APPLICATION OF HYDROXYAPATITE IN PROTEIN PURIFICATION

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Abstract

The precursors, $Na_2HPO_4.2H_2O$ and $CaCl_2.2H_2O$ are used for synthesizing pure hydroxyapatite which less of carbonate content. The high temperature of sintering, about 700^oC of temperature, is treated to minimize the carbonate group on hydroxyapatite surface. Carbonate content of hydroxyapatite which is sintered in 700^oC is less than 110^oC. It indicates an increasing temperature of sintering will increase crystallinity and decrease carbonate content of hydroxapatite. This method gave better way to resulting hydroxyapatite crystal. The patterns of band of SDS PAGE in resulting of Protein Purification by DEAE matrix is also appear in using hydroxyapatite matrix. The other band also appear in purification by hydroxyapatite matrix, it showed that hydroxyapatite not only has DEAE matrix characteristic as anion exchange but also has kation exchange characteristic. These patterns proof that hydroxyapatite could be used in protein purification as matrix as cation an anion exchange. Additionally, apatite matrix is abiding and can be used repeatedly more than one hundred times without contamination.

Keywords: hydroxyapatite, protein purification, wet method

1. Introduction

Hydroxyapatite is one type of calcium phosphate compound which are present in bone. Calcium phosphate is a mineral which consists of both calcium ions (Ca^{2+}) and orthophosphates (PO_4^{3-}), metaphosphates or pyrophosphates ($P_2O_7^{4-}$) and occasionally hydrogen or hydroxide ions. Calcium hydroxyapatite (CaHAp) has an important role in development of medical sciences as a basic components of hard tissues of bone and dental [1]. The applications of hydroxyapatite are not only limited as frames or motion devices but so many others, one of them is as a filter in protein purification to acquire a certain specific protein from protein complex.

The objectives of this research are analyzing the ingredient of carbonat in apatite composite on sintering variation and the effectiveness of hydroxyapatite as a matrix in protein purification. This analysis will inform what properties that apatite had if standed as a filter and how apatite can absorb the certain protein and pass through a specific protein.

2. Methods

The research was started by mixing up an apatite crystal which was formed in several steps. Apatite crystal was grown by dissolving compounds of calcium phosphate. The compounds came from precursors materials, they were Na_2HPO_4 and $CaCl_2$. Its Ca/P molar ratio is 1.67 [1]. The mass of precursors can be defined by calculation of molecule weight of $Na_2HPO_4$and $CaCl_2$. They are 177,99 gram/mol, 147,02 gram/mol, respectively [1]. 7.42 gram HAp was come from 8.94 gram Na_2HPO_4 and 12.33 gram $CaCl_2$. Amount of precursors were dissolved in 50 ml aquades. Finally, those precursors moved into beaker glass and buret, respectively.

Precipitation. Precipitation was performed after two solutions which are compounds of hydroxyapatite. Nitrogen gas was flowed into beaker glass which initially contained Na_2HPO_4 50 ml in order to avoid chemical reaction between solution and CO_2 which will result in carbonate content. It was variated in 5 and 10 minute. All parts of this precipitation set up were isolated and controlled under pysiologic condition, at temperature 80^oC, pH 7.4 and also stirred during precipitation process. It spent 5 hours to get precipitation result by setting a slowly current of streaming $CaCl_2$ solution. NH_3 ws used to control pH. As pH increased, NH_3 was dropped into the solution. In controlling the homogeneity of solution, stirring was held during 5 hours experiment, while precipitation has finished and temperature decreased into room temperature. The sample was left for about 24 hours, then rinsed by aquades triplo and dried in the furnace at

temperature 110^{o}C for 10 hours. Finally, the sample was made as powder by mortar. Then, HAp sample which was made in 5 minutes nitrogen streaming was sintered in 700^{o}C as annealing variation. Results of annealing is characterized by FTIR (Fourier Transform Infra Red Spectroscopy) and XRD (X-Ray Diffraction) characterization.

