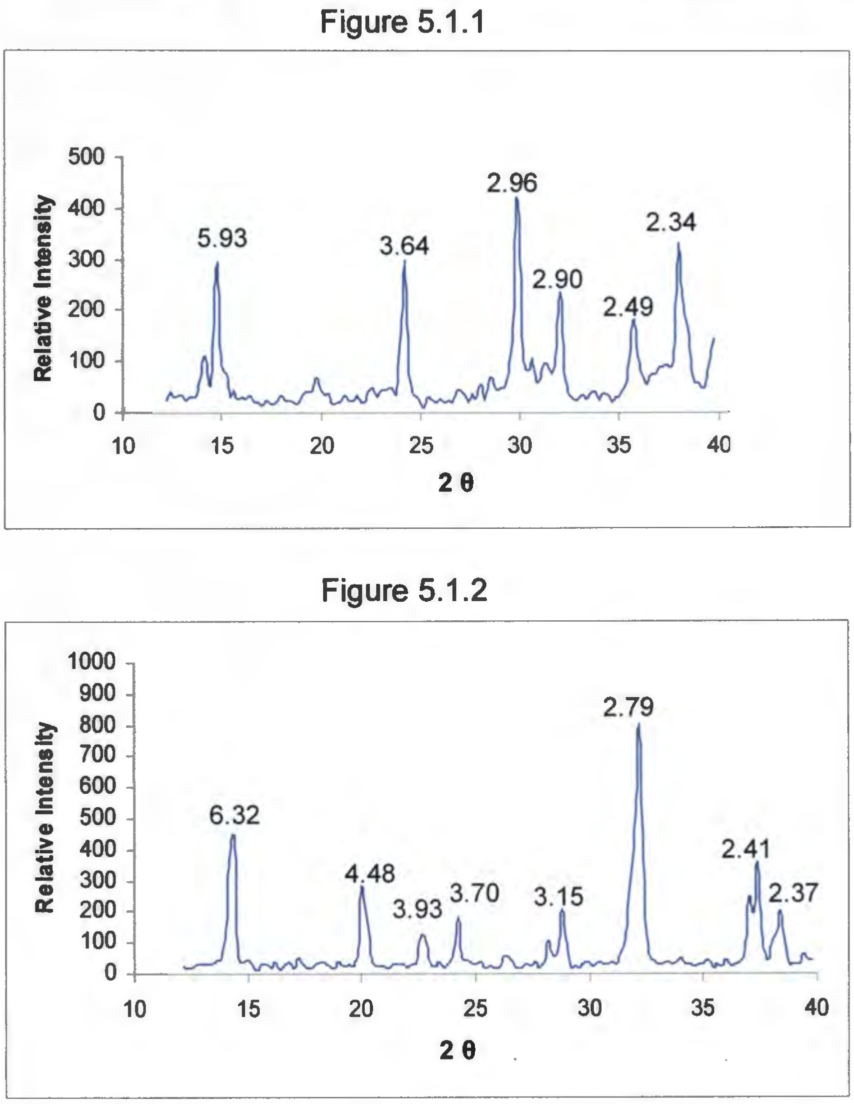


Figure 5.1.1

Figure 5.1.2

Figure 5.1: x-ray powder diffraction patterns of COM (Figure 5.1.1) and COD (Figure 5.1.2) crystals

Demineralization

The crystals were demineralized using 0.25 M EDTA (pH 8) (1 g/100 ml), dialyzed against distilled water and freeze-dried. This yielded a COM and COD crystal matrix.

5.2.2 Isolation of protein precipitate (PP) from urine

Protein precipitates were isolated using ammonium sulphate from the 24 hr urine samples which were collected from five healthy black and five white subjects using the method described in Chapter 4.2.2, page 74.

5.2.3 Protein concentration determination

The protein concentrations of the COM, COD and protein precipitates were determined using the Bradford method described in Chapter 2.2.3, page 38. The assays were performed in duplicate.

5.2.4 Bikunin isolation

Immunoaffinity chromatography

Trypsin CNBr-sepharose affinity column chromatography has been used for the isolation of bikunin because of the proteins ability to bind to trypsin (Médétognon-Bonissan *et al.* 1999; Yang *et al.* 2005). NP-HPLC has also been used (Höchstrasser *et al.* 1984) to separate bikunin. In the present study, immunoaffinity chromatography method was selected for the isolation of bikunin because previous studies have shown that immunoaffinity chromatography is an efficient method (Salier *et al.* 1983).

The principle of immunoaffinity chromatography is based on the highly specific interaction of an antigen with its antibody. It is therefore a type of chromatography that allows for the separation of a particular protein. In the present study bikunin was separated from a solution containing many different proteins. The affinity material is made from a solid substance such as Protein G beads to which the substrate of the protein (i.e. antibody) is covalently attached. A specific antibody binds only to the desired protein, even from an extract containing thousands of different proteins. In this study a specific monoclonal anti-Microglobulin (alpha 1) antibody (Abcam, UK) was used to bind to the bikunin from the urinary protein precipitate.

Column preparation

Protein G sepharose beads (0.25 g) were washed three times with 10 ml phosphate buffered saline (PBS, pH 7.5) and 14 mg of anti-Microglobulin (alpha 1) antibody was added. The protein G-antibody mixture was rotated overnight at 4 °C and washed three times with 10 ml PBS (pH 7.5). The antibody was cross-linked to the protein G using 20 mM dimethylpimelimidate in 3 M NaCl and 50 mM borate, pH 9 by rotating for 1 hr at room temperature. Binding of the antibody to Protein G was assessed using the Bradford assay. The preparation was washed three times with 0.2 M ethanolamine, pH 8 and resuspended in PBS. Glass wool was placed at the bottom of a 2.5 ml column and the Ab-protein G-Sepharose G slurry was transferred to the column. In order to test whether the antibody was cross-linked to protein G, the column was washed with 100 mM glycine, pH 2.8 at a rate of 0.5 ml/min and the absorbance of the eluent was measured at 280 nm.

Isolation of bikunin from the protein precipitate using immunoaffinity chromatography

The principle of column chromatography procedure is depicted in Figure 5.2. The protein precipitates from black and white subjects were independently applied to the immunoaffinity column at a flow rate of 0.5 ml/min, the unbound material was collected. The column was washed overnight with PBS. The bound bikunin was eluted using 100 mM glycine, pH 2 and the eluent was neutralized immediately by the addition of 1 M Tris, pH 9.5 to stabilize the pH. The column was preserved with 0.02 % sodium azide in PBS.

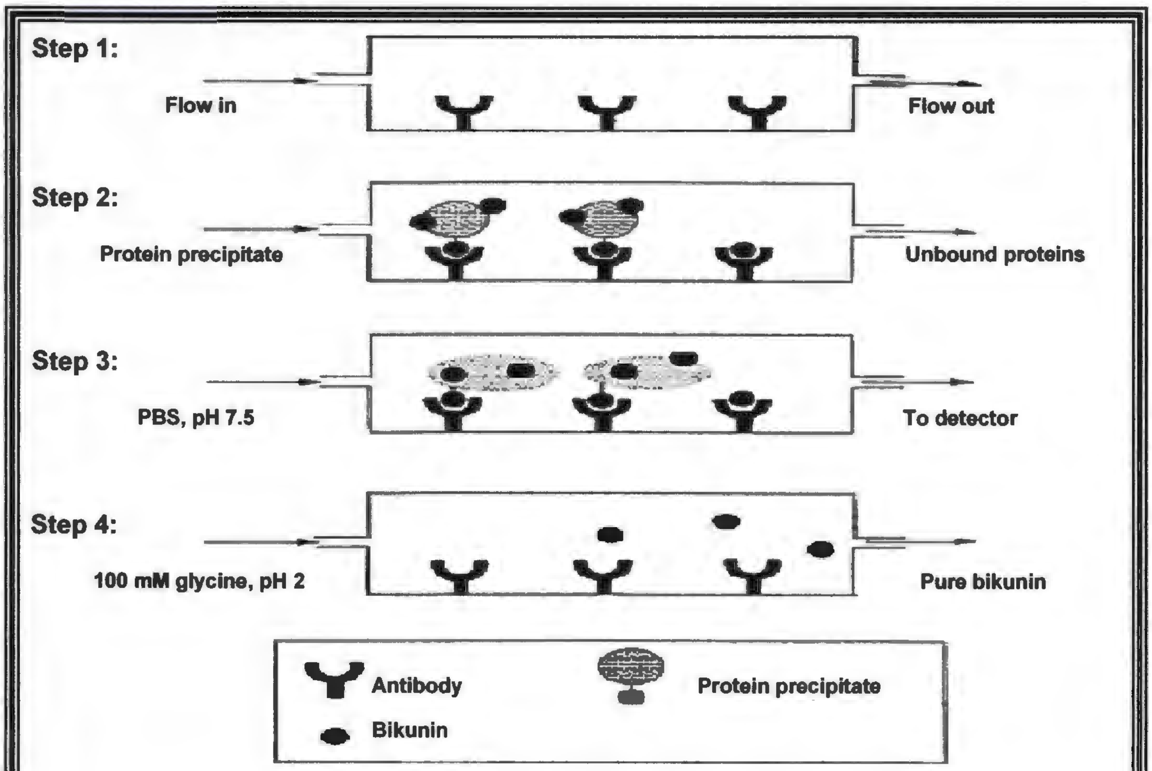


Figure 5.2: Immunoffinity column chromatography. Step 1: before sample loading. Step 2: loading of the protein precipitate and collection of the unbound proteins. Step 3: column wash with PBS. Step 4: elution of bound bikunin. Figure modified from http://tamuk.ed/fpllc/ pursamat/html

5.2.5 Characterization of protein extraction

SDS-PAGE

Proteins extracted from the urine of both population groups were analysed by 10 % SDS-PAGE using the method described in Chapter 2.2.3, page 39. A low range molecular weight marker was included (Bio-Rad). The samples were run under reducing and non reducing conditions (i.e. with and without mercaptoethanol). The gel was developed using a Coomassie Blue or silver staining procedure as described in Appendix 2.1.

Western blot

The SDS polyacrylamide gel and a nitrocellulose membrane were equilibrated in a blotting buffer (method in Appendix 1.1) for 15 min. The proteins from the gel were transferred to the nitrocellulose membrane at 300 mA for 1 hr with cooling. Non-specific binding sites on the membrane were blocked overnight with agitation using blocking buffer (Appendix 1.1). The membrane was incubated for 1 hr in the monoclonal anti-Microglobulin (alpha 1) which was diluted 1:1000 in blocking buffer. The blot was washed extensively with the same buffer and then incubated for 1 hr in anti-sheep IgG which was diluted 1:1000 in blocking buffer and incubated for an hour. After exposure to both antibodies, the blot was washed extensively with blocking buffer and the HRP (horse radish peroxidase) conjugate was detected using detection with a solution containing 4-chloro-1-napthol (Sigma) (Appendix 2.1) for approximately 15 min in a dark room.

Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry

Molecular weight determinations were performed on the purified bikunin isolated from both black and white subjects using MALDI-TOF MS (Perseptive Biosystems Voyager DE-PRO, USA) according to methods described by Egelhofer *et al.* 2002. Briefly, the proteins were separated on SDS-PAGE and the bands were excised and soaked in 50 µL acetonitrile and 25 mM NH_4HCO_3 for 15 min. The supernatant was discarded and the gel was soaked again for further 10 min in 50 µL 25 mM NH_4HCO_3. The bands were dried in a speedivac for 30 min and trypisin digested. The trypsin digestion method involves swelling of the gel pieces with 12.5 ng/µL trypsin (Promega) in 50 mM NH_4HCO_3 on ice. 10 µL of this solution was added initially followed by the addition of small amounts (1-2 µL) so that the gel fully rehydrates without leaving excess of trypsin solution. To keep the gel wet, the sample was covered with a minimum volume of 50 mM NH_4HCO_3 and incubated at 37 ^oC for 4 hrs. For the MALDI analysis 1 µL of the above sample was mixed with 1 µL MALDI-TOF MS matrix. The matrix was a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) in 50 % acetonitrile and 0.3 % TFA. The mixture (2 µL) was applied to a MALDI P100 gold sample plate and allowed to dry by gentle streaming with air to allow crystallization of the matrix and the sample. The sample was then analyzed

5.3 Results

5.3.1 Characterization of urinary protein extraction

Protein concentration

The table below presents the average protein concentration values of COM from three separate urine pools, COD from one pooled urine and PP from five pools of urine. The average total protein concentration of the PP was determined to be 20 and 16 mg/L for samples from black and white samples, respectively (Table 5.2). Owing to the fact that protein precipitation using 80 % ammonium sulphate saturation gave higher total protein yields than those obtained in the crystals matrices, PP was selected for further bikunin isolation.

Table 5.2: Total concentration of proteins in CaOx crystal matrices and protein precipitates

	Black subjects (mg/L)	White subjects (mg/L)
COM (n=3, pools)	10.8	11.6
COD (n=1, pools)	14.5	15.8
PP (n=5, pools)	20.0	16.0

SDS-PAGE and Western blotting

Figure 5.3 shows SDS-PAGE and Western blot of the protein precipitate isolated from the urine of black subjects and white subjects respectively. Bands ranging from 20-120 kDa were observed in the protein precipitates, irrespective of the race group from which they were derived. A band at 30 kDa corresponding to bikunin was observed on the Western blot, under both reducing and non-reducing conditions. Since the blot for both black and white subjects were qualitatively similar, only one gel and blot are presented.

Figure 5.3: SDS-PAGE (Figure 5.3.1) and Western blot (Figure 5.3.2) of protein precipitate isolated from the urine

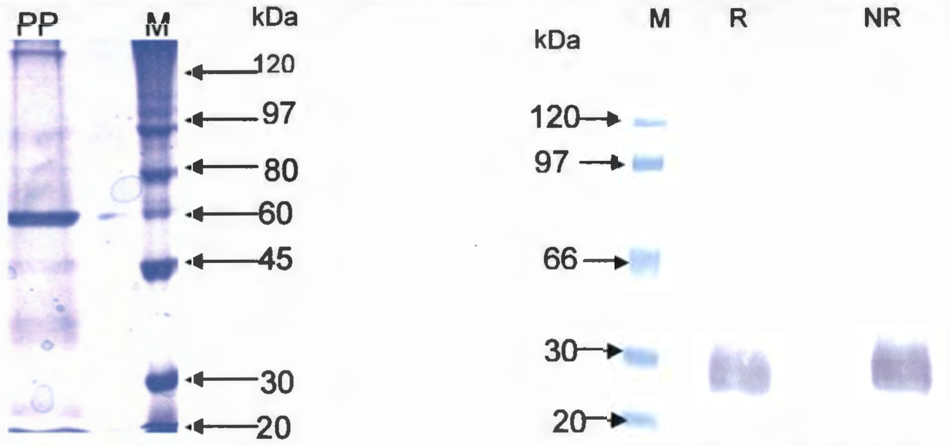


Figure 5.3.1: Protein precipitate (PP) isolated from the urine

Figure 5.3.2: Western blot of the Protein precipitate immunoblotted for bikunin. Lane M-marker, R-reduced conditions and NR-non- reduced conditions

Figures 5.4 and 5.5 show the SDS-PAGE and Western blot of COM and COD crystal matrix extracts obtained from the urine of black and white subjects, respectively. SDS-PAGE run under reducing conditions showed several protein bands in the range 30 - 120 kDa for both COM and COD crystals. 2 mg of protein was loaded on each lane for both B and W for both SDS-PAGE and Western blot. The bands observed were similar for both race groups. A 30 kDa band corresponding to bikunin was observed in both race groups' COM and COD crystals by immunoblotting.

Figure 5.4: SDS-PAGE (Figure 5.4.1) and Western blot (Figure 5.4.2) of COM crystals isolated from black and white subjects

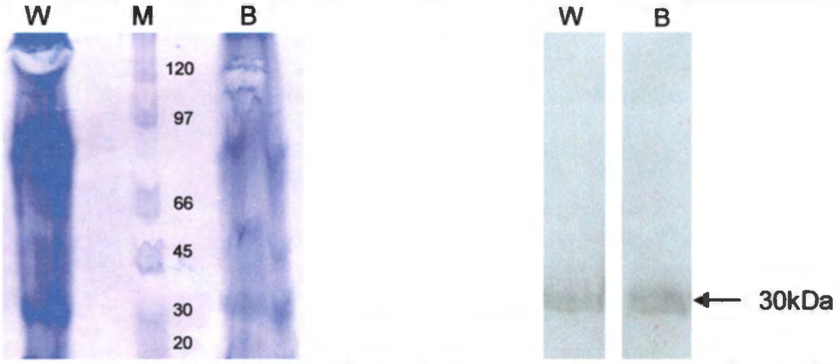


Figure 5.4.1: SDS-PAGE of COM crystals isolated from black (B) and white (W) subjects; M: low molecular weight marker.

Figure 5.4.2: Western blot of COM crystals isolated from black (B) and white (W) subjects immunoblotted for bikunin.

Figure 5.5: SDS-PAGE (Figure 5.5.1) and Western blot (Figure 5.5.2) of COD crystals isolated from black and white subjects

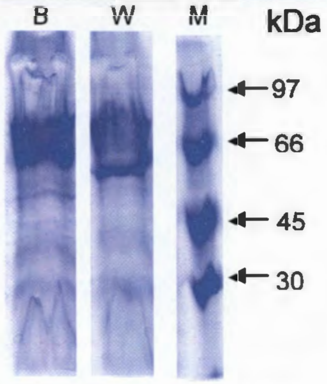


Figure 5.5.1: SDS-PAGE of the COD isolated from black (B) and white (W) subjects; M: low molecular weight marker.

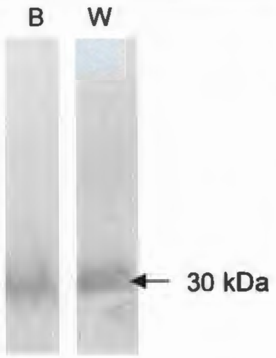


Figure 5.15.2: Western blot of COD crystals isolated from black (B) and white (W) subjects immunobloted for bikunin.

Comments:

The initial step in an attempt to purify bikunin involved the determination of the protein concentration of the proteins precipitated from the urines of black and white male subjects prepared using three different protein precipitation methods. These methods included the preparation of COM and COD crystals by adjusting the calcium concentration in the urine to 0.5 and 12 mM respectively, and the preparation of a protein precipitate using 80 % ammonium sulphate saturation. The protocol which produced best yields was selected for further bikunin isolation, namely protein precipitate. Western blotting confirmed the presence of bikunin in all of the protein precipitates isolated from the urines of both black and white male subjects, as shown by a broad band at 30 kDa.

5.3.2 Bikunin purification

Bikunin was purified from the protein precipitate using immunoaffinity chromatography. Three fractions were collected as shown in Figure 5.6. The first fraction (a) corresponded to the unbound proteins and was collected after the first absorbance increase. The column was washed extensively with a PBS buffer and this fraction (b) was collected. The bound bikunin fraction was then eluted as single peak (c) using glycine (pH 2.8).

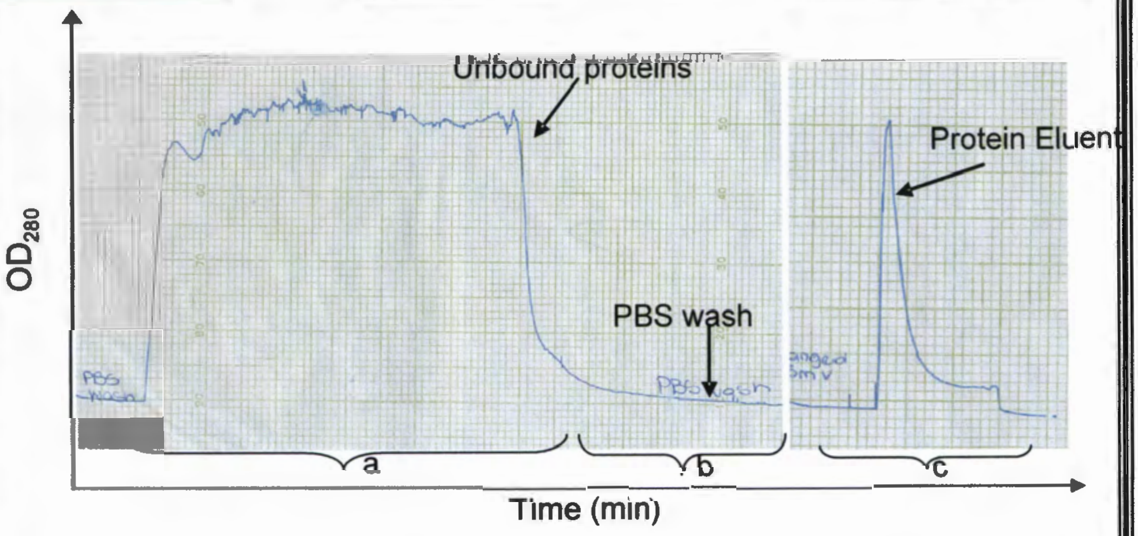


Figure 5.6: Elution profile for bikunin purification using immunoaffinity chromatography

Table 5.3 shows the bikunin concentration obtained from each batch of protein precipitate subjected to immunoaffinity chromatography. The protein concentration ranged between 0.36-0.64 mg/L and 0.11-0.70 mg/L for black and white subjects, respectively. Comparison of the mean concentration of bikunin in the two race groups did not reveal any significant differences.

Table 5.3: Bikunin concentration (mg/L) of urine from black (BB) and white (WB) subjects' urine

Batch	Bikunin concentration (mg/L)	Batch	Bikunin concentration (mg/L)
BB1	0.40	WB1	0.48
BB2	0.64	WB2	0.40
BB3	0.36	WB3	0.70
BB4	0.44	WB4	0.25
BB5	0.48	WB5	0.11
Average ± SE	**0.46 ± 0.079**	**Average ± SE**	**0.32 ± 0.079**

SDS-PAGE and Western blotting

Figure 5.7 shows the SDS-PAGE and Western blot of the bikunin isolated from the urine of black and white subjects using immunoaffinity chromatography. Under reducing conditions two bands at 30 and 60 kDa were observed. The isolated bikunin produced three major bands on SDS-PAGE at 120, 97 and 60 kDa (Figure 5.7.1). In order to confirm that the bands on the silver stained gels were due to bikunin, equivalent protein loads were immunoblotted for bikunin (Figure 5.7.2). The major bands on the gel also appeared on the Western blot. The band at 60 kDa as indicated by the arrow was faint. Since the blot for both black and white subjects were qualitatively similar, only one gel and blot is presented.

Figure 5.7: SDS-PAGE and Western blot of purified bikunin

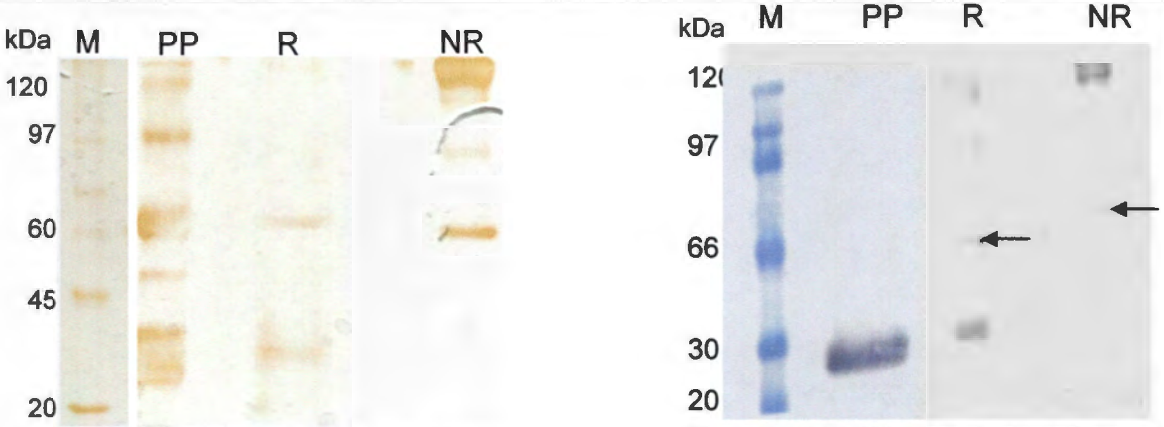


Figure 5.7.1: SDS-PAGE of the protein eluent; Lane NR- non reduced, lane R, reduced, lane PP- protein precipitate before column, M: low molecular weight marker.

