Production and Characterization of Scaffold made of Hydroxyapatite and Pectin of Green Cincau Leaf *(Premna Oblongifolia Merr)*

S Habibie[1*], Y Tristiyanti[2], D Gustiono[1], M E Harahap[1], S Y Chalid[2], D Effendi[1]
[1]*Center for Material Technology, Agency for The Assessment and Application of Technology – BPPT, Serpong, Indonesia*
[2]*Department of Chemistry, Faculty of Science and Technology, UIN Syarif Hidayatullah, Jakarta, Indonesia*
[*]*email : sudirman,habibie@bppt.go.id*

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Abstract

Scaffold is a 3-dimensional matrix created as a new bone cell growth medium made from natural polymers and bioceramics. The extracted pectin from green Cincau leaves (Premna oblongifolia Merr) and hydroxyapatite (HA) are used in the manufacture of scaffolds. Pectin was extracted using citric acid with variation concentration of 0, 0.1, 0.2 and 0.3% (w / v). The 3% (w / w) HA-pectin mixture, dried freeze using a freeze dryer. The characterization of extracted pectin and HA-pectin scaffold was then performed. The results showed that pectin of green Cincau leaves had low methoxyl content, which was 1.364 to 5.022%. The resulting scaffold has a pore size ranging from 8.25 to 115 µm while the scaffold resistance to the load, i.e. 0.03 to 0.15 MPa. The scaffold porosity that has been made is 15.33 to 40.97% while the density is 0.69 to 1.02 g/cm3

Keywords: *Green Cincau Leaf Hydroxyapatite, Pectin, Scaffold*

I. INTRODUCTION

Scaffold or commonly referred to as scaffolding is a 3-dimensional matrix that is used as a cell attachment. The scaffold is placed on the damaged bone so it can be used as a support for damaged bone. Scaffold is temporary and stimulates the formation of new bone tissue by diffusing nutrients to bone cells so that it is attached into the pores of the scaffold [1,2]. Scaffold will gradually degrade and be replaced by growing bone tissue [3].

According to Patel et al [1], the scaffold implanted into the body, has a condition that is biocompatible, not toxic, the mechanical properties corresponding to the tissue where the bone is damaged. Scaffold should also have pores that can support bone development, support of cells structure and become an extracellular matrix during regeneration.

Green Cincau leaves contain high fiber and gel formed polysaccharides. The gel formed from cincau leaves extract is caused by gel-formed polysaccharides in the form of low-methoxy pectin [4]. The pectin substance is composed of polygalacraconate acid and an esterified carboxyl group. According to Dougall et al [5], Pectin on cell walls is in the form of bonded with metal ions, especially divalent ions, so to be able to extract efficiently, pectin must be liberated from metal ions.

In this study, the scaffold is made from pectin extracted from green cincau leaves as an organic component and will be combined with hydroxyapatite in the form of inorganic components. Green Cincau leaves are used as raw material for making scaffold to know the potential of pectin extracted from green Cincau leaves. Citric acid is used as a pectin extract material because it has safe properties if consumed so it is not harmful if applied to the body [6]. This research is expected to make scaffold with pore size of 150 µm, resistance to pressure of 4 - 12 MPa and its porosity and density of 30-90% and 0.14-1.2 g/cm^3 respectively [7].

II. RESEARCH METHOD

A. Materials

The ingredients used were the green Cincau leaves (*Premna oblongifolia Merr*) which were picked from Bintaro area 9-Tangerang Selatan (Indonesia) and hydroxyapatite (BPPT-PTM). Hydroxyapatite is made from the extraction of limestone and diamonium sulphate, while citric acid (monohydrate for analysis) of

the KGaA Brand, ethanol 95% (absolute for analysis), $CaCl_2$ > 98% and sodium hydroxide (pellets for analysis) are obtained from the ingredients store chemistry.

