การยับยั้งรูปร่างผลึกแคลเซียมออกซาเลตโมโนไฮเดรตโดยใช้ออสทิโอพอนติน

Morphological Inhibition of Calcium Oxalate Monohydrate by Osteopontin

อำนวย วัฒนกรสิริ[1, 3]* และ คฑาวุธ ภาชนะ[2, 3]

[1]โครงการบัณฑิตศึกษา สาขาวิชาวิทยาศาสตร์สิ่งแวดล้อม คณะวิทยาศาสตร์ มหาวิทยาลัยบูรพา

[2]ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยบูรพา

[3]ศูนย์ความเป็นเลิศด้านอนามัยสิ่งแวดล้อม พิษวิทยาและการบริหารจัดการสารเคมี

Amnuay Wattanakornsiri[1, 3]* and Katavut Pachana[2, 3]

[1]Graduate School Program in Environmental Science, Faculty of Science, Burapha University

[2]Department of Chemistry, Faculty of Science, Burapha University

[3]Center of Excellence on Environmental Health, Toxicology and Management of Chemicals

บทคัดย่อ

ผลึกแคลเซียมออกซาเลต (CaOx) มีหลากหลายรูปแบบและรูปร่าง CaOx มีบทบาทสำคัญต่อกลไกทางชีวภาพของพืชและเป็นแหล่งก่อให้เกิดโรคนิ่วไต นิ่วไตประกอบด้วยผลึกแคลเซียมออกซาเลตโมโนไฮเดรต (COM) เป็นหลัก งานวิจัยนี้ได้ศึกษาการตกผลึก CaOx โดยใช้แคลเซียมคลอไรด์และโซเดียมออกซาเลตในสภาวะที่มีและไม่มีออสทิโอพอนติน (OPN) และผลของความเข้มข้นของ OPN และอัตราส่วนความเข้มข้นของ $[Ca^{2+}]/[C_2O_4^{2-}]$ ที่มีอิทธิพลต่อการก่อรูปร่างผลึกของ CaOx ผลึกที่เกิดขึ้นถูกจำแนกโดยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) และปริมาณ Ca^{2+} ในสารละลายที่ทำปฏิกิริยาถูกศึกษาโดยเครื่องอะตอมมิกแอบซอร์บชั่นสเปกโตรสโคปี (AAS) ผลการศึกษาพบว่าความเข้มข้นของ OPN และอัตราส่วนความเข้มข้นของ $[Ca^{2+}]/[C_2O_4^{2-}]$ มีผลต่อรูปร่างผลึกของ CaOx ซึ่งพบหลัก ๆ คือ เฮกซะโกนอล ออกตะฮีดรอล และเดนไดรท์ ปริมาณ OPN ที่เพิ่มขึ้นมีผลต่อการเพิ่มขึ้นของผลึกแคลเซียมออกซาเลตไดไฮเดรต (COD) ซึ่งมีรูปร่างแบบออกตะฮีดรอล การศึกษานี้แสดงให้เห็นว่า OPN สามารถยับยั้งการเกิดผลึกชนิด COM และมีผลทำให้เกิดผลึกชนิด COD ในหลอดทดลอง

คำสำคัญ : แคลเซียมออกซาเลต การตกผลึก ออสทิโอพอนติน โรคนิ่วไต กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด

Abstract

Calcium oxalate (CaOx) can be crystallized in several forms and morphologies. CaOx plays a functional role in plant physiology and is a source of chronic human disease called kidney stone, being primarily composed of calcium oxalate monohydrate (COM) crystals. In this research, we investigated the crystallization of CaOx by the precipitation of calcium chloride and sodium oxalate in the absence and presence of osteopontin (OPN). The effects of OPN concentration and $[Ca^{2+}]/[C_2O_4^{2-}]$ ratio on CaOx crystal forms and morphologies were studied. The crystals were characterized by scanning electron microscopy (SEM), and the quantities of Ca^{2+} in aqueous solution were detected by atomic absorption spectrometry (AAS). The results show that OPN concentration and $[Ca^{2+}]/[C_2O_4^{2-}]$ ratio affected the crystal morphologies that mainly were hexagonal, octahedral and dendrite. Higher OPN concentration increased the formation of calcium oxalate dihydrate (COD) crystals as the octahedral shape. This research shows the property of OPN to inhibit the formation of COM crystals, and to promote the induction of COD crystals simultaneously in tube experiment as an in-vitro method.

