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Extraction of Gelatin from Bovine Bone and its Use as Template in Synthesis of Mesoporous Silica

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Extraction of gelatin from bovine bone and its application as a template for synthesis of mesoporous silica have been studied. The bone was pretreated with acetic acid 4 % for 9 days, followed by sodium hydroxide 0.1 M for 24 h and hydrochloric acid 1 M for 1 h. The pretreated bovine bone was then refluxed in demineralized water for 5 h at 70, 80, 90 and 100 °C to extract gelatin. The gelatin was analyzed by FTIR and SDS-PAGE. The synthesis of mesoporous silica was performed by hydrothermal method. The product was analyzed by FT-IR, XRD, nitrogen gas adsorption and TEM. The result showed that the gelatin extracted at 80, 90 and 100 °C contained α and β-chains. However, the gelatin extracted at 70 °C contained only α-chains. The silica had pore diameter, specific surface area and pore volume of 6.08 nm, 384.21 m^2/g and 0.75 cm^3/g, respectively. The silica had wormhole-like mesoporous structure.

Keywords: Gelatin, Bovine bone, Mesoporous silica, Wormhole-like structure.

INTRODUCTION

Gelatin is a term used for all the collagen fractions that exceed an arbitrary minimum molecular weight of 30 kDa [1]. Molecular weight distribution, structure and composition of gelatin depend on conditioning process and its raw material. The raw material that usually used to gelatin production are pig skin, bovine skin, bovine bone, fish skin and fish bone [2-4].

Gelatin can be extracted by alkaline or acid pretreatment, or a combination of both, followed by thermal hydrolysis. The combination of alkali and acid pretreatment could provide higher yield of fish gelatin with better qualities than alkali or acid pretreatment alone, so it has become a widely accepted method for fish gelatin extraction [5]. Gómez-Guillén *et al.* [6] compared the viscoelastic and gelling properties of megrim skin gelatin extracted with alkali (0.2 M NaOH) and seven different organic acids (formic acid, acetic acid, propionic acid, lactic acid, malic acid, tartaric acid and citric acid). Zhou and Regenstein [7] used $Ca(OH)_2$ 0.1 M and three acids (acetic acid, citric acid and sulfuric acid with 0.01-0.1 M of hydrogen ions) as pretreatment to investigated the extraction yield and gel strength of pollock skin gelatin. The results were analyzed mainly based upon the pH of gelatin solutions.

Collagen content in dried bovine bone is about 30-40 %. Based on statistics data from the Central Bureau of Statistics Indonesia in 2013, the slaughtered cattle for consumption is more than one million heads/year. Cattle weight is about 500-700 kg with bone weight about 150 kg. Thus, total cattle bone weight reach 150,000 ton/year. This amount is quite significant for the bone to be used as collagen source for gelatin production.

Gelatin is an important functional biopolymer that has a broad application for food, material, pharmacy and photography industries. Another application of gelatin is a template for mesoporous silica. General template used for mesoporous silica is an ammonium quartener type surfactants. However, these surfactants are hard to degradate by environment. They may cause eutrophication of water and environmental pollution. In order to avoid this disadvantage, some natural polymers have attracted researchers' considerable attention [8-10].

Gelatin is a potential polymer to be used as a template in synthesis of mesoporous silica, because it contains lots of N–H functional groups. These groups tend to strongly interact with sylanol groups (Si–OH) on the silicate species *via* multiple hydrogen bonds. The pH of solution can affect the concentration of ammonium ions inside gelatin molecules, change the intensity of hydrogen bonds between gelatin molecules and tune conglomeration condition of gelatin. All of these factors would regulate pore size of the mesoporous silica. Moreover, gelatin has good biocompatibility, surfactivity, biodegradability and non-toxic [8].

Hydrothermal treatment is one of the most efficient methods to improve mesoscopic regularity of mesoporous material. After the solution reaction, the mesostructures undergo reorganization, growth and crystallization during hydrothermal treatment. The treating temperature of 95-100 °C is mostly used. This temperature is relatively lower than microporous treating temperature, because the mesostructures have assembled before the hydrothermal treatment [11].

Based on the above explanation, the authors undertaken extraction of gelatin from bovine bone using combination of base and acid pretreatment. The bovine bone source was produced in traditional markets in Yogyakarta, Indonesia. Gelatin with specific molecular weight distribution was used as a template for mesoporous silica synthesis. The synthesis was conducted using hydrothermal method. The product was then characterized by some instruments.

