Synthesis of Carbonated Hydroxyapatite Derived from Snail Shells (*Pilla ampulacea*): Effect of Carbonate Precursor to the Crystallographic Properties

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**Abstract.** This study aimed to investigate the effect of the amount of carbonate in synthesis to the crystallography properties of carbonated-hydroxyapatite (CHAp) using the snail shells as the calcium source. Snail shells were cleaned, milled, and sintered at 1000°C for 4 hours to get CaO powder. CHAp synthesized by co-precipitation method. The molar ratio CO$_3^{2-}$/PO$_4^{3-}$ was varied 0/6, 2/6, 3/6, and 6/6. Meanwhile, the molar ratio of Ca/P was kept at 1.67. After synthesis, the solution was aged, centrifugally washed, dried, and sintered at 400°C for 2 h in the air atmosphere. A scanning electron microscope (SEM) was used to observe the morphology of CHAp. An energy dispersive spectroscopy (EDS) was used to determine the chemical composition of CHAp. An X-Ray Diffractometer (XRD) was conducted to analyze the crystallography of CHAp. Fourier transform infrared (FT-IR) was used to evaluate the chemical structure of the CHAp. The XRD spectra of CHAp of all variations show they have apatite phase. There is no secondary phase observed in XRD spectra. The results of FTIR and EDS supported the XRD results. The increasing of carbonate amount in synthesis decreased the crystallinity and crystallite size of CHAp. The lattice parameter $a$-axis was decreased, but the lattice parameter $c$-axis was increased as the amount of carbonate increased in the synthesis process. The increasing of carbonate amount in synthesis decreases the crystallite size of CHAp. The results demonstrate that the amount of carbonate in synthesis affects the crystallography properties of CHAp.

1. **Introduction**

The most popular biomaterial for orthopedic is hydroxyapatite (HA) due to similarity to the mineral in bone [1]. Foreign ions easily substitute to the HA structure, hence many ions and traces found in biological apatite in bone, such as Ni$^+$, Mg$^{2+}$, Fe$^{2+}$, Zn$^{2+}$, silicate, and CO$_3^{2-}$. The human bone consists of 65 wt% carbonated hydroxyapatite (CHAp). CHAp contains carbonate ranging from 4 to 8 wt% according to the age [2]. Carbonate substitution to the HA crystal structure leads to a decrease of crystallinity and a higher solubility [3]. Those effects imply CHAp more soluble than pure HA in both in vitro and in vivo studies. This property makes CHAp can fully be resorbed and replaced by new bone. CHAp found to have better osteo-conductivity as earlier osteoblastic cells differentiation than sintered pure HA [4].

Many studies were conducted to develop CHAp using various methods and precursors. Landi et al. [5] synthesized CHAp using a co-precipitation method, while Ezekiel et al. [6] using a nanoemulsion...
method. These methods were carried out at a low temperature and were expected to yield B-type CHAp, which is the carbonate ions substitute the phosphate site in the crystal structure.

Several studies found the apatite, especially HA, can be extracted or synthesized using precursors derived from natural source, namely bovine bone, fish bone and scale, spongy marine, and eggshells [7]. One of the abundant bio-wastes found in nature is the shells from the snail species. Few studies were conducted to produce pure HA from snail shells [8, 9]. *Pilla ampulacea* is one of the common snail species that was found in the wetland such as on the rice field, river bank, and mangrove. Utilization of the snail shells as the precursors can minimize the cost of production of the bio-ceramics than using high pure chemical products for all precursors.

