Original Research Paper

Optimization of Conditions for Extraction of Pepsin-Soluble Collagen from Indonesian Local "Kacang" Goatskin by Response Surface Methodology

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Abstract: Indonesian local "Kacang" goat is an animal species widely known by consumers over the world which possesses many health effects. Goatskin is one of body parts which has potential as collagen sources. The optimal conditions for collagen extraction were determined by response surface methodology in this study. Multiple response optimization designs were applied to evaluate the effects of two independent variables (curing time of NaOH and hydrolysis time) on the pepsin-soluble collagen yield of Indonesian local "Kacang" goatskin. Correlation analysis of the mathematical regression model indicated that quadratic polynomial model could be employed to optimize the extraction of pepsin-soluble collagen. The optimal conditions to obtain the highest yield of pepsin-soluble collagen was without curing NaOH and 24 h time of hydrolysis.

Keywords: Indonesia Local "Kacang" Goatskin, Pepsin-Soluble Collagen, Extraction, Response Surface Methodology

Introduction

Indonesian local "Kacang" goatskin has a high population in Indonesian and it is not too exposed as collagen sources especially as healthy products. Previous research has many reported the extraction of collagen sources from by-products of bovine, pig, chicken, rabbit and fish. However, there was no information about collagen extraction from goatskin. Collagen is a product which highly demanded and it used in the cosmetic, pharmaceutics and food industry. Collagen can be found in all animal parts but is mainly concentrated the skin and bone (Nakamura *et al*., 2003). Collagen is the product of an almost continuous repeating of the Gly-X-Y-sequence and the most abundant structural protein has formed as a unique triple helix by three almost identical polypeptide amino acids chain (Pal *et al*., 2015; Gómez-Guillén *et al*., 2011).

Collagen extraction consist of two main steps, pretreatment of raw materials and extraction of collagen. Raw materials used for collagen preparation contains non-collagenous proteins, lipids, pigments, etc. It is important to remove impurities materials to increase the quality of the final extracted collagen (Regenstein and Zhou, 2007). NaOH is a widely used method to remove non-collagenous proteins and pigments (Pal *et al*., 2015; Benjakul *et al*., 2012).

The extraction process of collagen usually use acid and pepsin method with different time and concentration. The study reported variant amounts of collagen from fish skin and rabbit skin. PSC from Grass Carp skin has yield 25.5 and 19.8% with optimal condition 72 h time of hydrolysis (Wang *et al*., 2014). Catla skin which extracted using pepsin has yield 69.53% with a time of hydrolysis was 48 h (Pal *et al*., 2015). PSC yield from rabbit skin with enzymatic treatment for 48 h was 71% (Pimentel-gonzález *et al*., 2015).

Therefore, skin as a material to prepare collagen is an exciting attempt at adding the value of the collagen. The raw material process (time of pretreatment) and extracted of collagen (time of hydrolysis) was important variables affecting the extractability of collagen especially collagen from

Indonesian local "Kacang" goatskin. Response surface methodology is a statistical method used to quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equation (Triveni *et al.*, 2001). The basic principle of response surface methodology is the evaluation of the relationship between predicted values the dependent variable and the conditions of dependent variables (Edwards and Jutan, 1997). The experimental methodology can generate a mathematical model and optimize the process levels (Baş and Boyaci, 2007; Yang *et al.*, 2008). Publication of collagen extraction so far with response surface methodology is very limited. The objective of this work was to investigate the collagen extraction from local Indonesian "Kacang" goatskin which has a high population in Indonesian, mainly to the effects of above two variables on the yield of pepsin-soluble collagen by response surface methodology.

Materials and Methods

Materials

Indonesian local "Kacang" goatskin was obtained from local slaughterhouse with male sex and age in the range of one year. After being killed, the skins were washed, fleshing and unhairing to remove hair, fat, tissue and meat. Fresh skins were cleaned by washing with tap water, drained, cut into a small square of approximately 1 cm^2 and stored at -18°C until used for extraction.

