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## APPLICATION OF HYDROXYAPATITE IN PROTEIN PURIFICATION

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### Abstract

The precursors,  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  are used for synthesizing pure hydroxyapatite which less of carbonate content. The high temperature of sintering, about  $700^\circ\text{C}$  of temperature, is treated to minimize the carbonate group on hydroxyapatite surface. Carbonate content of hydroxyapatite which is sintered in  $700^\circ\text{C}$  is less than  $110^\circ\text{C}$ . It indicates an increasing temperature of sintering will increase crystallinity and decrease carbonate content of hydroxyapatite. 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*

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### 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 ( $\text{Ca}^{2+}$ ) and orthophosphates ( $\text{PO}_4^{3-}$ ), metaphosphates or pyrophosphates ( $\text{P}_2\text{O}_7^{4-}$ ) 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  $\text{Na}_2\text{HPO}_4$  and  $\text{CaCl}_2$ . Its Ca/P molar ratio is 1.67 [1]. The mass of precursors can be defined by calculation of molecule weight of  $\text{Na}_2\text{HPO}_4$  and  $\text{CaCl}_2$ . They are 177,99 gram/mol, 147,02 gram/mol, respectively [1]. 7.42 gram HAp was come from 8.94 gram  $\text{Na}_2\text{HPO}_4$  and 12.33 gram  $\text{CaCl}_2$ . 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  $\text{Na}_2\text{HPO}_4$  50 ml in order to avoid chemical reaction between solution and  $\text{CO}_2$  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  $80^\circ\text{C}$ , pH 7.4 and also stirred during precipitation process. It spent 5 hours to get precipitation result by setting a slowly current of streaming  $\text{CaCl}_2$  solution.  $\text{NH}_3$  ws used to control pH. As pH increased,  $\text{NH}_3$  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°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°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 porous disc on top of the settled gel bed by floating it into the liquid in column and push it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc [2].

There are five steps of purification using open column chromatography, equilibration, sample application, washing, elution and re-equilibration. Equilibration is used to lose impurities that might be adhere in hydroxyapatite matrix [2].

### 3. Results and Discussion

**Carbonate ion ( $\text{CO}_3^{2-}$ ) content analysis of hydroxyapatite by FTIR Spectrum.** Calcium Hydroxyapatite was resulted by wet method preparation of precursor  $\text{Na}_2\text{HPO}_4$  and  $\text{CaCl}_2$  which was prepared in nitrogen atmosphere that given before precipitation. The concentration ratio of  $\text{CO}_3^{2-}$  and  $\text{PO}_4^{3-}$  of hydroxyapatite is 1.67 in temperature of annealing and nitrogen gas variations. The nitrogen gas was varied in 5 and 10 minutes as HAp-1 and HAp-2, respectively. The temperature of sintering of hydroxyapatite which is made by 5 minutes nitrogen gas stream, HAp-1, varied in 110°C and 700°C as HAp-1 and HAp-3, respectively. The reason in using 5 minutes nitrogen gas of hydroxyapatite in sintering treatment is because HAp-1 has less of carbonate compound than HAp-2. Condition during precipitation was held at 80°C and pH 7.4. The chemical formula for this process of hydroxyapatite wet method preparation is followed as:  $10\text{CaCl}_2 + 6\text{Na}_2\text{HPO}_4 + 2\text{H}_2\text{O} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 12\text{NaCl} + 8\text{HCl}$

FTIR characterization is used to identify bunch of chemical compound. The infrared band positions and their assignments are summarized in Table 1. The spectral data indicate that  $\text{CO}_3^{2-}$  ion is present in HAp samples which is identified by characteristic single peak of the carbonate ions around 1402 and 865  $\text{cm}^{-1}$

which is attributed to the vibrational modes as the formation  $\text{CO}_3^{2-}$  by the reaction of adsorbed  $\text{CO}_2$ . The incorporation of  $\text{CO}_2$  into CaHAp exist only as  $\text{CO}_3^{2-}$  [3]. The bands around 1515  $\text{cm}^{-1}$  can be assigned to various C-O stretching vibration modes of  $\text{CO}_3^{2-}$  ions, substituted at phosphate site (type A) because the formation  $\text{CO}_3^{2-}$  by the reaction of adsorbed  $\text{CO}_2$  with surface  $\text{OH}^-$  ions of HAp is possible at high temperature. These FTIR results consider that  $\text{CO}_2$  is incorporated into A sites by the reaction with surface  $\text{OH}^-$  ions:  $2\text{OH}^- + \text{CO}_2 \rightarrow \text{CO}_3^{2-} + \text{H}_2\text{O}$ .