It is well-known that the properties of synthesized-CHAp depend on the parameters during the synthesis. One of the synthesis parameters that was needed to investigate is the effect of concentrations of carbonate ions. Few investigations have been conducted to investigate the effect of carbonate ions content on the physicochemical properties of CHAp. Ezekiel *et al.* [6] investigated the variation of molar ratio carbonate ion to phosphate ions using nanoemulsion method and yield A-type CHAp as the products. Lafon *et al.* [10] studied the AB-type CHAp synthesis by variations of carbonate/phosphate ions. Baig *et al.* [3] investigated the effect of carbonate content on CHAp to the various properties. Zapanta-legeross [11] studied the effect of carbonate content percentage in CHAp crystal structure on the lattice parameter. Their finding agrees that the different carbonate content in the crystal structure leads to different physicochemical properties, *i.e.*, crystallinity, the lattice parameters, solubility, and particle size.

In this work, CHAp was synthesized using a coprecipitation method. The calcium source was extracted from snail shells (*Pilla ampulacea*). The molar ratio of CO$_3^{2-}$/PO$_4^{3-}$ was varied 0/6, 2/6, 3/6, and 6/6. The properties of CHAp namely, morphology, crystallography, and chemical composition were evaluated in this study.

2. **Materials and Method**

2.1. Preparation of CaO powder
Snail shells (*Pilla ampulacea*) were got from a traditional market in Bali, Indonesia. The shells were pounded into small pieces and then were washed with water many times to remove any organic traces. Shells were dried at 80°C in the oven for 24 h, and then were milled for 1 minute using ball mill. The shells powder then calcined at 1000°C for 4 h to get calcium oxide.

2.2. Synthesis of carbonated hydroxyapatite
The synthesis of CHAp powder was performed starting from CaO derived from snail shells as a calcium source, (NH$_4$)$_2$HPO$_4$ as phosphate source and NaHCO$_3$ as carbonate source. The reactions carried out by using a coprecipitation method adapted from Landi *et al.* [5]. In the first step, 0.020 mol of CaO was dissolved in 50 ml distilled water for 1 h. The PO$_4^{3-}$/CO$_3^{2-}$ solution was made by dissolving 0.015 diammonium phosphate in 25 ml distilled water and the pH adjusted to pH 10–11 by adding ammonium hydroxide (25%), and then NaHCO$_3$ solution was added to phosphate solutions while stirred. The PO$_4^{3-}$/CO$_3^{2-}$ solution was dropwise to calcium-containing solutions while stirred and the temperature was set to 90°C. The molar ratio of CO$_3^{2-}$/PO$_4^{3-}$ was adjusted to 0/6, 2/6, 3/6 and 6/6. The samples were named CHAp 0/6, CHAp 2/6, CHAp 3/6, and CHAp 6/6, respectively. Otherwise, the molar ratio of Ca/P was fixed to 1.667 for all solutions. The final solution was kept stirring for 24 h while the temperature kept at 90°C, and then settled for the next 24 h. The precipitated CHAp was centrifugally washed, dried at 80°C for 24 h, and then sintered for 2 h at 400°C.

2.3. Characterizations of carbonated hydroxyapatite
The morphology of CHAp was observed by SEM. An EDS and FTIR were used to analyse the chemical composition and structure of CHAp, respectively. The crystallography properties of CHAp were evaluated by powder XRD. The crystallinity of CHAp was calculated using Eq. 1 from Landi *et al.* [12].
\[ X_c = 1 - \frac{V_{112/300}}{V_{300}} \]  

(1)

\( X_c \) is the relative crystallinity, \( V_{112/300} \) is the intensity of valley between the peak of (112) and (300), and \( V_{300} \) is the intensity of the peak of (300). The lattice parameters of CHAp were calculated using Eq. 2 [13].

\[ \frac{\lambda}{2\sin \theta} = \frac{1}{\sqrt{\frac{\lambda^2}{\sin^2 \theta} + \frac{4a^2}{3} + \frac{c^2}{3}l^2}} \]  

(2)

\( \lambda \) is the wavelength of X-ray (1.54 nm), \( \theta \) is the degree of the peak, \( h, k, l \) are the Miller indices of the peak, \( a \) and \( c \) are the lattice parameter of the crystal. The crystallite size of CHAp was calculated using Scherer’s equation (Eq. 3):

\[ t = \frac{kl}{\beta \cos \theta} \]  

(3)

\( t \) is the crystallite size, \( k \) is a constant (0.94), \( \beta \) is the full width at half maximum of the peak (002), and \( \theta \) correspond to the degree of the diffraction from the plane (002) is occurred [6].