Protein Purification. Previously, soy bean extraction is needed as a sample of protein. It is necessary to pack column for removal of fines in protein purification. By preparing the slurry in desired buffer, mix, and allow settling for approximately 5 minutes or enough time that the beads have settled. Decant off the suspension of fine particles and add phosphate buffer and re-mix. Repeat the process until particles settle within approximately 5 minutes and leave a clear supernatant. Since matrix is formed, set the position of second

Table 1. Band Absorption of Carbonate and Phosphate on CaHAp as Nitrogen gas Treatment Variation

Sample code	Temperature (^oC)	Time of N_2 (minutes)	Phospate absorption band (cm^{-1})			Carbonate absorption band (cm^{-1})	
			v_1	v_3	v_4	v_2	v_3
HAp-1	110	5	961	1031	564	874	1402
HAp-2	110	10	961	1030	563	856	1402

Table 2. Band Absorption of Carbonate and Phosphate on CaHAp as Sintering Temperature Treatment Variation

Sample code	Temperature (^oC)	Time of N_2 (minutes)	Phospate absorption band (cm^{-1})			Carbonate absorption band (cm^{-1})	
			v_1	v_3	v_4	v_2	v_3
HAp-1	110	5	961	1031	564	874	1515
HAp-3	700	5	959	1045	569	-	1420,1456,1513,1540

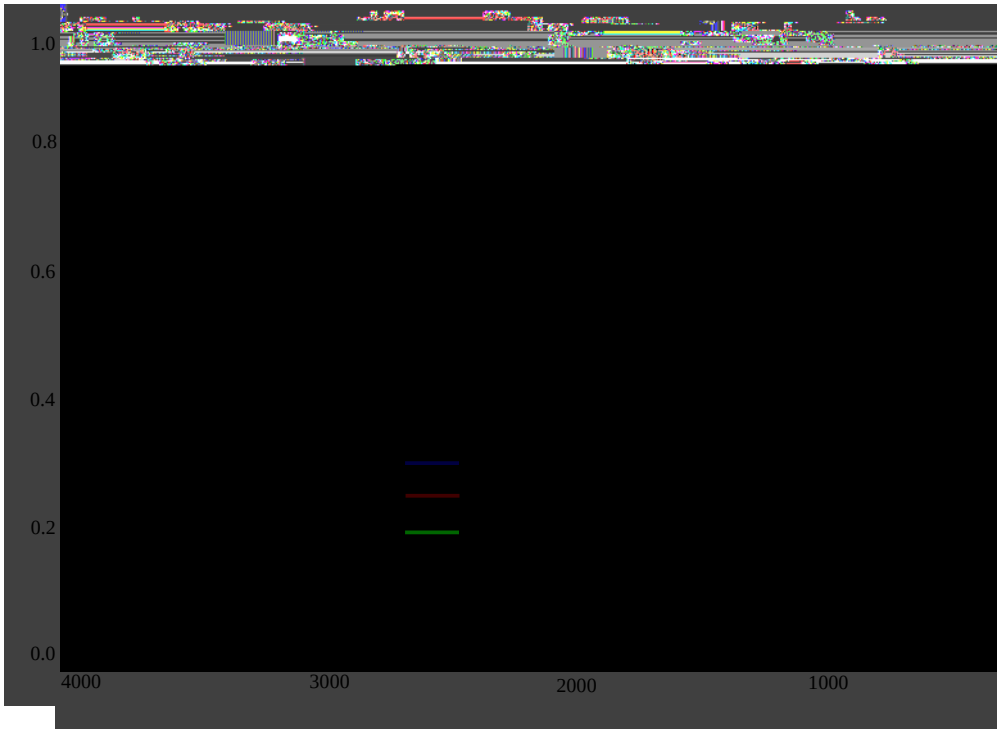

Figure 1. Collation of IR Transmitance Spectra of (a) CaHAp is Streamed by 5 Minutes Nitrogen Gas at 110^{o}C of Sintering; (b) CaHAp is Streamed by 10 Minutes Nitrogen Gas at 110^{o}C of Sintering; (c) CaHAp is Streamed by 5 Minutes Nitrogen Gas at 700^{o}C of Sintering

hydroxyapatite to engage negatively charged ion (electron) from the other atom and then, utilized as an electronegativity. The electronegativity of calcium is 1.00.