Keywords : Calcium oxalate, Crystallization, Osteopontin, Kidney stone, Scanning electron microscopy

Corresponding author. E-mail: amnuay1979@yahoo.com

Introduction

Kidney stone disease is a common chronic disorder in humans (Qiu *et al*., 2003). This disease occurs in 15-17% of the human population in northeast Thailand and is an important public health problem (Nimmannit *et al*., 1996; Yanagawa *et al*., 1997). High concentrations of calcium carbonate in water and a low-nutrient diet are contributing factors to this disease (Nimmannit *et al*., 1996). Kidney stones are commonly composed of calcium oxalate (CaOx), especially calcium oxalate monohydrate (COM) micro-crystals (Qiu *et al*., 2003; Sheng *et al*., 2004; Thongboonkerd *et al*., 2006). COM is the most thermodynamically stable form at room temperature in nature (Sheng *et al*., 2004; Yu *et al*., 2004). When food containing high oxalic acid, such as spiny pigweed (Amaranthus lividus L.) and cocoa (Theobroma cacao L.) are consumed, they cause the formation of CaOx crystals in urine. Generally, the generated crystal form is COM. Humans normally have biological control mechanisms to prevent COM crystallization in urine by inducing inhibitors that decrease nucleation, growth, and aggregation of COM crystals (Qiu *et al*., 2003; Oehlschlager *et al*., 2009). In particular, inhibitors in urine will transform COM to calcium oxalate dihydrate (COD) (Jung *et al*., 2004; Thongboonkerd *et al*., 2006).

In the past decade, interactions between stone crystals and organic matrixes, such as citrate, osteopontin and citrate, aspartate, glutamate, poly-(styrene-alt-maleic acid) (PSMA) and glycosaminoglycans have been investigated (Shirane *et al*., 1999; Qiu *et al*., 2003; Sheng *et al*., 2004; Yu *et al*., 2004). Osteopontin (OPN) was chosen for this study because urinary level of OPN has been shown to inhibit the growth and change the gross morphology of CaOx crystals. OPN is a single-chain protein (phosphorylated glycoprotein) with a peptide molecular mass of about 33 kDa and approximately 300 amino acid residues (Bayless *et al*., 1997; Mazzali *et al*., 2002). Normal human urine contains levels of OPN (>100 nM) that markedly inhibit several aspects of COM crystallization. OPN has an abundance of sequence domains rich in dicarboxylic acids (Qiu *et al*., 2003).

In the present study, we investigated the crystallization of CaOx crystals from aqueous solution in the absence and presence of OPN. The effects of OPN concentration and $[Ca^{2+}]/[C_2O_4^{2-}]$ ratio on CaOx crystal formation, and compositions were studied. In addition, we used atomic absorption spectrometry (AAS) for detecting the Ca^{2+} in the aqueous solution, and scanning electron microscopy (SEM) to describe the morphologies and surfaces of CaOx crystals as well as their compositions with energy dispersive X-ray spectroscopy (EDS).

Materials and methods

$CaCl_2$ and $Na_2C_2O_4$ solutions

All preparations and experiments were conducted at room temperature (25 °C), and all chemicals were analytical grade. $CaCl_2 2H_2O$ (ASP Ajax Finechem) and $Na_2C_2O_4$ (ASP Ajax Finechem) were from the same stock solutions of 1 M in deionized (dI) water. CaOx crystals were produced by using the required concentrations of $CaCl_2$ (1, 4 mM) and $Na_2C_2O_4$ (1, 4 mM) diluted from the stock solutions with dI water. With regard to the previous study (Pachana, 2008), the most appropriate $[Ca^{2+}]/[C_2O_4^{2-}]$ ratio is 1:1 because surface characteristics could not be distinctively classified the differences when comparing with 2:1 and 3:1 from SEM; the lowest Ca^{2+} concentration was chosen.

