Korean J. Chem. Eng., **28**(8), 1749-1753 (2011)
DOI: 10.1007/s11814-011-0022-8

RAPID COMMUNICATION

Biomineralization of calcium carbonate by adding aspartic acid and lysozyme

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(*Received 24 December 2010 • accepted 27 January 2011*)

Abstract−Calcium carbonate is one of the most abundant materials present in nature. Crystal structures of $CaCO_3$ become three polymorphic modifications, namely calcite, aragonite and vaterite. Polymorphic modifications are mediated by adding aspartic acid (Asp) and lysozyme. Lysozyme, which is a major component of egg white proteins, has influenced the calcification of avian eggshells. The influence of Asp and lysozyme on the crystallization of $CaCO_3$ was investigated by adding these additives and calcium chloride solution into sodium carbonate solution in a crystallization vessel. $CaCO_3$ crystals were analyzed by X-ray diffraction (XRD), field emission scanning electron microscopy (FE-SEM) and Fourier transform infrared spectrometry (FT-IR). XRD was used to select the intensities and crystal structure of specific calcium carbonate. SEM was employed for the analysis of the morphology of the precipitation and particle size. Two kinds of crystals were identified by FT-IR spectrum. Hexagonal crystals of vaterite were affected by the Asp in the crystallization solution. However, rhombohedral crystals of calcite by lysozyme were formed without any sign of vaterite.

Key words: Calcium Carbonate, Aspartic Acid, Lysozyme

INTODUCTION

$CaCO_3$ crystal is essential to form the frame of an organism and has contributed to synthesis of nanomaterials. In biomineralization, calcium carbonate ($CaCO_3$) is one of the most abundant mineral resources formed in the natural environment. $CaCO_3$ has increasingly attracted interest and industrial demand has increased for several years on account of physical and chemical properties of $CaCO_3$. Therefore, crystallization of calcium carbonate was extensively studied as it is widely used in the paper, paint, rubber and biomedical application [1]. The application of calcium carbonate particles is determined by several factors, such as particle morphology, specific surface area, particle size, particle size distribution [2]. Calcium carbonate exhibits three polymorphic modifications (rhombic calcite, needlelike aragonite and spherical vaterite) [1]. Crystal properties can be controlled by modifying the reaction medium using surfactants, suspensions, additives and precipitation conditions such as pH [3] and temperature. Calcite is usually formed at a high solution pH and low temperature, while vaterite and aragonite are formed at a low solution pH and high temperature. They are transformed to stable calcite since vaterite is unstable crystal morphology [4-6]. The ACC (amorphous calcium carbonate) forms in the initial stage of calcium carbonate reaction, and it is transformed into vaterite, aragonite and calcite [7-10].

The gas-liquid reaction of $CaCO_3$ is that CO_2 gas is absorbed into calcium hydroxide solution. $CaCl_2$-Na_2CO_3 represents liquid-liquid reaction. The liquid-liquid reaction for $CaCO_3$ crystallization makes both $CaCO_3$ crystals and NaCl byproduct as Eq. (1)

$$CaCl_2(aq)+Na_2CO_3(aq)\rightarrow CaCO_3(sol)+2NaCl(byproduct) \quad (1)$$

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This is because the reaction easily controls saturation concentration in solution and produces various morphologies of $CaCO_3$ crystallization. It is easy to produce biominerals from $CaCO_3$ crystallization reaction by a liquid-liquid reaction with organic materials.

Previous reports showed that the polymorph of calcium carbonate is dependent on the operating conditions of the crystallization, such as supersaturation [11], solution composition [12], pH [10], temperature [14], and presence of additives [15,16]. Biomineralization produces biomineral with good property by adding amino acid to $CaCO_3$ [17]. The outside shell of shellfish is organized by mainly calcite and $CaCO_3$ layer structure. This layer structure's fracture toughness is thousands times as strong as that of simple $CaCO_3$ crystals, while aragonite forms inside layer structure.

There are many studies of biopolymer which affect polymorph formation. Henderson et al. [18] discovered that glycine and alanine are used for crystal reaction of vaterite. Many proteins such as ansocalcin, lysozyme and lithostathine which affect calcium carbonate polymorph have been studied [19]. The studies of calcium carbonate polymorph using polysaccharide were presented by Xiao et al. and Arias and Fernandez [20]. Study of polymorph using nucleic acid was reported by Lukeman et al. [21].

