CHEMICAL ENGINEERING TRANSACTIONS
VOL. 63, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Jeng Shiun Lim, Wai Shin Ho, Jiří J. Klemeš

ISBN 978-88-95608-61-7; **ISSN** 2283-9216
DOI: 10.3303/CET1863118

Optimization of Acid Soluble Collagen Extraction from Indonesian Local "Kacang" Goat Skin and Physico-Chemical Properties Characterization

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Acid soluble collagen (ASC) from Indonesian Local of "Kacang" Goat skin was extracted and characterized. The skin was obtained from local slaughter house. Optimization of collagen extraction was investigated using one factor of response surface methodology. One hundred grams of skin was extracted with 0.5 M acetic acid for 24, 48, 72 h. The extract was filtered with Whatman No.1 paper. The collagen was precipitated by adding NaCl to final concentration of 2.6 M. The resulting sediment was collected by centrifuging at 7,000 g for 30 min and then re-dissolved in 0.5 M acetic acid. The resulting solutions were dialyzed with 0.1 M acetic acid and distilled water sequentially. The properties of Goat skin collagen were characterized by amino acid analysis, FT-IR, and scanning electron microscopy (SEM). The optimal condition to obtain the highest yield of acid soluble collagen was 48 h hydrolysis. The result of the research showed collagen contained 30.52 glycine residues/1,000 residues amino acid and this amino acid is a major amino acid residue in collagen. FT-IR spectra showed regions of amides A, I, II, and III were 3,433.04, 1,631.66, 1,550.65, and 1,404.07cm^{-1} respectively. The scanning electron microscopy of goat skin collagen revealed a complicated network. In conclusion "Kacang" goat skin displayed suitable source of collagen.

1. Introduction

Collagen is an important product and the most used in cosmetic, food, and pharmaceutics industry. The potential of collagen as supplements are proposed to complete the requirement of the body because synthesis of collagen will decrease of the older human body. Collagen in pharmaceutical industries as a carrier for protein, genes, and drug (Lee et al., 2001). Collagen in food industries can be used to produce an edible film of sausage casing. Collagen can be extracted from skin, bone, cartilage, tendon, and blood animals. However, collagen is produced mainly by chemical methods (alkali and acid hydrolysis) and enzymatic hydrolysis (Pal and Suresh, 2016). In the study, acid hydrolysis methods are the most used because of cost-effective and simple operative. Extraction of acid soluble collagen has varied methods and collagen characteristics obtained was different. In study by (Sionkowska et al., 2015), the yield of acid soluble collagen from the skin of B. Australis about 1,5 % on a wet weight basis with denaturation temperature was found to be 24 °C. (Wang et al., 2008) reported the predicted yield of ASC was 19.3 ± 0.5% with acetic-acid concentration of 0.54 M, a temperature of 24.7 °C and at time of 32.1 h. Different in this studies by (Li et al., 2013), ASC from the skin of Spanish mackerel with yields of 58.62 ± 1.21 % (on the dry weight) and 13.68 ± 0.35% (on the wet weight) with collagen characteristic of high solubility. Beside that extraction of collagen, the most used in the experiment from fish skin and calf skin, but collagen extraction from goat skin are very limited. So, objective of this work was to collagen extraction from local Indonesian "Kacang" goat skin which has a high population in Indonesian with acid hydrolysis methods and evaluated by using response surface methodology (RSM).

Please cite this article as: Rina Wahyuningsih, Rusman, Nurliyani, Ambar Pertiwiningrum, Abdul Rohman, Nanung A. Fitriyanto, Yuny Erwanto, 2018, Optimization of acid soluble collagen extraction from indonesian local "kacang" goat skin and physico-chemical properties characterization, Chemical Engineering Transactions, 63, 703-708 DOI:10.3303/CET1863118