The 605-419 and 961  $\text{cm}^{-1}$  bands are assignable to combination bands of  $\text{PO}_4^{3-}$  ions. The carbonate peak that was situated at 2000-2500  $\text{cm}^{-1}$  is resulted from the background carbon dioxide ( $\text{CO}_2$ ) within atmosphere [4]. The 1991, 2363  $\text{cm}^{-1}$  bands which is due to carbonate peak are detected only for the sample that obtained at 5 minutes nitrogen gas. The band of carbonate peak of HAp-1 at 1515  $\text{cm}^{-1}$  wavenumber is disappeared upon as raising nitrogen gas streaming in ten minutes but there were appear the other weak bands that consider to carbonate between 1500 until 1750  $\text{cm}^{-1}$ . This nitrogen treatment could not decrease the carbonate content of hydroxyapatite instead increasingly more as shown at 1402  $\text{cm}^{-1}$  wavenumber which has escalation from 0.25 to 0.30 of absorbance rate. The condition described above showed that by increasing nitrogen gas at hydroxyapatite sample preparation did not give a significant value of descent of carbonate content on hydroxyapatite. According to the FTIR result as nitrogen gas variation, the number of carbonate content on sample which is formed by the reaction of adsorbed  $\text{CO}_2$  as assigned at 1402 and 865  $\text{cm}^{-1}$  is not significant influenced by nitrogen gas treatment, although there was a little reducing value of carbonate which prove by reducing absorbance spectra at around 870  $\text{cm}^{-1}$  as shown in Appendix 5. The peak height of absorbance spectra of HAp-1 and HAp-2 at around 870  $\text{cm}^{-1}$  are 0.06 and 0.02, respectively. These wavenumber are assign to various C-O stretching vibration modes of  $\text{CO}_3^{2-}$ .

The mechanism of  $\text{CO}_2$  incorporation into CaHAP is markedly influenced by the solution pH during the preparation.  $\text{CO}_2$  incorporation into A sites ( $\text{OH}^-$  sites) is increased by raising the solution pH while that into B sites ( $\text{PO}_4^{3-}$  sites) is maximum at pH 5.94 and almost not detected at pH 9.42 [3]. It gives probability that the existence of  $\text{CO}_3^{2-}$  ions which is substituted into  $\text{OH}^-$  site is caused by raising pH in HAp-2 precipitation that could not be kept assuredly in remain same condition of HAp-1 precipitation although there were nitrogen gas streaming more than HAp-1.