The aim of the present work is to study the role of Asp and lysozyme on the precipitation of calcium carbonate. Morphology of $CaCO_3$ crystals is affected by additives such as aspartic acid (Asp) or lysozyme into calcium chloride and sodium carbonate solution. Lysozyme is a major component of egg white proteins and plentiful in eggshell membranes, which seems to be a modulator for $CaCO_3$ crystallization. Analytical investigations of $CaCO_3$ by FE-SEM, XRD and FT-IR were performed, as well as to discuss the mechanism. FE-SEM was used in order to analyze morphology and crystal size. XRD was used to measure peak intensities and the presence of $CaCO_3$ polymorph. Crystal morphology change with reaction time was identified with measured peak areas of XRD pattern and

FT-IR data.

EXPERIMENTAL

Anhydrous calcium chloride ($CaCl_2$) (purity≥99.0%) and sodium carbonate (Na_2CO_3) (purity≥99.0%) were purchased from Oriental Chemical Inc. (Korea) and Duksan Pharmaceutical Co. Ltd. (Korea), respectively. D, L-aspartic acids were purchased from Fluka (USA). Lysozyme from egg white (purity>95%, MW=14.3 kDa) was obtained from Sigma (USA). Distilled water was used to prepare aqueous solutions of $CaCl_2$ and Na_2CO_3 just before crystallization experiment.

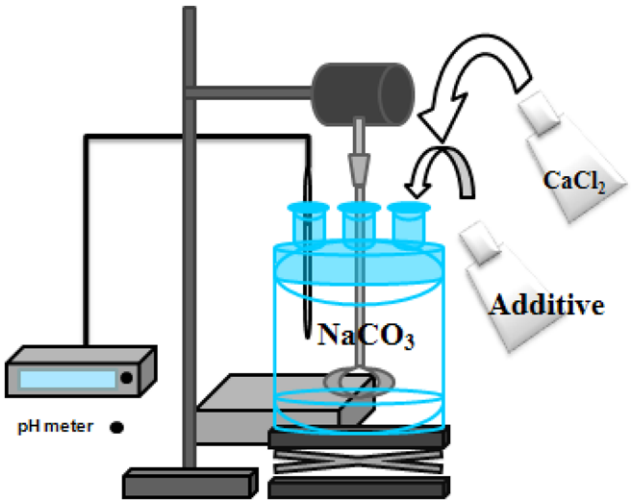

Fig. 1. Schematic diagram of experimental apparatus for biomineralization of calcium carbonate.

For the crystallization of $CaCO_3$ in the absence and the presence of Asp and lysozyme, the liquid-liquid reaction was applied under ambient laboratory conditions. This reaction is illustrated in Fig. 1. In a typical process, experiments were performed in a Pyrex vessel at 25 °C, and the working volume was 1.0 L. Control crystallization solutions were prepared by mixing equal volumes (500 mL) of 50 mM calcium chloride ($CaCl_2$) and 50 mM sodium carbonate (Na_2CO_3). To understand the influence of additives, a crystallization solution was prepared with 0.05 M aspartic acid (Asp) solution or 0.5 g/L, 1 g/L lysozyme. The stirring speed was controlled around 300 rpm. Samples were collected as 50 mL at various time intervals (5, 15, 120, 600 min). Samples of the product suspension were taken intermittently during reaction, and filtered immediately using a micromembrane filter with a 0.45 μm pore diameter. And then filtered samples were dried at 50 °C for 24 h. Details of experiments are given in Refs [22,23].

After being dried, the solid residues were characterized by SEM, on a JEOL JSM-7000F microscope for their crystal morphology and by multipurpose XRD for calculating the ratio of calcite to vaterite of $CaCO_3$. XRD (Rigaku D/MAX-2200 Ultima/PC, Japan) measurements were conducted using Cu Kα radiation (40 keV, 30 mA) to identify the composition of the crystals. The scanning step was 0.02° and 2θ ranges from 10° to 80°. In addition, infrared spectra were recorded with FT-IR. The dried $CaCO_3$ samples were ground with anhydrous KBr to produce pellets to facilitate the FT-IR characterization in the region of 4,000-600 cm^{-1} using a Shimadzu spec-