2. Literature review

Collagen is the major fibrous protein in the extracellular matrix of all multicellular animals, including animal skin, bone, cartilage, tendon, and blood and constitutes approximately 30 % of the total protein content. Collagen has a uniform triple helix structure of three polypeptide chains and every chain repeating of the Gly-X-Y sequence, which X is proline and Y is hydroxyproline (Gómez-Guillén et al., 2011). Most of the collagens used in this experiment reported from the animal skin are type 1 collagen, which form is a heterotrimer composed of two α1 (Gelse et al., 2003) and one α2 chain (Virginia et al., 2016). The absorbance peak derived collagen maximum at 230 – 240 nm and minimum peak at 280 nm with a characteristic peak of the amide I, II, II, and amide A, B. The amino acids in absorbance peak at 280 nm were tryptophan, tyrosine, and phenylalanine (Matmaroh et al., 2011). The microstructure of skin collagen was a fibrous network with thinner collagen fiber. Skin collagen is the most delicate network and proposed to feature more covalent bonds (Oechsle et al., 2016).

3. Methods and materials

3.1 Extraction of acid soluble collagen (ASC)

“Kacang” Goat skin was extracted using the method of (Wang and Yang, 2008) with slight modification. The skin was obtained from the local slaughter house. All of the skin cleaned by a fleshing process to remove flesh, connective tissue and fat, then the skins were shaved to remove most of the hair. One hundred grams of skin was precisely weighed. The skin was mixed with 0.1 M NaOH at 4 °C for 24 h at a ratio of 1:10 (w/v) to remove on non-collagenous proteins. The skin after removal non-collagenous proteins was washed with distilled water till the pH value was neutral. The insoluble matter was extracted with 0,5 M acetic acid for 24, 48, and 72 h. The extract was filtered with Whatman No.1 paper. The collagen was precipitated by adding NaCl to a final concentration of 2.6 M. The resulting sediment was collected by centrifuging at 7,000 g for 30 min and then re-dissolved in 0.5 M acetic acid. The resulting solutions were dialyzed with 0.1 M acetic acid for 24 h with a change of solution once per 3 h and finally a change used distilled water sequentially. The collagen was obtained by freeze-drying.

3.2 Characterization of the extracted collagen

3.2.1 One factor design

Response surface methodology was employed for experimental design, data analysis and model building with software Design Expert (Trial Version 10, Stat-Ease Inc., Minneapolis, Minnesota, USA). One factor design with one variable was used to determine the response pattern and then to establish a model. One variable used in this work was hydrolysis time (X) with three levels (24, 48, 72 h) while the dependent variable was the yield of acid soluble collagen (Table 2). The symbols and levels are shown in Table 1. Three replicates at the central point of the designed model were used estimate the pure error sum of squares. Experiments were randomized to maximize the effects of unexplained variability in the observed responses, due to extraneous factors.

Table 1: One factor design for acid soluble collagen from “Kacang” goat skin

Independent variable	*Symbol*	*Range and levels*		
		-1	*0*	*1*
Hydrolysis time (h)	*X*	*24*	*48*	*72*

Table 2: One factor design and response for the yield of acid soluble collagen from “Kacang” goat skin.

Experiments	Coded levels X Hydorolysis time (h)	Response Yield of acid soluble collagen (%)
1	-1	0.71
2	1	4
3	0	7.35
4	-1	0.71
5	1	4.18

3.2.2 Determination of amino acid

0.05 g sample was hydrolyzed in 5 mL of 6 N HCl in an evacuated and sealed tube at 116 °C for 24 h. Hydrolysate filtered with Whatman no.4 paper and pippeted 0.5 mL was dried with nitrogen. Dry hydrolysate was dissolved in 3 mL of 0.02 N HCl and centrifugated at 3,222 rpm for 15 min. Hydrolysate filtered with Millipore PTFE 0.45 µm paper and pippeted in 20 µL. Hydrolysate was dissolved in 140 µL Bora buffer ACCCQ Fluor and 40 µL ACC Fluor 2A, homogenization. Derivate was heated at 60°C for 10 min and injected in 5 µL to HPLC (System HPLC waters Alliance 2695). FT-IR spectra were obtained from 2 mg collagen in approximately 100 mg potassium bromide (K-Br). All spectra were obtained from 4000 to 1000 cm-1 by using a MBB3000 FT-IR spectrophotometer combined with the intuitive horizon MBTM FTIR software.