B. *Pectin Extraction*

Green Cincau leaves (*Premna oblongifolia, Merr*) are washed, then cut into small pieces. The cut leaves are then dried by oven at 600C for 24 hours and crushed using a blender to powder. The 500 ml aquadest was heated at 800C and then added citric acid, i.e. 0, 0.1, 0.2 and 0.3% (w/v). The hot citric acid solution was then added 25 g of Cincau leaf powder and stirred using stirrer for 15 minutes at a speed of 600 rpm, then filtered using a filter cloth to obtain gel of Cincau. The Cincau gel was isolated by adding ethanol 95% (v/v) with a volume ratio 1:1 and was allowed to sit overnight with a temperature of 50C. Cincau gel that has been soaked overnight then filtered using a cloth so that the resulting precipitate pectin. The pectin deposit is placed on a heat-resistant plastic wristwatch. Extracted pectin was dried in oven at 600C for 24 hours to produce dried pectin. Pectin is crushed using blender for 2 minutes to produce pectin powder.

C. *Production of Scaffold*

Scaffold is made from pectin gel with a concentration of 3% (w / v) for each variation of pectin sample (citric acid concentration, ie 0, 0.1, 0.2 and 0.3%) and mixed with hydroxyapatite (HA) with a concentration of 3% (v/w) so that the ratio of pectin concentration to HA is 50:50. The pectin powder that has been added aquadest is then stirred using a stirrer for 4 hours until the gel is homogeneous. The homogenous pectin gel is then added slowly with a hydroxyapatite slurry and stirred for 1 hour until the mixture is homogeneous. The mixture was added with $CaCl_2$ 0.03 M and stirred for 30 minutes. The mixture of pectin gel and hydroxyapatite was then molded into the mould and frozen with a freezer for 24 hours at -130C. The frozen scaffold is then dried freeze using freeze dryer for overnight at -350°C and pressure of 120 bar.

D. *Characterization of Pectin*

Pectin extracted from green Cincau leaves (*Premna oblongifolia, Merr*) was characterized to determine the nature of the resulting pectin, such as: yield of pectin, equivalent weight, methoxy content, and galacturonate acid level, degree of esterification and functional group and FTIR.

E. *Characterization of Scaffold HA-Pectin*

HA-Pectin Scaffolds are tested for SEM to determine surface morphology, porosity and density, as well as strength tests, degradation capabilities.

III. RESULTS AND DISCUSSION

A. *Pectin*

Increased citric acid concentration causes the increase of yield, methoxy content, galacturonat acid level and degree of esterification pectin of green Cincau leaves. However, the pectin equivalent weight of green Cincau leaves decreased as the concentration of citric acid increased.

B. *Yields*

Increased citric acid concentration, causing the yield of extracted pectin of green Cincau leaves also increased. Ca^{2+} ions that replace H^+ ions in citric acid cause the pectin to dissolve in water so that it can be extracted [8].

C. *Equivalent Weight*

Citric acid causes the polymer structure of pectin to be depolymerized [9], hydrolysis of glycosidic bond [10] and decrease of pectin equivalent weight [6]. The increase of acid concentration, causing many pectin polymer chains to be eliminated, thereby lowering the value of the equivalent weights obtained.

D. *Metoxyl Content*

Increased concentrations of citric acid lead to increased metoxyl levels of green Cincau leaves (Premna oblongifolia Merr). The increased acid concentration causes, the more glycosidic bonds in the esterified galacturonat acid are β-eliminated. The methoxyl present in carbon number 6 (C^{-6}), may be either demetoxylation or release methoxyl ($-OCH_3$). Demetoxylation occurs by methoxy or methyl ester ($-OCH_3$) undergoing hydrolysis at an alkaline atmosphere through a saponification process resulting in methanol [11]. Methoxyl content is inversely proportional to the result of determination of equivalent weight. The lower the equivalent weigh indicates the esterified galacturonat acid ($-COOCH_3$) increases so that the methoxyl ($-OCH_3$) levels increase as well.

E. *Galacturonat Acid Content*

Galacturonat acid is the basic structure of pectin in linear form that binds to other polysaccharides as a side chain [6]. The levels of galacturonat acid increase with the increase of citric acid concentration. This occurs because of the acid that causes the hydrolysis of non-uronic acids [10], so that the levels of galacturonate acid increase along with the hydrolysis of non uronic acid. The non-uronate compounds are D-galactose, L-arabinose and L-ramnose [12].