Osteopontin solutions

OPN (MERCK) was diluted with dI water to 0.25, 5, 10 and 50 g/L, which were used as the crystal modifier of OPN solutions.

Crystallization of CaOx crystals in absence and presence of osteopontin

In a typical experiment, $CaCl_2$ (1 mM, 20 mL)

was added to $Na_2C_2O_4$ (1 mM, 20 mL) in five beakers. Subsequently, the OPN was added to obtain the concentration of 0.25 to 50 g/L along with vigorous stirring for 1 minute. Then the mixtures were covered with a glass plate for 24 h until solutions crystallized. As a reference, the CaOx was prepared in the absence of OPN as well. The mixture consisting of $CaCl_2$ (4 mM, 20 mL) and $Na_2C_2O_4$ (4 mM, 20 mL) was prepared in the same way.

Detection and characterization

Quantities of Ca^{2+} in solution after filtration of the mixtures were determined by AAS (model AA-6501F, SHIMADZU). Dried CaOx crystals were characterized for their morphologies by SEM (model 1450VP, LEO) with an accelerating voltage of 20 kV. Chemical compositions of crystals were also characterized by SEM, with EDS as a detector.

Results and discussion

Effect of osteopontin concentration

The effect of the varying amount of OPN on the morphologies and the sizes of CaOx particles at 25 °C under the standard analysis conditions are exhibited in Figure 1. Figure 1A indicates that all CaOx particles were hexagonal plate-like in shape of COM (Qiu *et al*., 2003; Jung *et al*., 2004; Sheng *et al*., 2004; Thongboonkerd *et al*., 2006; Walton *et al*., 2005; Yu *et al*., 2005). When OPN increased in concentration, the amount of COM

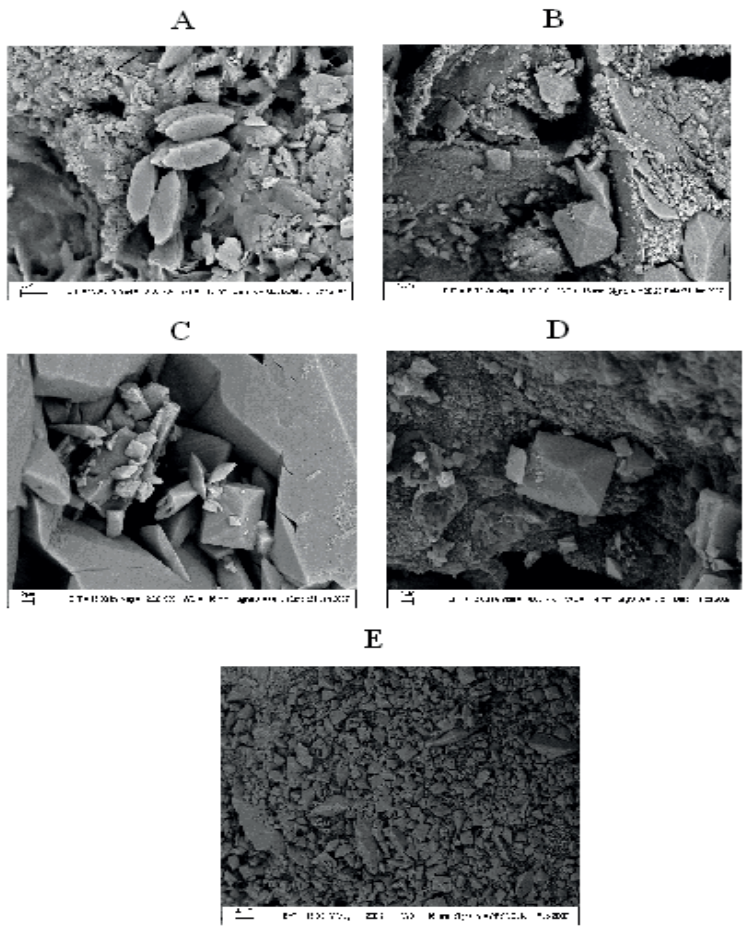

Figure 1. SEM micrographs of CaOx particles received from the absence and presence of OPN at 25 °C. [Ca^{2+}]: 1 mM and [$C_2O_4^{2-}$]: 1 mM, [OPN]: (A) 0, (B) 2.5, (C) 5, (D) 10 and (E) 50 g/L, the scale bars = 2 μm (A, B, C, D), 10 μm (E).

