The effect of casein addition in carbonate apatite mineral crystallization

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Abstract: Milk protein has been considered as one of the influenced factors in bone mineral crystallization. Bone mineral is mainly formed by carbonate apatite in which the crystallization process depends on the two main processes, nucleation and crystal growth. This study showed the formation of carbonate apatite by using precipitation method and observed the influence of casein, milk protein, in carbonate apatite nucleation through synthesis and crystal growth through immersion in SBF. The result showed that carbonate apatite type B was formed by using precipitation method with chicken eggshells as calcium precursor. The addition of casein concentration could promote the apatite nucleation and crystal growth with low casein concentration (≤ 7.5 g.l^{-1}) while high casein concentration addition plays role as an inhibitor.

1. Introduction

Generally, human body consists of soft tissue and hard tissue in which hard tissue plays important role in protecting vital organs inside. Bone is one of classified as hard tissue, composed of metabolically active cells, integrated into a rigid framework.[1] In bone growth, mineral in form of calcium is required regarding to the major component of bone. Calcium mineral can be sourced from foods and milks. In its growth, calcium could bind with some functional group such as carbonate, hydroxyl and phosphate most. Calcium (Ca) and phosphorus (P) are two milk micronutrients obtaining calcium phosphate that play important roles in bone structure as well as bone mass and strength.[2]

Calcium phosphate is performed in two phases; amorphous and crystal phase. The most stable calcium phosphate crystal is hydroxyapatite (HA). Hydroxyapatite belongs to the apatite family with chemical formula $Ca_{10}(PO_4)_6(OH)_2$. HA structure is very hospitable in allowing the substitutions of many other ions where carbonate (CO_3) can substitute either for the hydroxyl (OH) or the phosphate (PO_4) group and designated as Type A or Type B apatite respectively.[3]

The formation of calcium phosphate obtained from crystallization process. Crystallization is the formation of a solid particle (crystal) from fluid phase in which the crystals formed are structured with a repetitive arrangement of atoms, ions or molecules. Crystal formation consists of two stages: nucleation and crystal growth. Nucleation is the formation of a new solid phase (nuclei), homo or heterogeneously. One of the important factors in nucleation and crystal growth is supersaturation condition to get desirable crystal size distribution.[4] Despite of that, specific interactions between mineral crystals and organic and/or inorganic molecules also controls nucleation and crystal growth.[5]

This research aims to synthesize carbonate apatite by precipitation method with addition of casein. Casein with various concentration was added to observe the effect on crystallization process both nucleation and crystal growth.

2. Materials and methods

Carbonate apatite was prepared from analytical grade calcium chloride dehydrate ($CaCl_2.2H_2O$, Merck), di-ammonium hydrogen phosphate (($NH)_2HPO_4.2H_2O$, Merck), and sodium hydrogen carbonate ($NaHCO_3$, Merck). Meanwhile, casein was added in carbonate apatite synthesis by using sodium alginate as a template. Freeze dryer was used to obtain carbonate apatite/casein powder.

2.1. Synthesis of carbonate apatite/casein

Carbonate apatite was synthesized by using precipitation method with molar ratio 1.2 M : 0.72 M : 0.36 M of [Ca] : [P] : [CO_3]. Calcium chloride, di-ammonium hydrogen phosphate and sodium hydrogen carbonate was each dispersed in 100 ml distilled water. Then, calcium chloride dehydrate solution was dropped wisely to the mixture of di-ammonium hydrogen phosphate and sodium hydrogen carbonate solution at controlled rate under 300 rpm stirring. The precipitation undergo at pH 9 by adding sodium hydroxide. After completion of precipitation, casein was added ex-situ with various concentrations (0 g.l^{-1}, 2.5 g.l^{-1}, 5 g.l^{-1}, 7.5 g.l^{-1}, 10 g.l^{-1}, and 12.5 g.l^{-1}). Sodium alginate as a template was added into carbonate apatite/casein solution, aging overnight and then dried by using freeze dryer.