3. Results and Discussions

Figure 1. The XRD pattern of all variations of CHAp after sintering at 400°C for 2 h at (a) \( 2\theta = 20–60^\circ \), and (b) \( 2\theta = 24–37^\circ \)

Figure 1.a shows the diffraction pattern of all CHAp after sintering at 400°C are correspond to the apatite structure (PDF card number 090432). Therefore, all carbonate variations yield carbonated hydroxyapatite without secondary phases. Lafon et al. [10] reported the secondary phases such as calcium carbonate was yield at the molar ratio of \( \text{CO}_2^2/\text{PO}_4^3^- = 2 \). Figure 1.b shows the overlapping peaks at around 32° are due to the peak of the plane (211) and the peak of the plane (112). These overlapping peaks are the typical peaks of carbonated hydroxyapatite type B [5]. Figure 1.b) shows the XRD pattern of all samples at \( 2\theta = 24–37^\circ \). Obviously, the overlapping peaks at around 32° increasingly merge and are almost indistinguishable at CHAp 3/6 and CHAp 6/6. The broadening of these peaks mainly corresponds to a decreasing of crystallinity and crystallite size. This result is consistent with the
data of crystallinity and crystallite size, as summarized in Table 1 and illustrated in Figure 2.a. The crystallinity decreases from 70.71% at CHAp0/6 to 66.23% at CHAp0/6. The crystallite size decreases from 34.77 nm at CHAp 0/6 to 25.54 nm at CHAp 6/6. The increasing of carbonate ions substitute to the crystal structure results in perturbation of lattice. This perturbation leads to a lower reflection peak [6].

![Figure 2](image_url)

**Figure 2.** The relation of (a) the crystallinity and the crystallite size, and (b) the lattice parameters to the carbonate addition

The peak at around $\theta = 25.9^\circ$, which corresponds to the plane (002), shifted to a lower $\theta$ along the increasing of the carbonate addition during the synthesis. Contrary, the peak around $\theta = 33.2^\circ$, which relates to the plane (300), shifted to a higher $\theta$ as the amount of carbonate addition during synthesis was increased. The shift of these peaks strongly relates to the contractions of the lattice parameter. These contractions occur when the carbonate ions substitute to the phosphate or hydroxyl or both sites simultaneously in HA structure. The shift of peak of the plane (002) to the lower $\theta$ indicates the increasing of $c$-axis lattice parameter. Contrary, the peak of the plane (300) shifts to the higher $\theta$ means the decreasing of $a$-axis lattice parameter. These results are supported by the lattice parameter values which are summarized in Table 1 and illustrated in Figure 2.b. Table 1 shows, on the one hand, the $a$-axis lattice parameter decreases from 9.3345 Å at CHAp 0/6 to 9.2856 Å at CHAp 6/6 along the increasing of carbonate addition. On the other hand, the enhance of carbonate addition results in the enhance of $c$-axis lattice parameter from 6.848 Å at CHAp 0/6 to 6.874 Å at CHAp 6/6 along the increasing of carbonate addition. These results are the evidence of carbonate ions substituting to the phosphate ions [14]. A smaller size of the planar $\text{CO}_3^{2-}$ than the size of tetrahedral $\text{PO}_4^{3-}$ leads to the contraction of the lattice parameters [13].