EXPERIMENTAL

Bovine bone from various traditional market in Yogyakarta, hydrochloric acid analytical grade was purchased from Fluka (Japan), sodium hydroxide, glacial acetic acid, sodium silicate and sulfuric acid analytical grade were obtained from E. Merck (Germany).

Extraction of gelatin: The extraction of gelatin was carried out according to Zelechowska *et al*. [12] and Zhou and Regenstein [7] with slight modification. The bone was cleaned and washed with demineralized water. The prepared bone was then cut into small pieces (1-3 cm^2). Before gelatin extraction, bovine bone was soaked in an acetic acid 4 % with a bone/solution ratio of 1:2 (w/v) for 9 days. The bone was then washed with demineralized water until the pH of water become neutral. The clean bones were then mixed with 0.1 M NaOH solution (1:2, w/v). After 24 h, the mixture was separated and the solid portion was washed with demineralized water, followed by mixed with 1 M HCl solutions (1:3, w/v) for 0.5 h and filtered. The procedures of base and acid pretreatments were repeated twice. The pretreated bovine bone was then refluxed in demineralized water (1:6, w/v) for 5 h at 70, 80, 90 and 100 °C. The mixture was filtered and the solid was dried at 50 °C produced gelatin. The gelatin was then characterized by FTIR and SDS-PAGE.

Synthesis of mesoporous silica: The synthesis of mesoporous silica was done according to Hsu *et al*. [9] and Wang *et al*. [8] with slight modification. Gelatin and sodium silicate were dissolved in demineralized water at 40 °C. Sulfuric acid (0.1 M) was added to sodium silicate solution to get acidified silicate stock at pH 4. Gelatin solution was then added to the acidified silicate stock and the mixture was stirred for 1 h. The formed gel solution was transferred into autoclave and hydrothermally treated at 100 °C for 24 h. Finally, the product was filtered, washed with aquadest, dried at 80 °C and calcined at 550 °C for 5 h. The material was then analyzed by FTIR, XRD, GSA and TEM.

RESULTS AND DISCUSSION

Characterization of bovine bone gelatin: Fig. 1 showed five characteristic FTIR absorption band for polipeptide namely amide A, B and I-III. Amide I band (1700-1600 cm^{-1}) is mainly due to C=O stretching vibration (about 80 %) of the amide group coupled with in-plane NH bending (less than 20 %). Amide II (1575-1480 cm^{-1}) derives mainly from in-plane NH bending and CN stretching vibration and shows less protein conformational sensitivity compared with amide I, while other amide vibrational bands have less practical use in protein conformational studies. The amide III (1240-670 cm^{-1}) represented the combination peaks between C-N stretching vibrations and N-H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains. The amide A band (3600-3400 cm^{-1}) arises from the stretching vibrations of N-H and O-H group. Absorption band that arise at 3000-2800 cm^{-1} called amide B, this peak indicate assymetric stretching vibration of CH_2 [13].

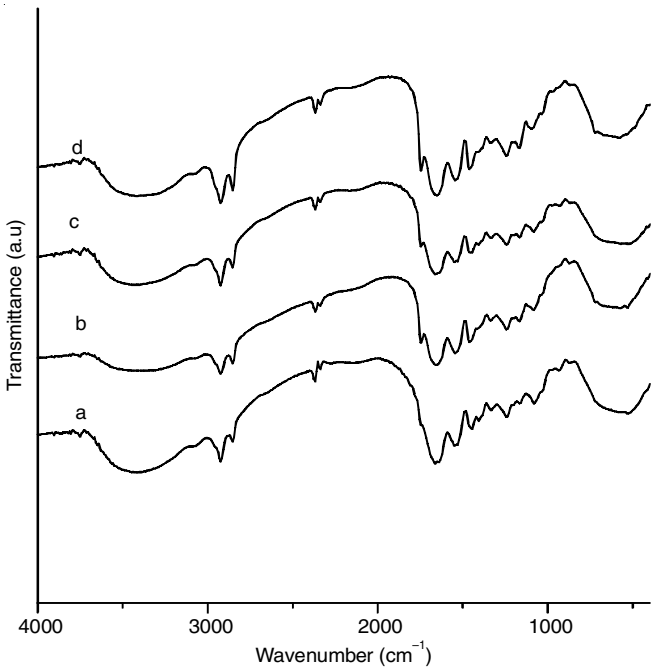

Fig. 1. FT-IR spectra of gelatin extracted from bovine bone at (a) 70 (b) 80 (c) 90 and (d) 100 °C hydrolysis temperatures

Fig. 1 showed that the sharpest functional groups band arise in extracted gelatin at 80 °C. It might be happened because gelatin denature to higher stage at higher temperature (90 and 100 °C), so gelatin molecule become smaller and decrease the spectra intensity of its functional groups. Amide I of all bovine bone gelatin appeared at the wavenumber 1651-1627 cm^{-1}. Higher frequencies of amide I bands is attributed to greater loss of molecular order of triple helix due to uncoupling of intermolecular cross-links and disruption of intra molecular bonding when gelatin was extracted at higher temperature or longer time [14,15].