F. *Degree of Esterification*

In the determination of equivalent weights, calculated galactic acid is non-esterified (-COOH). The degree of esterification determines the esterified galacturonate acid ($-COOCH_3$). Increased citrate acid

concentration causes the equivalent weight or galacturonate acid that does not undergo esterification is getting smaller. It causes galacturonate acid which undergoes esterification or degree of esterification increases with increasing of citric acid concentration. The increasing degree of esterification has a high methoxy content along with an increase in citric acid concentration.

G. IR Spectrum Analysis

Peak intensity shows that pectin extract of green Cincau leaves has low degree of esterification [13]. It is also proved on the characterization of pectin of green Cincau leaves which have low degree of esterification that is 12,656-13,750%. Pectin which has a low methoxy content can form a gel that is not soluble in water because of the presence of divalent ions that have a positive charge. The interaction between the carboxyl group at low ester levels with Ca^{2+} ions increases the formation of the egg-box structure.

In Fig. 1. It is shown that the peak intensity at the 1600 cm^{-1} wave area has a sharper peak than the peak at the 1700 cm^{-1} wave area. In Fig. 1 (a), the peaks of 1600 and 1315 cm^{-1} numbers can be determined as carboxyl functional groups ($-COO^-$) and bonds between C-H on the pyranose ring. Pectin with 0.1% citric acid concentration is presented in Fig. 1 (b), several wave numbers are shown, i.e. 1,612, 1,390 and 1,262 cm^{-1}. The wavelength can be determined as a carboxyl functional group ($-COO^-$), methyl (CH_3) of the ester group and ester group. The wave number 1600 and 1,316 cm^{-1} can be determined as carboxyl functional groups ($-COO^-$) and the bonds between C-H on the pyranose ring shown in Fig. 1 (c). Fig. 1 (d), is shown wave number 1,390 and 1,076 cm^{-1}. The wavelength can be determined as methyl (CH^3) of the ester group and the bond between C-O and C-OH on the pyranose ring.

H. Scaffold of HA-Pectin

Tests using SEM were only performed on HA-pectin Scaffolds to see surface morphology, pore size and scaffold density, scaffolds strength, and its degradability.

I. Morphology of HA-Pectin Scaffold

The surface pore size of the hydroxyapatite-pectin scaffold of green Cincau leaves has different sizes at the top and center. The top surface of the scaffold with a 0% citric acid concentration has a pore size of 110 µm while in the center has a pore size of 102 µm. Scaffold with 0.1% citric acid concentration also has a larger pore size at the top, which is 115 µm as shown in Figure 2. The morphology of the scaffold surface at the center is smaller, i.e. 44.1 µm.

Scaffold pore size measured at magnification 500 times. The pore size at the top of the scaffold with 0.2% citric acid concentration, i.e. 8.25; 26.7 and 14.6 µm. The scaffold surface in the center has a pore size of 65.6; 36.7; 32.3 and 29.1 µm. The pore size on the top of the scaffold with 0.3% citric acid concentration of 62.8 and 67.5 µm. At the middle surface of the scaffold has a larger pore size than the top, which is 115 µm.

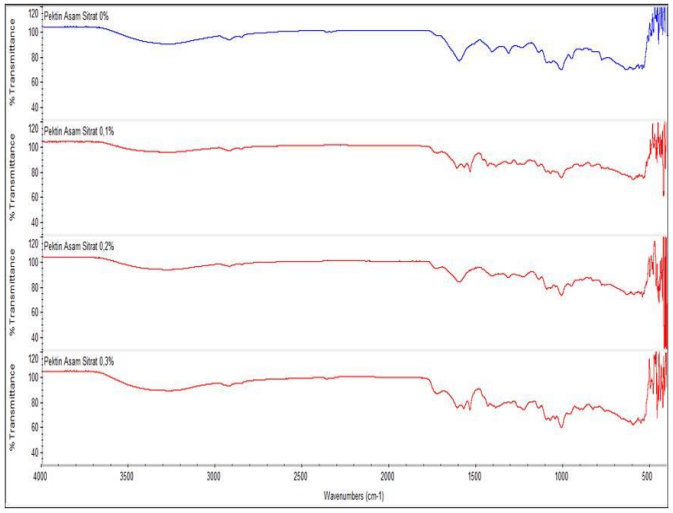

Figure 1. Spectrum IR pectin with concentration of citric acid (%), a = 0; b = 0,1; c = 0,2 and d = 0,3

