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Indonesian native chicken eggshell membranes as source for collagen: Optimum conditions and general characteristics

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Abstract

High quantity of Indonesian native chicken eggshell membrane is available as a by-product of the hatchery industry. The eggshell has a membrane-like interior, and still contains approximately 10% of the membrane's total protein. In the present work, membranes from Indonesian chicken eggs sourced from a hatchery could have the potential to be a source of collagen. Based on the interaction of two variables (enzyme concentration and hydrolysis duration), response surface methodology (RSM) with a central composite design was utilised to identify the optimum collagen extraction conditions. Using 5 M acetic acid and pepsin (85, 90, and 95 U/mg defatted skin) at 4°C for 72, 96, and 120 h, the collagen was extracted. The optimal conditions yielded 31% collagen with a hydrolysis duration of 120 h, and an enzyme concentration of 90 U/mg. The optimal yield was characterised using colour analysis, soluble protein, and FTIR. Collagen from the Indonesian native chicken eggshell membrane showed similar properties with standard collagen sources.

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Introduction

Numerous new varieties of livestock are now extensively available in Indonesia for the community's benefit. The community's high interest in maintaining native poultry, particularly for the hatchery business, indirectly resulted in an abundance of eggshells as by-products of the hatchery business. In general, eggshells have not been utilised extensively, despite many eggshell constituents containing excellent protein for further processing. Of the total weight of eggs (60 g), 10% is the eggshells (Nys and Guyot, 2011). The eggshell membranes lie between the liquid white egg and the compounds on the eggshell inner surface. Collagen constitutes as much as 10% of the total membrane protein as one of the membrane constituents. Biochemical, cytotoxicity, and genotoxicity experiments demonstrated in a previous study (Ruff *et al*., 2012) have shown that eggshell membrane collagen is safe for consumption, and does not induce autoimmune responses and allergic reactions. Therefore, the eggshell membrane has the potential to serve as an alternative source of collagen to sources derived from mammals.

To produce a high yield of collagen, it is critical to maximise the extraction process. Hydrolysis duration and enzyme concentration are crucial variables that influence the extractability of collagen. Response surface methodology (RSM) is an efficient experimental design instrument for optimising the production of collagen. A set of mathematical and statistical methods known as RSM is based on fitting a polynomial equation to experimental data to characterise the behaviour of a data set, with the objective of forecasting statistics. It works best when several factors influence an answer or relevant responses. To get the optimal system performance, the goal is to simultaneously optimise the levels of these variables. RSM is a statistical and mathematical technique utilised for optimising processes, products, or systems (Bezerra *et al*., 2008). It proved particularly valuable when handling complex systems where multiple variables influenced the outcome or response (Raissi and Eslami Farsani, 2009). It is widely used in both the creation and

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refinement of new items, as well as in the design of already existing ones. It is the impact of the independent variables on the processes, either separately or in combination. This experimental methodology not only analyses the impact of the independent variables, but also produces a mathematical model that explains the chemical or biochemical process (Farooq Anjum *et al*., 1997; Myers *et al*., 2016). The process levels between the independent and dependent variables anticipated values are optimised. The effects of sodium hydroxide concentration, alkali treatment time, enzyme concentration, and hydrolysis time on the optimum extraction of collagen from eggshell membranes were previously studied by Mohammadi *et al*. (2016). However, there is no information yet reported in the study regarding optimisation and general characteristics of collagen extracted from Indonesian native chicken eggshell membranes.

Therefore, the purpose of the present work was to optimise collagen extraction using membranes derived from Indonesian native chicken eggshells by manipulating enzyme concentration and hydrolysis time. RSM was the experimental design employed in this investigation. RSM facilitated the comprehension of the relationship between input variables (factors) and the output response, enabling the identification of optimal conditions (Gurumurthy *et al*., 2018). Selecting an experimental design that will specify which tests should be conducted in the experimental region under study is a prerequisite to using the RSM. For this, there are a few experimental matrices available. If there is no curvature in the data set, experimental designs for first-order models, such as factorial designs, can be utilised.

Materials and methods

Experimental details and treatments

Experimental material

Indonesian native chicken eggshell membranes was used as the source material for collagen production. It was manually removed by hand with care, and rinsed with distilled water. The pepsin enzyme was obtained from Sigma Aldrich Co. (St. Louis, United States); while sodium hydroxide, sodium chloride, and acetic acid were obtained from Merck (Darmstadt, Germany). Only analytical-grade reagents are used in the present work.

