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Synthesis of Hydroxyapatite from Rice Fields Snail Shell (*Bellamya javanica*) through Wet Method and Pore Modification Using Chitosan

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Abstract

Hydroxyapatite (HAp) was synthesized using calcium originated from rice fields snail shell, that was reacted with phosphate obtained from diammonium hydrogen phosphate. The resulted pore was modified using chitosan. The HAp was prepared using wet method. Chitosan was used as porogen due to its bioactivity and biocompatibility properties. Sonication time in the synthesis was varied in 2, 4, and 6 hours. The longer the synthesis time, the higher the purity of the HAp. Pore modification was applied to HAp with the highest purity. Pore modifications were conducted by adding 4% and 6% chitosan. Scanning electron microscope identification showed no significant difference in pore sizes using 4% and 6% chitosan. Fourier transform infrared spectrum of HAp with 6% chitosan showed almost similar functional groups with that of the unporogenated HAp. *In vitro* test of the porogenated HAp revealed its bioactivity.

Keywords: chitosan, hydroxyapatite, *in vitro* test, rice field snail shell, wet method

1. Introduction

The rise of traffic accidents that occurred recently led to high demand for material that can repair damaged bones. Accidents can cause damage such as cracked or fractured bones. Implantation in the damaged bone is a good treatment in restoring the bone function. Bone implants in human body can use a variety of alternative synthetic material of ceramic, metal, or polymer, such as powdered apatite. Powdered apatite that is implanted into the bone must meet some medical requirements, which are bioactive, biocompatible and non-toxic, and produce a great chemical bond to the bone tissue.

Hydroxyapatite with chemical formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ is one of the examples of powdered apatite. This material can be used as an artificial bone substitute which is implanted into human body. Porous HAp is applied as an artificial bone substitute, with the goal of repair and regeneration. Although bone tissue itself shows good bone

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regeneration ability, but if severe bone damage occurs, a treatment with bone graft would be difficult¹. As for the optimization of the pore, chitosan can be used as porogen.

Chitosan is a natural polymer of natural materials which have biodegradable, non-toxic, and biocompatible properties. Chitosan is expected to increase bioactivity, biocompatibility and mechanical properties of the composite. HAp's brittle and easily broken natures are expected to be eliminated with the use of chitosan as a biopolymer. Chitosan has been widely studied in a variety of biomedical fields such as tissue engineering of bone, blood vessels, and nervous systems. It was found that chitosan is not an ideal material for tissue engineering, thus bioactive properties of chitosan need to be used for particular techniques such as polymers. To increase bioactive properties of chitosan, it is usually combined with another bioactive materials. HAp synthesis and chitosan as porogen are expected to produce porous HAp with non-toxic property to the body.

This study aimed to make porous HAp from the waste of freshwater snail shells using chitosan as a porogen. Synthesis of HAp was conducted using wet method. The addition of porogen with different compositions was expected to produce HAp with the best pore, which is larger pore size and uniform. Afterwards, the phase analysis was conducted using X-ray diffraction (XRD), morphological analysis was conducted using Scanning Electron Microscopy (SEM), and the identification of functional groups with a Fourier Transform Infra Red (FTIR) Spectrophotometer. This study was also aimed to evaluate the apatite crystal growth *in vitro* using a simulated body fluid (SBF) solution.

2. Method

Hap synthesis in this study was conducted using wet method. The steps of the study consisted of (1) the identification and preparation of shell of rice field snail, (2) determination of calcium level in the shell powder using AAS, (3) HAp synthesis and HAp pore modification with gelatin, and (4) characterization by XRD, SEM, FTIR spectrophotometer, and *in vitro* test with SBF solution.

2.1. Preparation of shell of rice field snail

Shell of rice field snail was cleaned from dirt and sticking flesh, then sun dried. Dried shell was then ground to a fine powder, and analyzed using XRD to determine CaCO_3 phase contained therein. The next step, shell powder was calcined at 1000°C for 2 hours to produce CaO compound. Furthermore, CaO compound was converted to Ca(OH)_2 by left it in contact with air (water vapor) for 1 week at room temperature. For ensuring the formation of Ca(OH)_2 , an analysis using XRD was conducted.