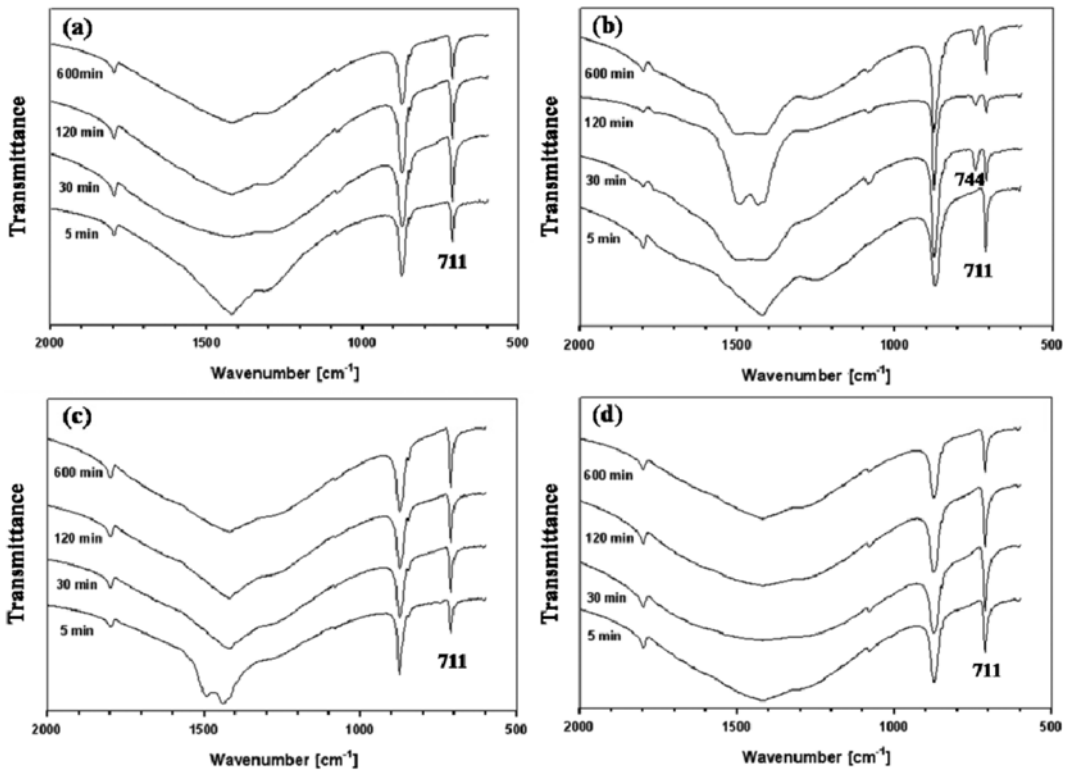

Fig. 2. Fourier transform infrared (FR-IR) spectra of $CaCO_3$ formed; (a) without additive (the control experiment), (b) with 0.05 M Asp, (c) with 0.5 g/L lysozyme, (d) with 1.0 g/L lysozyme. Note that the 711 and 744 cm^{-1} are the peaks of calcite and vaterite, respectively.

trophotometer (Rrestoge-21). From the data of the Joint Committee on Powder Diffraction Standards (JCPDS), it was compared with actual XRD data.

RESULTS AND DISCUSSION

The decomposition of Na_2CO_3 led to the ion of Na^+ and CO_3^{2-}, both of which subsequently diffused into the $CaCl_2$ solution. The formed Cl^- ions were neutralized by Na^+ ions. The formation of $CaCO_3$ precipitates caused the excess of Cl^- ions that were neutralized by Na^+.