Figure 5.7.2: Western blot; before column protein precipitate was loaded on the column lane (lane PP), protein eluent, from left lane NR (non-reduced) lane R (reduced) immunoblotted for bikunin.

Table 5.4: MALDI-TOF MS analysis of tryptic bikunin peptides from black (BB) and white (WB) subjects

Peptide residue	Theoretical m/z	Observed m/z			
No.		BB Band 1	BB Band 2	WB Band 1	WB Band 2
22-41*	2264.6**			2262.43	
23-41	2136.5**				2135.36
71-92*	2547.1**		2545.22		
78-88	1228.5**				1225.60
78-92*	1570.9	1568.74			
89-104*	1894.2**				1898.46
93-104	1409.7		1411.04		
93-121*	3204.7**		3205.45		
107-121	1699.9**				1697.90
107-126*	2354.7**				2351.83
107-129*	2600.9	2602.13	2601.27		
122-126	673.7	672.4	672.90		670.1
130-147*	2001.1	1999.20			

* Peptides resulting from incomplete trypsin cleavage.

** These peptides formed acrylamide adducts due to the presence of cysteine residues. The observed masses of these peptides increased by 71.1 kDa for every cysteine residue that was modified

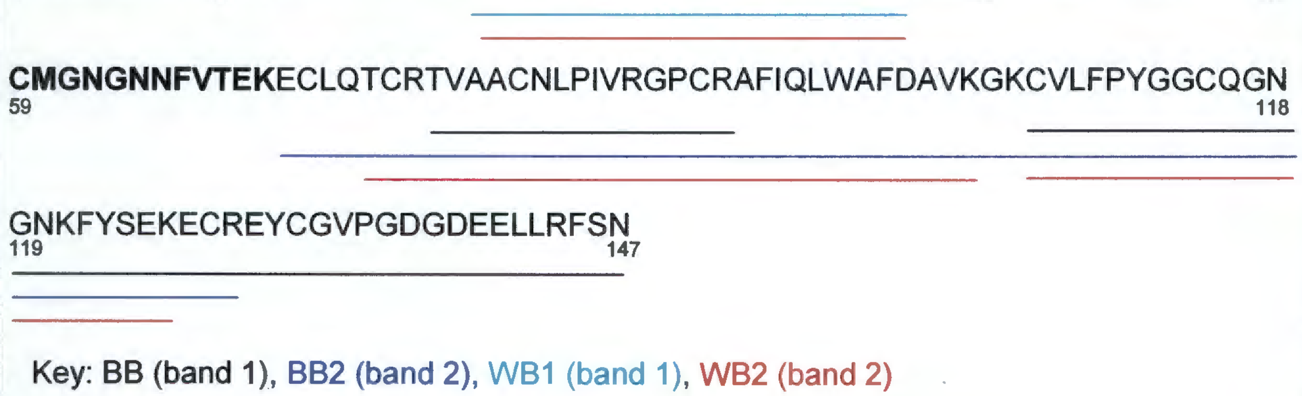


Figure 5.9: Bikunin sequence and amino acid coverage of BB and WB

The coloured lines indicated on the bikunin sequence above (Figure 5.9) show the coverage of the amino acids of each bikunin band analyzed by MALDI-TOF MS. Except for WB (band 1), which showed small amino acid coverage, BB (bands 1 and 2) and WB (band 2) demonstrated a large range of amino acid coverage. As depicted in Figure 5.10, bikunin has an *O*-linked glycan attached to the serine residue at position 10 and an *N*-linked glycan attachment to the asparagine at position 45 of the core protein. This glycosylation hindered the mass determination of the *N*-terminal peptide (1-21) and peptide (42-70) as the exact masses of these peptides are not known (highlighted in bold in Figure 5.9).

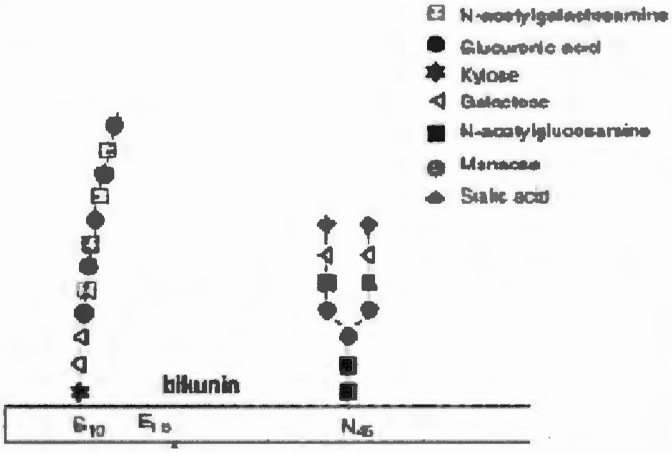


Figure 5.10: Structure of *N*-linked and *O*-linked glycans present on bikunin (Balduyck *at al.* 1986)

5.4 Discussion

This chapter focused on the description of the isolation of bikunin using immunoaffinity chromatography, a method chosen because it has been shown in previous studies to be efficient for protein isolation (Salier *et al.* 1983). Immunoaffinity chromatography was used successfully to isolate bikunin from the protein precipitate obtained from the urines of black and white male subjects. The 30 kDa mass seen on SDS-PAGE agrees with the mass reported for bikunin (Enghild *et al.* 1989; Morikawa *et al.* 1994; Suzuki *et al.* 2001; Okuyama *et al.* 2003) and the 60 kDa has also been reported previously (Muramatsu *et al.* 1980; Pratt *et al.* 1989; Slota *et al.* 1994; Suzuki *et al.* 2001). The mass at 60 kDa might be due to increased glycosylation and sulphation or variations in the glycosaminoglycans and polysaccharide side-chain (Yang *et al.* 2005). Another possibility is that bikunin could exist as a dimer which has been reported in numerous studies (Muramatsu *et al.* 1980; Pratt *et al.* 1989; Slota *et al.* 1994; Suzuki *et al.* 2001). Both bands on SDS-PAGE were confirmed to be bikunin by Western blotting (Figure 5.7, page 112) and MALDI-TOF MS (Table 5.4). The higher masses observed under non-reducing conditions are in agreement with those which has been previously reported. For example, Marengo *et al.* (1998) stated that the use of non-reducing conditions combined with increased exposure times during the development of gels and blots yields higher molecular weight dimer/ trimer forms of bikunin.

Of further interest was the yield of the bikunin eluted from the column. An average of 0.46 and 0.32 mg/L was obtained for bikunin isolated from the urine of black and white subjects, respectively. This concentration is within the published estimates of bikunin concentration in human urine (Nishino *et al.* 1989; Suzuki *et al.* 2001). Although bikunin excretion appears to be higher in black subjects, the difference is not significant. Bikunin is a "sticky protein" (Suzuki *et al.* 2001) and it is likely that a significant amount of it was lost in the isolation process due to it sticking on the surface of the walls of any apparatus used. The protein was considered to be pure since all bands observed on the

SDS-PAGE reacted with a monoclonal antibody and showed MALDI-TOF MS peaks corresponding to those reported for bikunin. Structural differences were observed in the bikunin isolated from the two race groups, for example bikunin isolated from black subjects showed a larger coverage of bikunin amino acids. Since some of the amino acids which easily bind calcium have been reported to play a role in the inhibitory activity of the proteins on CaOx crystallization (Franzen and Heinegard 1985; Chen *et al.* 1992; Denhardt and Guo 1993; Hunter *et al.* 1996), a larger coverage of amino acid by bikunin isolated from the black subjects suggest that bikunin might be a contributing factor in protecting the black South African population from the kidney stone disease. Although this is of major interest, further experiments are required to study the structure of bikunin isolated from black and white South African subjects.

Chapter 6

Crystallization studies of purified bikunin in urine and inorganic solution

6.1 Introduction

As described in section 1.7 of this thesis, several *in vitro* crystallization studies have demonstrated that bikunin is an inhibitor of CaOx crystallization (Nakagawa *et al.* 1985; Höchstrasser *et al.* 1984; Sorensen *et al.* 1990; Atmani *et al.* 1993a; Atmani *et al.* 1993b; Atmani *et al.* 1995) and, more specifically, that it modulates all three crystallization mechanisms, namely nucleation (Atmani *et al.*1999; Höchstrasser *et al.* 1984; Okuyama *et al.* 1999; Atmani *et al.* 1999); growth (Atmani *et al.* 1993a; Atmani *et al.* 1993b; Atmani *et al.* 1999; Okuyama *et al.* 2003) and aggregation (Atmani *et al.* 1999). Moreover, the observation that urinary concentrations of bikunin in SF are lower than those in healthy controls, has prompted speculations that this could be a contributing factor in CaOx stone pathogenesis (Médétognon-Benissan *et al.* 1999).

In the previous chapter, successful attempts to isolate bikunin from the urine of black and white South African subjects were described, albeit in smaller concentrations. The isolation of this protein from both race groups forms part of the rationale and strategy of this project and affords the opportunity to test the hypothesis that the protein derived from the urines of black subjects may be a more potent inhibitor of CaOx crystallization than that derived from white subjects. This chapter describes experiments which address this prospect.

Objectives

The objectives of this particular study were to conduct a variety of crystallization experiments (as described in Chapter 2) to test and compare the relative inhibitory activity of the bikunin protein isolated from the urine of healthy black and white male subjects and to examine how this activity might be influenced by the urine environment in which it operates.

6.2 Methods

6.2.1 Crystallization experiments

Urine collection and treatment

Single 24 hr urines were collected from each of five black and five white males and were treated using the method described in Chapter 2.2.1, page 30. All the crystallization experiments were all conducted at a final protein concentration of 0.5 mg/L.

Effect of bikunin on [^{14}C]-oxalate deposition kinetics

The effect of bikunin on [^{14}C]-oxalate deposition was determined as described in Chapter 2.2.2, page 32.

Effect of bikunin on crystal aggregation

The effect of bikunin on crystal aggregation was determined using the method described in Chapter 2.2.2, page 35.

Effect of bikunin on crystal nucleation

Nucleation inhibition by bikunin was tested using the method described in Chapter 2.2.2, page 36.

Effect of bikunin on particle formation kinetics

The particle formation (particle size, particle number and particle volume) experiments were conducted using the same protocol as that described in Chapter 2.2.2, page 37. SEM was also determined as described in Chapter 2.2.2, page 37.

6.3 Results

6.3.1 Effect of bikunin on [^{14}C]-oxalate deposition

These results demonstrate that addition of bikunin obtained from black (BB) and white (WB) subjects' urine reduced the percentage of [^{14}C]-oxalate deposition in both BUF and WUF as shown in Figure 6.1. The greatest decrease was observed in the crossed-over experiments i.e. in those experiments in which the protein from the one race group was tested in the ultrafiltered urine of the other race group. The typical figures show that gradients were the same in WUF, but that they decreased as protein was added in BUF. This suggests inhibition of the CaOx deposition rate by BB and WB in BUF. Data and plots for each of the five experiments is shown in Appendix 3 (Black subjects: Table C3.1: and Figure B3.1; White subjects: Table C3.2: Figure B3.2).

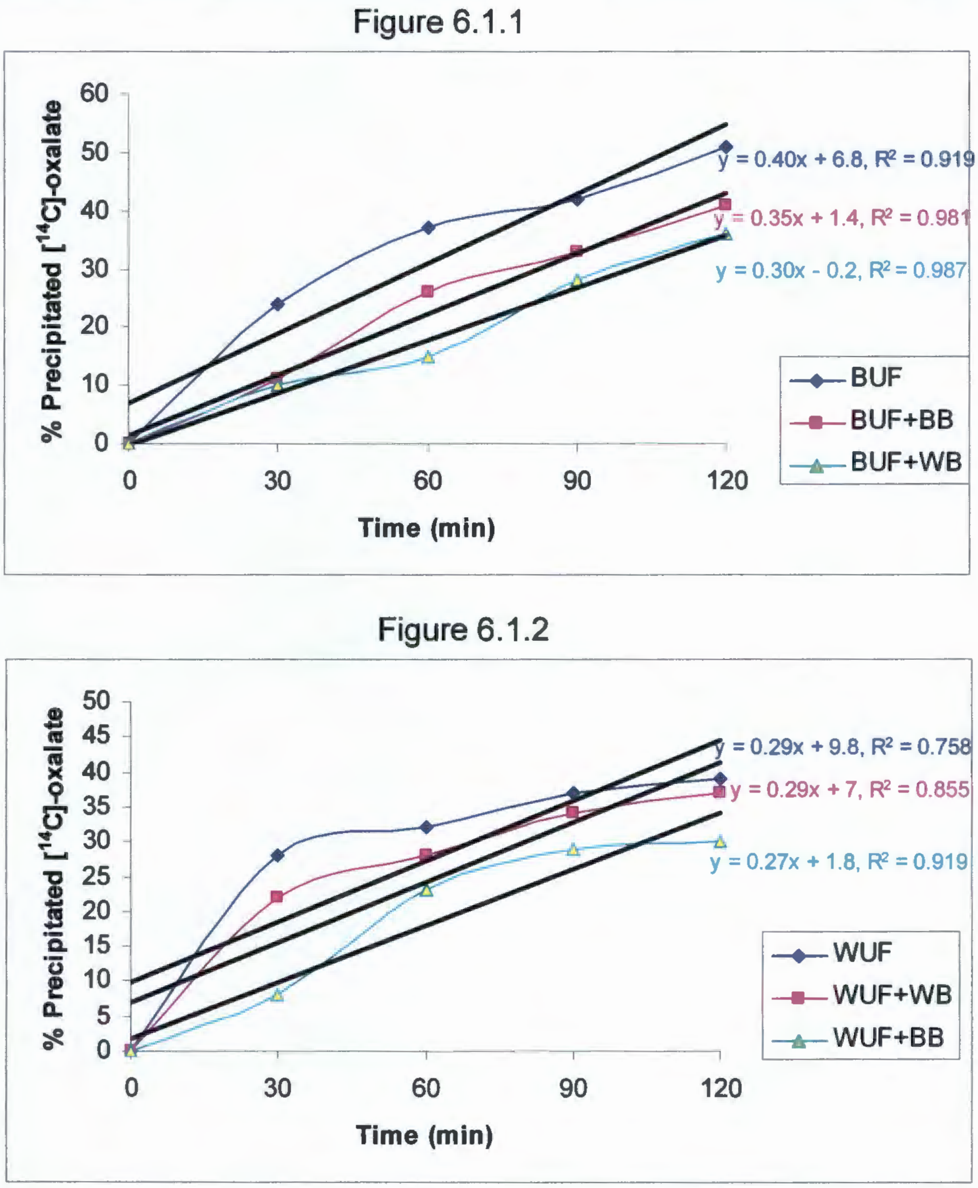


Figure 6.1: Typical plot of [^{14}C]-oxalate deposition experiment expressed as percentage precipitated

Comments:

These results have provided convincing evidence that bikunin reduces CaOx deposition irrespective of the race group from which it was derived. This suggests that bikunin is a strong inhibitor of CaOx crystal formation. Consistent reproducible differences were not observed between the two race groups. Interestingly, the magnitude of the effect of BB and WB in the CaOx deposition rate was greater in BUF. This suggests superior effect of the protein in the urine of the black subjects than in white subjects. As was also observed in Chapter 4, the cross-over combinations were the most effective in this experiment since they induced the lowest percent CaOx deposition.

6.3.2 Effect of bikunin on crystal aggregation

Addition of BB and WB in the COM crystal slurry induced an inhibition of crystal aggregation (Table 6.1). The percentage inhibitions by BB (range of 36-88 %, mean 70 %) and WB (range of 46-91 %, mean 75 %) were not statistically significant. Figure 6.2 shows a typical plot of absorbance vs time of COM crystal slurry before and after addition of BB and WB. The sedimentation rate of the COM slurry decreased greatly after addition of BB and WB as demonstrated by this figure. The plots for each experiment are shown in Appendix 3 (Black subjects: Figure C3.1; White subjects: Figure C3.2).

Table 6.1: Absorbance slopes of COM crystal slurries before and after addition of BB and inhibition of aggregation (% I_A)

Sample	Absorbance slope ($x10^{-5}s^{-1}$) (R^2) ± SE	% I_A
COM (n=5)	-370 (0.978) ± 15.2	
COM+BB1	-150 (0.970)	55
COM+BB2	-130 (0.878)	61
COM+BB3	-60 (0.924)	82
COM+BB4	-58 (0.943)	83
COM+BB5	-40 (0.931)	88
COM+BB6	-59 (0.980)	83
COM+BB7	-107 (0.869)	68
COM+BB8	-81 (0.981)	76
COM+BB9	-211 (0.914)	36
Average	**-100 (0.932) ± 18.6**	**70**
COM (n=5)	-333 (0.962) ± 15.8	
COM+WB1	-30 (0.801)	91
COM+WB2	-180 (0.889)	46
COM+WB3	-106 (0.919)	68
COM+WB4	-70 (0.982)	79
COM+WB5	-30 (0.979)	91
COM+WB6	-96 (0.958)	71
COM+WB7	-78 (0.968)	77
COM+WB8	-151 (0.908)	55
COM+WB9	-45 (0.985)	86
COM+WB10	-62 (0.989)	81
Average	**-85 (0.938) ± 15.7**	**75**

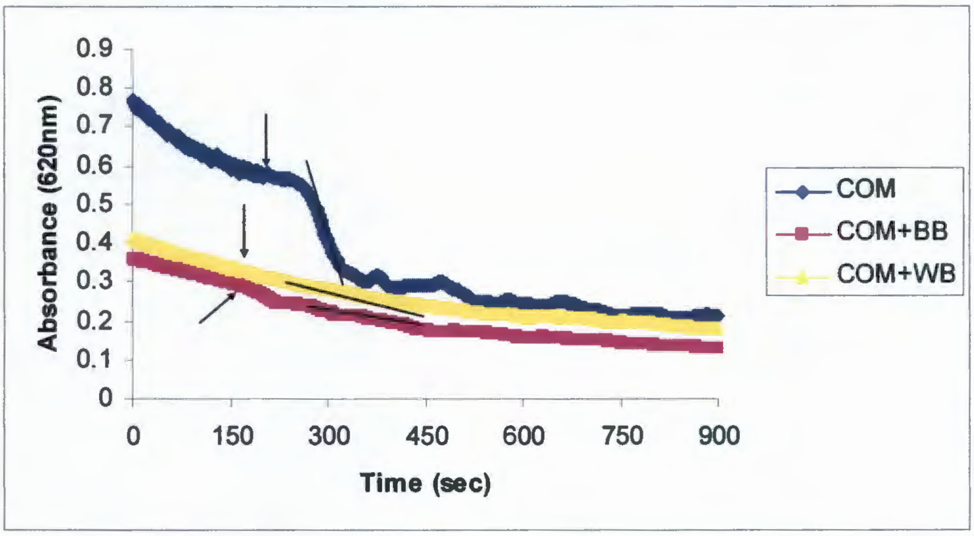


Figure 6.2: A typical plot of absorbance vs time for COM slurry before and after addition of BB and WB at a final concentration of 0.5 mg/L. The arrow indicates the times at which the stirring was stopped to induce aggregation.

Comments:

CaOx crystal aggregation was strongly inhibited by addition of BB and WB. Since this mechanism of crystal formation is a very important one in the formation of CaOx renal stones (Kok *et al.* 1993), it can be concluded that bikunin from both black and white subjects plays an important role in retarding stone formation. However, the results of this particular experiment were unable to establish whether either of the proteins (i.e. BB or WB) is superior to the other in this context.

6.3.3 Effect of bikunin on CaOx nucleation

These results demonstrated that the percent inhibition of CaOx crystal nucleation observed after addition of BB (range 20-60 %, mean 30 %) and WB (range 10-60%, mean 32 %) was not strong (i.e. only ~30%), nor was there a statistically significant difference between BB and WB. The values for each experiment are given in Table 6.2 and a typical plot of absorbance vs time of the control before and after addition of BB and WB is shown in Figure 6.3. The plots for each experiment are shown in Appendix 3 (Black subjects: Figure D3.1; White subjects: Figure D3.2).

Table 6.2: Absorbance slopes before and after addition of BB and WB and inhibition of nucleation (% I_n)

Sample	Absorbance slope ($x10^{-5}s^{-1}$) (R^2) ± SE	% I_n
Control (C) (n=5)	50 (0.869) ± 5.00	
C+BB1	40 (0.961)	20
C+BB2	30 (0.994)	40
C+BB3	40 (0.966)	20
C+BB4	40 (0.815)	20
C+BB5	40 (0.956)	20
C+BB6	20 (0.956)	60
Average	**40 (0.941) ± 3.41**	**30**
C+WB1	30 (0.969)	40
C+WB2	40 (0.876)	20
C+WB3	30 (0.951)	40
C+WB4	20 (0.986)	60
C+WB5	45 (0.968)	10
C+WB6	40 (0.996)	20
Average	**38 (0.957) ± 5.23**	**32**

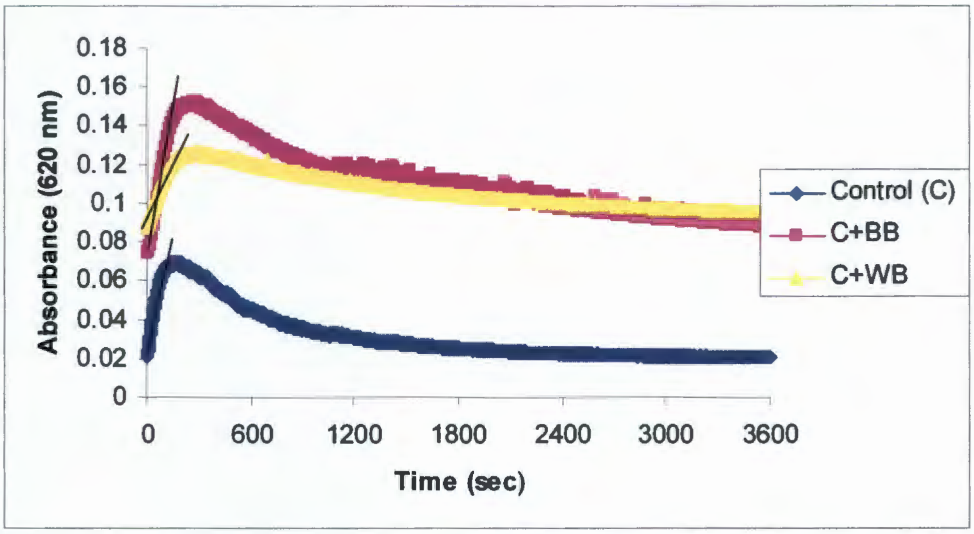


Figure 6.3: A typical plot of absorbance vs time for the control before and after addition of BB and WB at a final concentration of 0.5 mg/L.

Comments:

Nucleation, a process that is defined as the appearance of detectable crystals (Atmani and Khan 1999) was not strongly inhibited by the addition of BB and WB, nor was there a difference between the proteins from the two race groups. Thus, it can be concluded that while inhibition of CaOx nucleation may indeed be an important process in differentiating between the stone forming potential in the two race groups, urinary bikunin does not play a role in this regard.

6.3.4 Effect of bikunin on particle formation kinetics

Particle size distribution

In these experiments a size reduction as indicated by a distribution shift to smaller values was observed when BB and WB were added to BUF in three of five experiments (Table 6.3). Unlike BUF, WUF demonstrated a bimodal size distribution (Table 6.3). These modes were observed in three of five experiments. A decrease in the size of the smaller crystals was observed in four of five WUF experiments when BB and WB were added. Of the three experiments that showed a bimodal distribution in WUF, the larger particles demonstrated a decrease when WB was added. The plots for each of the five experiments are shown in Appendix 3 (Black subjects: Figures E3.1; White subjects: Figures E3.2).