Chemical

Sodium hydroxide and sodium chloride were purchased from Merck KgaA (Germany), acetic acid was purchased from Sigma-Aldrich (Germany) and pepsin from porcine gastric mucosa was purchased from Merck (Germany).

Extraction by Pepsin Soluble Collagen

Around one hundred grams of skin was thawed in room temperature for 1 hour and precisely weighed. The skin was mixed with 0.1 M NaOH at 4°C for 0 h, 24 h and 48 h ratio of 1:10 (w/v) to remove non-collagenous proteins. The insoluble matter was extracted with 0.5 M acetic acid containing 0.1% pepsin for 24, 48 and 72 h at 38°C. The extract was filtered with Whatman No.1 paper. The collagen was precipitated by adding NaCl at final concentration of 2.6 M. The pellet was collected by centrifuging at 7000 g for 30 min at 4°C and then re-dissolved in 0.5 M acetic acid. The resulting solutions were dialyzed against 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. The experiment was conducted for 5 replications for each treatment.

Table 1: Experimental range and values of the independent variables in the multiple responses optimize design for pepsin-soluble collagen from Indonesian local "Kacang" goatskin

Independent variables (h)	Symbols	Range and levels		
		-1	0	+1
Curing time of NaOH	X1	0	48	72
Hydrolysis time	X2	24	48	72

Multiple Response Optimization Designs

Response surface methodology was employed for experimental design, data analysis and model building with software Statgraphics Centurion version 15.2.11.0. The various responses optimize designs with two variables were used to determine the response pattern and then to establish a model. Two independent variables used in this study were curing time of NaOH (X1) and time of hydrolysis (X2) with three levels for each variable, while dependent variable was the yield of pepsin-soluble collagen. The ranges and center point values of two independent variables were based on the results of preliminary experiments in Table 1.

Results

Effects of Curing Time of NaOH and Hydrolysis Time on the Yield of Pepsin-Soluble Collagen

The effects of curing time of NaOH and hydrolysis time on the yield of pepsin-soluble collagen from Indonesian local "Kacang" goatskin was shown in Fig. 1. The yield of pepsin-soluble collagen was decreased with the extension time of treatment of NaOH and hydrolysis time with pepsin. Especially when the time was within the range treatment time of NaOH and hydrolysis time with pepsin 0 h, the effect of amount yield of pepsin-soluble collagen. Process treatment time and hydrolysis time can be causes of collagen hydrolyzed.

Model Fitting Data Analysis and Conditions for Optimum Responses

All 27 experimental points were evaluated and the results of the dependent variable (Y) for symbols and levels are shown in Table 2. This Table 3 shows each of the estimated effects and interactions. Also shown is the standard error of each of the effects, which measures their sampling error. Note also that the largest variance inflation factor (V.I.F.) equals 1.33333. For a perfectly orthogonal design, all of the factors would equal 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects.

This pane displays the regression equation which has been fitted to the data. The equation of the fitted model is:

$$Yield = 4.61111 - 5.72222X1 - 28.2778X2$$
$$+28.0667X1^2 + 2.25X1X2 + 25.0667X2^2 \quad (1)$$

where the values of the variables are specified in their original units.
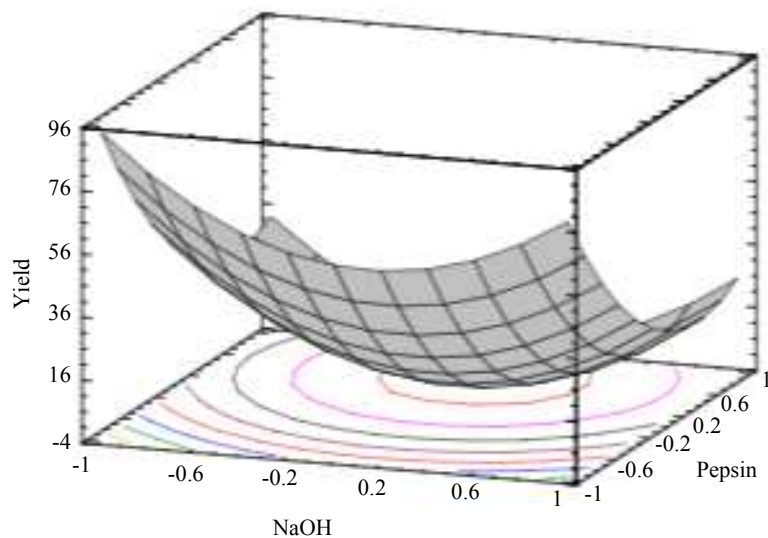