Treatments

Collagen was extracted from the eggshell membrane following modified methods described by Mohammadi *et al*. (2016). The eggshell membranes (5 g) were treated with 0.1 M NaOH at a ratio of 1:30 (w/v) for 24 h at 4°C to remove non-collagenous proteins. Once the pH was neutral, the samples were washed with distilled water, and extracted with acetic acid 0.5 M and pepsin (85, 90, and 95 U/mg of fat-free skin) at 4°C for 72, 96, and 120 h. It was then filtered with Whatman paper to remove particles that had not dissolved. To precipitate the solution, final NaCl concentrations of 2.6 M were employed. Centrifuging at 4,000 rpm for 30 min at 4°C was performed to collect the precipitate, and then redissolved in 0.5 M acetic acid at a 1:5 (w/v) solution ratio. The final solution was dialysed using a dialysis membrane with a weight cut-off in 14 kDa against 0.1 M acetic acid for 24 h at 4°C, with a solution change every 2 h, and then distilled water for 2 h at the same temperature. The solution was lyophilised for 24 h using a freeze-dryer. There were three replications of each treatment. The yield collection of collagens was calculated using the membranes precipitate from a sufficiently moist eggshell as shown in Eq. 1:

$$Yield\ (\%) = \frac{Weight\ of\ precipitate(g)}{Weight\ of\ wet\ eggshell\ membrane\ (g)} \times 100\% \quad \text{(Eq. 1)}$$

Statistical analysis and experimental design

The yield of total collagen was determined using two extraction variables: enzyme concentration and hydrolysis time, along with RSM and Design-Expert 7.0.0 (State-Ease Inc., Minneapolis, USA) software. It was utilised to compute the quadratic polynomial model's coefficients and optimise it with a statistically significant *p*-value of 0.05 or lower. A central composite design (CCD) with two independent variables at three levels was employed to ascertain the cumulative impact of the independent factors on the response. The range and levels of the variables assessed in the present work are shown in Table 1. The present work employed two independent variables, enzyme concentration (A) and hydrolysis time (B), each with three levels, and collagen yield as the dependent variable. All experiments were conducted in triplicates, and results reported as mean.

Colour analysis

The colour intensity of the sample was determined using a chromameter. The chromameter analysis employed a Konica Minolta (Singapore) device with a 3 mm measurement area and a Petri dish measurement type. The chromameter values were *L**: brightness; -*a**: greenish; +*a**: reddish, -*b**: blueness; and +*b**: yellowish, as indicated by the chromameter (Priyadarshini *et al*., 2017). The whiteness value was calculated using Eq. 2:

$$Whiteness = 100 - [(100 - L^*)^2 + (a^*)^2 + (9b^*)^2]^{\frac{1}{2}} \quad \text{(Eq. 2)}$$

Solubility determination

By modulating pH and NaCl concentration, it was possible to determine the solubility of extracted collagens following the procedures of Jongjareonrak *et al*. (2005) and Le Corre-Bordes *et al*. (2018) with minor modifications. The lyophilised collagens were dissolved with moderate agitation for 24 h in 0.5 M acetic acid to a final concentration of 3 mg/mL. A sample volume of approximately 8 mL was conveyed to a centrifuge tube. The pH was modulated across the range of 2, 4, 6, 8, and 10, with 6 N NaOH and 6 N HCl, with variations in the NaCl concentration with the range of 1, 2, 3, 4, 5, and 6%. The volume was brought to a total of 10 mL using distilled water. After 30 min of stirring at 4°C, the solutions were centrifuged at 10,000 *g* for 30 min at 4°C. Lowry's method determined the concentration of proteins in the supernatant (Lowry *et al*., 1951). Protein solubility was computed based on the pH value at which the maximum protein concentration was observed. The relative solubility of collagen was calculated using Eq. 3:

$$Relative\ solubility\ (\%) = \frac{Protein\ concentration\ of\ supernatant}{The\ highest\ protein\ concentration} \times 100 \quad \text{(Eq. 3)}$$

Fourier transform infrared spectroscopy (FTIR)

The sample was analysed using FTIR (Vertex 80, US) to demonstrate the functional groups and bond interactions in the extracted collagen. Potassium bromide was used to prepare the sample, which was then scanned between 650 - 4,000 wavenumbers (cm^{-1}).

Scanning electron microscopy (SEM)

The samples were coated with palladium for about 8 min with the sputtering system (Ion Sputter Hitachi MC1000, Japan). The image was taken by using a scanning electron microscopy from Japan (Hitachi SU3500) under vacuum at 20 kV and magnifications of 100×, 500×, and 1000×.