Similar experiments were carried out with and without additives to examine its effect on the nucleation and phase transformation of calcium carbonate crystals. The determination of the polymorph of $CaCO_3$ has mainly been conducted by using FT-IR. FT-IR spectra shown in Fig. 2(a) were obtained from the samples at different time intervals without any additive (control). Between the characteristic IR peaks for calcite (711, 875 and 1,087 cm^{-1}) and vaterite (744, 875 and 1,478 cm^{-1}), the peaks at 711 and 744 cm^{-1} were used to distinguish calcite and vaterite, respectively. These IR peaks are characteristic of the calcite at 711 cm^{-1} in Fig. 2(a). In comparison, FT-IR spectra obtained from $CaCO_3$ precipitates formed in the presence of 0.05 M Asp are shown in Fig. 2(b). In the precipitation process of $CaCO_3$, calcite was first formed. With the incubation time increasing to 30 min, ACC was transformed into a mixture of vaterite and calcite. From 30 min, the peak of vaterite at 744 cm^{-1} appeared, and then the peak of vaterite decreased. Because vaterite is the most unstable crystalline polymorph, it is transformed into the most stable calcite. So the peak of calcite increased with decreasing the peak of vaterite. Figs. 2(c) and 2(d) show the FT-IR spectra with 0.5 g/L and 1 g/L lysozyme, respectively. There were only the peaks of calcite, because lysozyme restrained the vaterite from being produced. In addition, with increasing the concentration of lysozyme from 0.5 g/L to 1.0 g/L, the transmittance of calcite peaks increased.

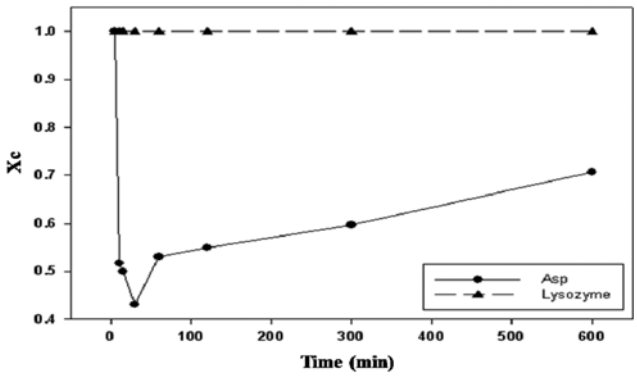

Fig. 3. Fraction of calcite calculated by measuring the weight ratio of calcite and vaterite peaks in FT-IR spectra; Xc=fraction of calcite, Xv=fraction of vaterite, Xc=1−Xv.

Fig. 3 illustrates that the fraction of weight ratio of calcite changes with Asp or lysozyme in FT-IR. Eq. (2) explains the calculation method by FT-IR spectral data in which calcite and vaterite peak area are obtained by measuring the peak area. The ratio Xc was calculated by Eq. (2). Xc means the percentage of calcite and Xv in Eq. (3) equals to 1 minus Xc.

$$Xc = \frac{\text{Calcite peak area}}{\text{Calcite} + \text{Vaterite peak area}} \tag{2}$$