**Table 1.** The crystallography properties and the weight fraction of carbonate of CHAp.

| Lattice parameter (Å) | c/a | Crystallinity (%) | Crystallite size (nm) |
|-----------------------|-----|-------------------|----------------------|
| HA *                  | 9.430 | 6.891 | 0.731 | - | - |
| CHAp 0/6              | 9.334 | 6.848 | 0.734 | 70.71 | 34.77 |
| CHAp 2/6              | 9.313 | 6.864 | 0.737 | 67.48 | 29.06 |
| CHAp 3/6              | 9.296 | 6.869 | 0.739 | 67.09 | 27.78 |
| CHAp 6/6              | 9.286 | 6.874 | 0.740 | 66.23 | 25.54 |

*PDF card number 090432

Figure 3a shows the infrared spectra of CHAp, which supports the XRD results of CHAp. Carbonated hydroxyapatite type-B strongly confirmed for all variations, thus shown by B-type $\text{CO}_3^{2-}$ substitution band at 875 cm$^{-1}$, and around 1410-1430 cm$^{-1}$, which are produced by stretching vibrations of $\text{CO}_3^{2-}$ [5].
The \( \text{CO}_3^{2-} \) band at 880 cm\(^{-1}\), which is a typical peak of A-type CHAp was not evidently found. It is expected since the low-temperature synthesize generates carbonate ions to substitute the phosphate site [5]. The typical spectral peaks of \( \text{PO}_4^{3-} \) ions for B-type CHAp were found at 575 and 1112 cm\(^{-1}\) [6]. The band at 1626 cm\(^{-1}\) and the broadband which is centred at 3609 cm\(^{-1}\) correspond to \( \text{H}_2\text{O} \) bond on lattice water and due to water absorption, respectively [6]. The area at 875 cm\(^{-1}\), which corresponds to \( \text{CO}_3^{2-} \), increases along the carbonate addition increases, as shown in Figure 3b. This result implies the enhance of carbonate during synthesis correspond to the increasing of carbonate in the CHAp crystal structures as observed in XRD results.

Figure 3. The infrared spectra of CHAp after sintering at 400\(^\circ\)C for 2 h (a) in range 4000-400 cm\(^{-1}\), and (b) the comparison of the area of the peak at around 875 cm\(^{-1}\) which corresponds to \( \text{CO}_3^{2-} \).

Figure 4a–4d shows the morphology of synthesized CHAp. Generally, the particles of CHAp agglomerated in micron sized. It seems the variation of the molar ratio of \( \text{CO}_3^{2-}/\text{PO}_4^{3-} \) has no observable effect on the morphology in the micron scale. The agglomerations were often observed during fabrication of nanoparticles. The high surface energy of nanoparticles causes interaction among nanoparticles to lower their surface energy. Thus, it leads nanoparticles to form agglomerations. The EDS spectra of CHAp generally show chemical compositions of CHAp. The number of carbon increases along with the carbonate addition during synthesis that supports the XRD and FTIR results. Interestingly, the carbon was also detected at CHAp 0/6. The carbon may come from the snail shells powder which is not perfectly converted to the CaO powder. A slight amount of Na was detected, which
is may arise from precursors. The substitution of Na atoms to the carbonated hydroxyapatite was known as a charge balancing to the carbonate ions in the crystal structure [13]. However, Na is one of the common traces that were found in biological apatite [2].

Figure 4. The morphology and EDS spectra of CHAp (a) 0/6, (b) 2/6, (c) 3/6, and (d) 6/6 after sintering at 400°C for 2 h. The scale bar = 5 μm.

4. Conclusions
B-type of carbonated hydroxyapatite successfully synthesized using calcium source from snail shells (Pilla ampulacea). There is no observable secondary phase observed. The variation of CO$_3^{2-}$/PO$_4^{3-}$ influences the crystallography properties, namely the lattice parameters and crystallite size of CHAp. The increasing of carbonate addition results in decreasing of $a$-axis lattice parameter and increasing of $c$-axis lattice parameter. The crystallinity and crystallite size decline, which are caused by the increasing of carbonate addition. Further studies were needed to evaluate the content of carbonate in CHAp using accurate tools and observe the morphology and size of particles in nanometer range as the effect of carbonate addition.

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