2.2. Characterization and analysis

Carbonate apatite/casein powder was characterized by using x-ray diffraction (SHIMADZU XRD700, λ Cu-Kα = 1.54060 Å) to observe the phase formed with 2θ = 25° − 40°. The functional group of obtained carbonate apatite/casein was analyzed by using fourier transform infrared (FTIR Bruker) with ranging wavenumber of 400 – 4000 cm^{-1}. Then, sample morphology and atomic percentage was analyzed by using scanning electron microscope-energy dispersive x-ray spectroscopy (SEM-EDS) with 10.000x magnification.

3. Results and discussion

Carbonate apatite was resulted by precipitation method with various concentration of casein ex situ-addition. Synthesis of carbonate apatite was conducted under supersaturation condition through homogeneous nucleation (spontaneous) and heterogeneous nucleation (induced by foreign particles) to promote the crystal growth.[5] The result of this method was powder with precipitate mass shown in Figure 1. The graph showed the effect of casein addition in precipitate mass obtained. It showed that higher casein concentration addition tended to be more precipitate mass obtained. Initially, the obtained mass without casein addition is 12.32 g then it is increasing with casein concentration, reaching maximum at 7.5 g.l^{-1} casein addition, and subsequently decreasing by casein addition > 7.5 g.l^{-1}. In short, as seen in graph, casein plays role as impurity, any substance other than the material being crystallized[6] that could effect on calcium carbonate nucleation in which casein addition with concentration ≤ 7.5 g.l^{-1} could be a promotor for carbonate apatite nucleation but above that concentration showed the inhibitor role.

3.1 X-Ray diffraction analysis

XRD pattern in Figure 2 showed the formation of carbonate apatite (CHA) either type A or type B for three samples regarding to JCPDS database No 35-180 (Type A-CHA) and 19-272 (Type B-CHA). The three samples (sample with casein concentration of 0 g.l^{-1}, 7.5 g.l^{-1}, and 12.5 g.l^{-1}) were selected based on the precipitate mass obtained in order to observe the phase formed along casein addition. In this study, casein plays role as impurity added in carbonate apatite synthesis. The presence of impurities in the sample could affect the rate of nucleation of a solution either as a promotor or inhibitor.[5] As mention above, casein acts as a promotor for crystal growth, clearly shown by the majority phase of type B-carbonate apatite below 7.5 g.l^{-1} casein concentration. In contrary, the higher casein concentration added (above 7.5 g.l^{-1}) tended to form type A-carbonate apatite. It happens due to the presence of solution-crystal interface that might delay the crystal growth process of type B-carbonate apatite. This result is in accordance with previous research stated that the coated mineral surface is slowing down the ionic diffusion to the crystal surface at high concentration of BSA protein.[5]

Crystallite size for the three samples was measured by using Debye Scherrer method shown in Table 1. The result showed decreasing of crystallite size along higher concentration addition. The decreasing of crystallite size appears in

sample with higher casein concentration of 7.5 g.l^{-1} and 12.5 g.l^{-1}. It showed the effect of casein addition on the nucleation process in case of adsorption ability on the substrate. As a result, higher casein concentration tended to slowing down the ionic diffusion to the coated crystal surface and resulting in small crystallite size.[5] Nevertheless, these results are in the range of bone crystallite size reported by Tadic and Epple (2003) on hkl (002) as 21–27 nm.[7]

Table 1. Crystallite size of carbonate apatite with various casein concentration.