4. Results and findings

4.1 Condition for optimum responses

The yield of ASC as a function of the independent variables was represented with mathematical model by the following equation:

$$Y = 7.35 + 1.69X - 4.95X^2 \quad (1)$$

Where Y is the yield of the ASC and X is the code variable for time hydrolysis time.

Table 3: Analysis of variance (ANOVA) for Response Surface Quadratic model of the yield ASC from "Kacang" goat skin

Source	Sum of square	Degrees of freedom	Mean square	F-value	P-value
Model	31,03	2	15,51	1915,21	0,0005
Residual	11,42	1	11,42	1410,42	0,0007
Lack of fit	19,60	1	19,60	2420,00	0,0004
Pure error	0,016	2	8,1		
Total	31,04	4			

The results of anova for response surface quadratic model are showed in Table 3. The coefficient and adjusted coefficient value of the model was 0.9995 (R2 = 0.9995) and 0.9990 (adj R2 = 0.9990), which confirmed was highly significant because it has good value between the predicted and experimental value of collagen yield. The coefficient of determination in anova is a ratio between of the explained variation to the total variation and measurement of the degree fitness (Nath and Chattopadhyay, 2007). The dependent variables in the model indicated a rich relevance if it has high value of R2 and when R2 approaches unity then the model can fit with an actual data (Sin et al., 2006). Besides it, the standard deviation (CV = 2.65) of the model is very highly reliable experimental value and high degree of precision (Song et al., 2011). The model also significant with P-value (Prob > F) and lack of fit were < 0.0005 and 0.0004. The effect of hydrolysis time in experimental was illustrated with using response surface methodology design and it was used to prediction of the yield (Liu et al., 2014). The optimal condition of ASC from "Kacang" goat skin by analysis with RSM shown the highest yield of acid soluble collagen was 48 h hydrolysis. The estimate for collagen content using calculations was 7.35% and using optimum condition for collagen content in experimental value was 7.74%, but in close agreement with the predicated value. The effect of hydrolysis time on the yield of ASC from "Kacang" goat skin is shown in Figure 1a. Collagen of hydrolysis time at 24 h has lower than at 48 h in yield. The yield of ASC was increased of the hydrolysis time at 24 h and decrease of yield at 48 h.