Table 6.3: Particle size (µm) of ultrafiltered urine from black (BUF) and white (WUF) subjects before and after addition of BB and WB at final concentrations of 0.5 mg/L

	BUF	BUF+BB	BUF+WB
Experiment 1	2.9	2.9	2.9
Experiment 2*	5.8	2.9	2.9
Experiment 3	6.9	8.7	6.9
Experiment 4*	5.4	4.4	3.5
Experiment 5*	8.0	5.5	7.0
	WUF	**WUF+WB**	**WUF+BB**
Experiment 1	5.8 and 15.3	2.9 and 13.11	4.4 and 15.6
Experiment 2*	19.3	14.9	15.3
Experiment 3*	17.5	16.0	16.4
Experiment 4	3.6 and 7.7	6.5 and 12.0	7.2 and 12.0
Experiment 5*	6.9 and 15.6	4.0 and 11.3	4.0 and 15.3

* Trends showing a decrease

Figure 6.4.1

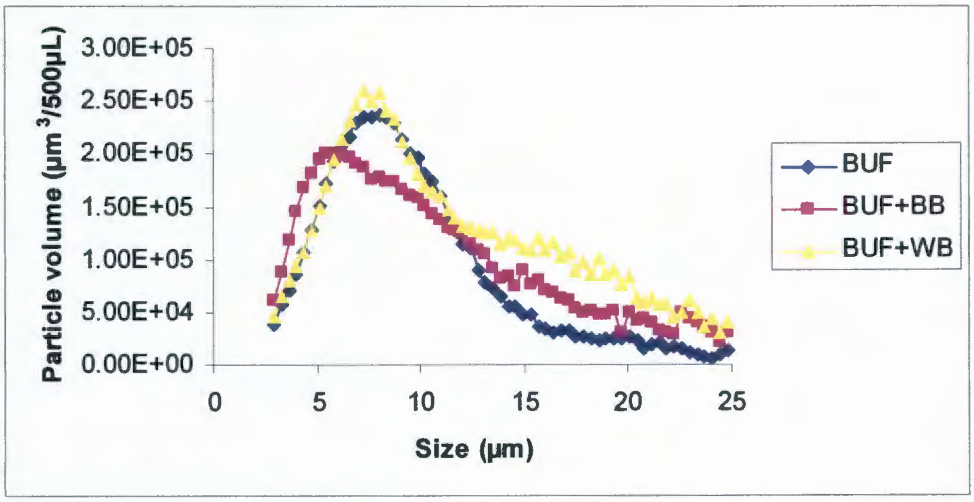


Figure 6.4.2

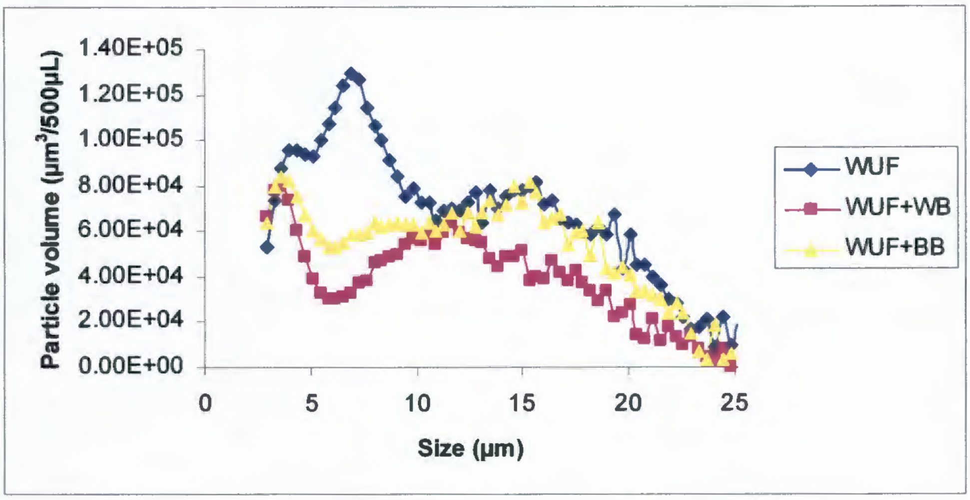


Figure 6.4: Particle size distribution in BUF (Figure 6.4.1) and WUF (Figure 6.4.2) before and after addition of BB and WB.

Comments:

Interesting findings emerged from these studies. For example, the particle size decreased when bikunin was added in both BUF and WUF. This suggests that bikunin isolated from the urine of black and white subjects inhibits CaOx growth. The observation that the particles in white subjects are larger and have bimodal distribution is not surprising and confirms a similar observation in Chapters 3. This is an unfavourable condition since larger particles form due to reduced or promotion of crystal aggregation (Hess *et al.* 1996) and this is synonymous with the high occurrence of the stones reported in the South African population.

SEM

The CaOx crystals precipitated after addition of Na_2Ox were viewed using SEM to identify their morphology and extent of aggregation. Figure 6.5 shows the micrographs of CaOx crystals deposited after addition of Na_2Ox before and after addition of BB and WB. As shown in Figure 6.5.1, the crystals deposited in the BUF have a predominantly COM crystal morphology while a mixture of COM and COD crystal morphology was observed in WUF (Figure 6.5.4). The crystal number decreased after addition of BB in BUF (Figure 6.5.2) and aggregates of crystals were seen after addition of WB (Figure 6.5.3). Of further interest was that the morphology of the crystals changed from COM to COD when both BB and WB were added (Figure 6.5.2, 6.5.3, 6.5.5 and 6.5.6). Changes in sizes after addition of BB and WB were not observed. The crystals were all viewed at 4K magnification except for Figure 6.5.6 which was viewed at 2.5K. This micrograph (Figure 6.5.6) is included to demonstrate morphology only.

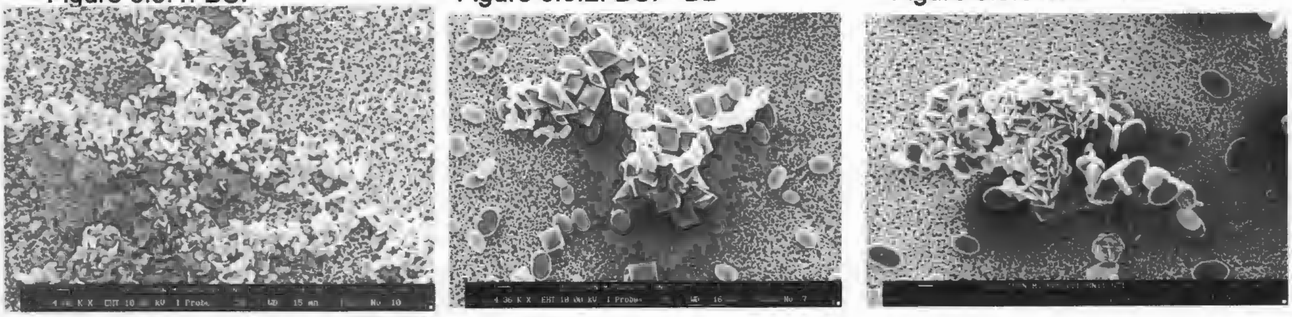


Figure 6.5.1: BUF

Figure 6.5.2: BUF+BB

Figure 6.5.3: BUF+WB

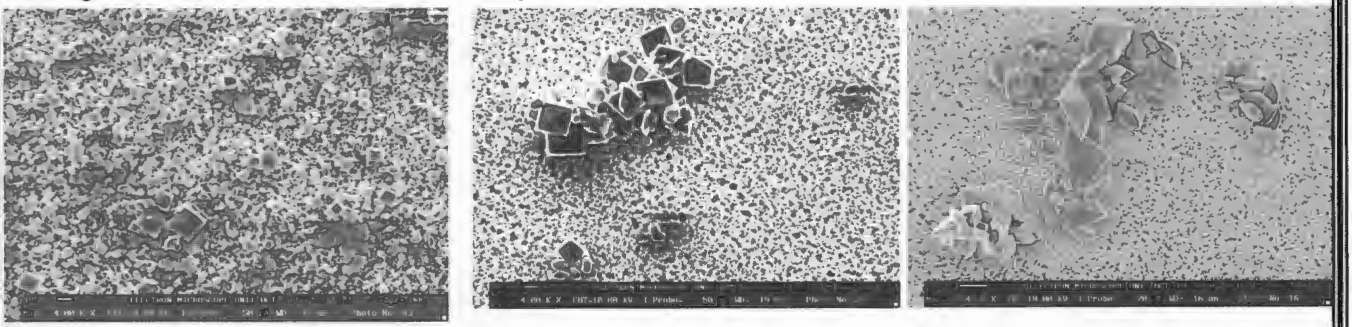


Figure 6.5.4: WUF

Figure 6.5.5: WUF+WB

Figure 6.5.6: WUF+BB

Figure 6.5: Scanning electron micrographs of CaOx crystals precipitated from BUF (Figure 6.5.1, mag. 4K) before and after addition of BB (Figure 6.5.2, mag. 4K) and WB (Figure 6.5.3, mag. 4K), and addition that of WUF (Fugure 6.5.4, mag. 4K) before and after addition of WB (Figure 6.5.5, mag. 4K) and after addition of BB (Figure 6.5.6 mag. 2.5K) at a final concentration of 0.5 mg/L

Comments:

As stated in other chapters, the Coulter Counter has limitations and therefore SEM was used to supplement the experiments involving this instrument. The SEM demonstrated that addition of WB unfavourably formed aggregates of crystals in BUF. *In vitro* CaOx crystallization under the conditions employed in the present study produced predominantly COM crystals in the ultrafiltered urine from black and white subjects. The morphology of the crystals changed from COM to COD after addition of BB and WB in both BUF and WUF. Since it has been reported that bikunin prevents adhesion of calcium crystal to renal tubular cells in human urine by blocking the binding of [^{14}C]-COM crystals to renal ephithelial cells (Ebisuno *et al.* 1999; Yang *et. al.* 2005), it is possible that it achieves this by changing their morphology to COD crystals, which has been reported to be less adherent to renal tubular cells (Wesson *et al.* 1998). This conversion may be regarded as favourable for another reason, namely that the higher positive charge on COD (due to greater number of calciums per unit cell) results in greater repulsive forces between crystals and therefore favors disaggregation (Webber 2003).

Particle number

An obvious trend was observed in the particle number determinations when bikunin was added to BUF. For example, a decrease in particle number (90-120 min) was observed in these experiments when both BB and WB were added. A consistent trend was not observed when bikunin was added to WUF, hence a typical plot for these experiments is not shown. Although the WUF experiment did not demonstrate any consistency, the plots for these experiments are shown in Appendix 3. Figure 6.6 shows a typical plot for particle number experiment of BUF before and after addition of BB and WB at 30 min intervals for 120 min. The rate of particle formation decreased on both BUF and WUF after addition of both BB and WB as shown in Figure 6.6. The tables and plots for each experiment for each pool at 30 min interval are shown in Appendix 3 (Black subjects: Tables D3.1 and Figures F3.1; White subjects: Tables D3.2 and Figures F3.2).

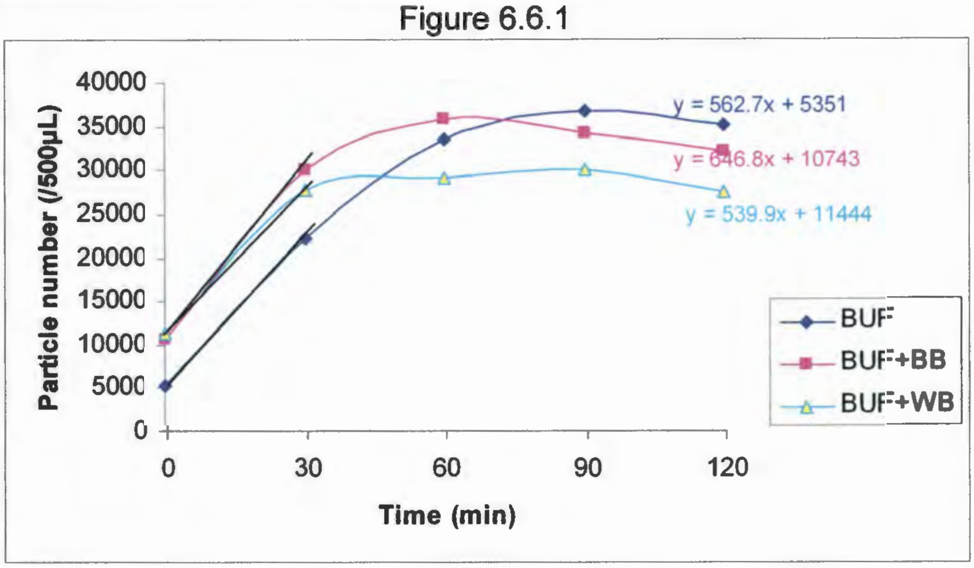


Figure 6.6.1

Figure 6.6: A typical plot of particle number of ultrafiltered urine from black subjects (6.6.1)

Comments:

Particle numbers tended to decrease after addition of BB in BUF but addition of bikunin in WUF did not show any obvious trend. As with the other experiments presented in Chapters 3 and 4, this type of trend is interpreted as either inhibition of nucleation or aggregation promotion. The latter process (viz promotion of aggregation) can be ruled out on the basis of the sedimentation experiments described in section 6.3.2 which demonstrated inhibition of this mechanism. Thus, the decrease in particle number after addition of bikunin is indicative of nucleation inhibition. This is consistent with the results observed in the experiments described in section 6.3.3 which demonstrated a weak inhibitory effect towards nucleation.

Particle volume

The particle volume determination demonstrated an increase in the particle volume when BB and WB were added to BUF and WUF. There were not significant differences observed between the two race groups ($p>0.05$). Figure 6.7 shows a typical plot of particle volume of ultrafiltered urine from BUF and WUF before and after addition of BB and WB at 30 min intervals for 120 min. The data and plots for experiments for each pool are shown in Appendix 3 (Black subjects: Tables E3.1 and Figures G3.1; White subjects: Tables E3.2 and Figures G3.2).

Figure 6.7.1

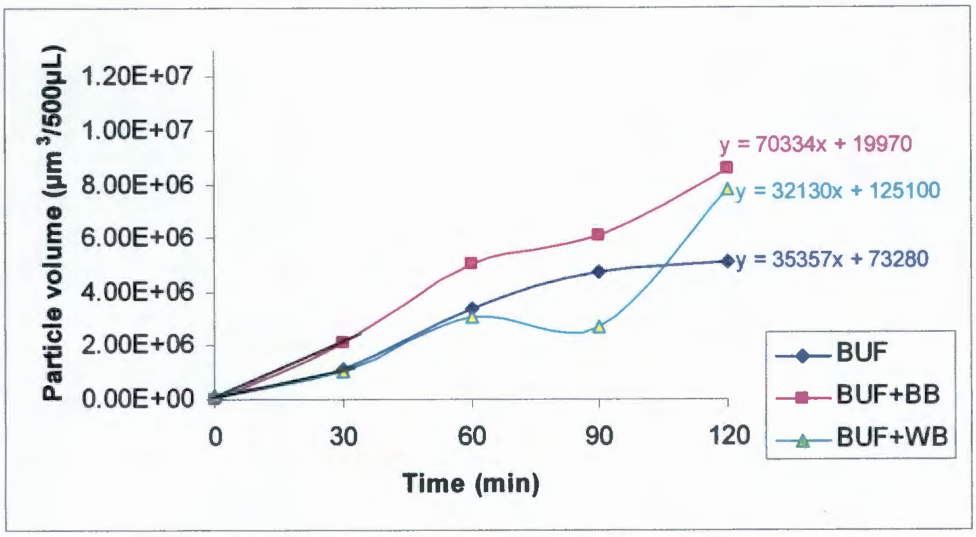


Figure 6.7.2

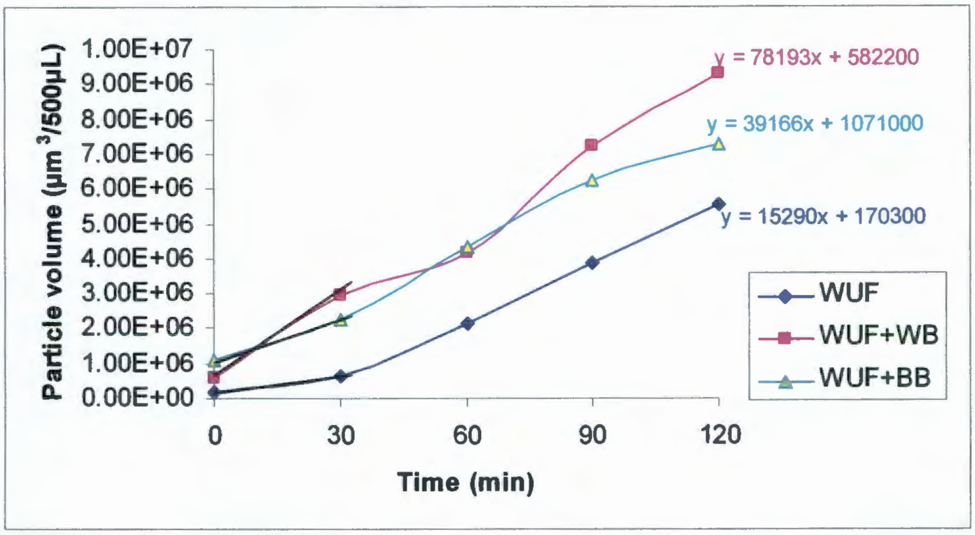


Figure 6.7: Typical plot of particle volume (µm^3/500µL) of BUF (Figure 6.7.1) and WUF (Figure 6.7.2)

Comments:

An increase in particle volume could mean an increase in the total mass of deposited crystalline material due to crystal nucleation or growth. Alternatively, it could be due to imperfect packing together of crystals causing pores containing fluid thereby generating larger volumes. However, none of these processes is consistent with the results of other crystallization experiments in this particular study.

6.4 Discussion

Following the successful isolation of bikunin, crystallization experiments were conducted to test the inhibitory activity of bikunin isolated from black and white South African male subjects. In the present study, two independent techniques (nucleation experiment and Coulter data) indicated that bikunin is an inhibitor of nucleation, albeit rather weak. This is in agreement with other reports which found bikunin to be an inhibitor of CaOx nucleation (Atmani *et al.*1998; Médétognon-Benissan *et al.* 1999). Further support for this notion was provided by the [^{14}C]-oxalate deposition experiments which demonstrated that bikunin is an inhibitor of CaOx crystal formation.

Coulter data also showed that addition of bikunin inhibited CaOx crystal growth. Growth in the context of crystallization is usually taken to mean the enlargement of crystals by direct incorporation of solution species in to the solid crystal lattice. The observation that bikunin inhibits CaOx growth is in agreement with the literature reports (Atmani *et al.* 1993; Okuyama *et al.* 2003). The present study also demonstrated that bikunin is a strong inhibitor of CaOx aggregation and this is also in agreement with other reports in the literature (Atmani *et al.* 1999).

Interesting findings also emerged from this chapter as it was suggested that the mechanism by which bikunin is able to reduce the risk of kidney stone formation is by altering the crystal morphology from COM to COD. The latter has been shown to have a low binding affinity for renal epithelial cells, thus reducing crystal retention in the collecting duct (Ebisuno *et al.* 1999; Yang *et. al.* 2005). This confirms the hypothesis that bikunin contributes to the ability of urine to inhibit *in vivo* crystallization. Literature has also indicated that bikunin alters the ratio of CaOx monohydrate (COM) to dihydrate crystallization (Wesson *et al.* 1998).

It needs to be noted that while SEM micrographs demonstrated an unfavourable crystal aggregate formation after addition of WB in BUF, addition of BB induced an inhibitory effect both in its native urine and in the cross-over experiment.

Furthermore the larger particles observed in the urine of the white subjects are consistent with the relatively higher stone occurrence in the South African white populations. This also confirms other findings in other experiments presented in other chapters.

While these experiments showed the inhibitory capacity of bikunin, they were unable to differentiate between the proteins from the two race groups. As such the findings described in this chapter serve to support the protective role of bikunin in CaOx urolithiasis, but do not explain the difference in stone incidence in the two South African race groups.

Chapter 7

Deglycosylation of bikunin and crystallization experiments in ultrafiltered urine from black and white subjects

7.1 Introduction

Glycosylation is a process whereby oligosaccharides are attached to proteins within the endoplasmic reticulum and Golgi apparatus of cells (Rudd and Dwek 1997). Bikunin is a highly glycosylated protein (Koide *et al.* 1981; Ebisuno 1999; Médétognon-Besissan *et al.* 1999; Yang *et al.* 2005). It has two glycosylation sites, namely an *O*-linked site whereby glycans are attached to a serine residue at position 10 of the core protein linked via the chondroitin sulphate that contains 15 disaccharide repeats, and an *N*-linked oligosaccharide at position 45 (Höchstrasse *et al.* 1981). The *O*-linked position is the more glycosylated of the two (Höchstrasser *et al.* 1981). The bikunin glycans account for approximately one third of the total molecular mass of the protein (Médétognon-Besissan *et al.* 1999; Ebisuno *et al.*1999; Yang *et al.* 2005).

Reports have indicated that bikunin's glycans are responsible for 70 - 90 % of its total inhibitory activity in urine (Koide *et al.* 1981; Médétognon-Besissan *et al.* 1999). However these studies only report the effect of this protein in inorganic solutions and not in urine. Since it was established in Chapter 3 that commercial bikunin (CB) has inhibitory effect towards CaOx crystallization, the study presented in this chapter describes an investigation into the inhibitory effect of this bikunin after the removal of glycans. In addition, since studies in the present project have demonstrated that the urine milieu affects the functionality of bikunin, the crystallization experiments involving deglycosylated protein were conducted in ultrafiltered urine from both race groups wherever appropriate, to further explore this synergistic relationship.

Objectives

- To deglycosylate bikunin using exoglycosylases
- To study and compare the inhibitory effect of glycosylated and deglycosylated commercial bikunin on particle size, [^{14}C]-oxalate deposition, crystal nucleation and aggregation as previously described in Chapter 3, 4 and 6, using ultrafiltered urine from both race groups where appropriate, to explore the synergistic relationship between functionality, structure and urine composition.
- To study the inhibitory effects on CaOx crystal aggregation using an additional technique - zeta potential, which has not yet been utilized in the other studies of this thesis

7.2 Methods

7.2.1 Bikunin deglycosylation

Enzymatic digestion

Commercial bikunin (CB) was enzymatically deglycosylated using a method described by Capon *et al.* (2003). Briefly, a control sample was prepared by incubating 400 µg CB in a 20 mM Gly/HCL pH 5.5, buffer containing 75 mM NaCl for 16 hrs at 37 °C. To remove the *O*-linked glycans, 400 µg CB was treated similarly after addition of 16 µL of chondroitinase ABC (2.5 U/ml) (Sigma-Aldrich) which removes the *O*-linked chondroitin sulphate chain. The solutions were then applied to a 10 kDa centricon membrane (Milipore) and centrifuged at 10 000 *g* for 11 min (Sorvall MC 12C centrifuge) to separate the released sugars and the partly deglycosylated protein. The sample protein on the membrane was then washed three times with 200 µL H_2O. To collect the partly deglycosylated protein, the membrane in the reservoir was inverted and centrifuged for ~10 sec.

Finally, to remove the *N*-linked glycans, the partly degycosylated protein was digested further using endoF2 (2.5 U/ml) and sialidase (2.5 U/ml) (Sigma-Aldrich). *Clostridium perfringens* sialidase (2.5 U/ml) (Sigma, Schnelldorf, Germany) removes α2-3, α2-6 and α2-8 linked sialic acids and *Chryseobacterium (Flavobacterium) meningosepticum* endoglycosidase F2 recombinant in *E. coli* (2.5 U/ml) (Sigma) cleaves between the two *N*-acetylglucosamine residues in the diacetylchitobiose core of the oligosaccharide. The remaining protein was then analyzed by MALDI-TOF MS, Nano-Electrospray ionization mass spectrometry (Nano-ESI-MS) and 12 % SDS-PAGE in order to determine the glycosylation status of bikunin. Henceforth, the partially deglycosylated bikunin will be referred to as DB.

MALDI-TOF MS analysis

1 µg of protein (1 µL) was mixed with 1 µL MALDI-TOF MS matrix. The matrix was a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) in 50 % acetonitrile and 0.3 % TFA. The mixture (2 µL) was applied to a MALDI P100 gold sample plate and allowed to dry by gentle streaming with air to allow crystallization of the matrix and the sample. The sample was then analyzed using a Perseptive Biosystems Voyager DE-PRO Biospectrometry Workstation with Delayed Extraction Technology.