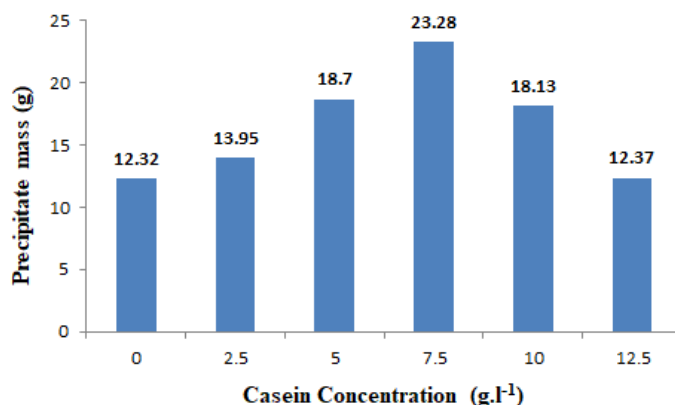| Sample	Average crystallite size (nm)
CHA-casein 0 g.l^{-1}	23.00
CHA-casein 7.5 g.l^{-1}	22.41
CHA-casein 12.5 g.l^{-1}	23.01

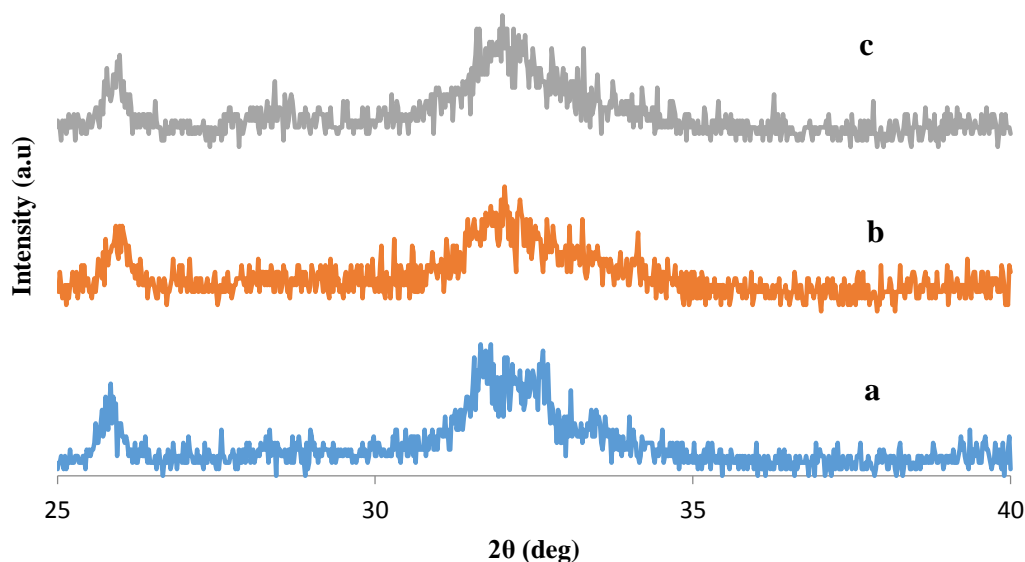**Figure 1**. Precipitation mass of carbonate apatite/casein with various concentrations.