Hydroxyapatite samples have a strong band at 1031 cm^{-1} that is assignable to vibration of PO_4^{3-}. H_2O observed in FTIR spectra by bands at 3431-2850 cm^{-1}. The band at around 3430 cm^{-1}, which is due to adsorbed water overlaps with the weak band at around 3600 cm^{-1} which is due to the O-H stretching vibrations of the surface P-OH groups. Further several bands appear around 1520 cm^{-1} assigne to various C-O stretching vibration modes of CO_3^{2-} ions [4]. Various temperature treatment is given to hydroxyapatite sample which is streamed by 5 minutes nitrogen gas because of its CO_3^{2-} content that mentioned the vibration mode in OH^- stretching bend was less than the other one. It was the reason of using HAp-1 in sintering treatment. Carbonate ions can disturb the precipitation

and impede the growth of apatite crystal [5]. The presence of CO_3^{2-} on hydroxyapatite structure influences the decomposition, sinterability, solubility and biological reactivity of calcium hydroxyaptite implantation materials [6]. The carbonate content is assigned at arround 870 and 1400 cm^{-1}. HAp-3 sample came from the HAp-1 sample that was sintered in 700^oC which is used to decrease the carbonate content on sample [4].

The heating of hydroxyapatite which has carbonate content commonly causes a decrease in carbonate number. Most of it is prone to decomposed into elements or smaller compounds such as metal oxidation and carbondioxide. The temperature that needed to remove carbonate is depend on how strong polarization CO_3^{2-} ions has. Positively charged ion has high density of charge and big distortion effect to negatively charged ion. It gives a little polarization effect so that the low temperature is needed to remove carbonate, while high temperature is needed by more positively charged ions. Hydroxyapatite has two positively charge ions on calcium.

The FTIR spectra of HAp-3 sample exhibit a significant value of change of carbonate content than HAp-1's. The reduction of carbonate content as an impact of sintering treatment was showed by dissapearing some peaks around at 870 cm^{-1}, 1402 cm^{-1}, 2250 cm^{-1} and reducing the peak absorbance of carbonate content around at 1456 cm^{-1}, while CO_3^{2-} ions which is subtitued at hydroxyl site around 1515 cm^{-1} still appear in less content than before. The peak height of absorbance of HAp-1 and HAp-3 at around 1456 cm^{-1} are 0.10 and 0.08. The hydroxyl group around at 3400 cm^{-1} also decrease as consequent of sintering treatment from 0.35 to 0.09 of absorbance rate. Phosphate bands around at 420, 470, 960 and 1035 cm^{-1} are not influenced by this sintering treatment [7].

Carbonate is such an impurity of hydroxyapatite that was originated by CO_2 adsorption or starting material. This research used Na_2HPO_4 and $CaCl_2$ as starting mat722 Tc56 T75.4u15(t722 Tc555 Tw[(o22 Tc( )-6.4(s1.42 7dw[(o0.3601 Tm-t0 Tw(dro)4.80 0 9.427 167.46002 Tw[(aT*Hii2d

The hydroxyapatite structure relative disposed to be more crystallized by increasing temperature treatment until the certain value of cut off which will conclude as poorly crystallized or amorphous for up to that certain value. The XRD pattern of hydroxyapatite which is experimented by Hidekazu in up to 900^{o}C showed no diffraction peaks characteristic of calcium phosphate which might be because this material is poorly crystallized or amorphous. On the other hand, some journals said that in 900^{o}C of treatment will increase the crystality of hydroxyapatite structure. This thermal behaviour of hydroxyapatite at higher temperature is very composition dependent. So that, the samples that made by different precipitation process will also require a temperature characteristic itself.