The pore size at the top is larger than the central pore size. This is because the top surface of the scaffold is located close to the air so the cooling rate is slower than at the bottom. The slow cooling rate causes the ice crystals formed in the freezing process to develop in size. The expanding ice crystals are then transformed into a gas phase resulting in a larger pore [14].

The pore size is not affected by the increase of citric acid concentration. It is proved in this study that the increase and decrease of irregular pore size to the increase of citric acid concentration. The morphological analysis of scaffold at 1000 times magnification, indicating the presence of uneven clumps. The clumps are hydroxyapatite (HA) which cannot disperse into pectin properly. This happens because of the way of making by mixing method. The added cross-linker works only on pectin by means of Ca^{2+} ions from $CaCl_2$ binding to a carboxyl group (-COO-) derived from pectin. The chemical bonds that occur between hydroxyapatite and pectin are ionic and hydrogen bonds. The chemical bond is likely to be weak when compared to the bonds that occur between the polymer and hydroxyapatite crystals blend made in situ [15].

J. Porosity and Density of Scaffold

Density of HA-pectin scaffold of green Cincau leaves has an average of 1.0211-1.4936 g/cm3. The percentage porosity of scaffold that has been made from HA-pectin of green Cincau leaves has an average value ranging from 15.3303-40.9739%.

Percent porosity at citric acid concentration 0% is higher than that at concentrations of 0.1, 0.2 and 0.3%. This is because the increase in the concentration of

citric acid causes polygalacturonate acid polymer to de-polymerize on glycosidic bonds that affect the ability of pectin in gel forming. The difficult of gel formed causes a decrease in the ability of pectin to absorb water, so that when freezing, the ice crystals are formed slightly and form a small pore structure [4].

According to Narbat et al. [16], the value of the density test results can show the strong power of the scaffold, so if the density is higher, then the higher the compressive strength also. The value of the porosity test results the higher, causing the strong power of the lower scaffold, because of the more pore formation.

K. Strength Scaffold

The results of the compressive test on the HA-pectin scaffold have a low value with an average of 0.03 to 0.147 MPa. Increasing the concentration of citric acid causes the scaffold pressure test results are smaller.

Scaffolds require strong resistance to retain their shape during the formation of bone tissue cells, resistant to pressure and pull after implantation into the body [14]. Increasing the concentration of citric acid causes the scaffold resistance to the load to be weak. The value of the low test result is caused by the formation of HA crystals that could not be dispersed into pectin properly. Hydroxyapatite can be easily moved or released from the polymer because the bond between pectin and hydroxyapatite is weak so that the compressive strength becomes low [15].

L. The Ability of Degradation

Degradation is an important scaffold parameter in tissue engineering. This is because, after being implanted into the body, the scaffold must be degraded as a result of the growth of newly formed bone tissue in place [16].

On the 7th day after immersion in the lactated ringer solution, the scaffold only experienced a slight degradation of its initial mass. Scaffold mass at the concentration of citric acid 0; 0.1 and 0.2% increased degradation on day 14. On day 21, scaffold was still degraded but not as much as at day 14. The scaffold is soaked in 1 M CaCl2 solution before soaking in ringer lactate solution to link gel tissue so that the scaffold can survive or degrade gradually during and after 21 days in the lactate ringer solution [13].

Scaffold degradation is caused by calcium ions which initially bind to pectin and are useful as crosslinkers in the scaffold, released by the presence of sodium and potassium ions present in the lactate ringer solution [17]. The pectin polymer chain which is not binding to the calcium ion then produces a carboxyl group (-COO-) so that the pectin is easily soluble. Soluble pectin causes the pectin polymer to degrade gradually and removed from the body through the process of excretion [18].