SDS-page electrophoresis

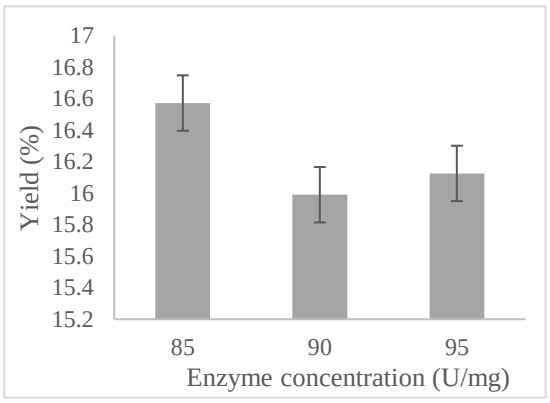
Electrophoresis patterns were measured following the method of Laemmli (1970). The lyophilised collagen was dissolved with 0.5 M Tris HCl buffer pH 6.8 to make 3 mg/mL. The sample was added with loading dye at a ratio of 1:1, and heated for 2 min. Polyacrylamide gel was prepared with 7.5% gradient gel and 4% stacking gel. The sample solutions were loaded into each gel, and electrophoresed for about 2 h at a constant voltage of 110 V. Gel after electrophoresis was stained (50% methanol, 40% H_2O, 10% acetic acid, and 1% Brilliant blue) and destained (50% methanol, 40% H_2O, and 10% acetic acid). Finally, the gel solution was changed with 10% acetic acid.

Results and discussion

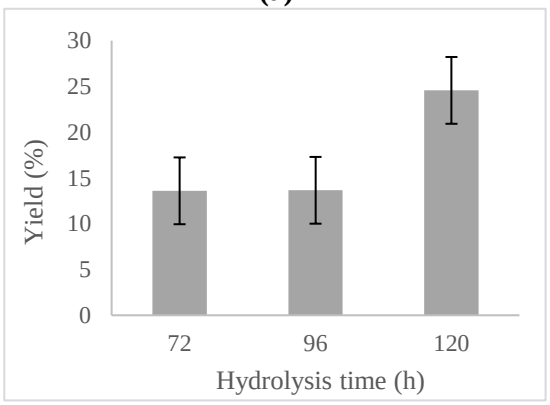
Effect of enzyme concentrations and hydrolysis times on extraction yields of collagen

Figure 1 depicts the impact of enzyme concentrations and hydrolysis times on the collagen extraction process. The optimal collagen extraction yield was determined by analysing the effect of enzyme concentrations of 85, 90, and 95 U/mg and hydrolysis durations of 72, 96, and 120 h. Figure 1 demonstrates that the enzyme concentration of 95 U/mg decreased the collagen yield when the hydrolysis duration was extended to 120 h, whereas the enzyme concentrations of 85 and 90 U/mg increased the collagen yield with an increase in hydrolysis time.

Figure 1a depicts the enzyme concentration effect on the collagen extraction yield. Enzyme concentrations of 90 U/mg were optimal for extraction because at this concentration, the enzyme could break down protein peptide bonds, thus the resulting yields were more optimal compared to concentrations of 85 and 95 U/mg. At an enzyme concentration of 85 U/mg, the enzyme did not optimally break down the crosslinks of the collagen peptide bonds, whereas at a concentration of 95



(a)



(b)

Figure 1. Effects of different **(a)** enzyme concentrations and **(b)** hydrolysis times on extraction yield of collagen from Indonesian native chicken eggshell membrane.

U/mg, the resulting yield was higher. But, if it was hydrolysed longer, the resulting yield was not optimal because the collagen protein bonds were destroyed, thus damaging the protein structure in collagen, especially the amino acid composition. The yield decreased as enzyme concentration increased. Mohammadi *et al*. (2016) found that a concentration of 45 to 60 U/mg of enzyme increased the yield. Other investigation conducted by Yu *et al*. (2011) found that an increase in enzyme concentration from 15 to 45 U/mg increased collagen yield from 22.5 to 30.9%, whereas the enzyme concentration used to produce collagen in the present work, which was more expensive industrial extraction method, was 15 - 45 U/mg.

Based on RSM, a maximum collagen yield (31%) was reported when the hydrolysis time was 120 h and the enzyme concentration was 90 U/mg; the length of the hydrolysis process has an impact on the collagen extraction process efficiency because pepsin can break the telopeptide crosslink that holds collagen in the eggshell membrane, causing the collagen to dissolve. In the study by Mohammadi *et al*. (2016), the optimal hydrolysis time for eggshell membrane yield PSC was 36 - 48 h, with a 29.6% yield. The present work revealed that collagen production from membranes of native chicken eggshells was too long because the source material was too desiccated, resulting in a sluggish mass transfer rate to the matrix. The mass transfer rate to the matrix is fundamental for the efficiency of collagen extraction. When a matrix of the eggshell membranes release rate is present in the extraction medium, collagen hydrolysis can be too long.