Electron spray mass spectrometer

Nano-Electrospray ionization mass spectrometry (Nano-ESI-MS) data was obtained using a quadrupole-time-of-flight (Q-TOF) (Ultima Global instrument, Waters/Micromass, Ltd, Manchester). Samples were prepared in 1:1 (v:v) methanol:water containing 1 % formic acid (Webber *et al.* 2006).

7.2.2 Crystallization experiments

Synthetic urine preparation

Synthetic urine (SU) was prepared by mixing NaCl (320 mM); NaH_2PO_4 (50 mM); $MgCl_2$ (7.52 mM); KCl (164.2 mM); $K_3C_6H_5O_7.2H_2O$ (4.34 mM); $(NH_4)_2SO_4$ (43.8 mM) and NH_4Cl (7.0 mM) in distilled water (Walton *et al.* 2005). The pH was adjusted to 6 using 5 M NaOH. To achieve a desired metastable limit, 50 mM Na_2Ox and 120 mM $CaCl_2$ were added to the filtered SU solution.

Effect of CB and DB on [^{14}C]-oxalate deposition

The effect of CB and DB on [^{14}C]-oxalate deposition in SU, BUF and WUF was tested after dosing with each protein at a final concentration of 0.5 mg/L using the method described in Chapter 2.2.2, page 32.

Effect of CB and DB on crystal aggregation

A COM crystal slurry was prepared as described Chapter 2.2.2, page 35 and sedimentation rates were determined after the addition of protein as follows: COM+CB, COM+DB, COM+SU, COM+SU+CB, COM+SU+DB, COM+BUF, COM+BUF+CB, COM+BUF+DB, COM+WUF, COM+WUF+CB and COM+WUF+DB in the ratio of 1:4 (Protein or urine: COM).

Effect of CB and DB on crystal nucleation

Separate solutions of 8.5 mM $CaCl_2$ and 1 mM Na_2Ox each containing 200 mM NaCl and 10 mM sodium acetate, pH 5.70 were prepared. The solutions were filtered (0.22 µm) before each experiment and the inhibitor was added to achieve a final concentration of 0.5 mg/L in the ratio of 2:1:2 ($CaCl_2$: Protein or urine: Na_2Ox) as follows: C+CB, C+DB, C+SU, C+SU+CB, COM+SU+DB, C+BUF, C+BUF+CB, C+BUF+DB, C+WUF, C+WUF+CB and C+WUF+DB.

Effect of CB and DB on particle size distribution

The urine was collected and pooled as described in Chapter 2.2.1, page 30. A Coulter Counter was used to determine the particle volume-size of SU, BUF and WUF before and after dosing with CB and DB at a final concentration of 0.5 mg/L using a method previously described in Chapter 2.2.2, page 37. SEM was used to view the crystals precipitated from the SU, BUF and WUF using a method described in Chapter 2.2.2, page 37. Three separate particle volume-size distribution measurements were made in SU. Five pooled urines from each race groups were collected for the particle volume-size distribution experiments.

Effect of CB and DB on zeta potential

Zeta potential, an indicator of the potential of a compound to reduce aggregation by action of its repulsive negative charge was measured using the method described by Hess *et al.* (1989). COM crystals were prepared and characterized as described in Chapter 2.2.2, page 33. COM slurry (0.03 mg/ml), which was used as a control, was prepared by the addition of COM crystals to a 10 mM sodium acetate buffer, pH 5.7, containing 270 mM NaCl. The slurry was equilibrated overnight with constant stirring (1100 rpm) at 25 °C. Thereafter, CB and DB were added at final concentrations of 0.5 mg/L and equilibrated for a further 2 hrs. The zeta potential (mV) of the COM slurry before and after addition of CB and DB was measured using a Zetasizer Nanoseries (Malvern Instruments, England). Five independent experiments were conducted.

7.3 Results

7.3.1 Bikunin deglycosylation

MALDI-TOF MS and Nano-ESI-MS

Two peaks at 22920 and 15694 were observed in the undigested protein (Figure 7.1). Treatment with chondroitinase ABC resulted in the reduction of the protein mass from 22920 to 18633 Da, a total mass loss of 4920 Da (Figure 7.2). The second peak at 16545 Da is almost similar to the 15694 Da signal before treatment with the enzyme. The protein mass after treatment with chondroitinase ABC, endoF2 and sialidase was 14922 Da, which represent a total mass loss of 7889 Da.

Figure 7.1: MALDI-TOF MS spectrum of CB

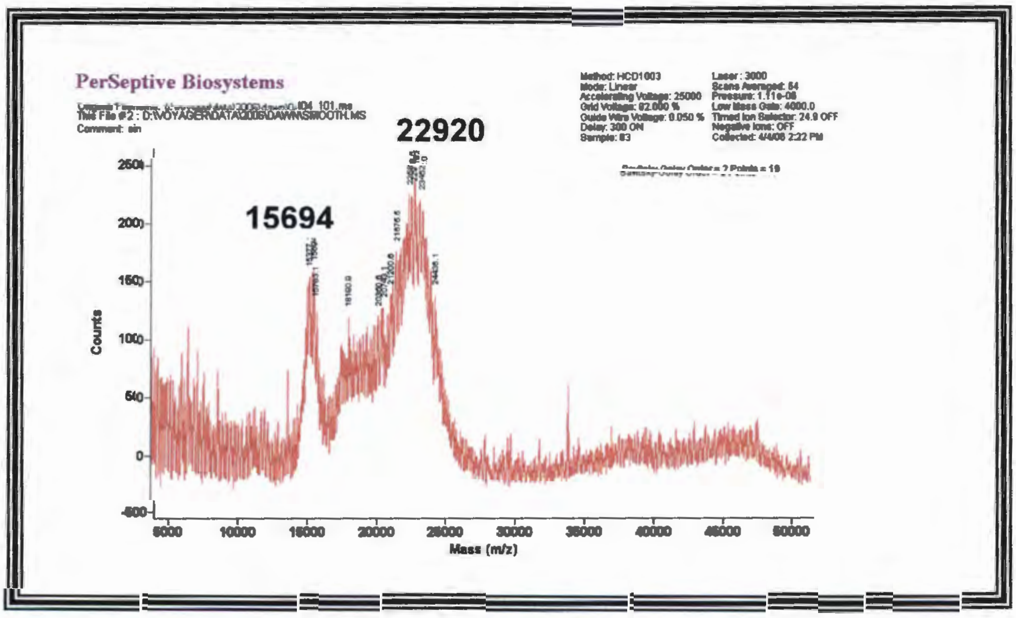


Figure 7.2: MALDI-TOF MS spectrum of CB after addition of chondroitinase ABC

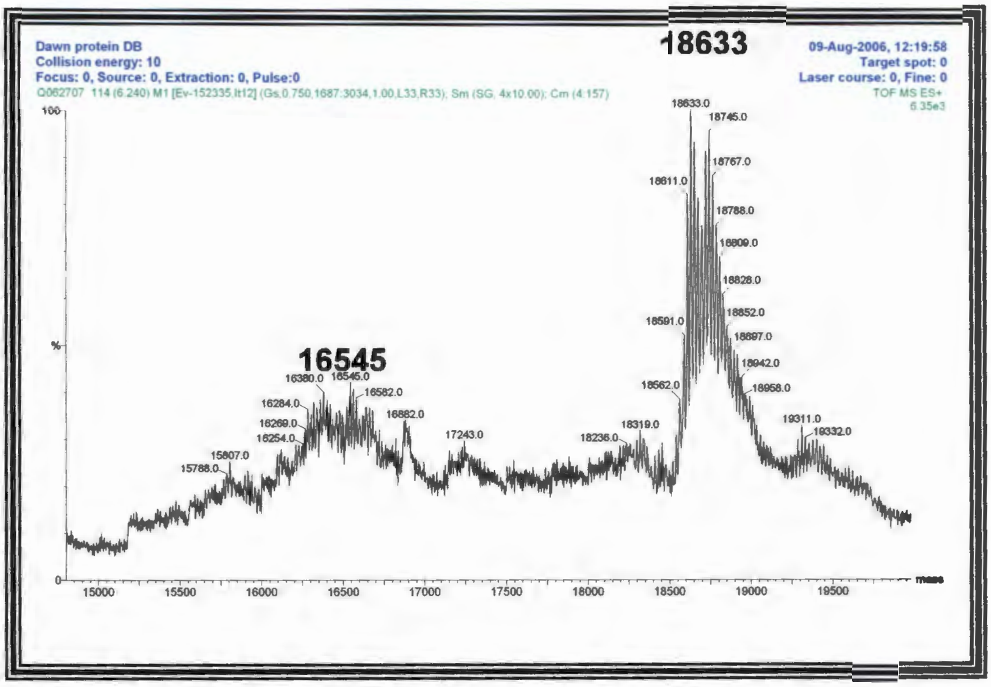
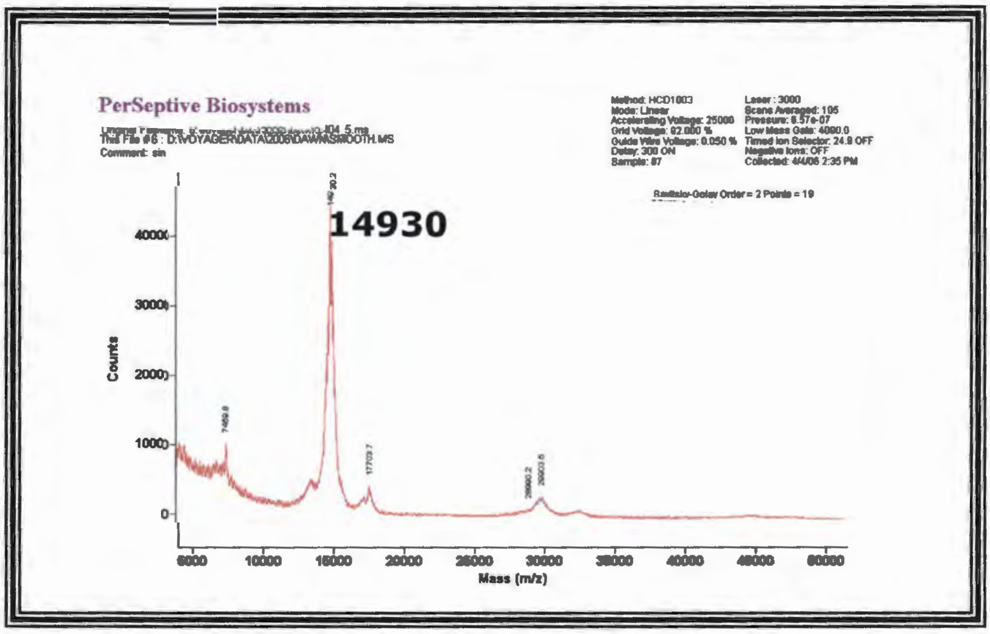


Figure 7.3: MALDI-TOF MS spectrum of CB after addition of chondroitinase ABC, endoF2 and sialidase



SDS-PAGE

In order to verify MALDI-TOF MS and Nano-ESI-MS results the digested proteins were analysed by 12 % SDS-PAGE. Figure 7.4 shows the gel of CB and the resulting products after enzyme digestion. The molecular masses of CB (lane 1) and that of the control sample in lane 2 incubated in buffer alone (lane 2) when run under reducing conditions were both 30 kDa. The mass of CB after treatment with chondroitinase ABC was 18 kDa under both reducing (lane 3) and non-reducing conditions (lane 3'). After treatment of CB with chondroitinase ABC, endoF2 and sialidase, three distinct bands were apparent at 15, 18 and 60 kDa under reducing (lane 4) and non-reducing conditions (lane 4').

Figure 7.4: SDS-PAGE of CB before and after enzyme digestion

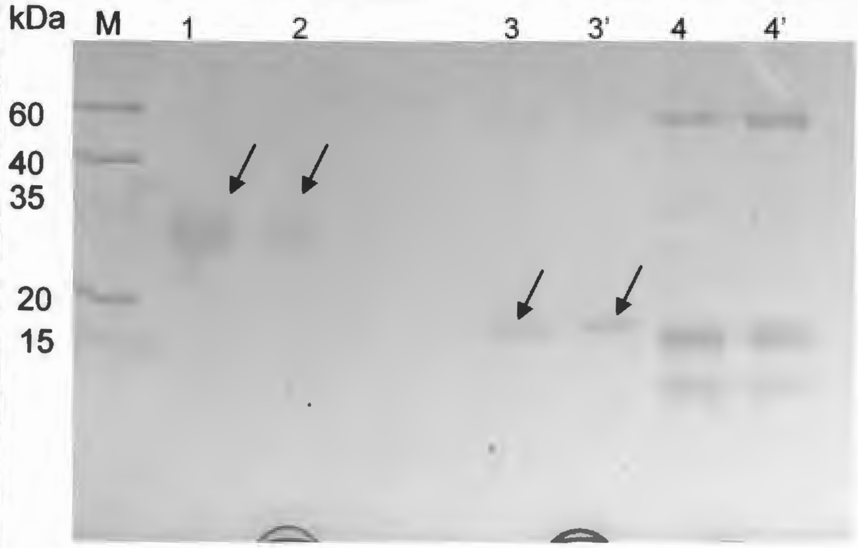


Figure 7.4: 12 % SDS-PAGE of CB (lane 1) and control (lane 2) before treatment with chondroitinase ABC digestion (lane 3 and 3') and treatment with chondroitinase ABC + endoF2 + sialidase (lane 4 and 4'). Lane M is the low molecular weight marker.

Comments:

Enzyme treatment of CB was monitored by MALDI-TOF MS, Nano-ESI-MS and SDS-PAGE. According to MALDI-TOF MS, CB has a mass of 22920 Da. However, a band at 30 kDa was observed on 12 % SDS-PAGE and this is consistent with the literature (Capon *et al.* 2003). It is likely that the extensive glycosylation of bikunin affects its migration on the gel. MALDI-TOF MS also showed an additional mass of 15694 Da suggesting the presence of a deglycosylated bikunin in the commercial bikunin preparation. However, this band was not observed on SDS-PAGE and this might be due to its low intensity.

Removal of the chondroitin sulphate chain from bikunin by chondroitinase ABC reduced the glycosylated proteins' mass from 22920 to 18633 Da. The latter mass has been reported previously after digestion with chondroitinase ABC (Imanari *et al.* 1992; Capon *et al.* 2003). The mass of bikunin treated with all three enzymes was 14930 Da, according to the MALDI-TOF MS which is the same as that predicted in the literature (Imanari *et al.* 1992; Capon *et al.* 2003; Yang *et al.* 2005). Surprisingly, the protein after treatment with all three enzymes also showed two additional bands at 18 and 60 kDa on SDS-PAGE. The band at 18 kDa is likely due to incomplete deglycosylation after addition of endoF2 and sialidase and the other band at 60 kDa could not be accounted for. Thus only the commercial bikunin and ABC treated protein were used for further crystallization experiments.

7.3.2 Crystallization experiments

Synthetic urine experiments

Effect of CB and DB on [^{14}C]-oxalate deposition

In this experiment, CB and DB had a different effect after addition to SU, for example DB reduced the deposition of [^{14}C]-oxalate to a lesser extent compared to CB. Figure 7.5 shows the typical plot of [^{14}C]-oxalate experiment before and after addition of CB and DB. The data and plots for each of the three experiments are shown in Appendix 4 (Table A4.1 and Figure A4.1).

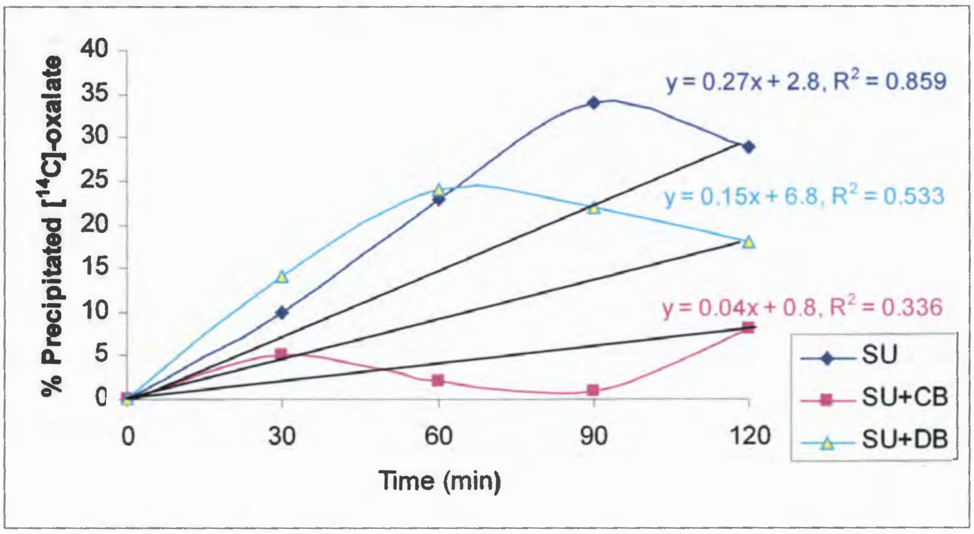


Figure 7.7: A typical plot of percent precipitated [^{14}C]-oxalate of SU before and after addition of CB and DB at final concentrations of 0.5 mg/L

Comments:

The speculation about the reduced inhibitory activity of CB after removal of glycans was confirmed by these results which showed that CaOx oxalate deposition inhibition by bikunin was greatly reduced after the removal of the glycans. This suggests that the CB's ability to inhibit CaOx crystal growth is largely due to the glycans.

Effect of SU, CB and DB on crystal aggregation

Addition of CB and DB on COM crystal slurry significantly inhibited CaOx crystal aggregation. However, DB (range 58 - 87 %, mean 74 %) reduced aggregation to a significantly lesser extent compared to CB (range 73-96 %, mean 87 %) as shown in Table 7.1 ($p<0.05$). A typical plot shown in Figure 7.6 demonstrates that the sedimentation rate was significantly reduced after addition of CB than when DB was added. The plots for each of these experiments are shown in Appendix 4 (Figure B4.1).

Table 7.1: Absorbance slopes of COM crystal slurries before and after addition of CB and DB and inhibition of aggregation (% I_A)

Sample	Absorbance slope ($x10^{-5}s^{-1}$) (R^2)	% I_A
COM (n=5)	-520 (0.994) ± 31.2	
COM+CB1	-40 (0.980)	92
COM+CB2	-20 (0.978)	96
COM+CB3	-60 (0.967)	88
COM+CB4	-40 (0.987)	92
COM+CB5	-110 (0.976)	79
COM+CB6	-140 (0.976)	73
Average	**-68 (0.978) ± 19.0**	**87**
COM+DB1	-190 (0.987)	63
COM+DB2	-70 (0.982)	87
COM+DB3	-90 (0.982)	83
COM+DB4	-220 (0.951)	58
COM+DB5	-90 (0.856)	83
COM+DB6	-150 (0.967)	71
Average	**-135 (0.954) ± 27.5**	**74**

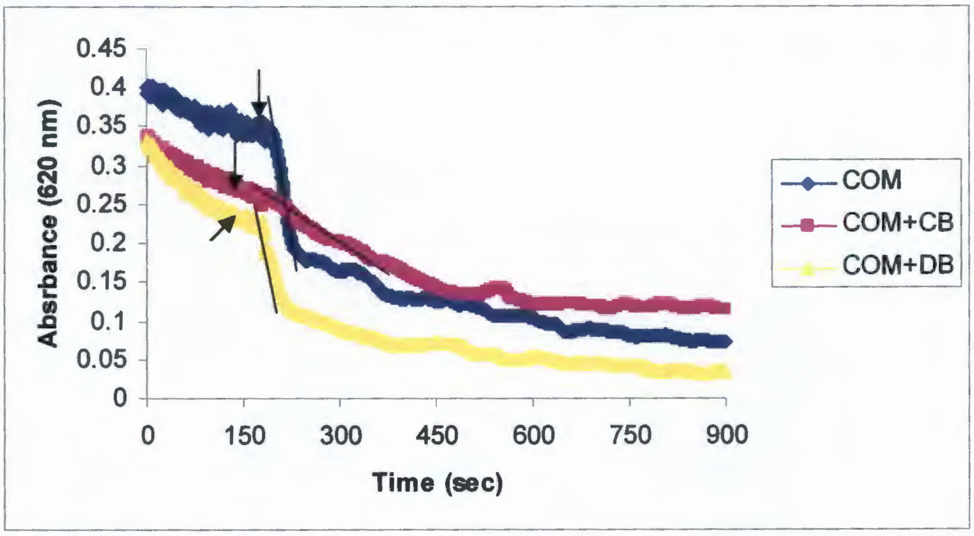


Figure 7.6: A typical plot of absorbance vs time plot of COM slurry before and after addition of CB and DB at final concentrations of 0.5 mg/L. The arrows indicate the time at which the stirring was stopped to induce aggregation

The effect of CB and DB was also tested in the presence of SU. The results show that there was no apparent change when DB was added with SU ($p>0.05$). The percent inhibition after addition of SU increased significantly when CB (87%) was added. A typical plot for these experiments is shown in Figure 7.7. The data and plots for each experiment are shown in Appendix 4 (Table B4.1 and Figures B4.2).

Table 7.2: Absorbance slopes and inhibition of aggregation (% I_A) of COM crystal slurries before and after addition of SU, CB and DB

Sample	Absorbance slope ($\times 10^{-5} s^{-1}$) (R^2)	% I_A
COM (n=5)	-520 (0.994)	
COM+SU (n=3, pools)	-173 (0.983)	67
COM+SU+CB (n=3, pools)	-67 (0.961)	87
COM+SU+DB (n=3, pools)	-243 (0.975)	53

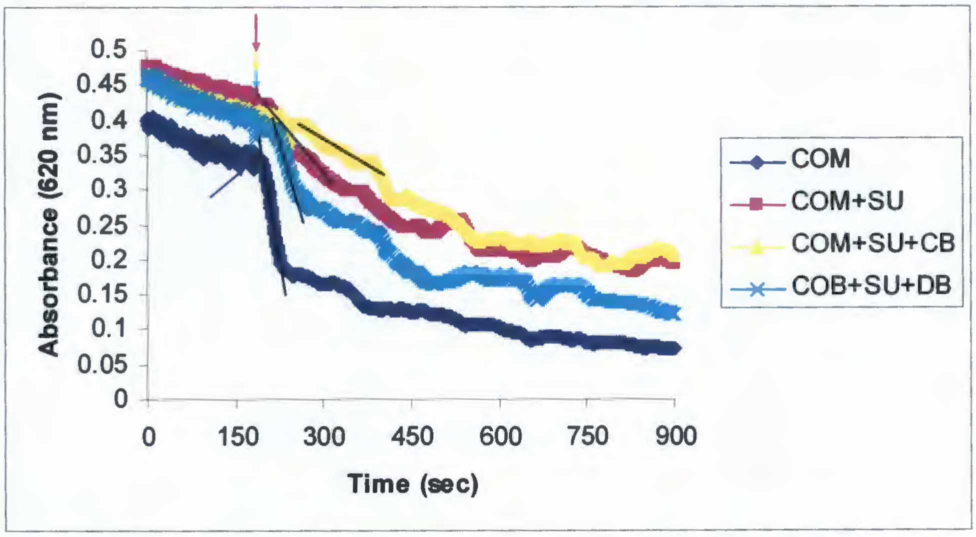


Figure 7.7: Typical absorbance vs time plot of COM slurry before and after addition of SU, CB and DB at final concentrations of 0.5 mg/L.

Comments:

These results indicate that both CB and DB have the ability to inhibit crystal aggregation when tested on their own, suggesting that aggregation inhibition by CB might not be entirely due to the CB glycans. However, DB's ability to inhibit CaOx crystal aggregation diminishes when SU is included in the mixture but a significant increase when CB was added. This is a further confirmation that bikunin glycans along with the nature of the synthetic urine are responsible for its ability bikunin to inhibit CaOx crystallization.

Effect of SU, CB and DB on crystal nucleation

This experiment demonstrated that there were no significant differences between the effect of CB and DB. The percent inhibition of nucleation by CB (range 25-75 %, mean 46 %) and DB (25-50 % mean 45 %), was not significantly different (Table 7.3). Figure 7.8 shows that nucleation was slightly delayed after addition both CB and DB. A typical plot for these experiments is illustrated in Figure 7.8 and plots for each experiment are shown in Appendix 4 (Figure C4.1).