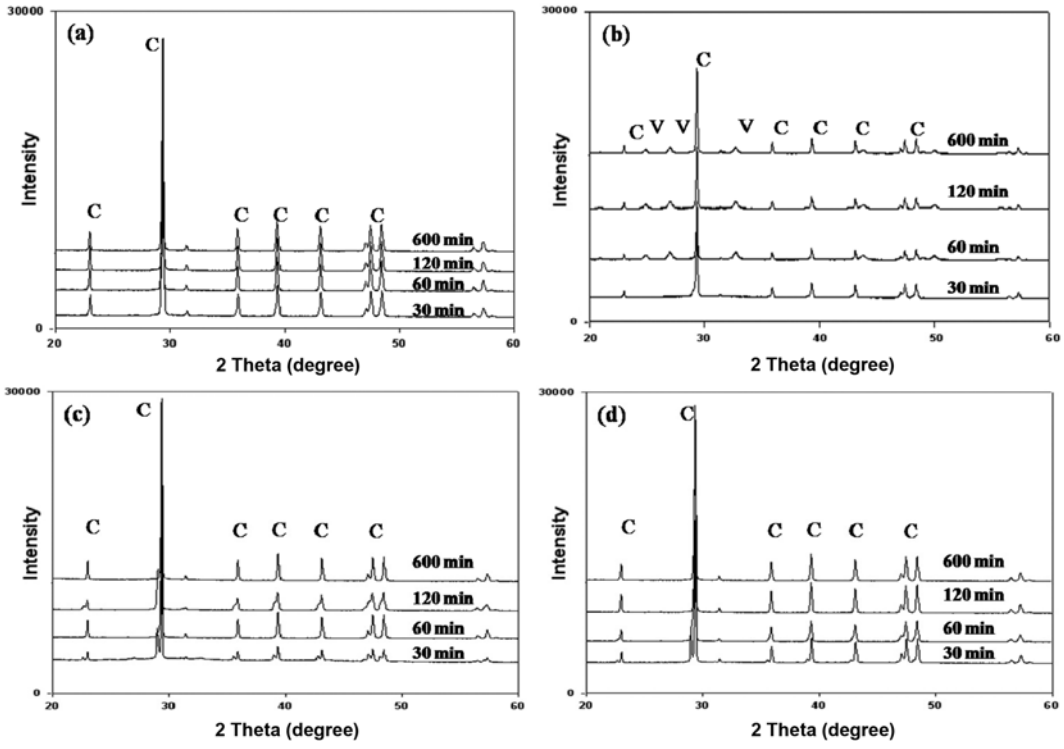

Fig. 4. X-ray diffraction (XRD) pattern of $CaCO_3$ precipitation (a) without additive (the control experiment), (b) with 0.05 M Asp, (c) with 0.5 g/L lysozyme, (d) with 1.0 g/L lysozyme; C=calcite, V=vaterite.

$$Xv = 1 - Xc \quad (3)$$

In the early phase, Xc rapidly decreased with Asp until 30 min. It means that vaterite was produced rather than calcite. After that, vaterite transforms to calcite Xc increases. This is because vaterite is unstable morphology among the $CaCO_3$ crystal. With the time increasing, Xc was constantly increased. However, Xc was dominant with lysozyme. We expect that Asp causes vaterite morphology and lysozyme induces calcite morphology.

With the aim to further characterize the phase composition of crystals, X-ray diffraction (XRD) measurement was performed for the collected precipitates. Fig. 4(a) shows the XRD pattern for the

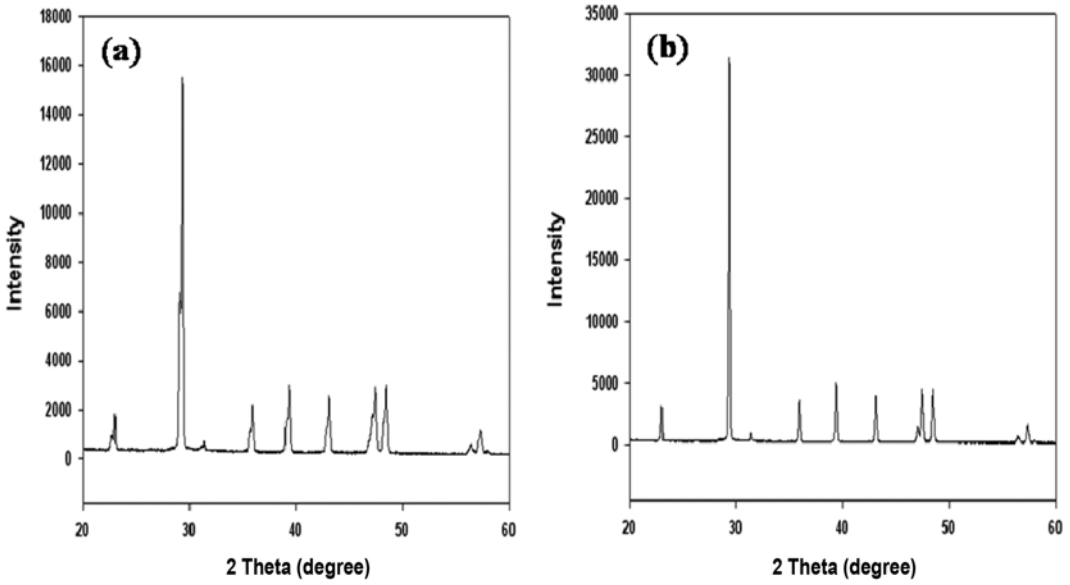

Fig. 5. X-ray diffraction (XRD) pattern of $CaCO_3$ precipitation collected at 120 min (a) with 0.5 g/L lysozyme, (b) 1.0 g/L lysozyme.