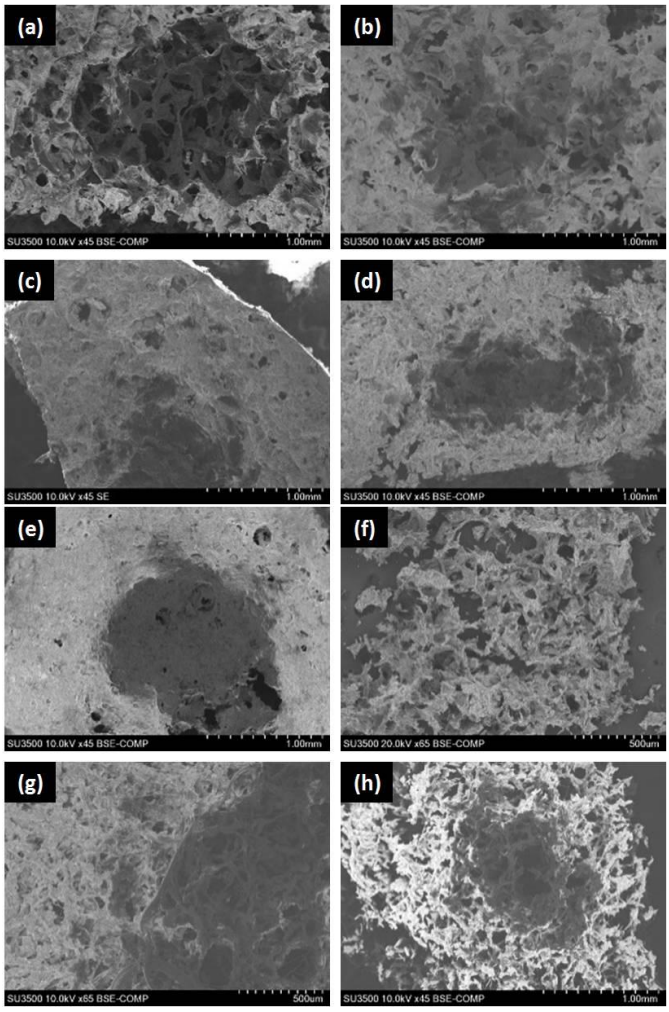

Figure 2. Morphology of scaffold with citric acid concentration, ie 0 (a,b); 0,1 (c,d); 0,2 (e,f) and 0,3% (g,h), at the top of scaffold (a,c,e,g) and at the middle (b,d,f,h) with magnification 45.

Scaffold that can be done degradation test only 3 variation of citric acid concentration that is 0; 0.1 and 0.2%. Scaffold of HA-pectin with 0.3% citric acid concentration could not be tested for degradation. This is probably because the scaffold contains pectin extracted with 0.3% citric acid concentration having a galacturonate polymeric that is much depolymerized [8]. This is evidenced by the destruction of scaffold with 0.3% citric acid concentration after soaking in a solution of its crosslinking agent, $CaCl_2$ 1 M.

IV. CONCLUSION

Pectin extract results from green Cincau leaf (Premna oblongifolia Merr) using citric acid showed that the increase of citric acid concentration could increase the amount of yields and metoxyl content, but the equivalent weight decreased with the increase of citric acid concentration. While the degree of esterification is relatively low despite the increased concentration of citric acid, it is also supported by the results of observation with FTIR. The observations by FTIR of the resulting pectin show there are carboxyl

groups and metal groups of the ester and ether groups, as well as the C-O and C-OH bonds of the pyranose chain. The result of morphological analysis and compressive strength of scaffold made by SEM observation, it still could not reach optimum and fragile pore size. The result of porosity analysis and the density of scaffold made has fulfilled the porosity range and sponge bone density and can experience degradation. The result also shows the difference of pore size between the surface part and the center of the scaffold where the surface part has a larger pore than the center of the scaffold. Between HA and pectin, there appears ionic bonding and hydrogen bonding, however the HA-pectin scaffold has low strength. The results of degradation testing showed that there was an increase in degradation with increasing days of observation and reached the maximum degradation on day 14, after which degradation decreased.

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