Figure 2. X-ray diffraction pattern of carbonate apatite/casein with casein concentration (a) 0 g.l^{-1}, (b) 7.5 g.l^{-1} and (c) 12.5 g.l^{-1}.
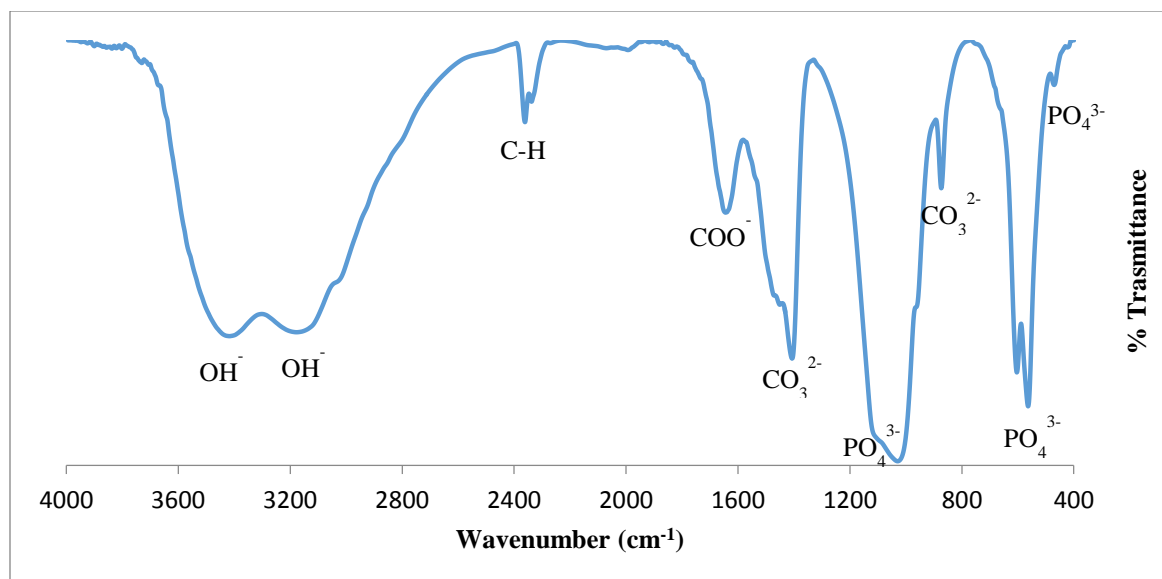
Figure 3. FTIR spectra of carbonate apatite/casein with casein concentration of 7.5 g.l^{-1}.

Table 2. Spectral region of carbonate apatite with casein concentration of 7.5 g.l^{-1}.

Functional Group	Wavenumber (cm^{-1})	
	CHA/casein 7.5 g.l^{-1}	Reference
PO_4^{3-}	470	430-460 (v_2)
	1029	1040 and 1090 (v_3)
	563	575-610 (v_4)
CO_3^{2-}	873	871 (v_2)
	1406	1410 (v_3)
OH^-	3417	3400-3700
COO^-	1644.56	1600-1700
C-H	2361.20	2850-2960

3.2 Fourier Transform Infrared (FTIR) analysis

Figure 3 showed the FTIR spectra of sample with casein concentration of 7.5 g.l^{-1}. This selection was due to the XRD results that proved the maximum casein addition as a promotor in nucleation process. The spectra showed the characteristic bands of carbonate apatite, sodium alginate and casein. The formation of carbonate apatite was obviously shown by the presence of carbonate (CO_3^{2-}), phosphate (PO_4^{3-}), and hydroxyl (OH^-) functional group. The presence of C-H group in a range of 2361.20 cm^{-1} belongs to sodium alginate.[8] Meanwhile, the characteristic band of casein was shown by the presence of amida I (COO^-) with wavenumber range of 1600-1700 cm^{-1}.[9]