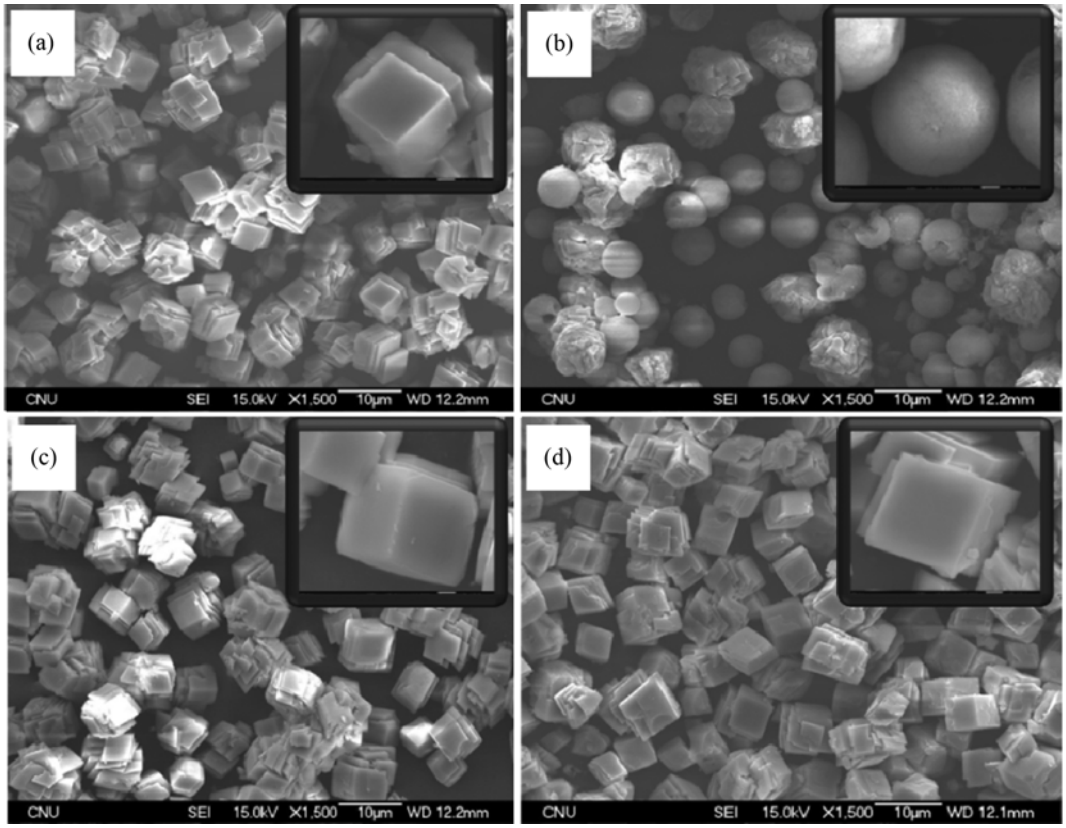

Fig. 6. SEM images of calcium carbonate crystal at 600 min; (a) without additive, (b) in the presence of 0.05 M Asp, (c) 0.5 g/L lysozyme (d) 1 g/L lysozyme.

collected precipitates without any additive. It is obvious that the crystalline phase mostly consists of calcite phase. Fig. 4(b) shows the XRD pattern with 0.05 M Asp in the presence of 0.05 M Asp; the mineral phase is composed of calcite and vaterite crystalline phase. From 120 min, intensity of XRD peaks decreased, because the unstable vaterite was transformed to the stable calcite. Figs. 4(c) and (d) show the XRD pattern with lysozyme. The mineral phase is exclusively composed of calcite crystalline phase, and no other crystalline phase was detectable. These results completely correspond with those gained from the FT-IR characterization. Moreover, it was surveyed that the concentration of lysozyme influences the crystalline phases of $CaCO_3$ (Fig. 5). The peak intensity of calcite was proportional to the concentration of lysozyme.

SEM is capable of revealing the morphological structure and orientation of crystals. SEM images of $CaCO_3$ precipitates collected with or without additives in Fig. 6. Fig. 6(b) show the morphology of spherical vaterite crystal in the presence of 0.05 M Asp. In addition, a phase transition is also observed with Asp, since vaterite crystals coexisted with calcite crystals. As the concentration of lysozyme was increased from 0.5 g/L to 1.0 g/L, only rhombohedral calcite crystals were observed. In addition, the morphological modification of rhombohedral calcite morphology became clearer (Fig. 6(d)). The morphology of rhombohedral calcite depends on the concentration of lysozyme.