2.2. Synthesis of Hydroxyapatite (HAp)

Ca(OH)_2 suspension from shell of rice field snail was drop wisely added with 0.3 M $(\text{NH}_4)_2\text{HPO}_4$ solution at $40\pm 2^\circ\text{C}$ temperature for 1 hour while stirred using a magnetic stirrer. The formed mixture was then sonicated to obtain a uniform HAp particle size. Sonication time was varied from 2, 4, and 6 hours. Sonication resulting solution was decanted for 24 hours at room temperature. The precipitate was centrifuged at 4500 rpm for 15 minutes and then rinsed with deionized water. The precipitate was then dried at 100°C for 3 hours. The dried precipitate was finely ground in a mortar, then put in a furnace at 900°C for 2 hours. The formed HAp powder was allowed to cool at room temperature. Hydroxyapatite obtained from three variations of sonication were tested and the best results were determined.

2.3. Hydroxyapatite Pore Modification Using Chitosan

This synthesis is stages were similar to HAp synthesis, but 4% chitosan (4 g) and 6% (6 g) chitosan in acetic acid solution were added into the mixture. The obtained HAp results were compared to HAp synthetic results obtained without chitosan addition. characterization by XRD, SEM, FTIR spectrophotometer, and *in vitro* test with SBF solution.

2.4. In Vitro Test with SBF solution

1 g sample was made into pellets, and then added in 100 mL SBF solution. Soaking was conducted at a predetermined time, which were 6 and 20 days. SBF solution resulted from soaking was pipetted 20 mL and then filtered with Whatman filter paper No. 40, and the filtrate was tested using AAS.

3. Results

3.1. Identification and preparation of freshwater snail shells

Freshwater snail is a freshwater mollusk whose flesh is popularly used as protein-and-minerals-rich food in various countries around the world including Indonesia. Freshwater snail shell is a waste of the flesh and yet has significant commercial utilization. This waste is rich in various minerals including calcium². Freshwater snail shells are used as raw materials in this study because it has a high calcium content. In addition to calcium, there are other phases contained in freshwater snail shells which can be seen on X-rays diffractogram prior to calcination process.

X-ray diffractogram of freshwater snail shells before being calcined in Figure 1 shows that freshwater snail shells contained CaCO_3 and $\text{Ca}_3(\text{PO}_4)_2$ phases. CaCO_3 phase with high intensity values contained in 2θ : 26.25° , 33.16° , and 45.88° values. This 2θ value matched JCPDS data for CaCO_3 , such as 26.213° , 33.128° , and 45.853° . X-ray diffractogram of freshwater snail shells above showed that CaCO_3 phase was the dominant component. Calcium contained in the shells of molluscs are generally existed in calcium carbonate form (Soido et al., 2009)³.

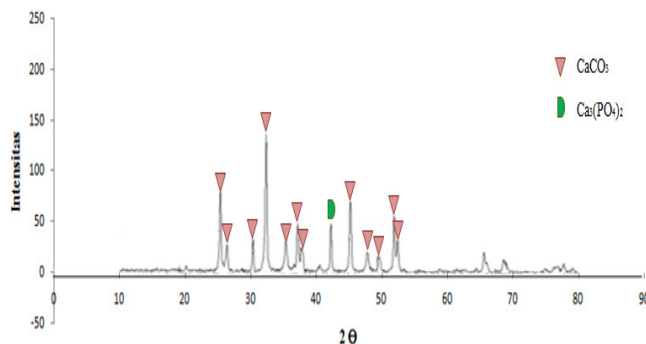
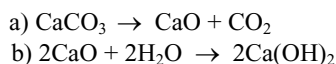


Fig. 1. Diffractogram of rice field snail shell before calcination

Calcium carbonate contained in the freshwater snail shells powder was converted into $\text{Ca}(\text{OH})_2$ through calcination and hydration processes for subsequently used as a source of calcium for HAp synthesis in this study. Calcination process was conducted to eliminate CO_2 gas and organic components of CaCO_3 (Adak and Purohit, 2011)⁴. The formed CaO compound was then converted into $\text{Ca}(\text{OH})_2$ through hydration process by letting CaO in contact with air by putting it in a humid place for a week. Here is calcination (a) and hydration (b) reactions that occurs:



Calcium carbonate that has been through calcination and hydration processes was then analyzed by X-ray diffraction for the ascertainment of $\text{Ca}(\text{OH})_2$ formation. After the calcination process at 1000°C for 2 hours and hydration for one week, the generated X-ray diffractogram (Figure 2) shows the formation of $\text{Ca}(\text{OH})_2$ phase and several other phases such as CaCO_3 dan $\text{Ca}_3(\text{PO}_4)_2$.

Calcium levels contained in the freshwater snail shells powder was measured using AAS. The analysis showed that the calcium levels contained in the freshwater snail shells powder was 88.54% and was an aragonite calcium .