Optimisation extraction of collagen

Model fitting and optimisation

In the present work, the experimental ranges and values of independent variables and levels in the RSM design for collagen from Indonesian native chicken eggshell membrane are illustrated in Table 1. The experimental design for optimising 13 trials using the CCD with two independent variables (A: enzyme concentration, and B: hydrolysis time) is presented in Table 2. The present work indicated that the collagen yield ranged from 10 - 31%. Maximum collagen yield (31%) was observed under conditions of enzyme concentration of 90 U/mg and hydrolysis time of 120 h. Mathematically, the response variable represented yield using Eq. 4:

$$Y = 14.17 - 0.33A + 5.50B - 3.25AB - 2.10A^2 + 6.40B^2 \quad \text{(Eq. 4)}$$

Table 3 displays the results of analysis of variance for the response surface. The model *F*-value of 3.48 indicated a 6.73% probability. Values of "Prob > F" less than 0.05 indicated significant

Table 1. Experimental ranges and values of independent variables and levels in the RSM design for collagen from Indonesian native chicken eggshell membrane.

Independent variable	Symbol	Coded factor level		
		-1	0	+1
Enzyme concentration (U/mg)	A	85	90	95
Hydrolysis time (h)	B	72	96	120

Table 2. Central composite structure of experiment and response for yield of collagen from Indonesian native chicken eggshell membrane.

Run order	Independent variable		Response extraction yield (R1, %)
	Enzyme concentration (A, U/mg)	Hydrolysis time (B, h)	
1	0	-1	15
2	0	0	13
3	0	+1	31
4	+1	-1	14
5	-1	1	27
6	0	0	18
7	-1	-1	12
8	+1	+1	16
9	0	0	10
10	+1	0	18
11	0	0	14
12	0	0	11
13	-1	0	11

Table 3. ANOVA for collagen yield quadratic model's response surface from Indonesian native chicken eggshell membranes.

Source	Coefficient	Sum of square	*df*	Mean square	*F*-value	*p*-value Prob > *F*
Model		337.78	5	67.56	3.48	0.0673
A-X1		0.67	1	0.67	0.034	0.8582
B-X2		181.50	1	181.50	9.35	0.0184
AB		42.25	1	42.25	2.18	0.1837
A2		12.22	1	12.22	0.63	0.4536
B2		113.01	1	113.01	5.82	0.0466
Residual		135.91	7	19.42		
Lack of fit		97.11	3	32.37	3.34	0.1374
Pure error		38.80	4	9.70		
Cor total		473.69	12			
Standard deviation	4.41					
CV	27.28					
PRESS	930.02					
Adj-R-squared	0.5081					
Adequate precision	5.846					

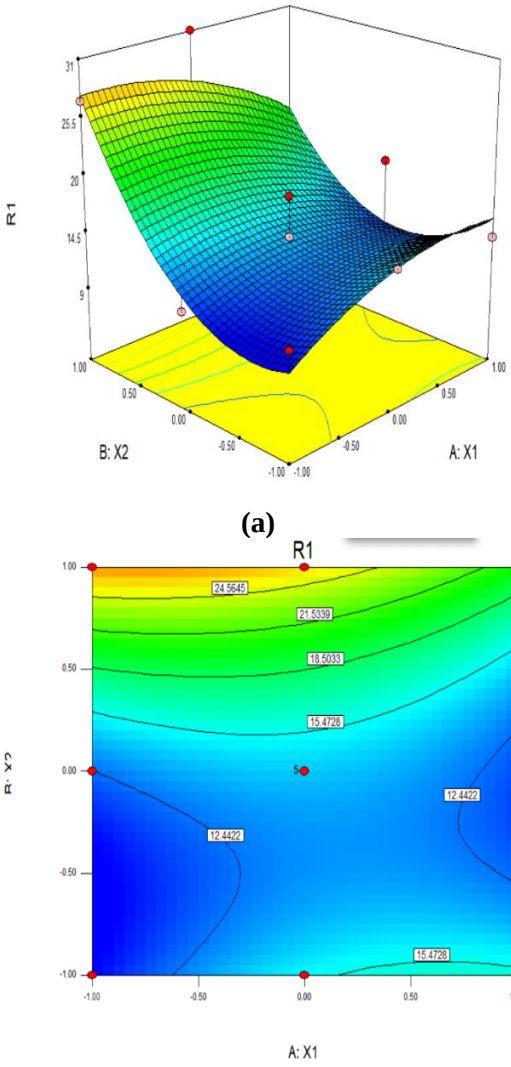
model terms. B and $square\ dare$ significant model parameters in this instance. The model parameters were not significant if their values exceed 0.1 points. The "Lack of Fit *F*-value" of 3.34 indicated that the lack of fit was negligible in comparison to the unadulterated error. A "Lack of Fit *F*-value" and a non-significant lack of fit had a 13.74% probability of being positive. A negative "Prediction of R-Squared" indicated that the mean predicted the data response more accurately than the current model. "Adequate Precision" is a signal-to-noise ratio meter. A ratio greater than four is preferred. The data ratio of 5.846 indicated that the signal was adequate. This model facilitated the design space navigation.