CONCLUSIONS

An experiment was conducted to control the polymorphism of calcium carbonate crystals by adding additives such as aspartic acid and lysozyme. To compare the influence of the additive, a control experiment was first performed and Asp or lysozyme was added into the experiment. In the presence of Asp, spherical vaterite crystals were produced; however, vaterite was transformed to calcite soon. The reason is that vaterite is unstable morphology and calcite is the most stable morphology. In the presence of lysozyme, there were only the peaks of calcite, because lysozyme restrained vaterite formation. Moreover, the concentration of lysozyme influences the crystalline phases of $CaCO_3$. The amount of calcite was proportional to the concentration of lysozyme.

ACKNOWLEDGEMENT

This research was supported by Korea Research Foundation Grant. The authors greatly appreciate the support for allowing the present work.

REFERENCES

1. C. Shivkumara, P. Singh, A. Gupta and M. S. Hegade, *Mater. Res. Bull.*, **41**, 1455 (2006).
2. K. Tamura and H. Tsuge, *Chem. Eng. Sci.*, **61**, 5818 (2006).
3. X. Sun, Y. Zhou, J. Ren, F. Cui and H. Li, *Appl. Phys.*, **7**, 75 (2007).
4. S. Bentov, S. Weil, L. Glazer and A. Berman, *J. Struct. Biol.*, **171**(2), 207 (2010).
5. L. Gago-Duport, M. J. I. Briones, J. B. Rodriguez and B. Covelo, *J. Struct. Biol.*, **162**(3), 422 (2008).
6. M. Faatz, F. Grohn and G. Wegner, *Mater. Sci. Eng.*, **25**(2), 153 (2005).
7. R. Beck and J. Andreassen, *Cryst. Growth*, **312**(15), 2226 (2010).
8. W. M. Jung, S. H. Kang, W. S. Kim and C. K. Choi, *Chem. Eng. Sci.*, **55**(4), 733 (2000).
9. S. H. Kang, I. Hirasawa, W. S. Kim and C. K. Choi, *J. Colloid Interface Sci.*, **288**(2), 496 (2005).
10. H. Wei, Q. Shen, T. Zhao, D. Wang and D. Xu., *Cryst. Growth*, **260**(3), 545 (2004).
11. J. Kawano, N. Shimobayashi, M. Kitamura, K. Shinoda and N. Aikawa, *J. Cryst. Growth*, **237**, 419 (2002).
12. Y. Li, T. Wiliana and K. C. Tam, *Mater. Res. Bull.*, **42**(5), 820 (2007).
13. G. Hadiko, Y. S. Han, M. Fuji and M. Takahashi, *Mater. Lett.*, **59**(19), 25192 (2005).
14. Y. S. Han, G. Hadiko, M. Fuji and M. Takahashi, *J. Cryst. Growth*, **289**(1), 269 (2006).
15. D. Zhao, T. Zhu, F. Li, Q. Ruan, S. Zhang, L. Zhang and D. Xu, *Mater. Res. Bull.*, **45**(1), 80 (2010).
16. J. Aizenberg, S. Albeck, S. Weiner and L. Addadi, *J. Cryst. Growth*, **142**(1), 156 (1994).
17. H. Wentao and F. Qingling, *Mater. Sci. Eng.*, **26**(4), 644. (2006).
18. G. E. Henderson and K. M. McGrath, *J. Cryst. Growth*, **244**(3), 369 (2002).
19. Y. Hu, Y. Ma, Y. Zhou, F. Nie, X. Duan and C. Pei, *J. Cryst. Growth*, **321**(10), 1741 (2010).
20. G. Frenandez and E. Castro, *J. Food Eng.*, **92**(1), 112 (2009).
21. P. S. Lukeman, W. B. Sherman, C. Micheel, A. P. Alivisatos and N. C. Seeman, *Biophys. J.*, **95**(1), 3340 (2008).
22. J. H. Kim, J. M. Kim, W. S. Kim and I. H. Kim, *Korean Chem. Eng. Res.*, **47**(2), 213 (2009).
23. J. H. Kim, S. M. Song, J. M. Kim, W. S. Kim and I. H. Kim, *Korean J. Chem. Eng.*, **27**(5), 1535 (2010).