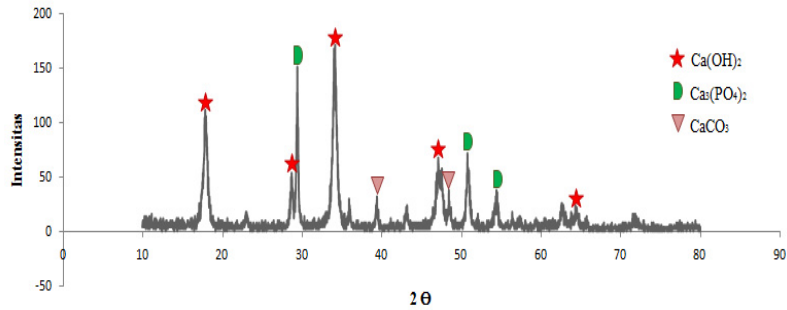
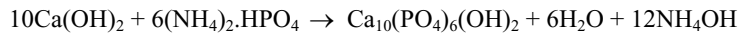


Fig. 2. Diffractogram of calcination and hydration results of rice field snail shell

3.2. HAp Synthesis

HAp synthesis in this study was conducted by the wet method. Use of the wet method is based on the availability of equipments that can be used, relatively low cost, and able to produce HAp with a fairly high degree of purity and produce water as a synthesis by-product⁷. HAp synthesis was conducted by mixing solution on 0.3 M $(\text{NH}_4)_2\text{HPO}_4$ suspension as prepared in the previous method at 40 ± 2 °C, pH was monitored but not corrected. Concentration ratio used was based on one HAp formation indicators, namely the ratio of Ca/P of 1.67. The reaction is as follows:



Sonication aimed to homogenize calcium mixture with phosphate. There are three variations of sonication time on HAp synthesis: 2, 4, and 6 hours (Figure 3). Time variation was conducted to see the best HAp synthesis results. Furthermore, centrifugation was conducted to separate the sediment.

Based on X-ray diffractogram in Figure 3, it shows that HAp was formed. X-ray diffractogram showed that the longer sonication time was, the resulting HAp would be more pure. X-ray diffractogram of synthesized hydroxyapatite with 2 and 4-hours sonication time showed that HAp phase was dominant, but there were other phases contained in the samples, such as CaCO_3 , CaO, $\text{Ca}(\text{OH})_2$, carbonate apatite (AKA) type A with $\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$ molecular formula and carbonate apatite (IMR) type B with $\text{Ca}_{10}(\text{PO}_4)_3(\text{CO}_3)_3(\text{OH})_2$ molecular formula, whereas this existance of another phase is not harmful to the body. Synthesized hydroxyapatite with 6-hours sonication time was able to obtain HAp with a fairly high purity, although AKB phase was still found. Therefore, this 6-hours sonication time was used to produce HAp which its pore will be modified using porogen.

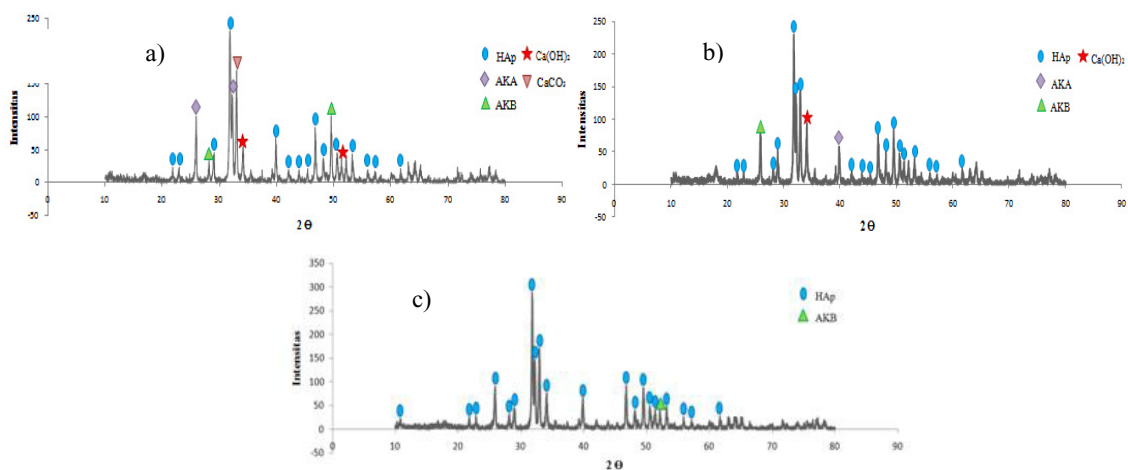


Fig.. 3 Diffraction of synthetic HAp with sonication time of 2 hours (a), 4 hours (b), and 6 hours (c)