RSM demonstrated the effect of enzyme concentrations and hydrolysis times during the collagen extraction process by optimising collagen extraction conditions. The present work employed a CCD experimental design with the following components: a fractional factorial design, a secondary design, and a central point (Bezerra *et al*., 2008). Wang *et al*. (2019) found that using the regression model equation in RSM, 3D response surface, and 2D contour plots, the two variables in the study can be used to predict the yield of collagen production. Insignificant interactions between the corresponding variables were represented by a circular contour plot, while significant interactions were represented by an elliptical contour plot (Muralidhar and Sarathy, 2006).

Figure 2a shows the 3D response surface, while Figure 2b shows contour plots for showing the effects of enzyme concentration and hydrolysis duration on the yield of collagen. The yield ranged from 10 - 31%. Maximum collagen yield (31%) was observed under conditions of enzyme concentration of 90 U/mg and hydrolysis time of 120 h. Mohammadi *et al*. (2016) determined that the optimal conditions for extracting collagen from eggshell membranes were sodium hydroxide concentration of 0.76 mol/L, alkali treatment time of 18 h, enzyme concentration of 50 U/mg, and hydrolysis time of 43.42 h, with a yield of 30.049%. With a hydrolysis time of 24 h by Wahyuningsih *et al*. (2018), they found a maximum yield of 93.94% from a sample of "*kacang*" goat skin from Indonesia. Dhakal *et al*. (2018) found the ideal circumstances for collagen extraction from chicken feet to be 28 h at a yield of 30.05%, while Arumugam *et al*. (2018) found the optimal conditions for collagen extraction from sole fish skin to be 32.32 h at a yield of 19.27 mg/g. The yield of the present work was identical to that of previous studies, even though enzyme concentrations and hydrolysis time were different. Several factors, including the origin and nature of the sample, the species of eggs used, and the quantity of protein in the sample, could have caused this.

Colour analysis

The outcome of the colour analysis is depicted in Figure 3. The eggshell membrane had coordinates of 90.42, -0.3, and 13.3 for *L** (brightness), *a** (+*a**: redness; -*a**: greenness), and *b** (+*b**: yellowness; -*b**: blueness). The whiteness value was 83.6%. This



(a)

(b)

Figure 2. (a) 3D response surface and **(b)** contour plots showing the effects of enzyme concentrations and hydrolysis durations on collagen yields. R1: yield of collagen (%); A: concentration of enzyme (U/mg); and B: hydrolysis duration (h).

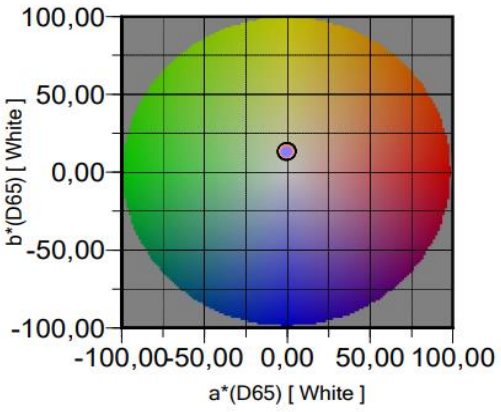


Figure 3. Colour measurement of collagen from Indonesian native chicken eggshell membrane.

indicated that the collagen from Indonesian native chicken eggshell membranes had a luminosity value of 90.42, with collagen from bone having the maximum brightness and collagen from scales having the lowest. According to Keskin *et al*. (2017), *L** value between 0 and 50 indicates darkness, while *L** value between 51 and 100 indicates luminosity. According to Jamilah *et al*. (2013), higher *L** value represents luminous sample. The second colour analysis based on the value of *a** (-) or the colour's intensity is greenness; the sample had a greenness colour intensity. The third colour analysis is based on the yellowness of the *b** component. The greater value of *b** is yellow, and the sample had this hue. The value of *b** indicated that the material is distinct from collagen. The number of pigments in the primary material that can be released during the curing process of sodium hydroxide affected the various colours of collagen. The quality of collagen is characterised by its white colour. Collagen having a near 100% whiteness value is of high quality. The luminance value of collagen is an essential parameter for determining its quality, as it can influence the pigment of the completed product.

Soluble protein concentration

pH effect on collagen solubility

The solubility of collagen was affected by the pH levels 2, 4, 6, 8, and 10. Figure 4 shows the effect of pH on collagen solubility. The collagen solubility of each sample increased as the pH decreased. Figure 4 indicates that collagen solubility in the treatment at pH 2, 4, and 6 was significantly greater than in the treatment at pH 8 and 10. At pH 8, the highest collagen solubility was found at 117 ± 0.04 ppm. The solubility of collagen decreased at pH 4, and increased between pH 6 and 8, but decreased again at pH 10. Li *et al*. (2013) reported that the collagen solubility of ASC derived from Spanish mackerel skin decreased at pH 7. According to Woo *et al*. (2008), the collagen solubility of ASC from yellowfin tuna dorsal skin increased at pH 4, stable at pH 5 to 6, then increased again at pH 7, and was comparatively stable up to pH 9. According to Kittiphattanabawon *et al*. (2005), the collagen of bigeye snapper (*P. tayenus*) dissolved least at pH 7 to 8, and most at pH 2 and 5. The value of the point of iso-electricity (pI) is affected by protein solubility; when pI is less than or greater than pH in a protein solution, it will increase protein solubility because of an increase in the repulsive force between the residues of protein molecules with positive or negative charges, whereas pI induces aggregation and precipitation due to interactions between hydrophobic sites (Wong *et al*., 1989; Strasburg and Xiong, 2017). The pH range for collagen is 6 - 9 (Strasburg and Xiong, 2017).