particles decreased, and those of COD gradually increased. Most CaOx particles were octahedral shape of COD (Jung *et al*., 2004; Walton *et al*., 2005; Yu *et al*., 2005). This is because OPN contains mainly single-chain proteins and dicarboxylic acids (Qiu *et al*., 2003). Hence, these functional groups of protein and acid could transform the structure of COM to COD. In conclusion, the higher concentration of OPN inhibits the formation of COM, and promotes the formation of COD.

After $CaCl_2$ reacted with $Na_2C_2O_4$ the crystallization of CaOx occurred so that the stable COM was the dominant phase. As the absence of OPN (Figure 1A), Ca^{2+} concentration in the aqueous solution was very low. When concentrations of OPN were added to the solutions, Ca^{2+} concentrations would increase following the OPN concentrations until reaching the maximum equilibrium; after that, the Ca^{2+} concentrations would gradually decrease as the OPN concentrations increased (Figure 2). Due to the reaction between OPN and Ca^{2+} ions in the aqueous solutions, COD then could be formed and increased with that of the OPN concentrations.

Surface structures of COM and COD differ in their affinities for cell membranes (Yu *et al*., 2004) COM has a higher affinity for renal tubule cells (Wesson *et al*., 1998) and for cell membranes as compared with COD (Mandel, 1994). Hence, a preferential adsorption to cell membranes of COM crystals induced kidney stones (Yu *et al*., 2004). In contrast, COD prevents kidney stones because it is easily excreted in urine (Wesson *et al*., 1998; Wang *et al*., 2006). It is suggested that OPN act as a good inhibitor for kidney stones once they induce the formation of COD. The presence of functional groups of phosphorylated glycoprotein with 300 amino acid residues (Bayless *et al*., 1997; Mazzali *et al*., 2002) and dicarboxylic acids (Qiu *et al*., 2003) in OPN may inhibit the formation of COM by reacting with calcium instead of oxalate.

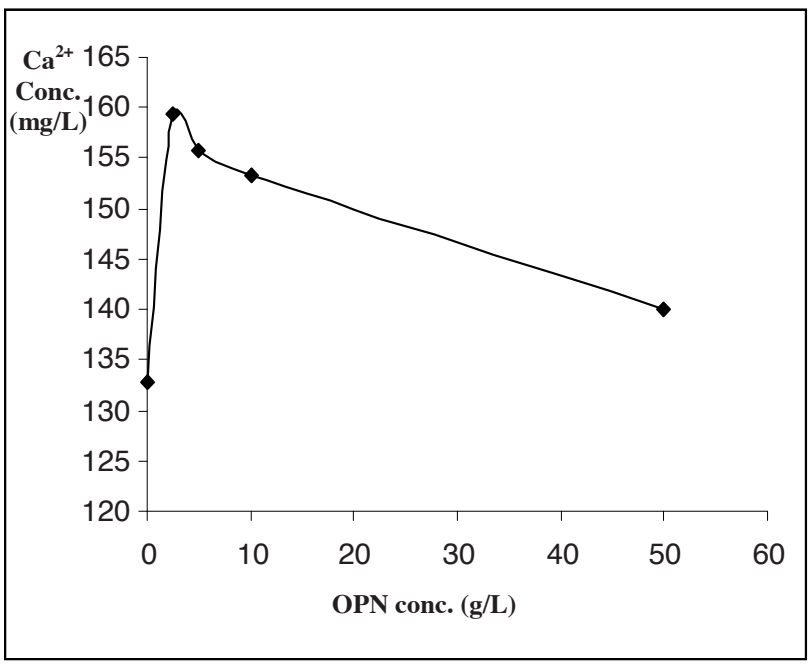

Figure 2. Ca^{2+} concentration in relation with OPN concentration from the reaction between $CaCl^2$ (1 mM, 20 mL) and $Na_2C_2O_4$ (1 mM, 20 mL) at 25 ˚C.