3.3. HAp Synthesis with Chitosan Porogen

Hydroxyapatite which is the main component of bone can be synthesized with mixing chitosan that serves as a porogen for pore modification. Porous hydroxyapatite has been used for artificial bone replacement. Its main use is to repair and regrowth the lost, damaged or changed tissues¹. Modification of porous HAp is conducted to enlarge and increase the number of pores on HAp. The use of chitosan as a porogen is due to its characteristic as an easily obtained natural polymer, biocompatible, bioactive, and safe when applied to humans. The addition of a chitosan porogen was conducted with 4% and 6% concentrations. The figure 4, below shows the X-ray diffraction of HAp with chitosan porogen and X-ray diffraction of chitosan.

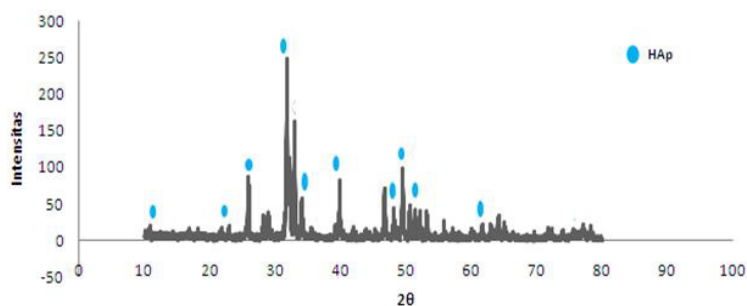


Fig. 4. Diffraction of 6% HAp-chitosan with 6-hour sonication time

X-ray diffraction of HAp with 6% chitosan porogen (Figure 4) shows that all peak angles is 2θ , both the low intensity or high intensity have complied with the JCPDS data. That diffraction only showed the formed HAp phase. This suggested that the addition of chitosan did not make any changes to HAp X-ray diffraction. This also means that the addition of chitosan did not cause structural form changes of HAp. Chitosan from composites has been lost when calcined at 1000 °C. Organic compounds would be degraded during heating on temperatures above 600 °C. Figure 5 shows chitosan X-ray diffraction which proves that on the diffraction of HAp with 6% chitosan porogen did not form X-ray diffraction of chitosan that have 2θ valued 20.362° and 10.527°

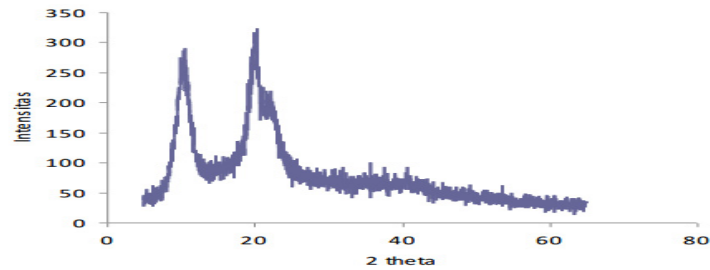


Fig.. 5 `Diffractogram of chitosan

Table 1. Particle size and lattice parameters of synthetic HAp and HAp-chitosan

| Sample | Particle size (nm) | Lattice parameter | |
|-----------------------------------|--------------------|-------------------|-------|
| | | a (Å) | c (Å) |
| HAp synthetic (6-hour sonication) | 41.959 | 9.413 | 6.883 |
| HAp-chitosan 6% | 37.606 | 9.473 | 6.922 |

The crystal structure of HAp is hexagonal. HAp lattice parameter values $a=b\neq c$. The calculation results of the lattice parameters shows both HAp with and without porogen has values that approaching a lattice parameter values as mentioned in the literature, namely the JCPDS values of the lattice parameters of a and c for HAp which are, respectively 9418 Å and 6884 Å. Hydroxyapatite added with 6% chitosan has smaller crystal size compared to HAp without porogen. The best results of SEM analysis will be further analyzed using XRD and FTIR. Identification of functional groups showed that overall FTIR spectra of HAp with 6% chitosan porogen resembled HAp without porogen. This result strengthened the previous XRD characterization results, which showed that chitosan addition only have little influence.