Temperature was defined as a measure of the vigor of motion, as kinetic energy, of the molecular particles in a substance. Increasing of temperature of system results in a greater kinetic energy because of the increased movement of the molecules. Consequently, at higher temperature there are more effective collisions and less of mean free path, so that chemical reaction being more perfectly conducted [15].

This study did not give XRD pattern of HAp-3 which has been treat by 700^{o}C because the amount of the sample is not enough to characterize, so that the phase that had by HAp-3 is in assumption that those peaks correspond to HAp-1 emphasize by adding the others attributes as temperature effects. The residu of sample that have been treatment is used to as a matrix in protein purification which is explained below.

Figure 2. Sample of HAp1

Figure 3. Sample of HAp2

Protein Purification Analysis. The purification in this research used open column chromatography by hydroxyapatite and DEAE matrix as a control. The ceramic hydroxyapatite columns were very high resolution that was obtained on analysis of protein [16].

Ceramic hydroxyapatite (CHT), $Ca_{10}(PO_4)_6(OH)_2$ is a sintered form of calcium phosphate that can be used to

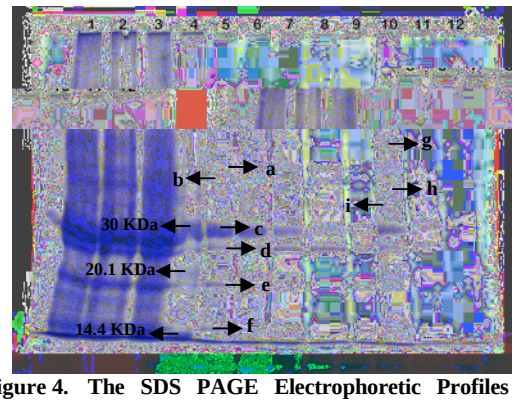

Figure 4. The SDS PAGE Electrophoretic Profiles of Purified Soybean that is Fractionationed on Hydroxyapatite and DEAE (anion exchange) Column. Crude extract of soybean (line1), unbound proteins of Hydroxyapatite column (line2), unbound proteins of DEAE column (line3), marker LMW (Low Molecular Weight) (line 4), elution of hydroxyapatite column in 0.3 M, 0.6 M, 0.8 M, and 1 M (line 5, 6, 7, 8), elution of DEAE column in 0.3 M, 0.6 M, 0.8 M and 1 M (line 9, 10, 11, 12)

carboxyls. This has been proven experimentally by evaluating the retention of proteins on which the carboxyls have been replaced by sulfo groups [19]. Further proof that carboxyl/C-site binding does not reflect a classical anion exchange interaction is found in the fact that binding capacity diminishes for acidic proteins with increasing pH [18-19, 22-23]. Phosphoryl groups on proteins and other solutes interact even more strongly with C-sites than do carboxyls [21]. This is reflected in extremely strong binding by phosphoproteins [24].

The outcome of hydroxyapatite can be compared to that of Ion Exchange Chromatography (this research used DEAE as control on anion exchange) should be regarded as a chromatographic joker sometimes offering surprisingly good results and excellent separation even between very closely related protein derivatives[7]. The adsorption depends on pH and ionic strength. The elution behaviour is a function of the isoelectric point of the protein leading to three different classes, (1) Basic proteins, which elute at similar, moderate concentrations of phosphate, fluoride, chloride or thiocyanate (0.1-0-3 M). Alternative low concentrations (<0.003 M) of Ca2+ or Mg2+ are used to elute the protein (2) Acidic proteins, which elute at about equal moderate concentrations of phosphate and fluoride but do not elute with Ca2+ and usually not with chloride (3) Neutral proteins, which elute with phosphate, fluoride and chloride and which do not elute with Ca^{2+} or thiocyanate [25].