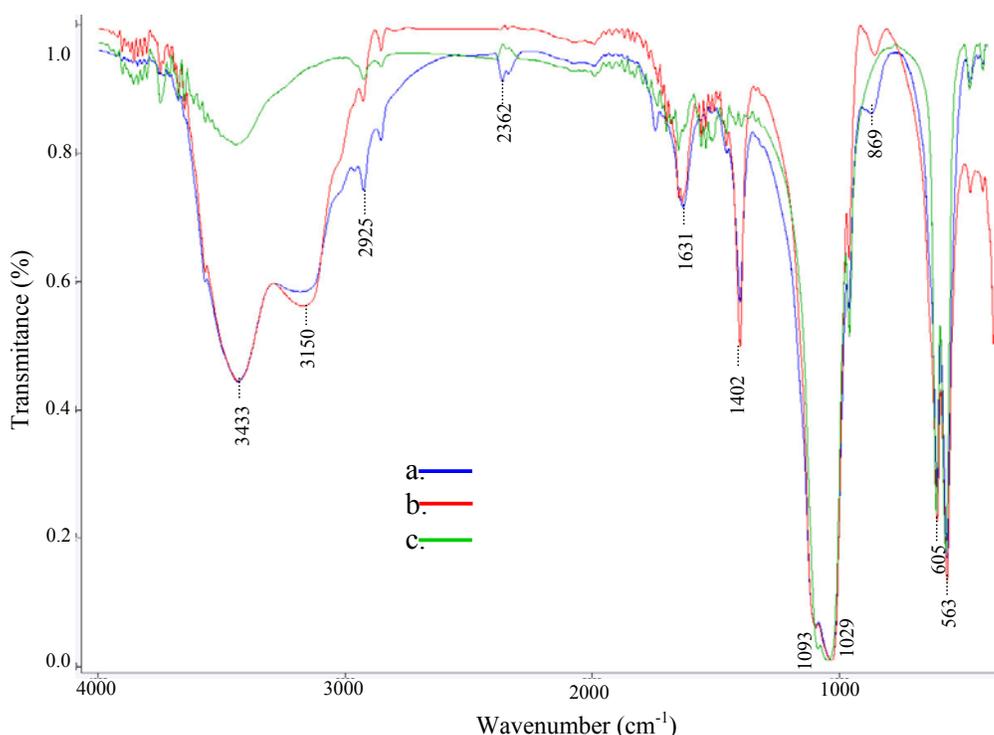
The substitution of  $\text{CO}_3^{2-}$  into  $\text{OH}^-$  and  $\text{PO}_4^{3-}$  sites as Carbonate Apatite tipe A and tipe B, respectively are influenced by tendency of calcium that is included in

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

| Sample code | Temperature (°C) | Time of N <sub>2</sub> (minutes) | Phosphate absorption band (cm <sup>-1</sup> ) |                       |                       | Carbonate absorption band (cm <sup>-1</sup> ) |                       |
|-------------|------------------|----------------------------------|---|-----------------------|-----------------------|---|-----------------------|
|             |                  |                                  | <i>v</i> <sub>1</sub>                         | <i>v</i> <sub>3</sub> | <i>v</i> <sub>4</sub> | <i>v</i> <sub>2</sub>                         | <i>v</i> <sub>3</sub> |
| 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 (°C) | Time of N <sub>2</sub> (minutes) | Phosphate absorption band (cm <sup>-1</sup> ) |                       |                       | Carbonate absorption band (cm <sup>-1</sup> ) |                       |
|-------------|------------------|----------------------------------|---|-----------------------|-----------------------|---|-----------------------|
|             |                  |                                  | <i>v</i> <sub>1</sub>                         | <i>v</i> <sub>3</sub> | <i>v</i> <sub>4</sub> | <i>v</i> <sub>2</sub>                         | <i>v</i> <sub>3</sub> |
| 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 Transmittance Spectra of (a) CaHAp is Streamed by 5 Minutes Nitrogen Gas at 110°C of Sintering; (b) CaHAp is Streamed by 10 Minutes Nitrogen Gas at 110°C of Sintering; (c) CaHAp is Streamed by 5 Minutes Nitrogen Gas at 700°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<sup>-1</sup> that is assignable to vibration of PO<sub>4</sub><sup>3-</sup>. H<sub>2</sub>O observed in FTIR spectra by bands at 3431-2850 cm<sup>-1</sup>. The band at around 3430 cm<sup>-1</sup>, which is due to adsorbed water overlaps with the weak band at around

3600 cm<sup>-1</sup> which is due to the O-H stretching vibrations of the surface P-OH groups. Further several bands appear around 1520 cm<sup>-1</sup> assigne to various C-O stretching vibration modes of CO<sub>3</sub><sup>2-</sup> ions [4]. Various temperature treatment is given to hydroxyapatite sample which is streamed by 5 minutes nitrogen gas because of its CO<sub>3</sub><sup>2-</sup> content that mentioned the vibration mode in OH<sup>-</sup> 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  $\text{CO}_3^{2-}$  on hydroxyapatite structure influences the decomposition, sinterability, solubility and biological reactivity of calcium hydroxyapatite implantation materials [6]. The carbonate content is assigned at around 870 and 1400  $\text{cm}^{-1}$ . HAp-3 sample came from the HAp-1 sample that was sintered in 700°C 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  $\text{CO}_3^{2-}$  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 disappearing some peaks around at 870  $\text{cm}^{-1}$ , 1402  $\text{cm}^{-1}$ , 2250  $\text{cm}^{-1}$  and reducing the peak absorbance of carbonate content around at 1456  $\text{cm}^{-1}$ , while  $\text{CO}_3^{2-}$  ions which is substituted at hydroxyl site around 1515  $\text{cm}^{-1}$  still appear in less content than before. The peak height of absorbance of HAp-1 and HAp-3 at around 1456  $\text{cm}^{-1}$  are 0.10 and 0.08. The hydroxyl group around at 3400  $\text{cm}^{-1}$  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  $\text{cm}^{-1}$  are not influenced by this sintering treatment [7].