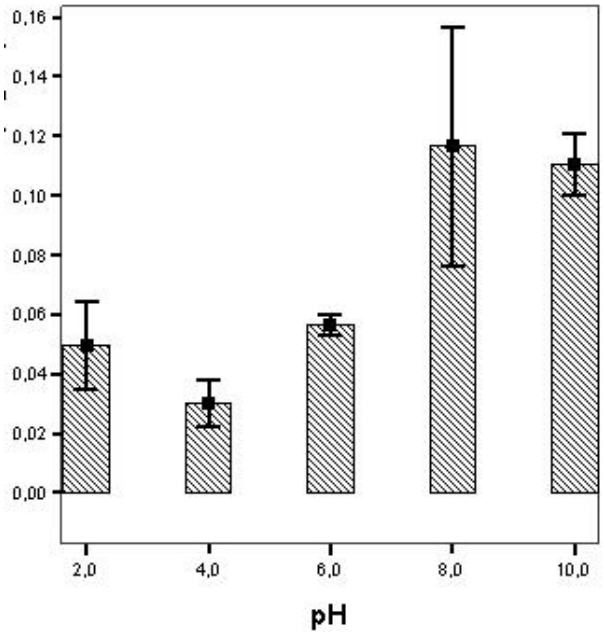


Figure 4. Collagen solubility from Indonesian native chicken eggshell membrane in different pH levels.

NaCl effect on collagen solubility

Figure 5 displays the solubility of collagen at various concentrations of NaCl, including 1, 2, 3, 4, 5, and 6%. The solution with the highest collagen solubility was 5% NaCl at 129 ± 0.001 ppm. According to Li *et al*. (2020), the capability of Nile tilapia epidermal collagen to be dissolved using chemical and fermentation processes was 2 and 1%, respectively. According to Li *et al*. (2013), the collagen solubility of ASC from the skin and bone of Spanish mackerel (*Scomberomorus phonics*) was constant in NaCl concentrations up to 2% (w/v), and decreased by 3% (w/v) and further. In the investigation conducted by Woo *et al*. (2008), the solubility of ASC derived from yellowfin tuna dorsal skin collagen increased as the concentration of NaCl increased from 1 to 2% (w/v). This could have been due to the structure of ASC from Indonesian "*kacang*" goat skin from the structure of fish collagen. In the present work, the collagen solubility decreased at a concentration of 3% NaCl, and increased at 5% NaCl. At 4% NaCl, the collagen structure was considerably more stable. Collagen solubility affects the salting-out in the extraction

process; so when NaCl concentration was increased, salting-out was affected. Due to increasing ionic strength, collagen solubility is decreasing, so salt ions compete with water and protein precipitates (Hall, 1996). Collagen solubility decreases when an increase in NaCl concentration causes the protein to precipitate as a result of chain aggregation, ionic salt competition with water, and the enhancement of hydrophobic-hydrophobic interactions (Jongjareonrak *et al*., 2005; Bae *et al*., 2008).

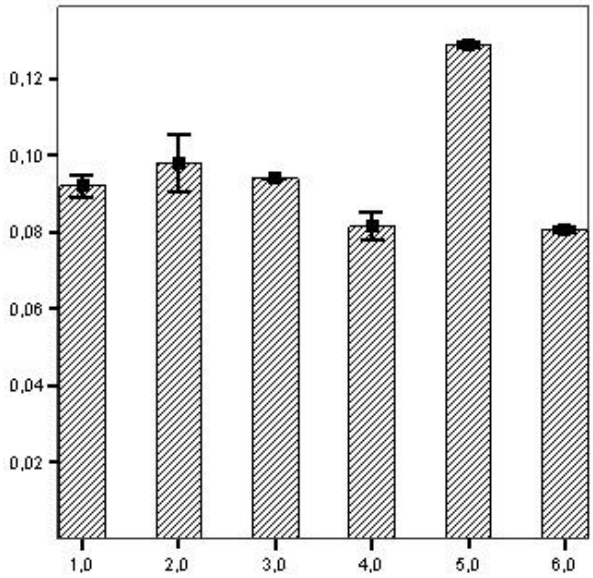


Figure 5. Collagen solubility from Indonesian native chicken eggshell membrane in different NaCl concentrations.