Table 7.3: Absorbance slopes and inhibition of nucleation (% I_n) before and after addition of CB and DB

Sample	Absorbance slope ($x10^{-5}s^{-1}$) (R^2) ± SE	% I_n
Control (C) (n=5)	40 (0.951) ± 3.31	
C+CB1	30 (0.983)	25
C+CB2	10 (0.983)	75
C+CB3	30 (0.995)	25
C+CB4	20 (0.987)	50
C+CB5	20 (0.986)	50
C+CB6	20 (0.981)	50
Average	**28 (0.986) ± 3.74**	**46**
C+DB1	20 (0.984)	50
C+DB2	30 (0.991)	25
C+DB3	20 (0.996)	50
C+DB4	20 (0.996)	50
C+DB5	20 (0.988)	50
C+DB6	20 (0.982)	50
Average	**22 (0.990) ± 1.67**	**45**

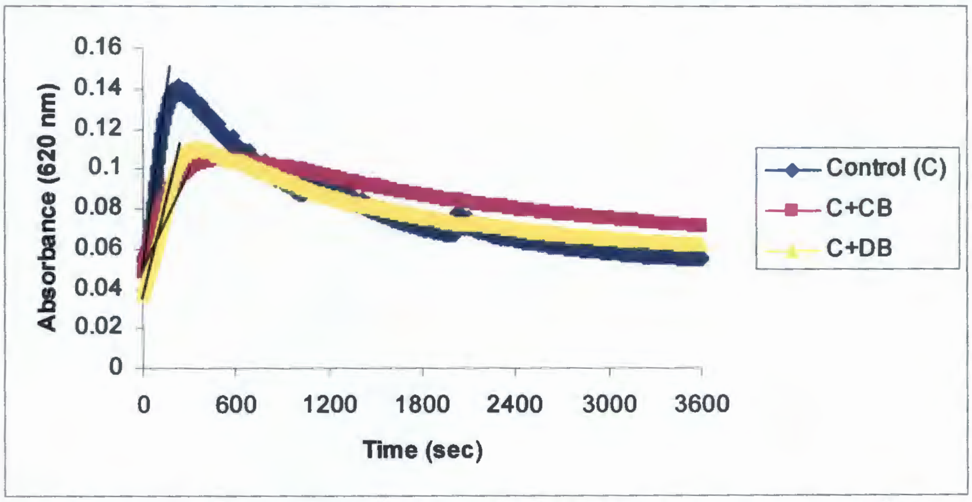


Figure 7.8: A typical plot of absorbance vs time of the control before and after addition of BB and WB at a final concentration of 0.5 mg/L.

The effect of CB and DB was also tested after inclusion of SU in the mixture. The highest inhibition was observed when only SU was tested (90 %, $p<0.05$). Addition of CB and DB did not increase the inhibition of nucleation indicating that the inhibition is largely due to the effect of SU. A typical plot for these experiments is shown Figure 7.9. The data presented in Table 7.4 shows the effect of CB and DB after addition SU. The data and plots for each experiment are shown in Appendix 4 (Table C4.1 and Figure C4.2).

Table 7.4: Absorbance slopes and percent inhibition of nucleation after addition of SU, CB and DB

Sample	Absorbance slope ($\times10^{-5}s^{-1}$) (R^2)	% I_n
Control (C) (n=5)	40 (0.951)	
C+SU (n=3, pools)	4 (0.842)	90
C+SU+CB (n=3, pools)	5 (0.956)	88
C+SU+DB (n=3, pools)	5 (0.987)	88

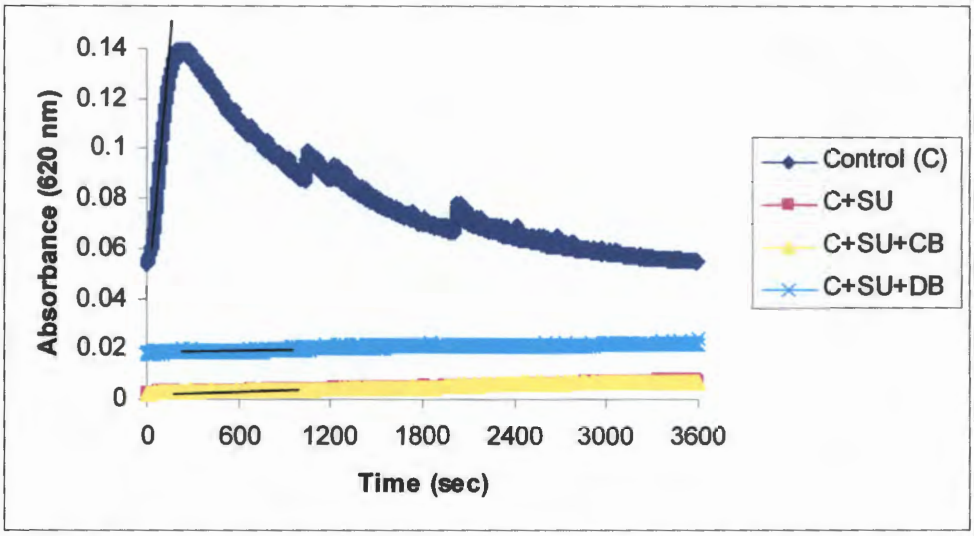


Figure 7.9: A typical plot of absorbance vs time of the control before and after addition of SU, CB and DB

Comments:

The results from this experiment demonstrate that CaOx crystal nucleation was only inhibited when synthetic urine was tested on its own. Addition of CB or CB did not induce any change. This is an indication that CB and DB do not inhibit CaOx nucleation, thus confirming findings in Chapters 3 and 6.

Effect of CB and DB on particle size distribution

A similar trend was observed in all three particle volume-size distribution experiments. As indicated by the distribution shift to the smaller size values in Figure 7.10, the particle size decreased significantly when CB was added to SU. A decrease observed when DB was added is not significant. Table 7.4 shows the particle size in SU before and after addition of CB and DB at a final concentration of 0.5 mg/L. A typical plot of particle volume-size distribution in the SU before and after addition of CB and DB is shown in Figure 7.10. The plots for the each experiment are shown in Appendix 4; (CB: Figures D4.1; DB: Figures D4.2).

Table 7.4: Modes of the particle size distribution (µm) of the SU before and after addition of CB and DB

Sample	SU	SU+CB	SU+DB
Experiment 1	2.91, 15.3	2.91, 7.19	2.91, 10.9
Experiment 2	2.91, 15.3	2.91, 7.19	2.91, 13.8
Experiment 3	2.91, 15.3	2.91, 10.9	2.91, 15.6

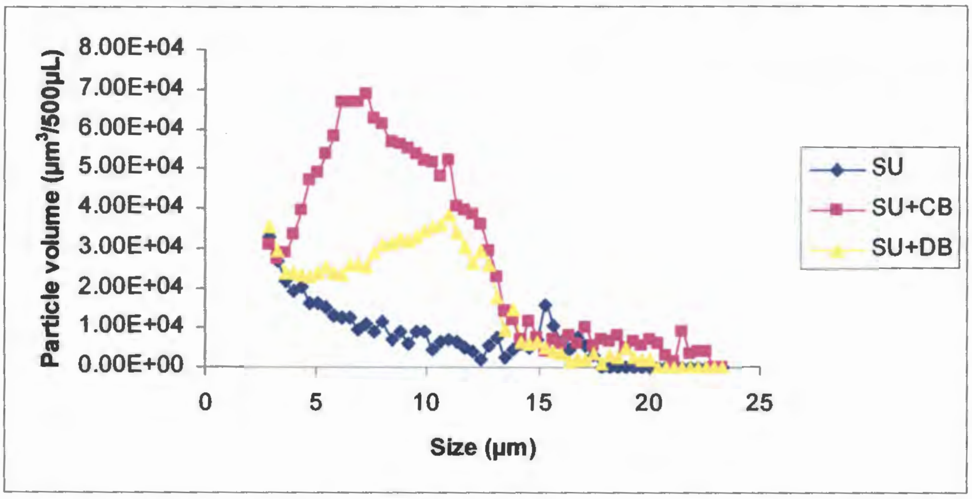


Figure 7.10: Typical plot of particle volume-size distribution of SU before and after addition of CB and DB at a final concentration of 0.5 mg/L at 120 min.

Comments:

A decrease in the particle size is an indication of growth inhibition. In all the experiments, addition of DB had reduced inhibitory effect compared to CB indicating that CB is a better particle growth inhibitor compared to DB.

SEM

The SEM micrographs in Figure 7.11 show the CaOx crystals that were precipitated from SU. All the SEM micrographs before and after addition of CB were viewed at 4K. SU had very few CaOx crystals and only COM crystal morphology was observed. The particle sizes appeared smaller in relation to SU after addition of CB (Figure 7.11.2) and DB did not have an effect of the particle size (Figure 7.11.3). No apparent change was observed in the number of particles after addition of both CB and DB.

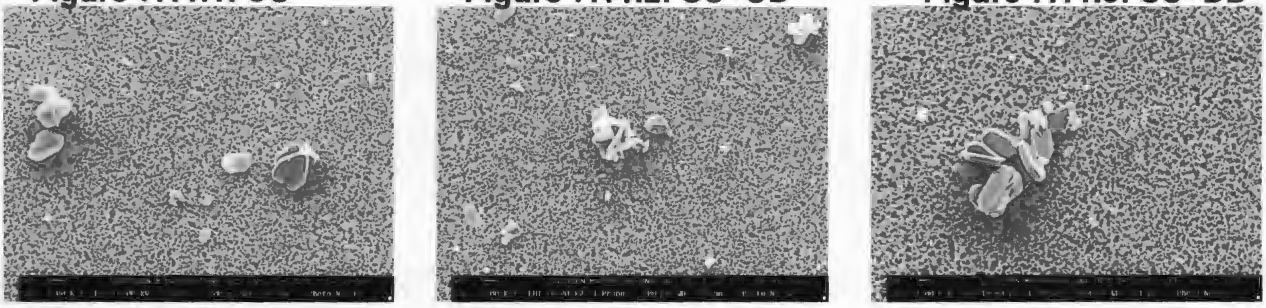


Figure 7.11.1: SU

Figure 7.11.2: SU+CB

Figure 7.11.3: SU+DB

Figure 7.11: SEM of SU before (Figure 7.11.1) and after addition of CB (Figure 7.11.2) and DB (Figure 7.11.3) at final concentrations of 0.5 mg/L viewed at 4K

Comments:

SEM confirmed the observation in the particle size distribution experiment where it was demonstrated that size decreases more after addition of CB but when DB was added there was no apparent change. It is therefore concluded that CB glycans are responsible for the inhibition of crystal growth thus confirming reports in the literature (Koide *et al.* 1981).

Ultrafiltered urine

Effect of CB and DB on [^{14}C]-oxalate deposition

There was no apparent change in the CaOx oxalate deposition in BUF after addition of DB, however a decrease in [^{14}C]-oxalate deposition was observed when CB was added. The CaOx oxalate deposited unfavourably increased upon addition of DB in WUF but decreased when CB was added. A typical plot depicting the inhibitory effect of CB and DB on the UF is shown below (Figure 7.12). The data and plots for each experiment are shown in Appendix 4 (Black subjects: Tables E4.1 and Figures F4.1; White subjects: Tables E4.2 and Figures F4.2).

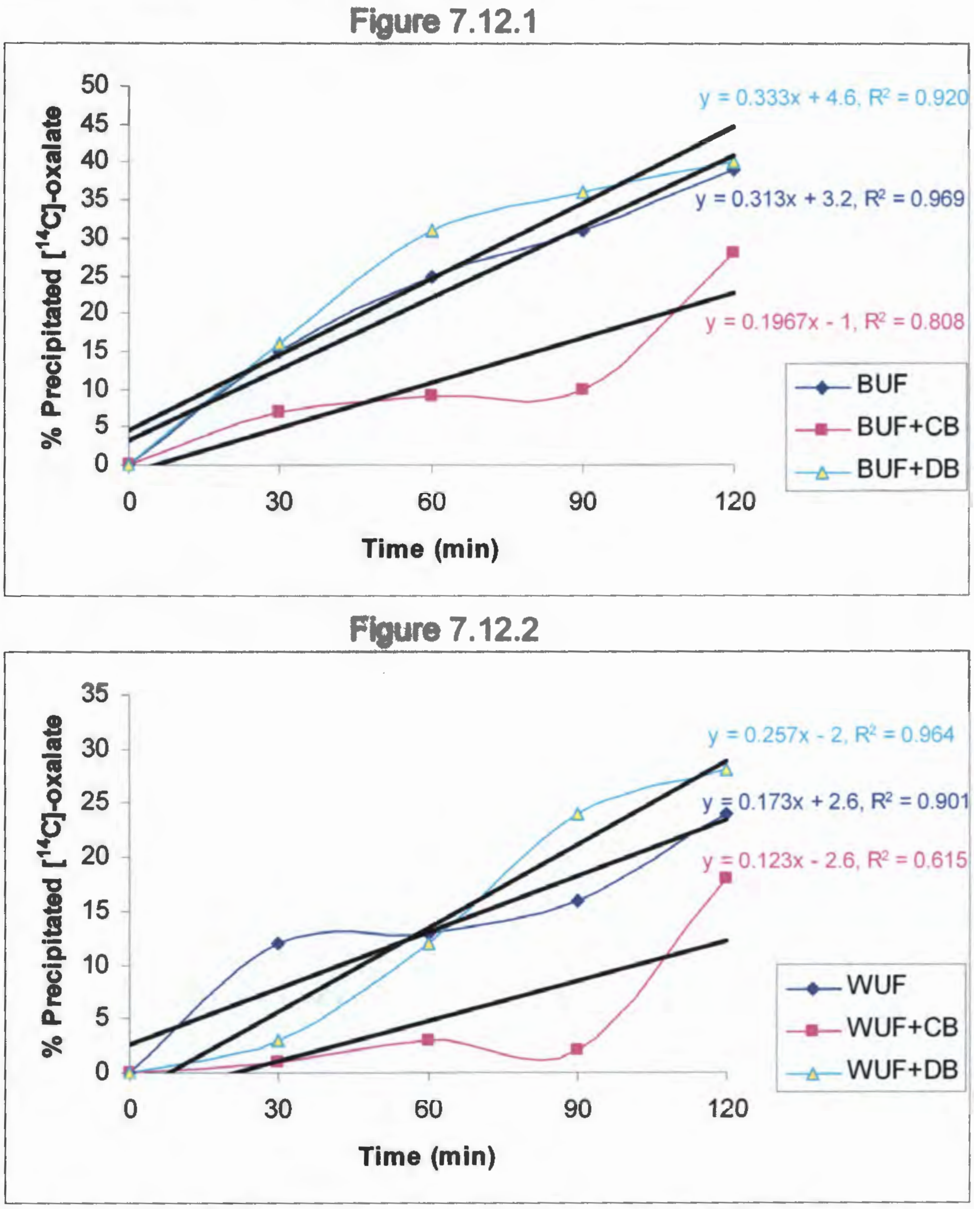


Figure 7.12.1

Figure 7.12.2

Figure 7.12: [^{14}C] - oxalate deposition of BUF (Figure: 7.12.1) and Figure 7.12.2) expressed as a percentage precipitated oxalate before and after addition of CB and DB

Comments:

Addition of DB unfavourably promoted the deposition of CaOx deposition while CB significantly reduced the [^{14}C]-oxalate deposited. This is an indication of CaOx crystal formation after the removal of glycans.

Effect of UF, CB and DB on crystal aggregation

The inhibition of CaOx cystal aggregation by CB was 87 % and this percent inhibition increased when BUF (95 %) and WUF (92 %) were included in the mixture (Table 7.5). The effect of DB was only 68 % but this percent inhibition increased favourably when UF was included in the mixture irrespective of the race group from which it was derived. Figures 7.13 show a typical plot of the aggregation inhibition experiments. The rate of sedimentation is reduced more when CB is added to the COM crystal slurry as indicated by flattening of the slope after addition of CB. The data and plots for each of these experiments are presented in Appendix 4 (Black subjects: Tables F4.1 and Figures G4.1, White subjects: Tables F4.2 and Figures G4.2).

Table 7.5: Absorbance slopes and inhibition of aggregation (% I_A) of COM crystal slurries before and after addition of CB and DB

Sample	Absorbance slope ($\times 10^{-5} s^{-1}$) (R^2)	% I_A
COM (n=5)	-520 (0.994)	
COM+CB (n=5)	-68 (0.978)	87
COM+DB (n=5)	-168 (0.954)	68
COM+BUF (n=5)	-164 (0.981)	69
COM+BUF+CB (n=5)	-28 (0.951)	95
COM+BUF+DB (n=5)	-128 (0.964)	75
COM+WUF (n=5)	-78 (0.976)	76
COM+WUF+CB (n=5)	-44 (0.961)	92
COM+WUF+DB (n=5)	-150 (0.981)	71

Figure 7.13.1

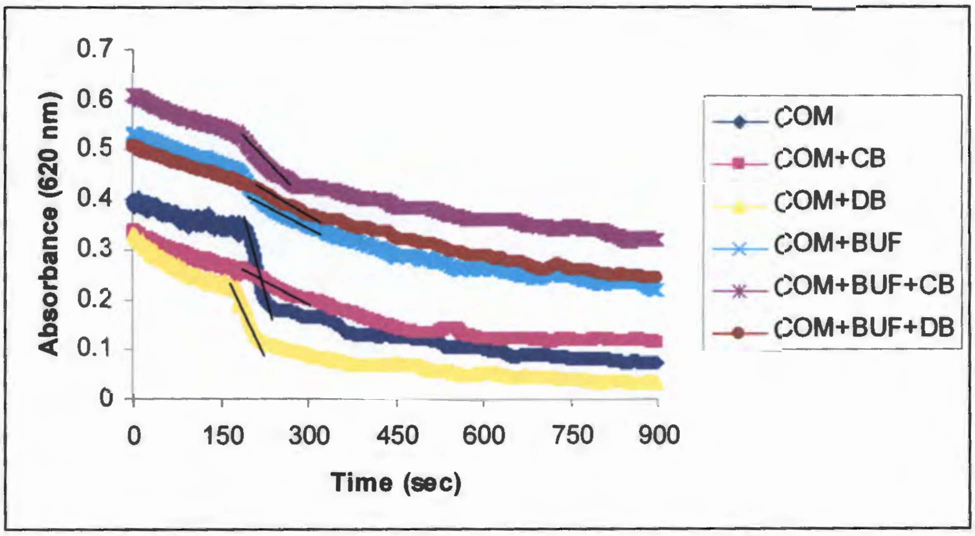


Figure 7.13.2

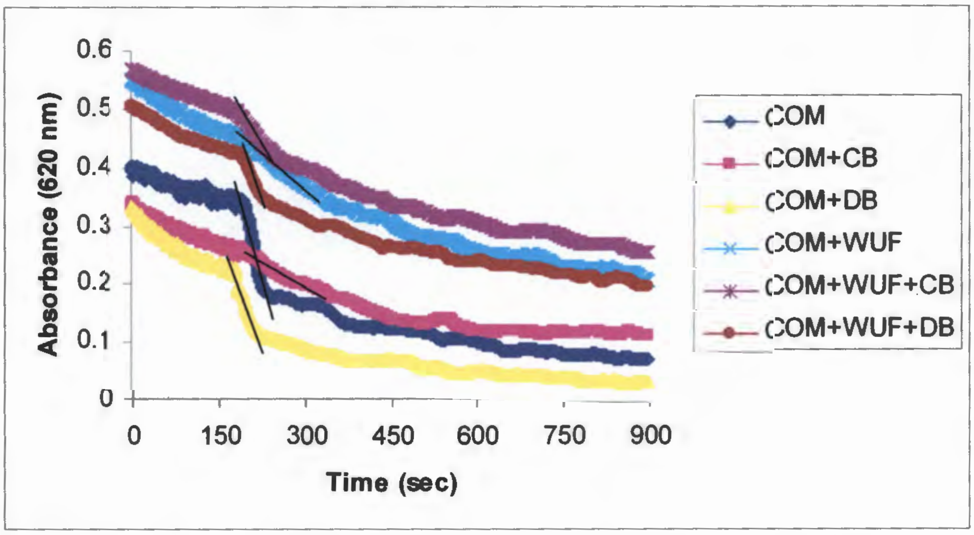


Figure 7.13: A typical plot of absorbance vs time of COM slurry before and after addition of BUF (Figure 7.13.1), WUF (Figure 7.13.2), CB and DB at a final concentration of 0.5 mg/L.

Comment:

The general principle that the urine milieu affects the inhibitory performance of bikunin was evident in the crystal aggregation experiment. The results demonstrate that the highest aggregation inhibition is achieved when CB is added with the urine. Addition of DB did not induce any change when tested with the urine.

Effect of UF, CB and DB on crystal nucleation

Addition of DB to the control inhibited nucleation by only 13 % and the mean percent inhibition by CB was 46 %. The percent inhibition significantly increased upon addition of both BUF and WUF. The plot in Figure 7.14 show the mean plots of the nucleation experiment before and after addition of the inhibitor. The plot in Figure 7.14 illustrate that nucleation is only delayed when UF is included in the reaction that CB and DB. Table 7.6 shows the mean data of the absorbance slope obtained from the plots presented in Appendix 4 (Black subjects: Tables G4.1 and Figure H4.1, White subjects: Tables G4.2 and Figure H4.2).

Table 7.6: Absorbance slopes and inhibition of nucleation (% I_n) before and after addition of CB and DB

Sample	Absorbance slope ($x10^{-5}s^{-1}$) (R^2)	(% I_n)
Control (C) (n=5)	40 (0.951)	
C+CB (n=5)	28 (0.986)	46
C+DB (n=5)	35 (0.990)	13
C+BUF(n=5)	3.4 (0.967)	92
C+BUF+CB (n=5)	7.0 (0.955)	83
C+BUF+DB (n=5)	3.2 (0.928)	92
C+WUF(n=5)	3.0 (0.947)	93
C+WUF+CB (n=5)	2.4 (0.914)	94
C+WUF+DB (n=5)	4.0 (0.933)	90

Figure 7.14.1

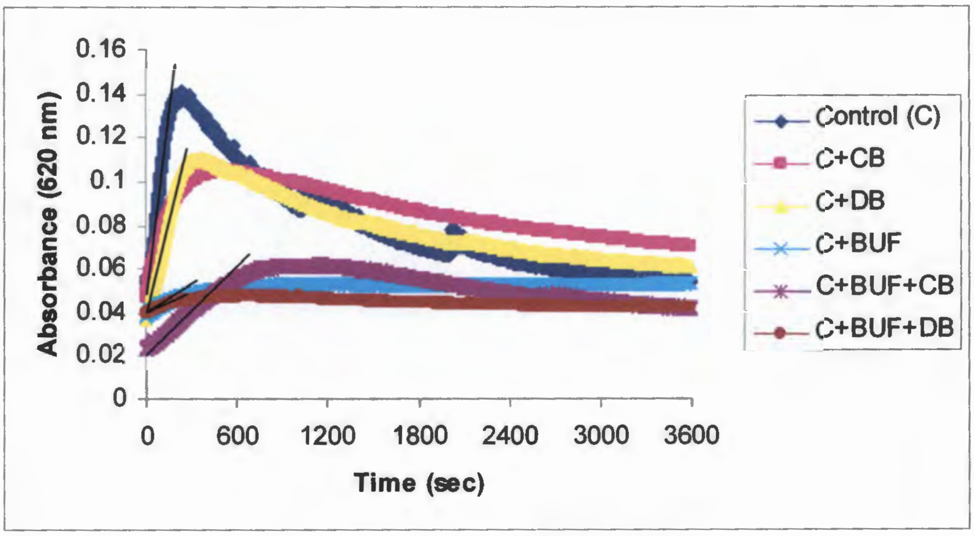


Figure 7.14.2

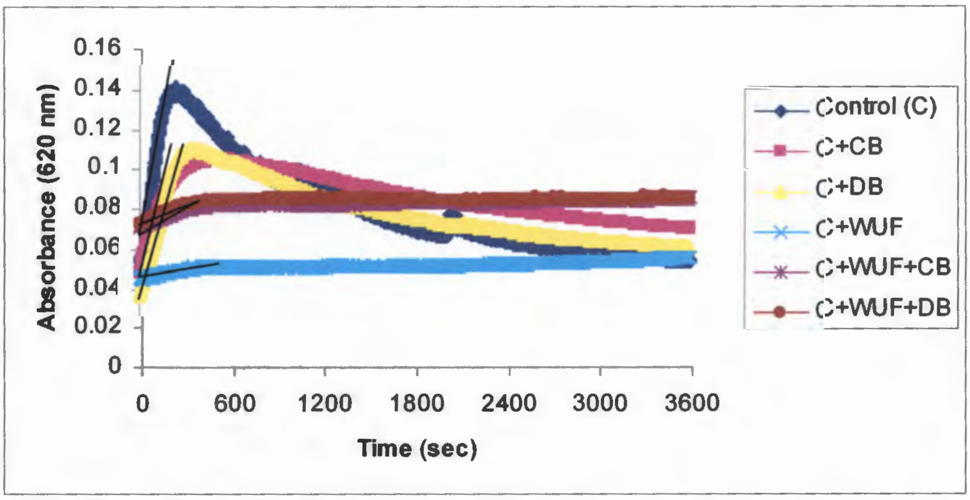


Figure 7.16: A typical plot of absorbance vs time of the control before and after addition of BUF (Figure 7.16.1), WUF (Figure 7.16.2), CB and DB at a final concentration of 0.5 mg/L.