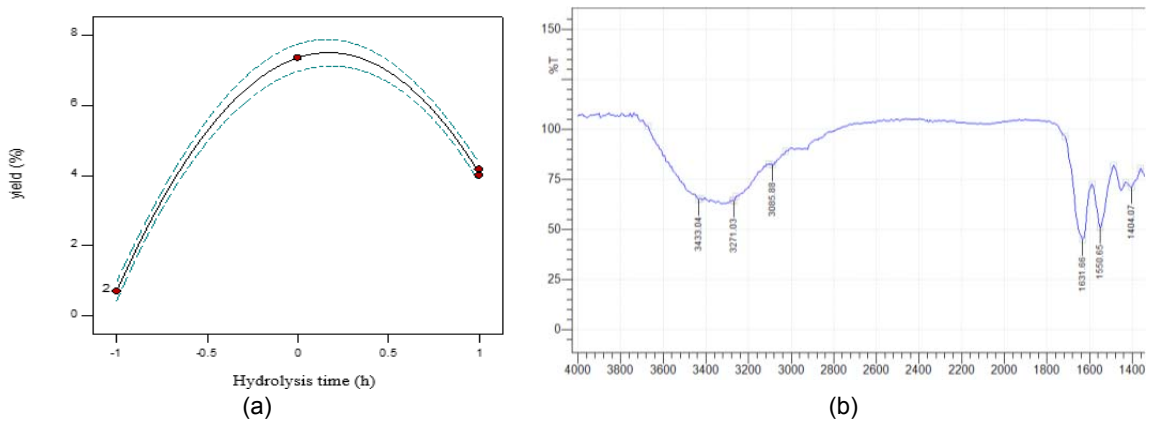
4.2 Amino acid composition

The amino acid composition of ASC from "Kacang" goat skin is presented in Table 4. The result of the research showed collagen contains 30.52 glycine residues/1,000 residues amino acid and this amino acid is a major amino acid residue in collagen. Amino acid contains large amounts of proline, alanine, glutamic acid, aspartic acid, and arginine, but did not contain histidine. Similar to other collagen from pig skin and calf skin showed of glycine is the most dominant amino acid were 340.7 residues/1,000 residues (pig skin) and 330.6 residues/1,000 residues (calf skin), and large amounts amino acids of proline, alanine, glutamic acid and arginine (Li, Wang, 2013). Amino acids are the most in collagen was glycine (Muyonga et al., 2004). Collagen contained glycine about 36% of all amino acid and suggest that collagen type 1. Glycine is the most abundant amino acid in collagen which is regularly spaced at every third residue throughout the central region of the α-chain in collagen (Alberts et al., 2002). Glycine of mammalian collagen is lower than to that fish skin. Fish skin

collagen from Tilapia (Oreochromis sp.) contained 378-390 glycine residues/1,000 residues (Huang et al., 2016). Amino acids of fish collagen are higher than to that mammalian collagen but low imino acids (proline and hydroxyproline). The function of imino acid is solubility, crosslinking ability, and thermal stability of collagen. Imino acids used usually in food and beverage industry (Hashim et al., 2015).

Table 4: Amino acid composition of acid soluble collagen from "Kacang" goat skin (residues/1,000 residues)

Amino acids	"Kacang" goat skin
Aspartic acid	3.32
Serine	1.68
Glutamic acid	3.50
Glycine	30.52
Histidine	0
Arginine	2.43
Threonine	0.45
Alanine	3.53
Proline	4.55
Cysteine	2.18
Tyrosine	0.05
Valine	1.34
Lysine	1.47
Isoleucine	0.54
Leucine	1.60
Methionine	0.31
Phenylalanine	0.94



(a) (b)

Figure 1: (a) Response surface plot showing the effect of hydrolysis time on the yield of ASC from "Kacang" goat skin. (b) FT-IR spectra of ASC from "Kacang" goat skin.

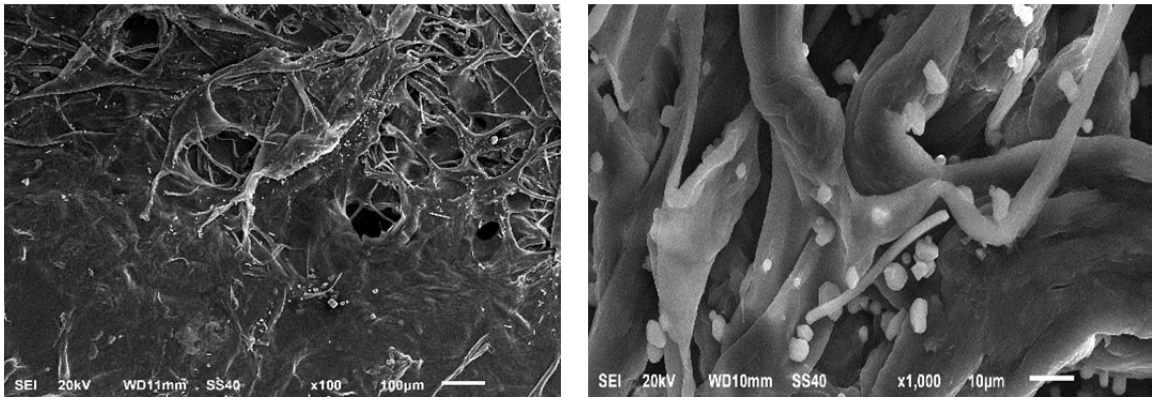
4.3 Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra of ASC from"Kacang" goat skin is shown in Figure 1b. The shape of polypeptide is known to be directly related the regions of amides I, II, and III. N-H streching vibration is related to amide A band (3,400-3,440 cm^{-1}). Stretching vibrations of carbonyl groups in peptides are related to amide I band (1,600 - 1,660 cm^{-1}). NH bending and CN stretching is associated to amide II band (± 1,550 cm^{-1}). CN stretching and NH associated with amide III band (1,320-1,220 cm^{-1}) is involved with the triple helix structure of collagen (Muyonga, Cole, 2004). FT-IR spectra in this study showed regions of amides A, I, II, and III were 3,433, 1,632, 1,551 and 1,404 cm^{-1}. In studies of calf skin by (Woo et al., 2008), regions of amides A, I, II, and III bands were 3,333, 1,660, 1,547 and 1,238 cm^{-1}. Similar to FT-IR spectra in studies of calf skin by (Li, Wang, 2013), who showed regions of amides I band (1,636 cm^{-1}) and amides II bands (1,545 cm^{-1}). FT-IR spectra of "Kacang" goat skin was similar to those of collagens from another mammalian collagen, respectively. Amide A band (3,433 cm^{-1}) of ASC from "Kacang" goat skin had more NH groups involved in hydrogen bonding. According to the report by (Doyle et al., 1975), band 3,400-3,440 cm^{-1} indicated a free stretching vibration and when the NH group of a peptide involved in hydrogen bonding. Amide I band (1,632 cm^{-1}) of "Kacang" goat