FTIR

Figure 6 depicts the amide peaks of amides A, B, I, II, and III in the FTIR spectrum of collagen from the chicken eggshell membranes. The was a relationship between collagen amides I, II, and III, and the polypeptide's conformation. The amide A peak at 3400 - 3440 cm^{-1} revealed N-H stretching vibration, the amide I peak at 1600 - 1660 cm^{-1} revealed peptides with carbonyl group stretching vibration, the amide II peak at 1550 cm^{-1} revealed CN stretching and NH bending, and the amide III peak revealed that collagen possessed a triple helix structure (Muyonga *et al*., 2004). Amide A peaked at 3291.49 cm^{-1}, amide B peaked at 2936.87 cm^{-1}, amide I peaked at 1642.99 cm^{-1}, amide II peaked at 1552.85 cm^{-1}, and amide III peaked at 1410.97 cm^{-1} in the present work. The FTIR spectra contained peaks that resembled those of collagen. N-H stretching vibration in collagen occurs at 3328 cm^{-1}, amide A at 3291.72 cm^{-1}, amide B between 3077.83 and 2944.65 cm^{-1}, amide I between 1625 and 1690 cm^{-1}, and amide II between 1543.03 and 1450.85 cm^{-1}. According to Arumugam *et al*. (2018), collagen from the epidermis of sole fish had amide I at 1650.77 cm^{-1}, amide II at 1541.81 cm^{-1}, and amide at 1238.08 cm^{-1}. Dhakal *et al*. (2018) stated that chicken feet collagen had amide A at 3464.31 - 3422.48 cm^{-1}, amide B at 2927.47 - 2852.74 cm^{-1}, amide I at 1639.42 - 1656 cm^{-1}, and amide II at 1555.06 - 1451.95 cm^{-1}. In other study by Song *et al*. (2021), amide A was detected at 3335 and 3326 cm^{-1}, amide B at 2930 cm^{-1}, amide I at 1652 and 1654 cm^{-1}, and amide II, III at 1550 and 1240 cm^{-1}. The present work's collagen had amide A peak similar to that of standard collagen from other sources, and are shown in Table 4, indicating that NH groups are present in the collagen structure; the amide B peak was lower than that of standard collagen, indicating the presence of CH_2 in the asymmetrical stretch; the amide I peak was within the range of normal collagen; and the amide II peak was higher than that of standard collagen, indicating CN stretching vibration and CH stretching of NH bond vibrations.

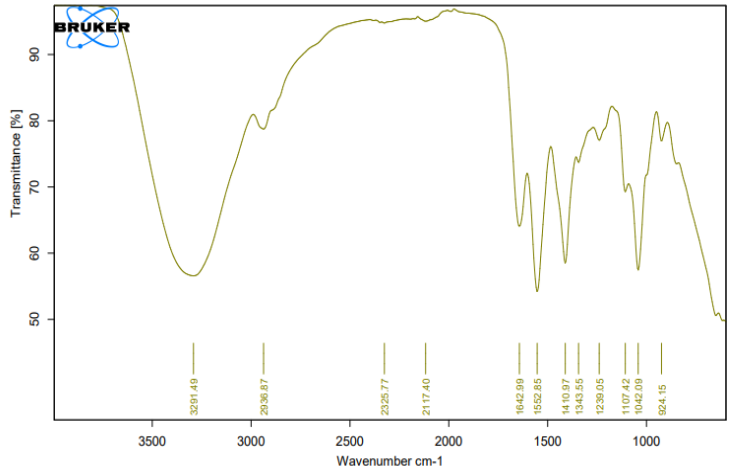


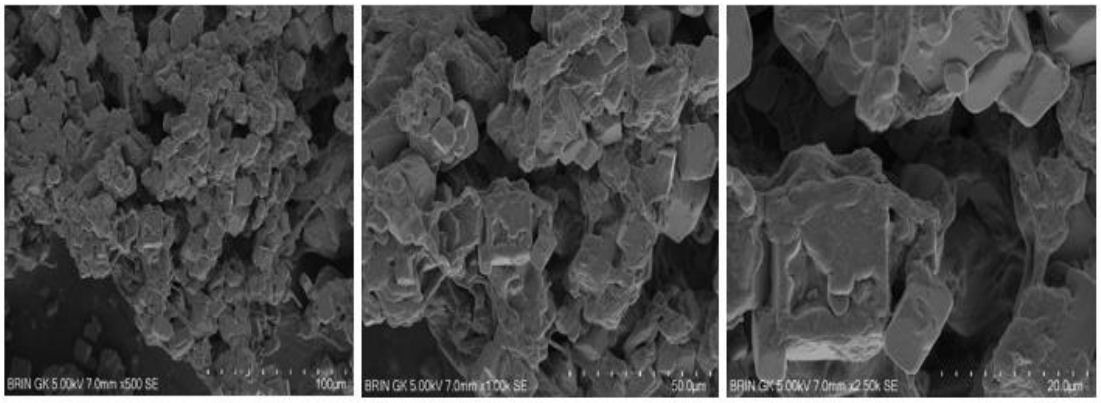
Figure 6. FTIR spectra of collagen from Indonesian native chicken eggshell membrane.