Comments:

The results of these experiments have shown that the urine rather than CB and DB inhibit CaOx crystal nucleation. The studies presented in other chapters have demonstrated that the urine milieu affects the functionality of the protein. However, the results obtained from the nucleation experiment in this study are disappointing, as there were no differences observed between BUF and WUF.

Effect of CB and DB on particle size distribution

Surprisingly, an unfavourable increase in particle size was observed after addition of CB and DB in BUF in four experiments of five experiments. Addition of DB resulted in a greater particle size increase in BUF. The particle size distribution in WUF was bimodal and these experiments showed an insignificant decrease in the particle number when CB and DB were added. Table 7.7 shows the table of the particle size values obtained from the particle size distribution plots. A typical plot for these experiments is shown in Figure 7.15. These plots demonstrate a shift in the particle volume-size distribution to the larger values in BUF and a decrease in WUF after addition of CB and DB. The plots from which the particle sizes in Table 7.7 were derived are shown in Appendix 4 (Black subjects: B 4.1, White B 4.2).

Table 7.7: Particle size (µm) of UF before and after addition of CB at a final concentration of 0.5 mg/L

Sample	BUF	BUF+CB	BUF+DB
Experiment 1	4.0	3.3	7.3
Experiment 2**	11.3	17.0	24.2
Experiment 3**	10.6	11.3	17.4
Experiment 4**	11.3	14.6	13.1
Experiment 5**	8.7	12.8	17.4
Sample	**WUF**	**WUF+CB**	**WUF+DB**
Experiment 1*	12.4 and 17.1	10.9 and 14.6	11.3 and 14.9
Experiment 2	8.7 and 13.5	10.6 and14.6	10.2 and 13.0
Experiment 3*	19.3 and 24.4	17.2 and 20.8	12.7
Experiment 4*	17.0	14.4	12.4
Experiment 5*	12.8 and 17.8	11.3 and 15.7	11.7 and 14.9

**Trend showing an increase
*Trend showing a decrease

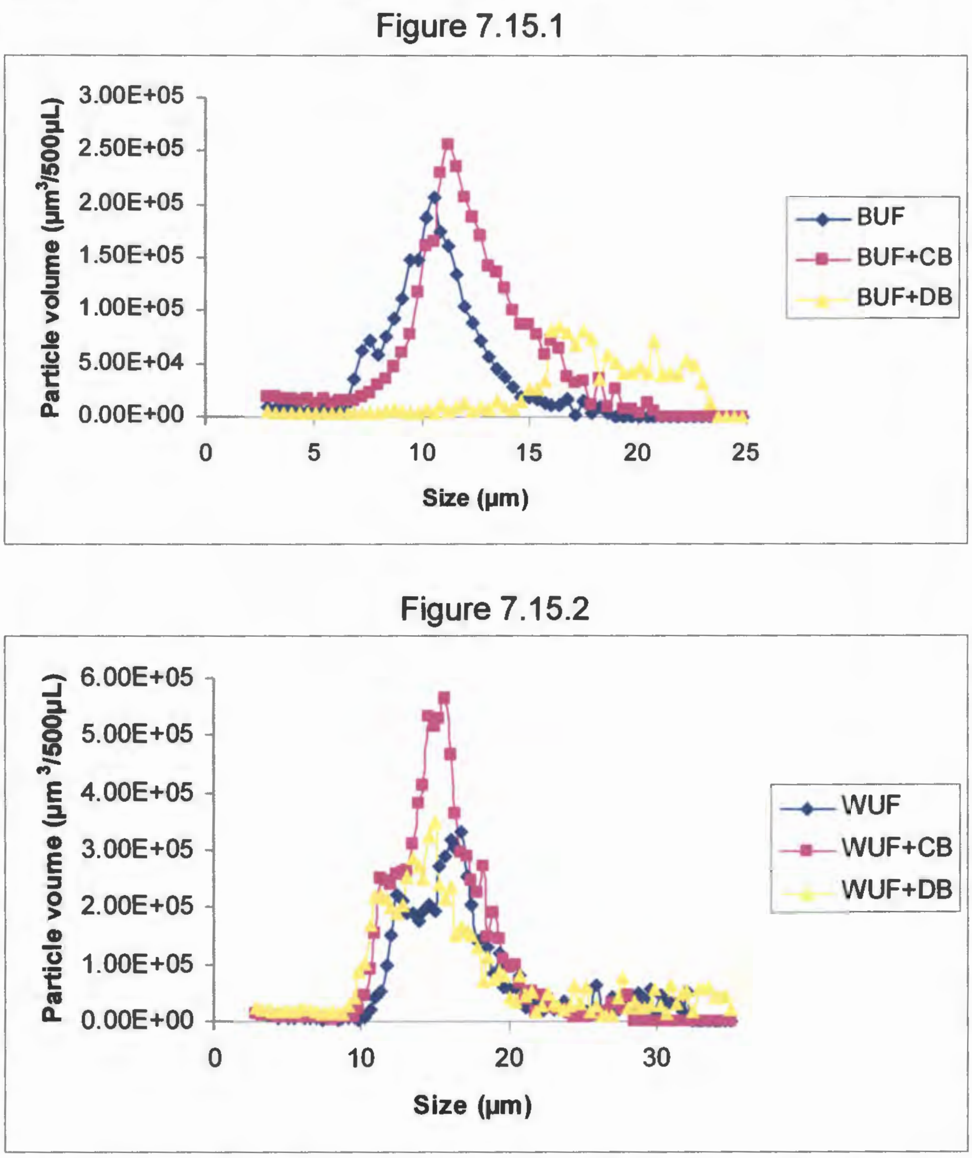


Figure 7.15: Typical plot of particle size distribution of BUF (Figure 7.15.1) and WUF (Figure 7.15.2) subjects before and after addition of CB and DB.

Comments:

Although the unfavourable increase in the particle size distribution is consistent with the reduced inhibitory activity of the deglycosylated protein, the results regarding the BUF experiment were surprising. For example, addition of CB in BUF resulted in a significant increase in the particle size indicating growth promotion. This observation is puzzling because it is inconsistent with the experiments in the previous chapters. However, the bimodal size distribution observed in the urine of white subjects is consistent and confirms a similar observation in other chapters. The general conclusion is that CB and DB do not significantly inhibit CaOx crystal growth.

SEM

The SEM pictures of the CaOx crystals precipitated from the UF are shown in Figure 7.16. The number of COD crystals observed in BUF decreased after addition of CB but the size greatly increased. An aggregate was also observed in BUF when DB was added. No apparent change in the particle number was observed after addition. The particle number and size decreased significantly in WUF when CB was added but the particle size appeared bigger when DB was added.

Figure 7.16.1: BUF **Figure 7.16.2:** BUF+CB **Figure 7.16.3:** BUF+DB

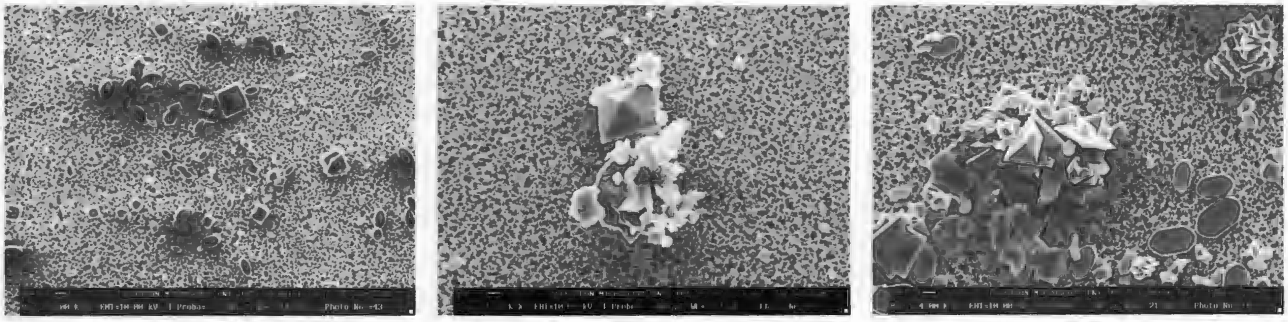


Figure 7.16.4: WUF **Figure 7.16.5:** WUF+CB **Figure 7.16.6:** WUF+DB

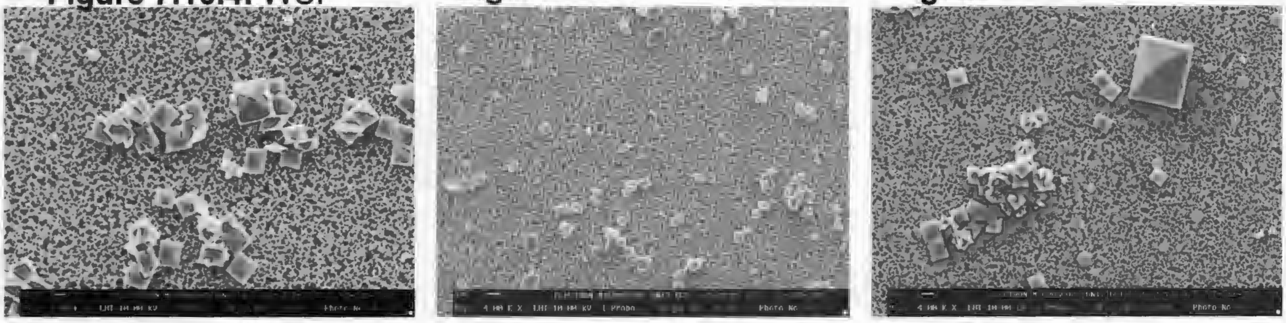


Figure 7.16: Scanning electron micrographs of CaOx crystals precipitated from BUF (Figure 7.16.1) before and after addition of CB (Figure 7.16.2) and DB (Figure 7.16.3), and addition that of WUF (Figure 7.16.4) before and after addition of CB (Figure 7.16.5) and after addition of DB (Figure 7.16.6) at a final concentration of 0.5 mg/L.

Comments:

It was observed that addition of DB resulted in an increase in the particle size and this observation was confirmed by the SEM micrographs which also showed an unfavourable increase in particle size after addition of CB in BUF indicating promotion of particle growth. The particles appeared smaller when CB was added to WUF and DB did not significantly reduce the particle size as there were no apparent changes observed.

Effect of CB and DB on Zeta Potential

Table 7.8 shows the zeta potential of the crystal slurry before and after addition of CB and DB. The average values are presented in Figure 7.17. The average zeta potential of the slurry before addition of CB and DB was -12.4 mV. The magnitude of the average negative charge of COM increased significantly to -16.6 mV after addition of CB (p=0.03) while there was no change after addition of DB (-12.7 mV, p=0.17).

Table 7.8: Zeta potentials of COM slurry in the absence and presence of CB and DB

COM (mV)	COM+CB (mV)	COM+DB (mV)
-12.8	-13.3	-12.7
-9.9	-17.8	-11.2
-13.9	-18.7	-16.0
-13.2	-12.3	-15.4
-12.5	-16.9	-13.9
-11.9	-	-
Average: -12.4 ± 0.56	**-17.5 ± 1.27**	**-13.8 ± 0.88**

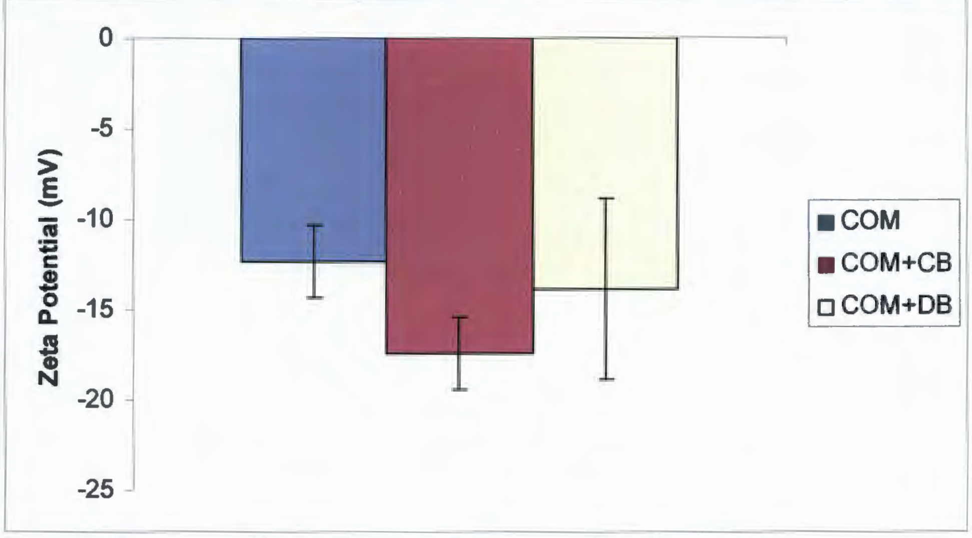


Figure 7.17: Average zeta potential of crystal slurry in the presence and absence of CB and DB

Comments

Inhibition of crystal aggregation is related to changes in the surface charge that occur when an inhibitor such as bikunin binds to crystals and therefore the surface charge becoming more negative. A shift to a greater magnitude in the negative zeta potential is regarded as being favourable as it indicative of stronger repulsive forces which would retard aggregation. This shift, which may thus be attributed to inhibition of aggregation, was significantly more pronounced after addition of CB. The presence of CB in the COM slurry increased its negative charge which could mean that CB exerted stronger repulsive forces than DB and therefore indicating that CB is a superior inhibitor of crystal aggregation than DB.

7.4 Discussion

Treatment of bikunin with chondroitinase has been reported to decrease the ability of bikunin to inhibit CaOx crystallization (Atmani *et al.* 1996; Yang *et al.* 2005). In the present study CB's *O*-linked glycans were successfully removed and deglycosylation with chondroitinase ABC resulted in the mass reduction of this protein from 22920 to 18 000 Da. This is in agreement with literature values (Ebisuno *et al.* 2003, Capon *et al.* 2003; Yang *et al.* 2005). The crystallization experiments on CB and deglycosylated bikunin (DB) were conducted in the synthetic urine and the ultrafiltered urine.

Synthetic urine studies

In order to test the inhibitory effect of CB and DB without any interference from other macromolecules that might be present in the urine, a neutral test solution was considered a necessity. Synthetic urine was prepared to mimic the urine and was used in these experiments for the first time as a neutral test solution. These experiments yielded results with a similar trend observed in all the experiments. The [^{14}C]-oxalate deposition experiment along with the particle size distribution experiment demonstrated that CB inhibited crystal growth in the synthetic urine. These experiments also provided convincing evidence that commercial bikunin's inhibition of CaOx crystal formation reduces greatly after the removal of the glycans. It was also shown that DB inhibited CaOx crystal aggregation and nucleation to a lesser extent than CB. These observations are consistent with the reports which indicate that bikunin's glycans are responsible for 70 - 90 % of its inhibitory activity (Koide *et al.* 1981; Médétognon-Besissan *et al.* 1999).

Ultrafiltered urine studies

As it was established in the synthetic urine that the inhibitory activity of CB reduces after the glycans have been removed, the same effect was also observed in the UF experiments. Since the study presented in this chapter was conducted to explore the differences between the effect of CB and DB in the urine of black and white subjects, the findings in this chapter in this regard are disappointing. Because there no significant differences were observed between BUF and WUF in all the experiments, the results from this chapter are disappointing and inconsistent with the findings in Chapter 3. Growth inhibition is usually shown by a decrease in the amount of CaOx deposited and smaller particle sizes after addition of an inhibitor. These experiments along with the SEM demonstrate that CB and DB do not inhibit CaOx crystal growth. The bimodal size distribution and larger particle is consistent with findings in other chapters and this notion is discussed in detail in these chapters. Nucleation was only significantly inhibited by addition UF and therefore a comment cannot be made on the participation of glycans in this mechanism.

In conclusion, the findings presented in this chapter suggest that chondroitin sulphate carbohydrate moiety of bikunin rather than the bikunin protein core are responsible for the inhibition of calcium oxalate crystallization. The findings in this study are in agreement with previous studies that have demonstrated that glycosaminoglycans such as chondroitin sulphate are inhibitor of CaOx growth and aggregation (Suzuki *et al.* 2001; Grases *et al.* 1991).

Chapter 8

Conclusion and Future outlook

8.1 Conclusion

Since the effect of bikunin on CaOx crystallization in the urine of South Africa's population groups has not been previously undertaken, the objective for this PhD project was to isolate, characterize and extensively study the effect of this protein from the two race groups on CaOx crystallization with a view to gaining insights into why stone incidence is rare in the black population group. It is therefore appropriate to review the extent to which this objective was achieved.

A study comparing the composition of the urine from black and white male subjects revealed a few significant differences and confirmed findings in other studies involving the two South African race groups (Chapter 2). For example, urinary calcium was found to be 46 % lower in black subjects than in white subjects. Owing to the fact that 85 % of kidney stone is composed predominantly of calcium compounds (Ito *et al.* 1992) these results suggest that differences in the composition of the urine may provide insight into the stone rarity in the South African black population. Since higher sodium has been reported to aggravate the development of kidney stone (Blacklock 1982; Robertson and Peackok 1983), it was surprising to observe that black subjects excreted less sodium compared to white subjects. The higher metastable limit observed in black subjects' urine demonstrates that the urine of black subjects can tolerate higher concentration of supersaturation without the formation of CaOx crystals. Although the urinary chemical composition and CaOx metastable limits results mentioned above are indeed related to the lower incidence, these parameters lie within the normal range and therefore they alone cannot account for the stone incidence differences observed in the two South African race groups.

As a lead-in to investigating the proposed objective, a series of crystallization studies were undertaken involving the commercially available bikunin. The crystallization experiments provided compelling evidence that commercial bikunin is an inhibitor of crystal aggregation, growth and nucleation.

Different crystal matrices were precipitated from the urine to investigate the presence of bikunin for further purification. The presence of bikunin in COM, COD crystals and proteins precipitated using ammonium sulphate was confirmed in all these crystal matrix extracts using SDS-PAGE. However, Bradford assay demonstrated a higher total protein concentration in the proteins precipitated using ammonium sulphate. As such this method was used for further experiments and for bikunin isolation. The crystallization experiments were conducted on the protein precipitate containing bikunin. The results from these experiments demonstrated conclusively that proteins included in the precipitate (amongst which is bikunin), are potent inhibitors of CaOx growth, aggregation and nucleation crystallization, irrespective of the race groups and that these effects occurred in both the urine and inorganic solution.

Bikunin itself was successfully isolated from the protein precipitated from the urine of black and white South African male subjects using immunoaffinity chromatography. The isolated protein was characterized with SDS-PAGE, Western blotting and MALDI-TOF MS. Although SDS-PAGE showed qualitative similarity between bikunin isolated from the urine of black and white subjects, MALDI-TOF MS showed a larger coverage of bikunin amino acids by bikunin isolated from black subjects. This suggests possible structural differences in the bikunin isolated from the urine of the two race groups.

In addition to investigating the structural differences of bikunin isolated from the two race groups, the inhibitory activity of BB and WB was compared using a series of crystallization experiments (Chapter 6). These results revealed that bikunin was able to retard CaOx crystallization irrespective of the race group from which it was derived. In particular bikunin was found to be a strong inhibitor of CaOx aggregation and growth rather than crystal nucleation. Nucleation has been reported to lower urinary supersaturation without producing particles of significant sizes, therefore it has been considered as a phenomenon of uncertain significance for stone formation (Coe *et al.* 1991, Hess *et al.* 1995). However it is a vital precondition for further formation of larger particles within the urinary tract which ultimately might form a stone. Of further significance was the observation that bikunin alters the COM crystal morphology to COD. As it has been explained before, COD does not adhere to the epithelial cells. Thus, it appears as if bikunin is a major protective factor in kidney stone disease largely by altering the morphology from COM to COD (Chapter 6).

Of great importance is that the results from this study provided key evidence in support of the original hypothesis of this thesis, namely that bikunin from the black subjects might play a major role in protecting this race group from kidney stone. This emerged from the observation that bikunin isolated from white subjects promoted crystal aggregation when added in the BUF, but the bikunin from the black subjects was able to retard CaOx crystallization, both in its native urine and in the cross-over experiment.

Differences were for the first time observed when comparing the effect of commercial bikunin and isolated bikunin. For example, the particle size increased when CB was added to both BUF and WUF but a decrease was observed after addition of BB and WB. Also, a morphology change was observed after addition of BB and WB but that was not the case after addition of CB to UF. This suggests structural differences between these two different forms of bikunin. Further experiments are required to clarify the mechanism.

The contribution of the glycans, to the activity of bikunin was also investigated after enzymatic removal of chondroitin sulphate chains by comparing the inhibitory activity of this protein before and after removal of the glycans. In all the experiments, a decrease in inhibition was observed when the deglycosylated protein was added, demonstrating that bikunin deglycosylation reduces potency of the proteins inhibition of CaOx crystallization. Since several of these experiments were conducted in real urine for the first time, the results are extremely noteworthy.

An interesting feature which was common in almost all the studies presented in this thesis regarding the differences between urine of black and white subjects was that white subjects have predominantly COD morphology while black subjects have predominantly COM morphology (Chapters 3, 6 and 7). This observation is not surprising since COD forms at higher calcium concentration and this supports the evidence that kidney stone disease is rare amongst the black South African population. Another interesting common feature is the larger particle size along with the bimodal size distribution observed in the urine of the white subjects and not in blacks. It is reasonable to suggest that larger particles form due to reduced inhibition of crystal growth and aggregation. This also is consistent with the stone formation being prevalent in South African white population.

Several experiments involving the different forms of bikunin demonstrated a synergistic dependence on the urine milieu. Furthermore, in some cases, the ultrafiltered urine from black subjects (BUF) had a more favourable effect than that of WUF. These effects were particularly apparent in the crystallization experiments involving commercial bikunin ([^{14}C]-oxalate deposition, sedimentation and Coulter experiments, page 72) and purified bikunin ([^{14}C]-oxalate deposition and particle number, page 133) and are reported here for the first time. As has been discussed elsewhere in this thesis, similar synergistic relationship between protein functionality and urine composition have been reported for UPTF1, THP and Albumin. Of interest is the absence of these effects in the study involving deglycosylated protein suggesting that bikunin glycans play a major role in the proteins' ability to inhibit CaOx crystallization.

The differences in the urines of the two race groups coupled with crystallization experiment results from this thesis leads to the conclusion that bikunin may be a potential role-player in protecting the South African black population against CaOx lithogenesis. The importance of this protein in the pathogenesis of urolithiasis, can be attributable to the concentration, degree of glycosylation and its ability to alter the crystal morphology.

8.2 Future Outlook

Although this thesis provided more understanding on the remarkably low stone incidence in the South African black population, the role of this protein still warrants further studies in conjunction with other aspects, amongst these is diet. Studying the structure of bikunin and the effect of bikunin isolated from the urine of stone formers may also provide some more insight.