Amide III band of bovine bone gelatin was detected at 1242 cm^{-1} which was associated with loss of triple-helix state of the molecules and transformation of α-helical to random coil structure due to denaturation of collagen to gelatin [16]. Amide A band that derives from the stretching vibration of N-H group appeared at 3410-3402 cm^{-1}. The position of the band in amide A region shifts to lower frequencies might be happened because N-H group of shorter peptides are involved

in hydrogen bonding [15]. Amide II region appeared at 1543 cm^{-1}, while amide B appeared at 2924 cm^{-1}.

Table-1 showed the molecular weight distribution of extracted gelatin. The weight distribution was calculated with standard curve from SDS-PAGE data. Extracted gelatin at 100, 90 and 80 °C temperature has molecular weight range 35-200 kDa, 26-181 kDa and 28-181 kDa, respectively. This result show that bovine bone gelatin consists of a mixture of polypeptide representing collagen type I with α-chains, β chains (two covalently cross-linked α-chains) and γ-chains (three covalently cross-linked α-chains) together with higher and lower molecular weight fragments.

TABLE-1
MOLECULAR WEIGHT DISTRIBUTION
OF EXTRACTED GELATIN

Sample	Molecular weight range (kDa)
Gelatin 70	31-141
Gelatin 80	28-181
Gelatin 90	26-181
Gelatin 100	35-200

Gelatin extracted at 70 °C has narrower molecular weight distribution range than another gelatin, that is 31-141 kDa. This result showed that gelatin 70 components was not as complex as other gelatin. Gelatin 70 was choosen as mesoporous silica template because it has higher contents of α-chains. The gelatin α-chains consist of polar and non-polar regions. The 'non-polar' regions are made up from the tripeptide Gly-Pro-R, where R is a non-polar amino acid, predominantly hydroxyproline. These 'non-polar' regions are interspersed with polar regions, which are relatively deficient in both proline and hydroxyproline. The presence and distribution of the charged, polar and non-polar amino acids provides gelatin with unique properties. Gelatin is easily dissolved in water at the right conditions due to the presence of charged amino acids and forms colloidal solutions. Due to its chemistry, gelatin is a multifunctional hydrocolloid with considerable surface activity [17]. Besides, gelatin 70 used less energy than gelatin 80, 90 and 100, so it's compatible with green chemistry principle.

Characterization of mesoporous silica: The FTIR spectra of gelatin extracted at 70 °C, mesoporous silica prepared with gelatin templating before and after calcination were presented in Fig. 2.

The FTIR spectra of gelatin (Fig. 2a) was characterized by a large band around 3410 cm^{-1} that corresponds to NH stretching vibrations coupling with OH. A band at 2924 and 2854 cm^{-1} attributed to CH_2 symmetric and assymetric stretching vibrations. The two bands at 1635 and 1543 cm^{-1} are corresponding to C=O stretching and NH bending (primary amine), respectively [9,18]. There were 2924, 2854 and 1543 cm^{-1} band at mesoporous silica before calcination spectrum (Fig. 2b). These data showed that there still a plenty of gelatin in the mesoporous silica framework. Wang *et al.* [8] reported that the content of gelatin in mesoporous silica reach 20 wt. % after hydrothermal treatment. At Fig. 2c, the 2854 and 1543 cm^{-1} bands was not shown. It was proven that calcination process was effective to eliminate gelatin from mesoporous silica framework [9,18].

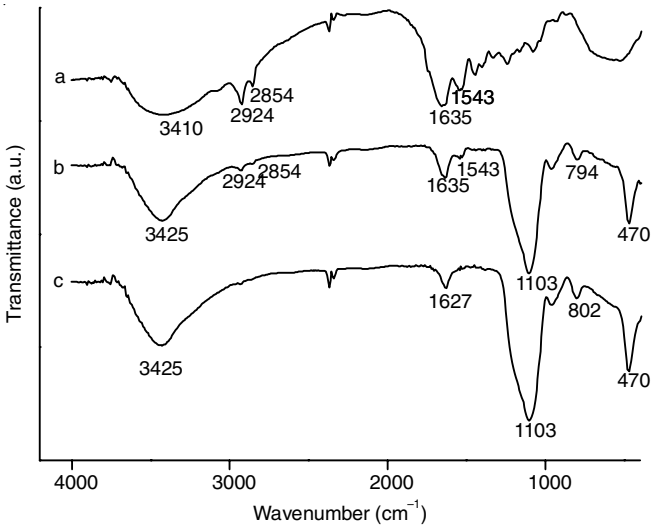

Fig. 2. FT-IR spectra of (a) Gelatin (b) Mesoporous silica before calcination (c) Mesoporous silica after calcination