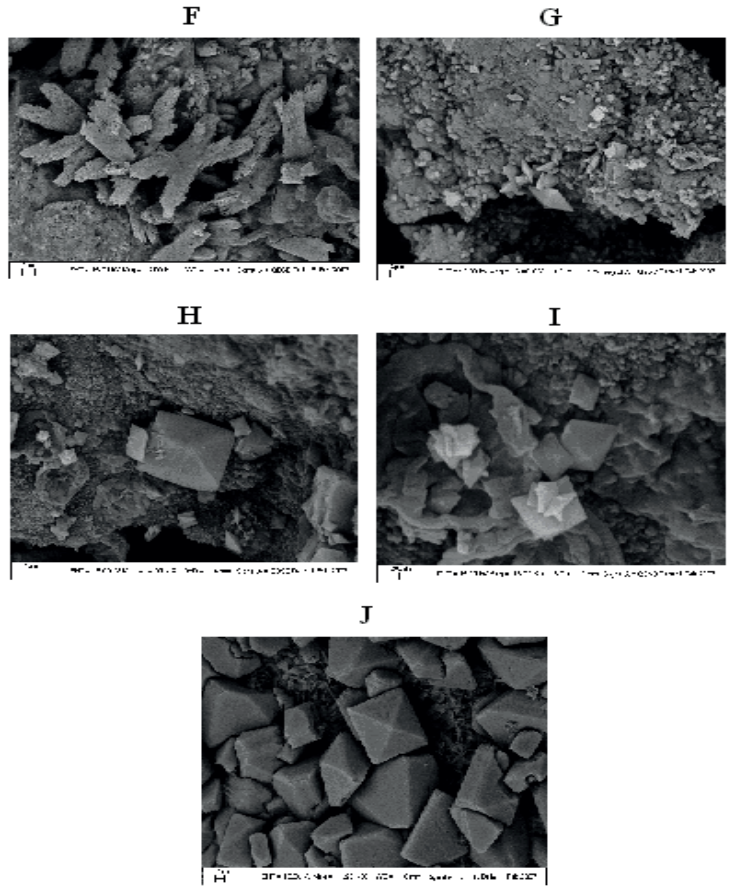

Figure 3. SEM micrographs of CaOx particles received from the absence and presence of OPN at 25 °C. $[Ca^{2+}]$: 4 mM and $[C_2O_4^{2-}]$: 4 mM, [OPN]: (F) 0, (G) 2.5, (H) 5, (I) 10 and (J) 50 g/L, the scale bar = 2 μm (F, G, H, I, J).

The compositions of crystals from $CaCl_2$ and $Na_2C_2O_4$ were characterized with EDS and found to consist of carbon (C), oxygen (O), calcium (Ca) and gold (Au). Au was found because the crystals had to be sealed with Au before detecting by SEM. The presence of C, O and Ca confirmed the SEM result.

Effect of $[Ca^{2+}]/[C_2O_4^{2-}]$ ratio

Varying $[Ca^{2+}]/[C_2O_4^{2-}]$ ratio would change the CaOx crystals and the CaOx-modifier crystals reaction, leading to morphological variation of CaOx crystals (Yu *et al*., 2004). At the higher $[Ca^{2+}]/[C_2O_4^{2-}]$ ratio of 4:4, Figure 3F shows the COM particles to be dendritic in shape (Petrova *et al*., 2004; Thongboonkerd *et al*., 2006), compared with the hexagonal shape when the ratio is 1:1 (Figure 1A). The amount of COD particles of octahedral shape gradually increased with OPN concentration instead of COM particles as same as the ratio of 1:1. The compositions of crystals in the ratio of 4:4 were characterized also by EDS that the chemical elements were the same as the ratio of 1:1.

Conclusion

Our findings clearly indicated that the factors OPN concentration and $[Ca^{2+}]/[C_2O_4^{2-}]$ ratio affected morphologies of CaOx crystals. Main crystal morphologies found were hexagonal, octahedral and dendrite. Higher OPN concentration increased the formation of COD crystals as the octahedral shape. OPN may act

as a good inhibitor to control CaOx crystals and to prevent kidney stones since they induce COD crystals that are easily excreted in urine.

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