Isolation and characterization of collagen from local goat bone using pepsin hydrolysis

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Abstract. Goat bone is one of by-product which has not commonly used in Indonesia. Collagen is one of proteins which contained a bone which characterizing its uniq function. The purpose of experiment was to isolate and characterize collagen from local goat bone with pepsin enzymatic hydrolysis in various concentration. The experiment consisted of bone preparation, Isolating by leaching method, and the hydrolysis of collagen bone using pepsin enzyme in various concentration (0.1; 0.3; 0.5; and 1%). Variables observed were collagen yield, soluble protein, pH, Fourier Transform Infrared Spectroscopy (FTIR) spectra, thermal stability using Differential Scanning Calorimetry (DSC), and molecular weight using Sodium Dodesyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The results showed that soluble protein concentration of collagen solution was not affected by pepsin concentrations. The soluble protein of 0.1; 0.3; 0.5 and 1 % of enzym were 0.203±0.013; 0.244±0.045; 0.295±0.065; and 0.257±0.066 mg/ml for, respectively. The results of collagen yield was significant, and it were 7.12; 7.54; 13.3; dan 8.81 %. The results of pH showed significant, it was 6.37; 5.96; 6.88; 5.92. The FTIR spectra showed that all of the sample has not changed into gelatin. The thermal stability in DSC analysis showed that the collagen start to gelation at 56.72 to 57.40 °C and Tmax for each sample were 128.20; 189.32; 131.35; 124.43 °C, respectively. In conclusion, collagen could be isolated from goat bone using enzymatic treatment and showed the fine properties as well as collagen from skin.

1. Introduction

Increased consumption of goat meat in Indonesia also produces by-products that are underutilized. This problem in the results obtained or this waste is only necessary without being specific to increasing its economic value. One of the results of by-product that has not been widely used is goat bone. Until now it was published at a time when it was still very limited as a food flavoring ingredient such as soup, ingredients for making buttons, dice, knife algae, and so on. Bones also contain collagen which can be utilized in the food, pharmaceutical, health and cosmetics industries. Collagen is one of the most distributed proteins in the skin, bones, and other parts of the human body and animals [1]. The collagen industry in Indonesia is still very rare and the quality of collagen made is not in accordance with the needs of collagen and gelatin. These things are related to the use as well as the community's unknown economic value of collagen, besides that it is still very rare for domestic innovations related to this collagen.
The process of isolating collagen has been carried out on several ingredients produced by livestock and fish. Gao et. al [2] conducted a study on the characterization of collagen from sheep bones isolated by acidic and enzymatic methods, in addition nalinanon et al. [3] reported a collagen that can be extracted with proteolysis enzymes using pepsin. Until now there is no information which give clear and appropriate method to isolate collagen from bone. The character of collagen obtained from bone was very little information compared to collagen from the skin. Therefore it is necessary to conduct a study on the feasibility of goat bones in Indonesia so that they can compete with the quality and quantity of collagen on the market with the method of making collagen and what factors increase the quality and collagen, from these two things a combination will be obtained for get high-quality collagen.

2. Materials and methods

2.1. Preparation of goat bone
The preparation of goat bone follows [4] with modification. The fresh goat bones were obtained from a Giwangan slaughterhouse in Yogyakarta, Daerah Istimewa Yogyakarta, Indonesia. Bones were placed in polyethylene bags with ice cube. The terminals of bones were cut off using a hammer and bone marrows were removed manually. Bones were then broken into small pieces 0.5 until 1.0 cm in length and stored at −25°C until used. First, The bones were submerged in HCl 5% for 3 days. The prepared bones washed with cold distilled water for 3 hours. The bones were first soaked in 0.1 M NaOH with a sample:alkali solution ratio of 1:5 (w/v) for 24 hours to remove non-collagen proteins, and changing the solution every 6 hours. The residue was washed with distilled water until pH neutral. After being washed with distilled water, The bones were decalcified with 0.5 M EDTA–2Na solution in pH 7.5 for 5 days, and the solvent replaced every 12 hours. Fat was removed with detergent at sample:detergent solution ratio 1:10 (w:v) overnight, and then the samples were washed with distilled water fully.