Carbonate is such an impurity of hydroxyapatite that was originated by  $\text{CO}_2$  adsorption or starting material. This research used  $\text{Na}_2\text{HPO}_4$  and  $\text{CaCl}_2$  as starting material to scant a carbonate content on sample in order to get pure hydroxyapatite which would be used as a matrix in protein purification for the next step of this research. So that, if there was any carbonate content in this research's hydroxyapatite sample, it is only caused by incorporated  $\text{CO}_2$  from the air during precipitation process that can not be avoided perfectly.

In order to obtain CaHAP samples free of  $\text{CO}_3^{2-}$  ions we must synthesize them in an atmosphere without  $\text{CO}_2$  using reagents containing  $\text{CO}_3^{2-}$  ions. However, it is rather difficult in a usual synthesis of CaHAP to completely avoid contamination with  $\text{CO}_2$  during the solution preparation, reaction and the separation,

washing and drying of the products. Therefore, it should be recognized that CaHAP particles synthesized in aqueous systems without special care will contain  $\text{CO}_3^{2-}$  ions [3].

**Hydroxyapatite analysis by using XRD Characterization.** The XRD pattern of HAp which is precipitated by nitrogen stream in during 5 minute (HAP-1) does not have significant difference from XRD pattern of HAp which is precipitated during 10 minutes nitrogen gas (HAP-2). This comparison is identical with FTIR result as explained above. Figure 2 and 3 show the X-Ray diffraction patterns of dried product of 5 and 10 minutes nitrogen gas streaming before precipitation process which is calculated by Philips Leo 420i Oxford. In both cases there is only HAp. XRD patterns of all the materials fitted that of hydroxyapatite group (JCPDS 9-432), (JCPDS 24-0033), (JCPDS 74-0565), (JCPDS 74-0566), (JCPDS 73-1731), (JCPDS 73-0294), (JCPDS 72-0293), (JCPDS 72-1243), (JCPDS 76-0694). The hydroxyapatite peaks were broad in  $2\theta$  on 10 – 100° of range. Dashed lines show the lattice planes of pure apatite crystal that constitute as a crystal phase [8].

The apatite phases were essentially not influenced by change of the nitrogen gas streaming, since the quantity of  $\text{CO}_2$  incorporated into HAp particles was small which is kept by isolation area during precipitation process. The constantly phase above is supported by lattice parameter which have high accuracy of lattice correspond to hydroxyapatite. Hydroxyapatite has a hexagonal structure. Patterns of hexagonal crystals can also be indexed by graphical methods or cohen's method, since the hexagonal unit cell, like the tetragonal, is characterized by two parameters, a and c. The plane-spacing equation is

$$\frac{1}{d^2} = \frac{4}{3} \left( \frac{h^2 + hk + k^2}{a^2} \right) + \frac{l^2}{c^2} \quad (1)$$

Nevertheless, the tendency shows that those sample have a few number of AKA component phase as attribute of incorporation  $\text{CO}_2$  into hydroxyapatite particles that is adjacent to hydroxyapatite phase. Supporting evidence is shown in the FTIR spectra as  $\text{CO}_3^{2-}$  wavenumber. Therefore, the impurity that included in sample is decreased by reheating treatment at 700°C to get purer hydroxyapatite.

Heating of hydroxyapatite commonly causes a decrease in weight as yield of absorbed water that removed from the surface at temperatures less than 200°C and from pores at temperatures up to 900°C [9-12]. Carbonate that occupies either the hydroxide site or phosphate site can also be relieved by heating at a temperature of 700°C [13-14].

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°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°C of treatment will increase the crystallinity 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°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.