References

- Aaggarval S., Tandon C. D., Forouzandeh M., Singla S. K., Kiran R. and Jethi R. K. (2000) Role of biomolecules from human renal stone matrix on COM crystal growth. *Mol. and Cell Biochem.* **210 (1-2):** 109
- Allie S. and Rodgers A. L. (2003) Effects of Calcium carbonate, magnesium oxide and sodium citrate bicarbonate health supplements on the urinary risk factors for kidney stone formation. *Clin. Chem. Lab. Med.* **41(1):** 39
- Allie S. and Rodgers A. L. (2005) Prophylactic and therapeutic properties of a sodium citrate preparation in the management of calcium oxalate urolithiasis: randomized, placebo-controlled trial. *Urol. Res.* **33:** 116
- Andersen D. A. (1973) "Environmental factors in the aetiology of urolithiasis" In: "Urinary calculi: Proceedings of international symposium on renal stone research" Madrid 1972 eds: Cifuentes Delatte. L., Rapado. A., Hodgkinson. A., Karger. Basel. 130
- Asplin J. R., Arsenault D., Parks J. H., Coe F. L. and Hoyer J. R. (1998) Contribution of human uropontin to inhibition of calcium oxalate crystallization. *Kidney Int.* **53:** 194
- Astrup P. and Nissen U. (1964) Urinary trypsin inhibitor (mingin): transformation into a new trypsin inhibitor by acid hydrolysis or by sialydase. *Nature.* **203:** 255
- Atmani F., Lacour B., Drueke T. and Daudon M. (1993) Isolation and purification of a new glycoprotein from human urine inhibiting calcium oxalate crystallisation. *Urol. Res.* **21:** 61
- Atmani F., Lacour B., Strecker G., Parvy P., Drueke T. and Daudon M. (1993) Molecular characteristic of uronic-acid-rich protein, a strong inhibitor of calcium oxalate crystallization in vitro. *Biochem. Biophys. Res. Commun.* **191:** 1158
- Atmani F., Lacour B., Jungers P., Drüeke T. and Daudon M. (1994) Reduced inhibitory activity of uronic-acid-rich protein in the urine of stone formers. *Urol. Res.* **22:** 257

- Atmani F. and Khan S. R. (1995) Characterization of uronic-acid-rich inhibitor of calcium oxalate crystallization isolated from the rat urine. *Urol. Res.* **23:** 95
- Atmani F and Khan S. R. (1999) Role of urinary bikunin in the inhibition of calcium oxalate crystallization. *J Am Soc Nephrol* **10:** S385
- Atmani F., Mizon J. and Khan S. (1996) Identification of uronic-acid-rich protein as urinary bikunin, the light chain of inter-α-inhibitor. *Eur. J. Biochem.* **236:** 984
- Atmani F., Mizon J. and Khan S. R. (1996) Inter-alpha-inhibitor: a protein family involved in the inhibition of calcium oxalate crystallization. *Scan. Micros.* **10:** 425
- Atmani F., Glenton P. A. and Khan S. R. (1998) Identification of proteins extracted from calcium oxalate and calcium phosphate crystals induced in the urine of healthy and stone forming subjects. *Urol. Res.* **26:** 201
- Atmani F. and Khan S. R. (1999) Role of urinary bikunin in the inhibition of calcium oxalate crystallization. *J. Am. Soc. Nephrol.* **10:** S385
- Balduyck M., Hayem A., Kerckaert J. P., Mizon C. and Mizon J. (1982) Isolation of human urinary trypsin inhibitor. *Biochem. Boiphys. Res. Commun.* **109:** 1247
- Balduyck, M., Mizon, C., Loutfi, H., Richet, C., Roussel, P., and Mizon, J. (1986) *Eur. J. Biochem.* **158:** 417
- Bell D. J., Hoare M. and Dunnill P. (1983) 'The Formation of Protein Precipitates and Their Centrifugal Recovery. *Adv. Biochem. Eng.* **26:** 1
- Beukes. G. J., De Bruiyn. H. and Vermaak. W. J. H. (1987) Effect of epidemiological factors on the composition and racial distribution of renal calculi. *Br. J. Urol.* **60:** 1
- Berg C., Larsson L. and Tiselius H. G (1990) Effect of different doses of alkaline citrate on urine composition and crystallization of calcium oxalate. *Urol. Res.* **18:** 13
- Bichler K. H., Kirchner C. H., Ideler V. (1976) Uromucoid excresion of normal individuals and stone formers. *Brit. J. Urol.* **47:** 733
- Binnette J. P., Binnette M. B., Gawinowicz. M. A. and Kendrick. N. (1996) Urinary stone proteins: An update. *Scanning Microsc.* **10:** 509

- Blacklock N. J. (1969) Patterns of urolithiasis in the Royal Navy. In Hodgkinson A., Nordin B. E. C. eds. Renal Stone Research Symposium. London. J and A. Churchill Ltd. pp 33
- Blacklock N. J. (1982) "Epidemiology of urolithiasis" in: "Scientific foundations of urology" 2nd ed. Eds: Chisholm G. D., Williams D. I., Heineman. London. pp 235
- Blomen L. J. M. J. (1982) Growth and agglomeration of calcium oxalate crystals. PhD. Thesis. University of Leiden.
- Boeve E., Cao L.C., Verkoelen C. F., Romijn J., De Bruijn W. and Schroder F. (1993) Glycosaminoglycans and other sulphated polysaccharides. *World. J. Urol.* **12:** 43
- Borghi L., Meschi T., Amato F., Novarini A. And Giannini A. (1996) Urine volume, water and recurrence in idiopathic calcium nephrolithiasis: a 5-years randomized prospective study. *J. Urol.* **155:** 839
- Bourguignon J., Sesboüé R., Diarra-Mehrpour M., Daveau M. and Martin J. P. (1989) Human inter-α-trypsin inhibitor. Synthesis and maturation in hepatoma HepG2 cells. *Biochem. J.* **261:** 305
- Boyce W. H. (1968) Organic matrix of human urinary concretions. *Am. J. Med.* **45:** 673
- Boyce W. H. and Garvey F. K. (1956) The amount and nature of the organic matrix in urinary calculi: a review. **37:** 1589
- Boyce W. H., King J. S. Jr and Fielden M. L. (1962) Total non-dialysable solids (TNDS) in human urine XIII. Immunological detection of a component peculiar to renal calculous matrix and to urine of calculus patients. *J. Clin. Inves.* **41:** 1180
- Boyce W. H. (1968) Organic matrix of human urinary concretions. *Am. J. Med.* **45:** 673
- Bradford M. M. (1976) A rapid and sensitive method for the quantitation of the quantitation of microgram quantities of protein utilization the principle of protein dye-binding. *Anal. Biochem.* **72:** 248

- Breslau N. A., Padalino P., Kok D. J., Kim Y. G. and Pak C. Y.C. (1995) Physiocochemical effects of a new slow-release potassium phosphate preparation (Urophs-K) in absorptive hypercalciluria. *J. Bone. Min.* **10:** 394
- Broadus A. E., Insogna K. L. and Lang R. (1984) Evidence for disorder control for 1.25-dihydroxyvitamin D production in absorptive hypercalciuria. *New Engl. J. Med.* **311:** 73
- Brutus W. J., Gay L and Insogna K. L. (1994) Dietary hypercalciuria in patients with calcium oxalate kidney stones. Am. J. Clin. Nutr. **60**:429
- Butler W. (1989) The nature and significance of osteopontin. *Connect. Tissue. Res.* **23:** 123
- Butt A. J. (1956) Historical survey of etiology factors in renal lithiasis. In Butt A. J. ed. Etiology Factors in Renal Lithiasis. Illinois. Charles C Thomas. pp6.
- Capon C., Mizon C., Lemoine J., Rodié-Talbére P. and Mizon J. (2003) In acute inflammation, the chondroitin-4-sulphate carried by bikunin is not only longer; it is also undersulphated. *Biochimie.* **85:** 101
- Chiriboga J. (1963) Some properties of an oxalic oxidase purified from barley seedlings. *Biochem. Biophys Res Comm* **11:** 277
- Curhan G. C., Willett W. C., Rimm E. B. and Stampfer M. J. (1993). A prospective study of dietary calcium and other nutrients and the risk of symptomatic kidney stones. *N. Engl. J. Med.* **328:** 833
- Curhan G. C., Willett W. C., Speizer F. E., Spiegelman D. and Stampfer M. J. (1997). Comparison of dietary calcium with supplemental calcium and other nutrients as factors affecting the risk for kidney stones in women. *Ann. Int. Med.* **126:** 497
- Clarke E. (1968) A history of the stone. Hosp med. 1065
- Coe F. L. (1983) Uric acid and calcium oxalate nephrolithiasis. Kid. Int. **24**:392
- Coe F. L., Parks J. H. and Nakagawa Y. (1991) Protein inhibitors of crystallization. *Semin. Nephrol.* **11:** 98
- Coe F. L., Evan A. and Worcester E. (2005) Kidney stone disease. *J. Clin. Invest.* **115:** 2598

- Conte A., Roca P. K., Genestar C. and Grases F. (1989) The relationship between orthophosphate and pyrophosphate in normal subjects and in patrients with urolithiasis. *Urol. Res.* **17 (3):** 173
- Conte A., Pizá P. and Garcia-raja A. (1999) Urinary lithogen risk test: usefulness in the evaluation of renal lithiasis treatment using crystallization inhibitors (citrate and phytate). *Arch. Esp. Urol.* **52:** 305
- Craig T. A. Rodgers A. L. and Brandt W. (1999) Inhibitory properties of Tamm Horsfall mucoprotein isolated from two different population groups. Proceeding of the 8th European symposium on urolithiasis, Institute of semeiotica medica, Parma, Italy, eds. Borghi L., Meschi T., Briganti A., Schianchi T. and Novarini A. 261
- Craig T. A. Rodgers A. L. and Brandt W. (2001) Inhibition of calcium oxalate crystallization by Tamm Horsfall mucoprotein (THP) from two different population groups. Proceeding of the 9th European symposium on urolithiasis, The Netherlands eds. Kok D., Rominj H. C., Verhagen P. C. M. S. and Verkoelen C. F. 59
- Cupisti A., Morelli E., Lupetti S., Meola M and Barsotti G. (1992) Low urinary citrate excretion as main risk factor for recurrent calcium oxalate nephrolithiasis in males. Nephron. **61:** 73
- Dawson C. J., Grover P. K., Kanellos J., Pham H., Kupczyk, Oates A. and Ryall R. L. (1998) Inter-alpha-inhibitor in calcium stones. *Clin. Sci.* **95:** 187
- Dean C., Kannelos J., Pham H., Gomes M., Oates A., Grover P. and Ryall R. (2000) Effects of inter-α-inhibitor and several of its derivatives on calcium oxalate crystallization *in vitro*. *Clin. Sci.* **98:** 471
- Dermas J. and Tawashi R. (1973) Dissolution and growth of calcium oxalate monohydrate. Effect of magnesium and pH. *Biochim. Biophys. Acta.* **313:** 256
- Doremus R. H., Teich S. and Silvis P. X. (1978) Crystallization of calcium oxalate from synthetic urine. *Invest. Urol.* **15:** 469
- Doyle I. R., Ryall R. and Marshall V. R. (1991) The incorporation of urinary proteins into calcium oxalate crystals precipitated from human urine: A highly selective phenomenon. *Clin. Chem.* **37:** 1589

- Doyle I., Marshall V., Dawson C. and Ryall R. (1995) Calcium oxalate crystal matrix extract: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in undiluted human urine in vitro. *Urol Res.* **23:** 53
- Durrbaum D. and Rodgers A. (2001) A study of crystal matrix extract and urinary prothrombin fragment 1 from a stone-prone and stone-free population. *Urol. Res.* **29:** 83
- Dryer R. L. and Routh J. I. (1963) Determination of serum inorganic phosphorus. *Clin. Chem.* **4:** 191
- Ebisuno S., Morimoto S. and Yoshida T. (1986) Rice-bran treatment for calcium stone formers with idiopathic hypercalciuria. *Brit. J. Urol.* **58:** 592
- Ebisuno S., Nishihata M., Inagaki T., Umehara M. and Kohjimoto Y. (1999) Bikunin prevents Adhesion of calcium oxalate crystal to renal tubular cells in human urine. *J. Am. Nephrol.* **10:** S436
- Edyvane K., Ryall R. and Marshall V (1987) The effect of serum on the crystallization of calcium oxalate in whole human urine: Inhibitiom disguised as apparent promotion. *Urol. Res.* **15:** 87
- Egelhofer V., Gobom J., Seitz H., Giavalisco P., Lehrach H. and Nordhoff E. (2002) Protein identification by MALDI-TOF MS peptide mapping: A new strategy. *Anal. Chem.* **74 (8):** 1760
- Elliot J. (1973) Structure and composition of urinary calculi. *J. Urol.* **109:** 82
- Elliot J. and Rabinowits I. (1980) Calcium oxalate crystalluria: crystal size urine. J. Urol. **121:** 324
- Enghild J., Thogersen I. B., Pizzo S. V. and Salvesen G. (1989) Analysis of inter-α-trypsin inhibitor and a novel trypsin inhibitor, pre-α-trypsin inhibitor, from human plasma. *J. Biol. Chem.* **264:** 15975
- Erturk E., Kierman M. and Schoen S. R. (2002) Clinical association with urinary glycosaminoglycans and urolithiasis. *Urology.* **59:** 495
- Ettinger B., Pak C. Y. C., Citron J. T., Thomas C., Adams-Huet B. and Vangessel A (1997) Potassium–magnesium citrate is an effective prophylaxis against recurrent calcium oxalate nephrolithiasis. *J. Urol.* **158:** 2069
- Faarvagn H. J. (1965) Urinary trypsin inhibitor in man (mingin). *Scand. J. Clin. Lab. Inv.* **17:** 1

- Fernandez F. J. and Kahn H. L. (1971) Clinical methods for atomic absorption spectroscopy. *Clin. Chem. Newsl.* **3:** 24
- Ferner C. D., Barilla D. F., Townsend J. and Pak C. Y. C. (1978) Effect of magnesium oxide on the crystallization of calcium salts in urine in patients with recurrent nephrolithiasis. *J. Urol.* **120:** 399
- Fleish H. and Bisaz S (1962) Isolation from urine of pyrophosphate, a calcification inhibitor. *Am. J. Physiol.* **203:** 671
- Finlayson B. (1978) Physicochemical aspects of Urolithiasis. *Kidney Int.* **13:** 344
- Finlayson B. and Reid F. (1978) The expectation of free and fixed particles in urinary stone disease. *Invest. Urol.* **15:** 442
- Finlayson B., Khan S. R. and Hackett R. L. (1984) Mechanism of stone formation- an overview. *Scan. Micros.* **111:** 1419
- Fossati P., Prencipe L. and Berti G. (1980) Use of 3,5-dichloro-2-hydroxybenzenesulphonic acid/4-aminophenazone chromogenic systems in direct enzymatic assay of uric acid in serum and urine. *Clin. Chem.* **26:** 227
- Fries E. and Blom A. M. (2000) Bikunin - not just a plasma proteinase inhibitor. *The Inter. J. Biochem. & Cell Biol.* **32:** 125
- Gaby A. R (1996) Commentary. *Nutr. Healing.* **1:** 10
- Gebhard W. and Hochstrasser H. (1986) Inter-α-trypsin inhibitor and its colese relatives. In Proteinase Inhibitors (Barret A. and Salvesen G. eds.) pp390-401, Elsevier Science publishers, in Amsterdam
- Gebhard W., Hochstrasser H., Fritz J. J. Enghild S. V., Pizzo and Salvesen G. (1990) Structure of the inter-α-inhibitor and pre-α-inhibitor: current state and proposition of a new terminology. *Biol. Chem. Hoppe-Seyler.* **371:** 13
- Gill W., Silver M. and Roma M. (1984) Superaturation levels and crystallization rates of calcium oxalate from urines of normal humans and stone formers determined by a ^{14}C-oxalate technique. *Invest. Urol.* **12:** 203
- Goldfarb S. (1994) Diet and nephrolithiasis. *Annu. Rev. Med.* **45:** 235
- Grant A. M. S., Baker L. R. L. Neuberger A. (1973) Urinary Tamm Horsfall glycoprotein in certain kidney disease and its content in renal and bladder calculi. *Clin. Sci. Med.* **44:** 377

- Grases F. and March P. (1989) Determination of phytic acid based on inhibition of crystalline growth of calcium oxalate monohydrate. *Anal. Chim. Acta.* **219:** 89
- Grases F., Costa-Bauza A., March J. G. and Masárová L. (1991) Glycosaminoglycans, uric acid and calcium oxalate urolithiasis. *Urol. Res.* **19:** 375
- Grases F. and Llobera A. (1996) Determination of phytic acid in urine by ICP Atomic Emission Spectrometry. *Anal. Letters.* **29:** 1193
- Grases F, Costa-Bauza A. (1999) Phytate (IP_6) is a powerful agent for preventing calcifications in biological fluids: usefulness in renal lithiasis treatment. *Anticancer Res.* **9:** 3717
- Grases F., March J. G., Prieto R. M., Simonet B. M., Costa-Bauza A., Garcia-Raja A. and Conte A. (1999) Urinary phytate in calcium oxalate stone formers and healthy people. Dietary effects on phytate excretion. *Scand. J. Urol. Nephrol.* **34:**162
- Grover P. K., Moritz R. L., Simpson R. J. and Ryall R. L. (1998) Inhibition of growth and aggregation of calcium oxalate crystal in vitro, a comparison of four human proteins. *Eur. J. Biochem.* **253:** 637
- Grover P. K and Ryall R. L. (1999) Inhibition of calcium oxalate crystal growth and aggregation by prothrombin and its fragments in vitro. *Eur. J. Biochem.* **263:** 50
- Gruber W. and Möllering H. (1966) Citrat-lyase und bestimmung von citrat. Biochemische Zeitschrift. **346:** 85
- Hallson P. C., Rose G. A. and Sulaiman S. (1983) Raising urinary citrate lowers calcium oxalate and calcium phosphate crystal formation in whole urine. *Urol. Int.* **38:**179
- Harangi F., Gyorke Z. and Melegh B. (1996) Urinary glycosaminoglycans excretion in healthy and stone forming children. *Pediatric Nephrol.* **10 (5):** 555
- Hastings A. B., Mclean F. C., Eichelberger L., Hall J. L. and Da Cesta E. (1934) The ionization of calcium, magnesium and strontium citrates. *J. Biol. Chem.* **107:** 351

- Hess B., Zipperle L. and Jaeger P. (1993) Citrate and calcium effects on Tamm-Horsfall glycoprotein as a modifier of calcium oxalate crystal aggregation. *Am. J. Physiol.* **265:** 784
- Hess B., Nakagawa Y. and Coe F. L. (1989) Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am. J. Physiol. **257:** F99
- Hess B., Nakagawa Y. Parks J. H. And Coe F. L. (1991) Molecular abnormality of Tamm-Horfsfall glycoprotein in calcium oxalate nephrolithiasis. *Am. J. Physiol.* **260:** F569
- Hess B., Zipperle L. and Jaeger P. (1993) Citrate and calcium effect on Tamm-Horsfall glycoprotein as a modifier of calcium oxalate crystal aggregation. *Am. J. Physio.* **265**: F784
- Hess B., Meinhart U., Jaggi L., Zipperle L., and Jaeger P. (1995) Reduced carbohydrate content of Tamm-Horsfalll glycoprotein (THP) from severely recurrent renal calcium stone formers (RCSF). *J. Am. Soc. Nephrol.* **6:** 949
- Hess B., Meinhart U., Zipperle L., Giovanoli R. and Jaeger P. (1995) Simultaneous measurements of calcium oxalate crystal nucleation and aggregation: impact of various modifiers. *Urol. Res.* **23:** 231
- Hess B. and Kok D. J. (1996) Nucleation, growth, and aggregation of stone-forming crystals. In Kidney stones: Medical and surgical management. Coe F. L., Favus M. J., Pak C. Y. C., Parks J. H. and Preminger G. M., editors. Lippincott-Raven Publishers, Philadelphia, 3
- Hess B., Ryall R. L., Kavanah J. P., Khan S. R., Kok D., Rodgers A. L. and Tiselius H. (2001) Methods for measuring crystallization in urolithiasis research: Why, how and when. *Eur. Urol.* **40:** 220
- Hesse A., Classen A., Knoll M., Timmermann F. and Vahlensieck W. (1986) Dependence of urine composition on the age and sex and healthy subjects. Clin. Chim. Acta. **160:** 79
- Hesse A., Siener R., Heynek H. and Jahnen A (1993) The influence of dietary factors on the risk of urinary stone formation. Scan, Micros. **7:** 1119
- Höchstrasser K. and Wachter E. (1979) Kunitz-type proteinase inhibitors derived by limited proteolysis of inter-α-trypsin inhibitor, I. Determination of the amino acid sequence of the antitryptic domain by solid-phase Edman degradation. *Biol. Chem. Hopper-Seyler.* **373:** 1019

- Höchstrasser, K., Schonberger O.L., Rossmanith, I. and Wachter, E. (1981) *Hoppe Seylers Z. Physiol. Chem.* **362:** 1357
- Höchstrasser K., Reisinger P., Albrecht G. J., Watcher E. and Schönberger O. L. (1984) Isolation of acid-resistant urinary trypsin inhibitors by high performance liquid chromatography and their characterization by *N*-terminal amino-acid sequence determination. *Hopper Seyler Z. Physiol. Chem.* **365:** 1123
- Hosting D. H., Wilson J. W. L., Liedtke R. R., Smith L. H. and Wilson D. H. (1985) Urinary citrate excretion in normal persons and patients with idiopathic calcium urolithiasis. *J. Lab. Clin. Med.* **106:** 682
- Hodgkinson A (1962) Citric acid excretion in normal adults and in patients with renal calculus *Clin. Sci.* **23:** 203
- Howard J. E. and Thomas W. C. (1958) Some observations on rachitic rat cartilage of probably significance in the etiology of renal calculi. Trans. *Am. Clin. Clin. Climatol. Assoc.* **70:** 94
- Hoyer J. P. (1994) Role of uropontin in urinary calcium stone formation. *Urolithiasis.* **2:** 253
- Imanari T., Shinbo A, Ochioi H, Ikei T, Koshiishi I. And Toyoda H. (1992) Study of proteoglycans having low-sulphated chondroitin-4 sulphate in human urine and serum. *J. Pharmacobio-Dyn.* **15:** 231
- Ito H., Suzuki F., Yamaguchi K., Nishikawa Y. and Kotake T. (1992) Reduction of urinary oxalate by combined calcium and citrate administration without increase in urinary calcium oxalate stone formers. Clin. Nephrol. **37:** 14
- Iwata H., Nishio S., Wakatsuki A., Ochi K. and Takeuchi M. (1985) Architecture of calcium oxalate monohydrate urinary calculi. *J. Urol.* **133:** 334
- Jiang L., He L. and Fountoulakis M. (2004) Comparison of protein precipitation methods for sample preparation prior to proteomic analysis.
- Johansson G., Backman U., Danielson B., Ljunghall S. and Wikstrom B. (1980) Magnesium metabolism in renal stone disease. *Inv. Urol.* **18:** 93

- Johansson G., Backman U., Danielson B., Fellstrom B., Ljunghall S. and Wikstrom B. (1980) Biochemical and Clinical Effects of the prophylactic treatment of renal calcium stones with magnesium hydroxide. *J. Urol.* **124:** 770
- Jones W. and Resnick M. (1990) The characterization of soluble matrix proteins in selected human renal calculi using two-dimensional polyacrylamide gel elecrophoresis. *J. Urol.* **144:** 1010
- Kavanagh J. P. and Rao P. N. (1994) Calcium oxalate supersaturation of urine: which factors should be measured? *Dept. Urol.* 418
- Khan S. R. and Kok D. J. (2004) Modulators of urinary stone formation. *Frontier in BioSci.* **9:** 1450
- Kobayashi H., Shibata K., Fujie M., Sugino D. and Terao T. (1998) Identification of structural domains in inter-α-trypsin inhibitor involved in calcium oxalate crystallization. *Kidney. Inter.* **53:** 1727
- Kohri K., Garside J. and Blacklock N. J. (1989) The effects of glycosaminoglycans on the crystallization of calcium oxalate. *Br. J. Urol.* **63:** 584
- Kohri K., Umekawa T., Kodama M., Katayama Y., Ishikawa Y., Takada M., Katoh Y., Kataoka K., Iguchi M. and Kurita T. (1990) Inhibitory effect of glutamic acid and aspartic acid on calcium oxalate crystal formation. *Eur. Urol.* **17:** 173
- Konya E., Umekawa T., Iguchi M. and Kurita T. (2003) The role of osteopontin on calcium oxalate crystal formation. *Euro. Urol.* **43:** 564
- Koide T., Takemoto M., Itatani H., Takaha M. and Sonoda T. (1981) Urinary macromolecular substances as natural inhibitors of calcium oxalate crystal aggregation. *Invest. Urol.* **18:** 382
- Kok D. J., Papapoulos S. E., Blomen L. J. M. J. and Bijvoet O. L. M. (1988) Modulation of Calcium-Oxalate Monohydrate crystallization kinetics *in vitro*. *Clin. Chim. Acta* **112:** 349