Fig. 1: Response surface plots for optimization of pepsin-soluble collagen from Indonesian local "Kacang" goatskin. NaOH (X1, curing time of NaOH, h), Pepsin (X2, hydrolysis time, h), Yield (Y, contents, %)

Table 2: Multiple responses optimize design and the response for the yield of pepsin-soluble collagen from Indonesian local "Kacang" goatskin

| | Coded levels | | Response |
| | --- | --- | |
Experiment	X1 Curing time of NaOH (h)	X2 Hydrolysis time (h)	Yield (Y) of pepsin-soluble collagen (%)
1	-1	-1	58.00
2	-1	0	31.00
3	-1	+1	17.00
4	-1	-1	133.00
5	-1	0	46.00
6	-1	+1	15.00
7	-1	-1	156.00
8	-1	0	10.00
9	-1	+1	9.00
10	0	-1	14.00
11	0	0	9.77
12	0	+1	45.00
13	0	-1	12.00
14	0	0	15.71
15	0	+1	33.00
16	0	-1	13.00
17	0	0	12.42
18	0	+1	37.00
19	+1	-1	146.00
20	+1	0	23.00
21	+1	+1	10.00
22	+1	-1	112.00
23	+1	0	17.00
24	+1	+1	9.00
25	+1	-1	46.00
26	+1	0	24.00
27	+1	+1	6.00

Table 3: Estimated coefficients of the fitted quadratic polynomial equation for the response of Y (contents, %) based total error of degree of freedom

Effect	Estimate	Standard error	V.I.F
Average	4.61111	15.9904	
X1	-11.44440	17.5166	1.00000
X2	-56.55560	17.5166	1.00000
X1X1	56.13330	30.3396	1.00000
X1X2	4.50000	21.4533	1.00000
X2X2	50.13330	30.3396	1.00000
Block	3.86889	20.2264	1.33333
Block	-2.41778	20.2264	1.33333

X1: Curing time of NaOH (h), X2: Hydrolysis time (h)

Table 4: Analysis of variance for the response surface quadratic model of the yield of pepsin-soluble collagen from Indonesia local "Kacang" goatskin

Source	Sum of squares	Df	Mean square	F-ratio	P-value
X1	589.3890	1	589.3890	0.43	0.5214
X2	14393.4000	1	14393.4000	10.42	0.0044
X1X1	4726.4300	1	4726.4300	3.42	0.0799
X1X2	60.7500	1	60.7500	0.04	0.8361
X2X2	3770.0300	1	3770.0300	2.73	0.1149
Blocks	51.5693	2	25.7846	0.02	0.9815
Total error	26233.9000	19	1380.7300		
Total (corr.)	49825.5000	26			

X1: Curing time of NaOH (h), X2: Hydrolysis time (h)

The ANOVA (Table 4) partitions the variability in yield into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 1 effects have P-values less than 0.05, indicating that it was significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 47.3484% of the variability in Yield.

The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables is 34.8123%. Standard error of the estimate shows was the standard deviation of the residuals to be 37.1582. Mean Absolute Error (MAE) of 25.5298 is the average value of the residuals. P-value is greater than 5.0%; there is no indication of serial autocorrelation in the residuals at the 5.0% significance level. The predicted content of the dependent variable (Y, contents) was 93.9944% from calculations using the model equation derived from the above optimal conditions. This study the optimal conditions to obtain the highest yield of pepsin-soluble collagen was curing time of NaOH 0 h and hydrolysis time 24 h.