Wavenumber that appeared at 1103, 802 and 470 cm^{-1} for the silica material is related to condensed silica framework. Wavenumber peak at 802 and 470 cm^{-1} can be indicated as Si-O-Si symmetric stretching and deformation, while 1103 cm^{-1} is related to vibrational strething Si-O-Si bridges. The silanol hidrogen bonding arise at 3425 and 1627 cm^{-1} bands. Based on the FTIR spectrum, it can be concluded that mesoporous silica has been successfully formed.

The XRD pattern of the silica was presented in Fig. 3. Here, it can be observed that the silica is amorphous, as indicated by the single broad peak at 2θ 20° that arise from the lack of any ordered crystalline structure.

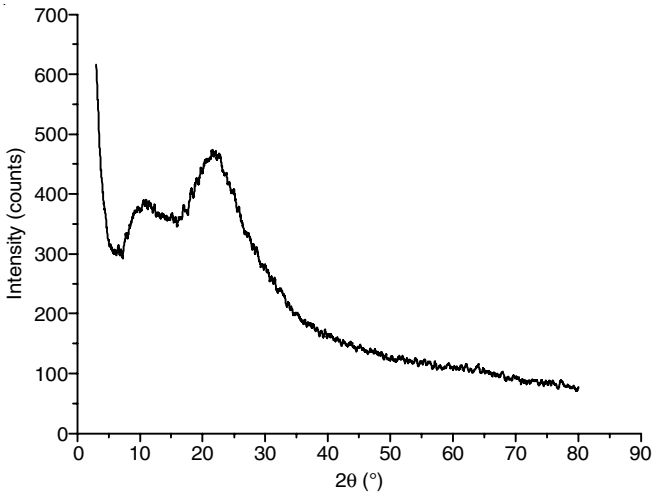

Fig. 3. XRD pattern of mesoporous silica

The nitrogen adsorption-desorption isotherms of the mesoporous silica prepared from bovine bone gelatin was shown in Fig. 4a. The adsorption–desorption isotherms was close to type IV isotherm according to the IUPAC classification. The type IV isotherm is characterized by the disappearance of saturation limit with a hysteresis. This type of isotherm indicates an indefinite multilayer formation after completion of the monolayer and that the obtained materials are mesoporous. The hysteresis type of bovine bone gelatin

samples can be classified as an H2 type. This hysteresis shape involves a vapour-percolation threshold of the boundary curve occurring at a P/P_0 value of approximately 0.42, reflecting the steep evaporation curve from pores presenting steric hindrance, in which the interphase becomes mechanically unstable. This sudden evaporation from pores, also called the cavitation phenomenon, consists of the nucleation of bubbles within the liquid-like condensed phase, thus allowing an abrupt release of almost all of this latter phase to the bulk vapour surrounding the sample [18].