Table 4. FTIR spectra of collagen from other sources and assignment.

Region	Peak wave number (cm^{-1}) of collagen source					Assignment
	Standard collagen	**Native chicken eggshell membranes (this study)**	**Lamb feet (Ata *et al.*, 2022)**	**Rabbit skin (Toniasso *et al.*, 2022)**	**Chicken skin (Zhou *et al.*, 2016)**	
Amide A	3400 - 3440	3291.49	3303 - 3307	3433	3308	A free stretching vibration in the NH
Amide B	2920	2936.87	2922 - 2924	2925	2932	Asymmetric stretching of the CH2 stretching vibration
Amide I	1600 - 1700	1642.99	1631 - 1636	1652	1629	Connected to the carbonyl groups stretching vibrations along the polypeptide backbone
Amide II	1550 - 1600	1552.85	1547 - 1549	1558	1548	Coupled with a C-N stretching vibration are N-H flexion vibrations
Amide III	1200 - 1400	1410.97	1236 - 1237	1238	1242	Vibrations of N-H flexion combined with a vibration of C-N stretching

SEM

The microstructures of collagen from Indonesian native chicken eggshell membranes are shown in Figure 7. They were globular, strongly bound, hard, and had small fragments. Study from Song *et al*. (2021) observed that the microstructures of collagen from the Nile tilapia skin were porous, loose, and fibrillar. Collagen has filament's fibrous structure, irregularity, and density (Arumugam *et al*., 2018). Chicken collagen has thin, clear fibres, and crosslinking in molecule of the structure (Oechsle *et al*., 2016).



(a) **(b)** **(c)**

Figure 7. Morphology of collagen from Indonesian native chicken eggshell membranes: **(a)** scale bar 100 µm × 500; **(b)** scale bar 500 µm × 1,000; and **(c)** scale bar 200 µm × 2,500.

SDS-PAGE

The SDS-page patterns of collagen from Indonesian native chicken eggshell membranes are shown in Figure 8, and have γ, β, α1, and α2 chain with molecule weight 218.51 - 88.34 kDa. The molecular weight of collagen in the present work was similar with collagen from other sources and indicated as type I collagen. The α1 and α2 chains of molecular weight are typical of type I collagen (Gelse *et al*., 2003). Study from Faralizadeh *et al*. (2021) reported that collagen from calf skin had α1 and α2 chains with molecular weight of 100 kDa, and dimer (β chain) of 240 kDa. The molecular weights of skin collagen and swim bladder collagen from Gulf corvina were 117 - 110 kDa and 116 - 109 kDa, with dimers (β chains) of 200 kDa (Cruz-López *et al*., 2021).

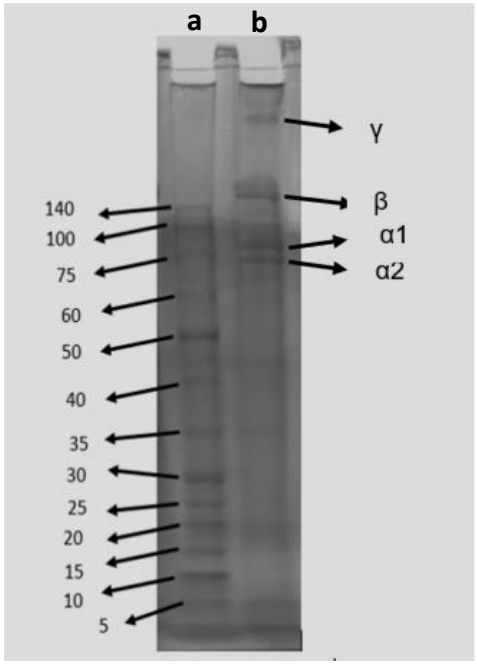


Figure 8. Molecular weight of collagen from Indonesian native chicken eggshell membrane: **(a)** protein marker; and **(b)** collagen from Indonesian native chicken eggshell membrane.

Conclusion

The present work found that Indonesian native chicken eggshell membranes yielded 31% collagen with a concentration of enzyme of 90 U/mg, and a hydrolysis duration of 120 h. Based on the colour, soluble protein content, FTIR, SEM, and SDS-page analyses, the collagen shared similarities with other collagen sources such as rabbit skin, fish skin, calf skin, goat skin, and lamb feet. Therefore, Indonesian native chicken eggshell membranes have the potential to serve as collagen raw material, and can supplement other raw materials commonly used to produce collagen.

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