Discussion

Collagen extraction consists of two main steps, pretreatment of raw materials and extraction of collagen. The purpose of pretreatment is performed to facilitate the contaminant removal (non-collagenous proteins, fats, pigments, hairs, meat and so on) and maximum collagen extraction (Pal and Suresh, 2016). NaOH is alkaline

pretreatment to remove the non-collagenous proteins and pigments (Pal *et al*., 2015; Benjakul *et al*., 2012). In this study was a show of pretreatment with NaOH can affect the yield of collagen. Curing of NaOH in this study not successful significant because of the longer curing time then, the less of the yield of collagen produces. Extension of NaOH of raw material from Indonesian local "Kacang" goatskin can effect of collagen remove and causes of decreasing the yield of collagen produces.

Previous research founded acid soluble collagen from Black tilapia has yield production was 5.97% with 0.75 M of acetic acid lower than yield production of PSC (Sahubawa and Putra, 2011). Extraction of collagen using enzymatic extraction process has been developed to maximize of collagen yield. Various enzymes to extraction of collagen such as pepsin, trypsin and collagenase have been used to maximize the yield of collagen (Benjakul *et al*., 2012; Regenstein and Zhou, 2007). The enzyme used in the most to the extraction of collagen is pepsin and can be used either in combination with acetic acid or alone after initial extracting of collagen within acetic acid. This study showed that use of limited pepsin to extraction of collagen can be increased the yield of collagen (Liang *et al*., 2014; Wang *et al*., 2014; Pal *et al*., 2015; Mahboob, 2015; Chuaychan *et al*., 2015; Jeevithan *et al*., 2014; Veeruraj *et al*., 2013; Tamilmozhi *et al*., 2013). Potential method extraction of collagen with a limited of pepsin such as pepsin can hydrolyse the non-collagenous proteins and also can be easily removed using salt presipitation and dialysis to improve purity of collagen, pepsin can hydrolyse the

telopeptides of collagen that may be resulting in improvement of the extraction efficiency and pepsin can be significant effect problem in food and pharmaceutical applications because pepsin digestion may reduce the antigenicity caused by telopeptide in collagen (Duan *et al.*, 2009; Matmaroh *et al.*, 2011; Benjakul *et al.*, 2012; Wang *et al.*, 2014). The based on before study showed that the addition of NaOH as a pretreatment before extraction of collagen could great affect the yield of collagen produces because, without the addition of NaOH, pepsin was can to hydrolysis non-collagenous proteins.

The optimal predicted yield of pepsin-soluble collagen from Indonesia local "Kacang" goatskin in this study was 93.9944% with optimum condition of treatment time of NaOH 0 h and hydrolysis time 24 h. The efficiency of extraction was mass transfer rate of analyze the matrix plays (Bartle *et al.*, 1991). The mass transfer rate is controlled by the diffusion process which is time-dependent and the recovery of analyzing keeps increasing along with the extension of time (Wang *et al.*, 2008). This result indicated that there was abundant collagen in Indonesian local "Kacang" goatskin.

Conclusion

On the basis of this result, in conclusion this study of the two variables (curing time of NaOH and hydrolysis time) showed a significant effect in the extraction of pepsin-soluble collagen. The optimal conditions to obtain the highest yield of pepsin-soluble collagen is without curing of NaOH (NaOH 0 h) and hydrolysis time of pepsin 24 h. The predicted yield of pepsin-soluble collagen was 93.9944% which was in agreement with the actual value ($p < 0.05$) indicating that it was significantly different from zero at the 95.0% confidence level. The results of this study were helpful for the production of pepsin-soluble collagen from Indonesian local "Kacang" goatskin.

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Author's Contributions

Rina Wahyuningsih: Record the data of the experiment in the field, analysis of the data and contributes to the writing of the article.

Rusman, Nurliyani, Ambar Pertiwiningrum, Abdul Rohman and Nanung Agus Fitriyanto: Reviewing it critical for significant intellectual content.

Yuny Erwanto: Research idea proposes, experiment supervision and final approval of the version to be submitted and any revised version.

Ethics

All of the authors confirm that this article is original and no ethical issues are concerned with the present article.

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