Figure 6 shows the infrared spectra of HAp with 6% chitosan porogen which contained OH bond on vibrational waves of 3571 and 632 cm^{-1} , while synthesized HAp showed OH bond on the vibrational waves of 3571 and 632 cm^{-1} . In this study, the phosphate groups bound on HAp with 6% chitosan porogen appeared at wavenumbers of 571, 602 and 962 cm^{-1} , whereas HAp without porogen can be seen at 570, 602 and 962 cm^{-1} (Figure 6). According to Pattanayak et al. (2005)⁶, a phosphate group bond with highest intensity contained at 1000-1100 cm^{-1} wavenumber. In this study, phosphate groups bond with the highest intensity was at the wavenumbers of 1054 cm^{-1} for HAp with 6% chitosan porogen and 1059 cm^{-1} for HAp without porogen. Compound functional group of Ca-O phase of HAp with 6% chitosan porogen and HAp without porogen did not significantly different, which was found on the vibrational waves of 1423 and 1458 cm^{-1} . According to Pattanayak et al. (2005)⁶ compound functional group of Ca-O phase is found on the vibrational waves from 1400 to 1700 cm^{-1} .

3.4. HAp Morphology

SEM analysis was conducted to determine HAp morphology with or without chitosan. The physical characteristics of porous HAp include pore size, pore morphology and pore uniformity will affect bone growth into the implant¹.

Figure 7 shows SEM results of HAp without porogen. Analysis result that conducted at 15000× magnification showed fine particles that formed aggregates with uneven size and showed a very small pore size which was around 0.09 to 0.40 μm after measurement.

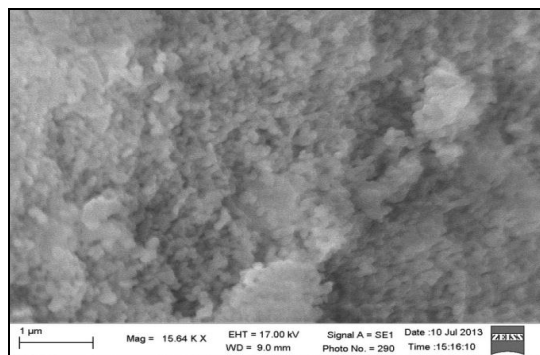


Fig. 6. SEM analysis result of synthetic HAp (6-hour sonication) at 15000× magnification

Figure 7a shows SEM photograph of HAp with 4% chitosan porogen that was performed at 15000× magnification. The formed pores were around 0.14-0.24 μm. Although the resulting pores are still too small, but according to the results of this study, there has been a change in the sample pore size by using chitosan as a porogen.

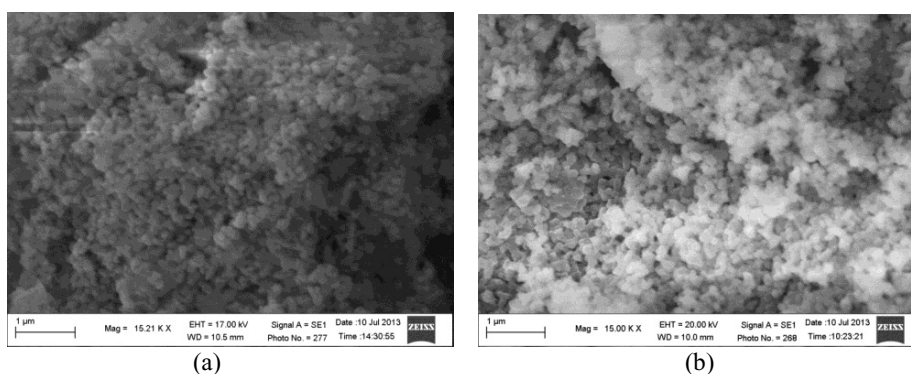


Fig. 7. SEM analysis result of synthetic HAp with 4% (a) and 6% (b) chitosan porogen at 15000× magnification

The results of SEM analysis of HAp with 6% chitosan porogen (Figure 7b) were performed at 15000× magnification showed a porous HAp structure that was slightly clear than that of HAp with 4% chitosan porogen, although the difference was not significant. The formed pores were slightly larger, which was about 0.17-0.65 μm. If the pore size is less than 10 μm, it will block the cell growth, 15-50 μm can stimulate the fibro vascular growth, 50-150 μm can produce osteoid formation and the size of more than 150 microns allows the mineralization⁷.

According to Chen et al. (2002)⁸, hydroxyapatite-chitosan composite will generate a small pore thus the pore particle size turns small as well, hence forming nano-hydroxyapatite which has a particle size of 20-30 nm in length and 50-60 nm in wide. HAp crystal size obtained in this study was equal to 37.606 nm which approached nano-hydroxyapatite size. Nano-hydroxyapatite can function as a metal coating for bone implants.