The role of casein as promotor in carbonate apatite nucleation was confirmed in the sample with casein addition of 7.5 g.l^{-1}. It is clearly shown by the presence of CO_3^{2-} functional group in a range of 873.45 cm-1 and 1406.52 cm^{-1}. Those two carbonate spectral bands are overlapped contributions from the A and B carbonate species. Nevertheless, previous research reported that Type B carbonate has characteristics bands at about 1455, 1410 and 871 cm^{-1}.[10,11] Detailed spectral region of carbonate apatite/casein shown in Table 2.
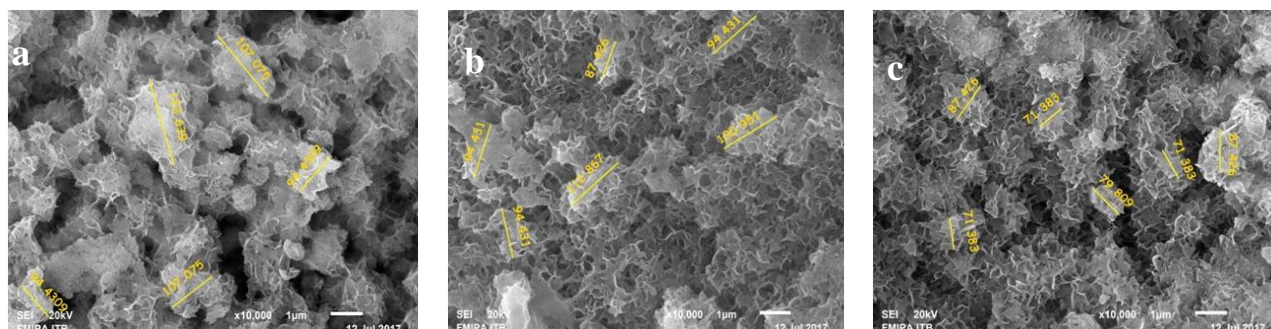
Figure 4. SEM micrograph of carbonate apatite/casein with casein concentration of (a) 0 g.l^{-1}, (b) 7.5 g.l^{-1} and (c) 12.5 g.l^{-1} with 10.000x magnification.

Table 3. Particle size of carbonate apatite with various casein concentrations.

Spot	Average particle size (nm)		
	CHA/casein 0 g.l^{-1}	CHA/casein 7.5 g.l^{-1}	CHA/casein 12.5 g.l^{-1}
1	107.7	100.95	87.43
2	123.64	87.43	71.38
3	94.43	94.43	79.81
4	107.7	94.43	71.38
5	94.43	94.43	71.38
	105.33±15.67	97.42±23.50	78.13±24.48

Table 4. Mineral concentration and Ca/P molar ratio of carbonate apatite/casein.

Sample	Calcium (%)	Phosphor (%)	Ca/P
CHA-casein 0 g.l^{-1}	18.05	10.58	1.32
CHA-casein 7.5 g.l^{-1}	23.56	12.96	1.41
CHA-casein 12.5 g.l^{-1}	21.74	12.95	1.30

3.3 Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy (SEM-EDS) analysis

The three samples obtained from precipitation method showed similar morphology as coral-like carbonate apatite morphology (Figure 4). The SEM micrograph showed agglomeration of coral-like morphology that is characteristic of carbonate apatite. The mean particle size of three samples was measured by using Fiji ImageJ and the result is quite different shown in Table 3. The result showed that higher casein concentration tended to decrease particle size. It emphasized the effect of casein addition in carbonate apatite nucleation that could inhibit the crystal growth indicated by smaller particle size.

The role of casein as promotor in carbonate apatite nucleation was confirmed in the sample with casein addition of 7.5 g.l^{-1}. Table 4 proves the increasing of calcium (Ca) and phosphor (P) concentration in the sample with 7.5 g.l^{-1} of casein that showed higher adsorption of calcium and phosphor ion. The presence of 75 g.l^{-1} casein showed increase of mass, high carbonate, high Ca/P, smaller crystal size, that means casein inducing precipitation of new carbonate apatite.

4. Conclusion

Casein addition has significant effect in carbonate apatite crystallization both nucleation and crystal growth. It plays dual role, as promotor and inhibitor, depends on the casein concentration added in the solution. Casein plays role as promotor with lower concentration (≤ 7.5 g.l^{-1}) and higher concentration (> 7.5 g.l^{-1}) shows the inhibitor one. It is clearly confirmed by precipitate mass obtained, XRD pattern, FTIR spectra, and SEM-EDS result. The decreasing precipitate mass obtained with higher casein concentration (> 7.5 g.l^{-1}) proved the inhibitor role in carbonate apatite nucleation as well as shown by the majority phase of B type-CHA in XRD pattern. In addition, EDS result showed the effect of casein addition as promotor in carbonate apatite nucleation with lower casein concentration (≤ 7.5 g.l^{-1}) resulting in Ca/P ratio of 1.41.

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