skin suggesting the more interaction of C=O with adjacent chains via hydrogen bond of the formers. C=O stretching of the carboxyl group from acetic acid (Kamińska and Sionkowska, 1996) (Farrell et al., 2001) reported the following secondary structures in the amide 1 band regions: β-turn (1,660-1,700 cm^{-1}), α-helix (1,645-1,659 cm^{-1}), irregular structure (1,640-1,644 cm^{-1}), and β-sheet (1,620-1,640 cm^{-1}). Amide II bands of ASC from "Kacang" goat skin was found at the wavenumber of 1,551 cm^{-1}, which indicated that N-H involved in bonding with adjacent α-chains. Mammalian collagen had lower degrees of molecular order than fish collagen (Payne and Veis, 1988). The amide III bands (1,404 cm^{-1}) of ASC from "Kacang" goat skin shown triple helix structure. Wagging vibrations from VH2 groups of the glycine backbone and proline side-chains of combination peak between C-N stretching vibrations and N-H deformation from amide linkages as well as absorption arising (Jackson et al., 1995). Extracted collagen from skin had a higher degree of intermolecular cross-link and molecular order than from bone. The extent of collagen from skin had higher peptide chain unwinding but low existence hydrogen bonding that collagen from bone (Li, Wang, 2013).

4.4 Scanning electron microscopy

Microstructure analysis of collagen using scanning electron microscopy with scale bar 10 μm and 100 μm. In this studies structure of ASC from "Kacang" goat skin had display fibrous structure. The fibrous network had structure is delicate bonding and complex with small fragments (Figure 2). According to the report by (Oechsle, Akgün, 2016), chicken collagen had similar structure with goat skin which thin and clear collagen fibers that also of crosslinking allows the collagen molecules. Fibrous structure correlation with decreasing shear thinning behavior might exist. A study by (Zhou et al., 2016), collagen structure from fish skin had white fiber and loose of the crosslink, it causes by dehydration influence that effect of occurrence polymer condensation.



(a) (b)

Figure 2: SEM of collagen from "Kacang" goat skin (a) scale bar 100 μm, (b) scale bar 10 μm

5. Conclusion

The result in this study was demonstrated an optimum extraction conditions can be adjusted to ASC from "Kacang" goat skin. The collagen has almost similar characteristic and physicochemical properties with collagen from other by-product source. ASC from "Kacang" goat skin can be used as potential substitutes for mammalian or fish collagen. It the advantages can be utilises in functional food, pharmaceutics, and tissue engineering.

Acknowledgements

This research work is supported by DIKTI Ministry of Research, Technology, and Higher Education Indonesia.

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