Microcrystals with a size of 190-230 μm from porous structure allows the blood vessels and the connective tissues enter in between the pores, thus can stimulate bone growth. Pores with minimum size of 100 μm required for porous implant materials in order to function properly, as it can form a new bone, because the connective tissue and blood vessel will grow in the pore in between the implant and the bone at that size. Synthesis result of HAp without porogen and with the addition of porogen in this study can not be applied for medical purpose due to its small pore size and less uniform pore distribution. HAp pores with irregular shape and size can lead to lower porosity of the resulting HAp which caused incompact HAp structure, thus if used as implants it becomes brittle or easily broken.

3.5. In Vitro Test

SBF (simulated body fluid) solution is a solution containing ions that have an approximately equal composition to human body fluids. SBF solution can be used as a medium for the growth of apatite crystals in *in vitro* test. *In vitro* test is conducted to determine the bioactive properties of HAp material which is characterized by the growth of apatite crystals. Apatite crystal growth requires calcium and phosphate ions⁹. Sample immersion in SBF solution was conducted for 20 days with calcium concentration observations conducted on day 6 and day 20. According to Sharma et al. (2009)¹⁰ the first step in apatite crystal growth is seen after seven-days immersion, because at that time period Ca^{2+} precipitation is occurred.

Calcium concentration can be identified using AAS. Calcium concentration in the initial SBF solution was 3.43 ppm. Calcium concentrations of HAp without porogen, HAp with 4% chitosan porogen and HAp with 6% chitosan porogen after immersion for 6 consecutive days were 8.78, 6.82 and 6:07 ppm, respectively. Calcium concentration of HAp without porogen, HAp with 4% chitosan porogen and HAp with 6% chitosan porogen after immersion for 20 consecutive days were 10.93, 10.88 and 12.59 ppm, respectively.

Figure 8 shows that after the immersion in SBF solution, both HAp and HAp with or without porogen would produce higher calcium concentrations than the initial SBF solution. This is caused by the chemical potential difference between samples with SBF solution. Ion exchange process occurs between samples and SBF solution, ie the sample releases Ca^{2+} ions into the SBF solution, thus Ca^{2+} ions in the SBF solution increases (Sharma et al., 2009)¹⁰. Hydroxyapatite with 6% chitosan porogen resulted higher calcium concentrations compared to HAp with 4% chitosan porogen and HAp without porogen. The analysis results showed the speed of HAp in the porous structure in improving bioactive properties. Pores presence will facilitate the ions exchange between the sample and the SBF solution. Chitosan will inhibit the release of Ca^{2+} , but because chitosan has been lost in the calcination process, then chitosan can not inhibit the release of Ca^{2+} .

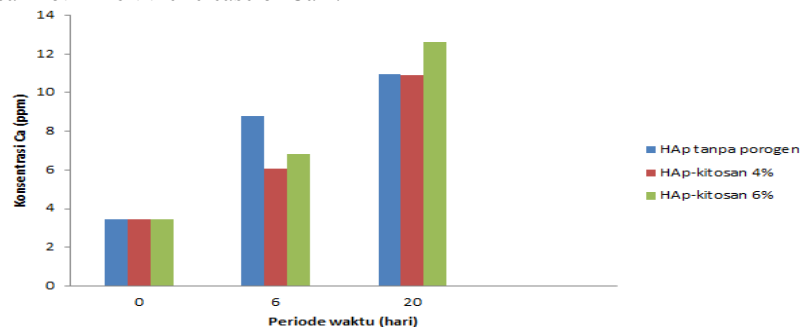


Fig. 8. Calcium concentration in SBF solution to time period of soaking

Conclusions

Hydroxyapatite synthesis with $\text{Ca}(\text{OH})_2$ freshwater snail shells powder as a raw material has been successfully conducted using the wet method. The XRD analysis results showed that HAp phase was formed in the sample with a fairly high degree of purity. SEM characterization showed the pores with the size of 0.17-0.65 μm which clearly visible on HAp with 6% chitosan porogen. The addition of chitosan could reduce particle size and obtain uniform pores. Functional groups identification of HAp with porogen by FTIR showed that FTIR spectra of HAp with chitosan porogen resembled HAp without porogen. *In vitro* study showed that the longer the immersion time was, the release of Ca^{2+} would be increased.

Acknowledgments

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