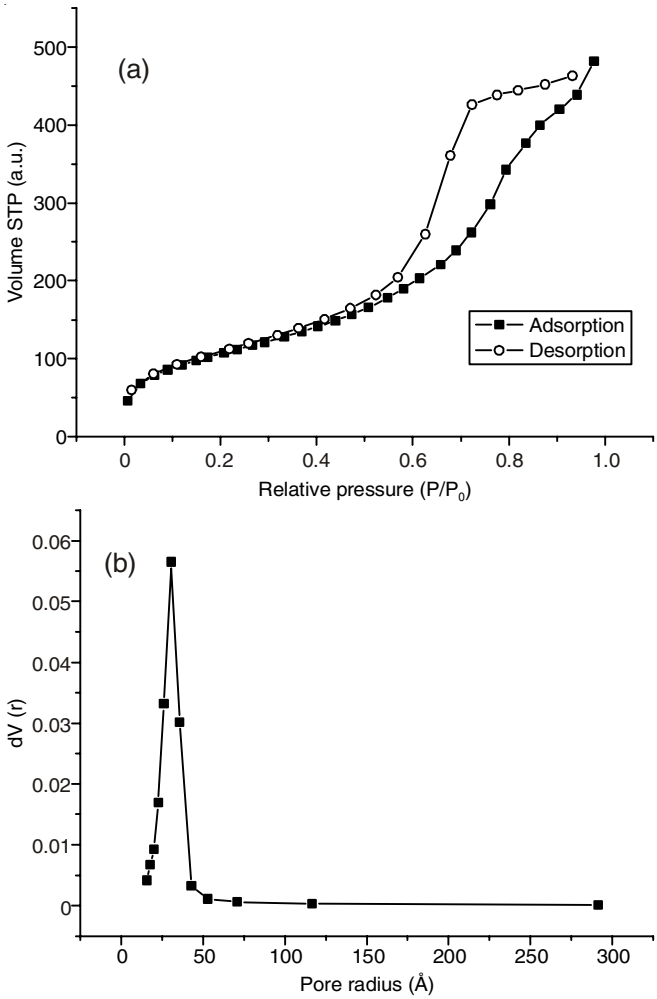

Fig. 4. (a) Nitrogen adsorption-desorption isotherm of mesoporous silica (b) BJH pore size distribution of mesoporous silica

Pore size distribution of the silica material presented in Fig. 4b was calculated by the BJH method. It was shown that mesoporous silica has the narrowest peak at 30 Å. The sharp peak indicated that the material has high uniformity pore size. Synthesized mesoporous silica has pore diameter of 6.08 nm, according to Barret-Joyner-Halenda (BJH) method. Specific surface area of the silica material was calculated by Brunaeur-Emmett-Teller (BET) method and the result was 384.21 m^2/g. Pore volume, determined by nitrogen adsorption at relative pressure (P/P_0) 0.977, of mesoporous silica was 0.75 cm^3/g. It is apparent that bovine bone gelatin can play an effective role as a structure-directing agent in an aqueous sodium silicate system to form mesoporous silicas.

Fig. 5 exhibited TEM images of the synthesized silica. The TEM image showed that the silica has a wormhole-like mesoporous structures. The mean mesopore sizes of the silica was about 6 nm, which was compatible with the results calculated from nitrogen adsorption-desorption isotherms.

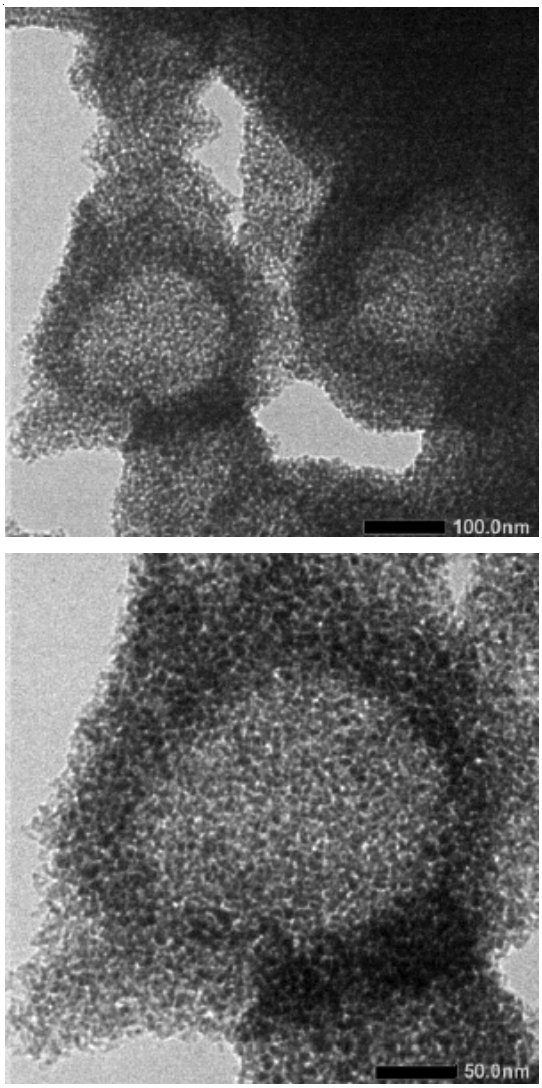

Fig. 5. TEM images of mesoporous silica

Conclusion

Gelatin extracted at 70 °C was used as mesoporous silica template because it has the narrowest molecular weight distribution of α-chains content. An amorphous mesoporous silica has been successfully prepared using bovine bone gelatin as a template. Based on the nitrogen adsoprtion-desorption, the synthesized silica have pore diameter, specific surface area and pore volume of 6.08 nm, 384.21 m^2/g and 0.75 cm^3/g, respectively. According to the TEM image, the silica has a wormhole-like mesoporous structure.

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