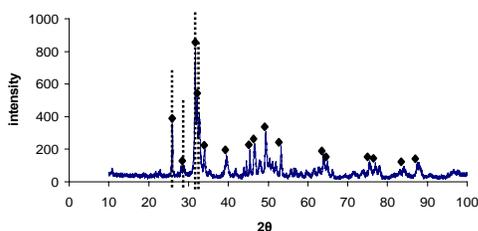


Figure 2. Sample of HAp1

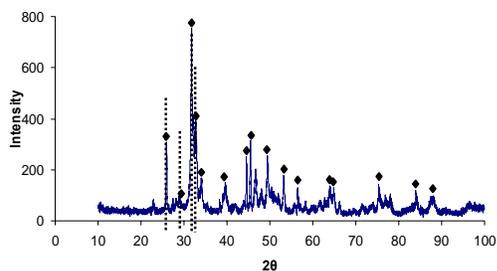


Figure 3. Sample of HAp2

**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].

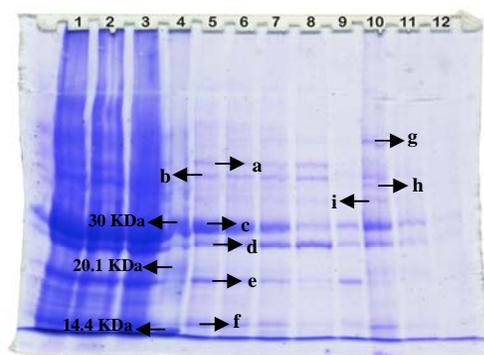
Ceramic hydroxyapatite (CHT),  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  is a sintered form of calcium phosphate that can be used to separate and purify proteins, enzymes, nucleid acids, viruses, and other macromolecules. Its unique mechanism of separation involves both electrostatic interactions and the formation of calcium coordination complexes [17]. Ceramic hydroxyapatite column is abiding, could be used repeatedly more than 100 times without any problem or contamination. So that, the hydroxyapatite is an effective purifier that could be decrease an error which might be caused by the defect of matrix. Basicly, protein is involved as two parts, basic and acidic proteins on amino and carboxyl groups, respectively, which have different characteristics.

Figure 4 shows the result of SDS PAGE that contained by marker LMW (low Molecular Weight), crude ekstract of soybean, unbound of HAp and DEAE (Anion Exchange) matrix, and the elution of HAp and DEAE (Anion Exchange) matrix. The marker LMW that is resulted on SDS electrophoresis is not clear to be as a reference in calculating of molecular weight that gained on the other lines.

However, it is estimated by combining its band to LMW reference. The vagueness of marker might be caused by any contamination of protein from before and after lane from SDS marker. The unbound of HAp is prone to be less amount of proteins, so that its bands could be distinguished (Figure 4) than the others, crude extract and unbound of DEAE, which have hoarding patterns which showed that there were a lot of protein that could not be bound on matrix.

For instance, this suggested that most of proteins that included in soybean is bounded on hydroxyapatite matrix which is contained by positively and negatively charged sites on crystal calcium ions (C-sites) and triplets of crystal phosphate (P-sites), respectively. This point explained that hydroxyapatite matrix is more attractive than DEAE as an anion exchange chromatography which appears to contain numerous bands unbound fraction.

In binding protein, amino groups are attracted to P-sites but repelled by C-sites. The situation is reversed for carboxyls [18-21]. Although amine-binding to P-sites and the initial attraction of carboxyls to C-sites are electrostatic, the actual binding of carboxyls to C-sites involves formation of much stronger coordination complexes between C-sites and clusters of protein



**Figure 4. The SDS PAGE Electrophoretic Profiles of Purified Soybean that is Fractionated 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<sup>7</sup>. 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 Ca<sup>2+</sup> or Mg<sup>2+</sup> 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 Ca<sup>2+</sup> and usually not with chloride (3) Neutral proteins, which elute with phosphate, fluoride and chloride and which do not elute with Ca<sup>2+</sup> 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  $\beta$ -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.  $\alpha$ -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 seen in Figure 4, a (44.78 kDa) refers to  $\beta$ -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  $\alpha$ -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 outcome 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<sub>2</sub>. 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 repeatedly more than one hundred times.

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