Crude extract fraction is thicker than elution fractions of hydroxyapatite, it describes that the purification is presence in hydroxyapatite matrix. The thicker bands of protein in crude extract shows there were a lot of proteins which have characterization that could not be binded in hydroxyapatite. Soybean products is differentiated qualitatively for patterns of soluble proteins were evidence by SDS PAGE. Molecular mass of β-conglycinin subunits ranged from 42 to 85 kDa; acidic and basic subunits ranged from 60 to 85 and 42 to 53 kDa, respectively. Glycinin polypeptides were in the range of 20 to 42 kDa, and acidic and basic polypeptides ranged from 30 to 42 kDa and from 20 to 25 kDa, respectively. Lectin subunits (25 to 30 kDa) were expected to appear between the acidic and basic polypeptides of glycinin. α-Conglycinin (19 to 22 kDa) [26]. Ferritins subunits were in the ranges of 28 and 26.5 kDa [27].

According to SDS (Figure 4), there were not a discrepancy that could be provoked further investigation. The patterns of band in elution fraction of hydroxyapatite is identical with DEAE's because 0.3, 0.6, 0.8, 1 M phosphate buffer chloride which were used to elute the protein is a solution that could outcome basic proteins (amino groups) that were attracted to P-sites in hydroxyapatite. It can be seeen in Figure 4, a (44.78 KDa) refers to β-conglycinin subunits (basic protein) that have ranged from 42-53 KDa [26], c (28.49 KDa) refers to ferritins that in range of 28 KDa [22], fortunately both ferritins in 28 and about 26 KDa appeared in this research, e (19.8 KDa) refers to α-conglycinin that have ranged from 19-22 KDa [26].

Its evidence described that hydroxyapatite could be used as a matrix that can bind proteins and also proof that hydroxyapatite has positively charged as same as DEAE which is an anion exchanger. Binding in hydroxyapatite, pattern of c (28.4 KDa) band appear in 0.3-1 M of hydroxyapatite elution, there were a decreasing of density. Content of c band in 1 M elution is less than others because the amount of its protein had been outcame in 0.3-0.8 M of elution. It showed that c band which refers to ferritin only need a low concentration of elution so that the binding ferritin in hydroxyapatite matrix is weakly. On the other hand, d band (25.25 KDa) which refers to ferritin appear in less content at 0.3 M and more out come at 1 M of elution. It seems that this second kind of ferritin which is 25.25 KDa has a stronger binding on hydroxyapatite than other one because it could be entered in high molarity of elution.

The patterns of whole of band on hydroxyapatite elution is almost similar but the quantity is different. The band of DEAE elution is unique, there are a distinction of pattern in 0.6 M of elution that appears more band, although still any similar band of ferritins. The explanation above shows that the polypeptide of

soybean in 28.4 and 25.25 KDa is appear on both hydroxyapatite and DEAE elution. It is infers that its proteins is in same of charge in such as basic protein which can only elute by sodium phosphate buffer. In the case of the absence of acidic protein in SDS it is known that basic and acidic molecules of protein are adsorbed into different crystalline surfaces of hydroxyapatite and because of only sodium phosphate buffer in the range of 0.1 to 1 M that used in this elution while an acidic proteins will not elute in sodium phosphate buffer, even at concentrations more than 3 M. Elution requires displacers with stronger affinity for C-sites, such as phosphate, citrate or fluoride ions. Basic protein bind to a negatively charged site and acidic proteins to a positively charged site it is known that basic protein has positive charge that could be binded into negatively charged site on hydroxyapatite surface and vice versa for acidic protein.

4. Conclusion

The pure hydroxyapatite in less content of carbonate is yielded from wet method preparation in high temperature of sintering that could decrease carbonate content. The sintering temperature which can liberate carbonate depends on how strong polarization of carbonate on hydroxyapatite surface has. To free carbonate on hydroxyapatite is difficult except its preparation was performed in vacuum area without CO_2. The hydroxyapatite is an effective pseudo affinity on protein purification process because the affinity of hydroxyapatite is more than other matrix which can bind a positively and negatively charged protein. Additionally, the use of hydroxyapatite as a matrix is more abiding, it can be used repeatly more than one hundred times.

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