- Levine B. S., Rodman J. S., Wiernerman S., Bockman R. S. and Lane J. M (1994) Effect of calcium citrate supplementation on urinary calcium oxalate saturation in female stone formers: implication for prevention of osteoporosis. *Am. J. Clin. Nutr.* **60:** 592
- Lewandowski S., Rodgers A. and Schloss I. (2001). The influence of a high-oxalate / low-calcium diet on calcium oxalate renal stone risk factors in non-stone forming black and white South African subjects. *Br. J. Urol.* **87:** 307
- Lonsdale K. (1968) Epitaxy as a growth factor in urinary calculi and gallstone. *Nature* **217***:* 56
- Li M. K., Blacklock N. J. and Garside J. (1985) Effects of magnesium on calcium oxalate crystallization. *J. Urol.* **133:** 123
- Li M. K., Kavanagn J. P. and Prendiville P. (1986) Does sucrose damage kidneys? *Br. J. Urol.* **58:** 353
- Lemann J. jr. Piering W. P. and Lennon E. J. (1969) Possible role of carbohydrate-induced calciuria in calcium oxalate kidney-stone. *N. Engl. J. Med.* **280:** 232
- Ljunghall S., Christensson T. and Wengle B. (1977) Prevalence and incidence of renal stone disease in healthy screening programme. *Scand. J. Urol. Nephrol.* **41:** 39
- Malki N., Balduyck M., Maes P., Capon C., Mizon C., Han K. K., Tartar A., Fournet B. and Mizon J. (1992) The heavy chains of human plasma inter-α-trypsin inhibitor: the isolation, their identification by electrophoresis and partial sequencing. *Biol. Chem. Hoppe-Seyler.* **373:** 1009
- Malyankar U. M., Almedia M., Johnson R. J., Pichler R. H. and Giachelli C. M. (1997) Osteopontin regulation in cultured rat renal epithelial cells. *Kidney Int.* **51:** 1766
- March J. G., Simonet B. M., Grases F. and Salvador A. (1998) Indirect determination of phytic acid in urine. *Anal. Chim. Acta.* **367:** 63
- Marengo S. R., Resnick M. I. and Yang L. (1998) Differential expression of urinary inter-α-trypsin inhibitor trimers and dimmers in normal compared to active calcium oxalate stone forming men. *J. Urol.* **159:** 1444

- Massey L. K., Roman-Smith H. and Sutton R. L. (1993) Effect of dietary oxalate and calcium on urinary oxalate and risk of formation of calcium oxalate kidney stones. *J. Am. Diet. Assoc.* **93:** 901
- McCormack M., Dessureault. J and Guitard. M. (1991) The urine specific gravity dipstick: a useful tool to increase fluid intake in stone forming patients. *J.Urol.* **146:** 1475
- Médétogen-Benission J., Tardivel S., Hennequin C., Daudon M., Drueke T. and Lacour B. (1999) Inhibitory effect of bikunin on calcium oxalate crystallization in vitro and urinary bikunin decrease in renal stone formers. *Urol. Res.* **27:** 69
- Menon M. and Mahle C. J. (1983) Urinary citrate excretion in patients with renal calculi. *J. Urol.* **129:** 1158
- Mensah P. D. N. A. (2004) Investigation of the crystallization inhibitory properties of albumin isolated from the urine of black and white South Africans. MSc thesis, University of Cape Town, South Africa
- Merhoff V. F., Holmes R. P. and Assimos D. J. (2001) The renal excretion of oxalate following an oxalate load. In Kok D. J., Romijn H. C., Verhagen P. C. and Verkoelen C. N (eds.). Eurolithiasis, 9th European Symposium on urolithiasis, Shaker Publishing, The Netherland. 120
- Meyer A. S., Finlayson B. and DuBois L. (1971) Direct observation of urinary stone ultrastructure. *Br. J. Urol.* **43:** 154
- Meyer J. L. and Smith H. L. (1975) Growth of calcium oxalate crystals. II. Inhibition by natural urinary crystal growth inhibitors. *Invest. Urol.* **13:** 36
- Mizon C., Piva F., Queyrel V., Balduyck M., Hachulla E. and Mizon J. (2002) Urinary bikunin determination provides insight into Proteinase/ Proteinase inhibitor imbalance in patients with inflammatory disease. *Clin. Chem. Lab. Med.* **40 (6):** 579
- Modlin M. (1968) The aetiology of renal stone: A new concept arising from studies on a stone-free population. *Ann. Roy. Coll. Surg (Eng).* **40:** 155
- Moore C. A. and Bunce G. E (1964) Reduction in frequency on renal calculus formation by oral magnesium administration. A preliminary report. *Invest. Urol.* **2:** 7

- Morii M. and Travis J. (1985) The reactive site of human Inter-α-inhibitor is in the amino-terminal half of the protein. *Biol. Chem. Hoppe-Seyler.* **366:** 19
- Morikawa M., Yachiku S. and Arima S. (1994) isolation and purification of a high molecular weight substance from human urine which inhibits calcium oxalate crystal growth. *Int. J. Urol.* **1:** 216
- Morse R. M. and Resnick M. I. (1989) A study of the incorporation of urinary macromolecule on crystals of different mineral composition. *J. Urol.* **141:** 641
- Muramatsu M., Mori S., Matsuzawa Y., Horiguchi Y., Nakanishi Y. and Tanaka M. (1980) Purification and characterization of urinary trypsin inhibitor, UTI68, from normal human urine, and its cleavage by human uropepsin. *J. Biochem.* **88:** 1317
- Nakagawa Y., Abram V. and Kezdy F. J. (1983) Purification and characterization of the principle inhibitor of calcium oxalate monohydrate crystal growth in human urine. *J. Biol. Chem.* **258:** 12594
- Nakagawa Y., Renz C. L., Ahmed M. and Coe F. L. (1991) *Am J Physiol Renal Physiol.* **260:** F243
- Nguyen N. U., Dumoulin G., Wolf P. J., Betenylay S (1986) Urinary calcium and oxalate excretion in response to oral glucose load in man. *Horm. Metab. Res.* **18:** 869
- Nguyen N. U., Dumoulin G., Wolf P. J., Betenylay S (1989) Urinary calcium and oxalate excretion during oral fructose or glucose load in man. *Horm. Metab. Res.* **21:** 96
- Nguyen N. U., Henriet M. T., Dumoulin A., Windmer A. and Regnard J. (1994) Increase in calciuria and oxaluria after a single chocolate bar load. *Hormo. Meta. Res.* **26:** 383
- Nguyen N. U., Dumoulin G., Henriet M. T. and Regnard J. (1995) Increase in urinary calcium and oxalate after fructose infusion. *Horm Metab Res.* **27(3):** 155
- Nicar M. J., Skula C., Sakhaee K. and Pak C. Y. C (1983) Low citrate excretion in nephrolithiasis. *Urol.* **21:** 8
- Nishino N., Aoki K. And Toruka Y. (1989) Measurement of urinary trypsin inhibitor in urine, plasma and cancer tissue of patients with stomach cancer. *Haemostasis.* **19:** 112

- Nordin B, Need A. and Morris H. eds. (1993) Metabolic bone and stone disease, Churchill Livingstone, Edingburg
- Odum L. (1990) Inter-α-inhibitor and pre-α-trypsin inhibitor in health and disease. Determination by immunoelectrophoresis and immunoblotting. *Biol. Chem. Hoppe-Seyler* **371:** 1153
- Ogawa Y (1994) Impact of sodium-potassium citrate on the diurnal variation in urinary calcium oxalate and calcium phosphate saturation levels in normal individuals. Br. J. Urol. **73:** 136
- Okuyama M., Yamaguchi S. and Yachiku S. (2003) Identification of bikunin isolated from human urine inhibits calcium oxalate crystal growth and its localization in the kidney. *Inter. J. Urol.* **10:** 530
- Pak C. Y. C. (1987) Citrate and renal calculi. *Miner. Electro. Metab.* **13**:257
- Pak C. Y. C. (1991) Citrate and renal calculi: new insight and future directions. *Am. J. Kidney. Dis.* **17**:420
- Pak C. Y. C. (1994) Citrate and renal calculi: an update *Miner. Electro. Metab.* **20**:371
- Pak C. Y. C., Ohata M. and Holt K. (1975) Effect of diphosphate on crystallization of calcium oxalate in vitro. *Kidney. Int.* **7**: 154
- Pak C. Y. C., Smith L. H., Resnick M. I. and Weinerth J. L. (1984) Dietary management of idiopathic calcium urolithiasis. *J. Urol.* **131**: 850
- Parivar F., Low R. K. and Stoller. M. L. (1996) The influence of diet on urinary stone disease. *J. Urol.* **155**: 432
- Piesse J. W. (1985) Nutritional factors in calcium containing kidney stones with particular emphasis on vitamin C. *Int. Clin. Nutr. Rev.* **5**:110
- Prat C. W., Swaim M. W. and Pizzo S. V. (1998) Inflammatory cells cells degrade inter-α-inhibitor to liberate urinary proteinase inhibitors. *J. Leuko. Biol.* **45**: 1
- Reddy S. T., Wang C., Sakhaee K., Brinkley L., and Pak C. Y. C. (2002) Effect of low-carbohydrates high-protein diets on acid-base balance, stone-forming propensity and calcium metabolism. *Am. J. Kidney stone.* **40:** 256
- Rhoades, R. and Richard P. (2002). *Human Physiology* (4th ed.). Brooks/Cole.
- Ramello A., Vitale C. and Marangella M. (2000) Epidemiology of nephrolithiasis. *J Nephrol.* **13** (3): S45

- Roberts S. and Resnick M (1986) Glycosaminoglycans content of stone matrix. *J. Urol.* **135**:1078
- Robertson W. G., Peacock M. (1972) Calcium oxalate crystaluria and inhibitors of crystallization in recurrent renal stone formers. *Clin. Sci.* **43:** 499
- Robertson W. G., Peacock M. Heyburn P. J., Marshal D. H., Clark P. B. (1978) Risk factors in calcium stone disease of the urinary tract. *Br. J. Urol.* **50**: 449
- Robertson W. G. and Nordin B. E. C. (1982) Physico-chemical factors governing stone formation. In Scientific foundation of urology. Williams D. I. and Chisholm G. D, editors. William Heinemann Medical Books Ltd, London. 194
- Robertson W. G. and Peacock M. (1983) Review of risk factors in calcium oxalate urolithiasis. *World. J. Urol.* **1**: 114
- Robertson W. G. (1987) Diet and calcium stones. *Min. Electr. Metab.* **13**: 228
- Robertson W. G. and Hughes H. (1994) Epidemiology of urinary stone disease in Saudi Arabia. In : Ryall R., Bais R., Marshall V. R., Rofe A. M., Smith L. H. and Walker V. R. eds. Urolithiasis 2. New York and London: Plenum press. 453
- Robertson W. G., Whitfield H. N., Unwin R. J., Mansell M. A., Neild G. H., Mostafid. H and Longhorn S. E. (2000) Proceedings of 9th International Symposium of Urolithiasis. Eds. Rodgers. A. R., Hibbert. B. E., Hess. B., Khan. S. R., Preminger. G. 305
- Rock R. C., Walker W. G. and Jennings C. D. (1986) Nitrogen metabolites and renal function. In Textbook of Clinical Chemistry. Tietz NW, editor. WB Saunders, Philadelphia. 1278
- Rodgers A. L. (2006) The riddle of kidney stone disease: lessons from Africa. *Urol. Res.*
- Rodgers A. L. (2006)
- Rodgers A. L. (1997) Effect of mineral water containing calcium and magnesium on calcium oxalate urolithiasis risk factors. Urologia Internationalis. **58**: 9

- Rodgers A. L. and Lewondowski S. (2002) Effect of 5 different diets on urinary risk factors for calcium oxalate kidney stone formation evidence of different renal handling mechanism in different race groups. *J. Urol.* **168**: 931
- Rofe A. M., Bais R. and Conyers R. A. J. (1986) The effect of dietary refined sugars and sugar alcohols on renal calcium oxalate deposition in ethylene glycol-treated rats *Food. Chem. Tox.* **24 (5):** 397
- Rose G.A. and Sulaiman. S. (1982) Tamm-Horsfall mucoproteins promote calcium oxalate crystal formation in urine: Quantitative studies. *J. Urol.* **127**: 177
- Route P., Daveau M. and Salier J. P. (1992) Electrophoresis pattern of the Inter-α-trypsin inhibitor family proteins in human serum, characterized by chain-specific antibodies. *Biol. Chem.* **267**: 20765
- Rudman D., Kutner M. H., Redd S. C., Waters W. C., Gerron G. G. and Bleier J. (1982) Hypocitratiria in calcium nephrolithiasis. *J. Urol.* **168**: 931
- Russell R. G. G., Bisaz S. and Fleisch H. (1976) The influence of orthophosphate on the renal handling of iorganic pyrophosphate in man and dogs. *Clin. Sci. Mol. Med.* **51:** 435
- Ryall R. L., Harnett R. M. and Marshal V. R. (1981) The effects of urine, pyrophosphate, citrate, magnesium and glycosaminoglycans on the growth and aggregation of calcium oxalate crystals in vitro. *Clin. Chim. Acta.* **112**: 349
- Ryall R. L., Hibberd C. M. and Marshall V. R. (1986) A method for studying inhibitory activity in whole urine. *Urol. Res.* **13**: 285
- Ryall R. L, Grover P. K., Harnett R. M., Hibberd C. M and Marshal V. R. (1989) Small molecular weight inhibitors. In: Walker. V. R., Sutton. R. A. L., Cameron. E. C. B., Pak. C. Y. C., Robertson. W. G. eds. Urolithiasis. Plenum. New York. London. pp 91
- Ryall R. L., Harnett R. M., Hibberd C. M., Edyvane K. A. and Marshal V. R. (1991) Effects of chondroitin sulphate, human serum albumin and Tamm-Horsfall mucoprotein on oxalate crystallization in indiluted human urine. *Urol. Res.* **19**: 181
- Ryall R. L. (1997) Urinary inhibitors of calcium oxalate crystallization and their potential role in stone formation. *World. J. Urol.* **15**: 155

- Ryall R. L. (2000) The mystery of macromolecules: Modulators, matrix and mineralization. Urolithiasis 2000, edited by Rodgers A., Hibbert B., Hess B., Khan S. and Preminger G. 99
- Sakhaee K., Baker S., Zerwekh J., Poinder J., Garcia-Hernander P. A. and Pak C. Y. C (1994) Limited risk of kidney stone formation during long-term calcium citrate supplementation in nonstone forming subjects. *J. Urol.* **152:** 324
- Salier J. P., Sesboüre R., Vercaigne D., Bourguignon J. and Martin J. P. (1983) Inter-α-trypsin inhibitor (ITI): use of new antisera for qualitative studies and discrete quantification of ITI and its derivatives. *Anal Biochem.* **133**: 336
- Schepers M. S. J., van der Boom B. G., Romijn J. C., Schröder F. H. and Verkoelen C. F. (2002) Urinary crystallization inhibitors do not prevent crystal binding. *J. Urol.* **167**: 1844
- Shigara H., Min W., VanDusen W. J., Clayman M. D., Miner D., Terrell C. H., Sherbotie., Foreman J. W., Przysiecki C., Neilson E. G. and Hoyer J. R. (1992) Inhibition of calcium oxalate crystal growth *in vitro* uropontin: Another member of the aspartic acid-rich protein superfamily. *Proc. Natl. Acad. Sci.* **89:** 426
- Shum D. K. Y., Gohel M. D. I. and Tam P. C. (1999) Hyaluronans: Crystallization-promoting activity and HPLC analysis of urinary excretion. *J. Am. Soc. Nephrol.* **10**: S397
- Schwille P. O., Rumenapf G., Kohler R. and Sorgel F. (1987) Effect of acute oral sodium potassium citrate load in healthy males-outlook for treatment of patients with calcium containing renal stones. *Urol. Int.* **42:** 81
- Schwille P. O., Schmiedl A., Herrmann U., Fan J., Gottlieb D., Manoharan M. and Wipplinger J. (1999) Magnesium, citrate, magnesium citrate, and magnesium-alkali citrate as modulators of calcium oxalate crystallization in the urine: observations in patients with recurrent idiopathic calcium urolithiasis. *Urol. Res.* **27**:117
- Schwille P. O., Rumenapf G., Wolfel G. and Kohler R. (1988) Urinary pyrophosphate in patients with recurrent urolithiasis and in healthy controls: a reevaluation. *J Urol.* **140**: 239

- Scott R. and Mowart W. (1977) The prevalence of calcified upper urinary tract stone disease in random population-Cumberland health survey. *Br. J. Urol.* **49**:589.
- Scurr D. S. and Robertson W. G. (1986) **(a)** Modifiers of calcium oxalate crystallisation found in urine. Studies on their mode of action in artificial urine. *J Urol.* **136**: 128
- Scurr D. S. and Robertson. W. G. (1986) **(b)** Modifiers of calcium oxalate crystallisation found in urine. Studies on the role of Tamm-Horsfall mucoprotein and of ionic strength. *J Urol.* **136**: 505
- Shah P. J. R. (1980) Unprocessed bran and its effect on urinary calcium excretion in idiopathic hypercalciuria. *Br. Med. J.* **281**: 426
- Sharma S. S., Vaidyanathan S. K. Thind and Nathan R. (1992) Urinary excretion of inorganic pyrophosphate by normal subjects and patients with renal calculi in north-west India and the effect of Diclodefac sodium upon urinary-excretion of pyrophosphate in stone formers. *Urol. Int.* **48:** 404
- Shulman N. R. (1955) A proteolytic inhibitor with anti-coagulant activity separated from human urine and plasma. *J. Biol. Chem.* **213**: 655
- Slota A., Sjöquist M., Wogast M., Alston-Smith J. and Fries E. (1994) Bikunin in rat plasma, lymph and bile. *Biol. Chem. Hopper-Seyler.* **375**: 127
- Söhnel O. and Grases F. (1995) Calcium oxalate monohydrate renal calculi formation and development mechanism. *Adv. Colloid. Interface Sci.* 59:1
- Sorensen S., Hansen K., Bak. S. and Justesen S. J. (1990) An unidentified macromolecular inhibitory constituent of calcium oxalate crystal growth in human urine. *Urol. Res.* **18**: 373
- Soucie J. M., Thun M. J., Coates R. J., McClellan W. and Austin H. (1994) Demographic and geographic variability of kidney stones in the United States. *Kidney Int.* **46**: 893
- Sugiki M., Sumi M., Maruyama M., Yoshida E. and Mihara H. (1989) Clearance and distribution of acid-stable trypsin inhibitor (ASTI). *Enzyme* **42**: 31
- Sutor D. J. and Scheidt S. (1968) Identification standards for human urinary calculus components, using crystallographic methods. *Br. J. Urol.* **40:** 22

- Suzuki M., Kobayashi H., Kageyama S., Shibata K., Fujie M. and Terao T. (2001) Excretion of bikunin and its fragments in the urine of patients with renal stones. *J. Urol.* **166:** 268
- Tamm I. and Horsfall F. L. (1950) Characterization and separation of an inhibitor of viral hemagglutination present in the urine. *Pro. Soc. Exp. Biol. Med.* **74:** 108
- Trinchieri A., Mandressi A and Luongo P. (1991) The influence of diet on urinary risk factors for stones in healthy subjects and idiopathic renal calcium stone formers. *Br. J. Urol.* **67:** 230
- Trudeau D. L. and Freier E. F. (1967) Determination of calcium in urine and serum by atomic adsorption spectrophotometry. *Clin. Chem.* **13:** 101
- Vermont V. (1937). The occurrence of renal calculi and their possible relation to diet. *J. Am. Med. Assoc.* **109:** 857
- Walton R. C., Kavanagh J. P., Heywood B.R. and Rao P. N. (2005) The association of different urinary proteins with calcium oxalate hydromorphs. Evidence for non-specific interactions. *Biochim. Biophys. Acta.* **1723:** 175
- Webber D., Rodgers A. L. and Sturrock E. D. (2002) Synergism between Urinary Prothrombin Fragment 1 and urine: A comparison of inhibitory activities in stone-prone and stone-free population groups. *Clin Chem Lab Med.* **40:** 930
- Webber D. (2003) Urinary prothrombin fragment 1: a potential role-player in the protection of South African blacks from calcium oxalate kidney stone disease. PhD thesis, University of Cape Town, South Africa
- Webber D., Radcliffe C. M., Royle L., Tobiasen G., Merry A. H., Rodgers A. L., Sturrock E. D., Wordmald M. R., Harvey D. J., Dwek R. A., Rudd P. M. (2006) Sialylation of urinary prothrombin fragment 1 is implicated as a contributory factor in the risk of calcium oxalate stone formation. *FEBS. J.* **273:** 3040
- Welshman S.G. and McGeown M. G. (1972) A quantitative investigation of the effects on the growth of calcium oxalate crystals of potential inhibitors. *Br. J. Urol.* **47:** 237

- Werness P. G., Brown C. M., Smith L. H. and Finalyson B. (1985). EQUIL2: a basic computer program for the calculation of urinary supersaturation. *J. Urol.* **134:** 1242
- Wesson J. A., Worscester E. M., Wierssner J. H., Mandel M. S. and Kleinman J. G. (1998) Control of calcium oxalate structure and cell adherence by urinary macromolecules. *Kidney Int.* **53:** 952
- Whalley N. A., Moraes M. F. B. G., Shar T. G., Pretorius S. S. and Meyers A. M. (1998) Lithogenic risk factors in the urine of black and white subjects. *Br. J. Urol.* **82:** 785
- Whalley. N. A., Moraes. M. F. B. G., Shar. T. G., Pretorius. S. S. and Meyers. A. M. (1998) Lithogenic risk factors in the urine of black and white subjects. *Br. J. Urol.* **82:** 785
- Willis J. B. (1967) Determination of calcium and magnesium in urine by atomic absorption spectroscopy. *Anal Chem.* **33:** 556
- Wise R. O. and Kark A. E. (1961) Urinary calculi and serum calcium levels in Africans and Indians. *S. Afr. Med. J.* **35:** 47
- Worcester E. and Beshensky A. (1995) Osteopontin inhibits nucleation of calcium oxalate crystal. *Ann. N. Y. Acad. Sci.* **760:** 375
- Wray W., Boulikas T., Wray V. P. and Hancock R. (1981) Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118:** 197
- Xu Y., Carr P. D., Guss M. and Ollis D. L. (1998) The crystal structure of bikunin from the inter-α-inhibitor complex: a serine protease inhibitor with two kunitz domain. *J. Mol. Biol.* **276:** 955
- Yang Y., Grover P. K., Motitz R. L., Simpson R. J. and Ryall R. L. (1995) Is nephrocalcin related to the urinary derivative (bikunin) of inter-α-inhibitor? *Br. J. Urol.* **25:** 425
- Yang L., Resnick M. I. and Marengo S. R. (2005) A simple procedure for isolating microgram quantities of biologically active bikunin from human urine. *Brit. J. Urol. Intern.* **96:** 647
- Yoshioka T., Koide T., Utsunimiya M., Itatani H., Oka T., Sonoda T (1989) Possible role of Tamm-Horsfall glocoprotein in calcium oxalate crystallization. *Br. J. Urol.* **64:** 463

- Yuki Y., Nomura K and Kirihara M. (1993) Charge isomers of urinary bikunin (trysin inhibitor). *Biochim. Biophys. Acta.* **1203:** 298
- Zimmerman D., Voss S., von Unruh G. E and Hesse A (2003) Influence of magnesium and absorption and excretion of oxalate. **31 (2):** 84