2.2. Collagen extraction
Goat bones were soaked in 0.5 M acetic acid containing rabbit lung pepsin with a solid:solvent ratio of 1:10 (w/v) at 4°C for 3 days with stirring. The extracting solution was centrifuged at 5000rpm for 30 min at 4°C, and the pellet was re-extracted under the same conditions. NaCl with a concentration of 2.0 M was used to salt out collagen. The sequent precipitate was centrifuged at 5000rpm for 30 min. The sediment was dialysed in 0.1 M acetic acid for 1 day and pure water for 2 days with dialysate changed every 12 hours. Solvent that containing collagen were freeze-dried and collagen powder were obtained [4].

2.3. Sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS PAGE)
SDS-PAGE analysis was performed as previously described [5]. Samples were dissolved in destilled water. Protein concentration was determined by Lowry method. A volume of collagen solution was added with a same volume of 2× loading buffer with a sample:loading buffer ratio of 2:1 (v:v) and boiled for 10 minutes. Electrophoresis was performed using 10% gradient gel. After electrophoresis, Coomassie Brilliant Blue R-250 (Sigma-Aldrich, USA) were used to stain overnight, and then destained. The gels were imaged using Canon Pixma G2010 scanner.

2.4. Fourier transform infrared spectroscopy (FTIR)
Fourier transform infrared spectroscopy (FTIR) of the nanofibers were recorded using a Nicolet iS10 instrument (Thermo NicoletLtd., USA) at a resolution more than 0.7 cm⁻¹ with the wavenumber range of 4000-400 cm⁻¹ [6].

2.5. Statistical analysis and graphical work
The graphical work data carried out by using Ms. Excel. The statistical results data analyzed by one-way analysis of variance (ANOVA) followed by Duncan’s New Multiple Range Test (DMRT) to
3. Results and discussion

3.1. Collagen
The yields obtained for pepsin soluble collagen (PSC) of goat bones were 7.12; 7.54; 13.3; 8.81%. Based on the data above shows that the treatment with the greatest yield on the addition of the enzyme pepsin as much as 0.5% is 13.3 grams, and the smallest is 7.12 grams at the treatment of 0.1% enzyme. Directional statistical analysis and Duncan’s multiple range test, show that the four treatments are significantly different, this is because the more enzymes are given, the more proteins are hydrolyzed into simpler peptides at certain levels. Yield collagen that is not in line with the analysis of dissolved protein indicates that the content of collagen powder obtained still contains other substances such as minerals derived from bone. [7] states that the high and low levels of collagen are influenced by the type of raw material, the solvent used, pretreatment treatment, and the size of the collagen protein before extracts are obtained. This is related to the opinion of [8] which states that the enzyme pepsin works on peptides that have bonds with aromatic compounds or carboxylic L-amino acids. Pepsin has the specificity of cutting amino acids in the terminal C of the amino acids Phenylalanine and Leucine. Pepsin does not cut through the amino acids Valin, Alanin, and Glycine. [9] states that with the hydrolysis process, higher levels of collagen are obtained. [8] also proved that the enzyme pepsin was able to reduce the molecular weight of proteins into simple peptides through SDS-PAGE analysis which ultimately affected the yield value.

3.2. Sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS PAGE)
The molecular weight of local goat bone collagen with different level pepsin enzymatic hydrolysis can be seen in Figure 1 and Table 1.

![Figure 1. Result of SDS-PAGE base different concentration pepsin enzyme for hydrolysis](image-url)
The results of reading protein molecular weights using SDS-PAGE showed that the samples were hydrolyzed with a concentration of 0.1% pepsin enzyme addition; 0.3%; 0.5%; and 1% have relatively the same molecular weight. Protein with the addition of the enzyme prptease has a lower molecular weight, this is because when the hydrolysis process the protein will split into shorter peptides so that it will reduce its molecular weight. SDS will wrap the protein chain that is bound to the same negative charge to form SDS-protein. The SDS-protein complex has identical charge density and moves on the gel based only on protein size, the larger SDS-protein complex has lower mobility compared to the smaller SDS-protein complex [10]. The SDS-PAGE results also show the corresponding α1, α2, and β chains [11]. The molecular weight of the sample protein can be seen in the following table.

**Table 1.** Molecular weight of local goat bone protein collagen in different level of pepsin enzymatic hydrolysis.

| Protein marker | MW of marker | Molecular weight (%) |
|----------------|--------------|----------------------|
|                | 0.1          | 0.3                  | 0.5                  | 1.0       |
| I              | 245          | -                    | -                    | -         |
| II             | 180          | -                    | -                    | 147.91    |
| III            | 140          | 121.34               | 122.74               | 125.03    | 133.35    |
| IV             | 100          | 105.68               | 106.91               | 108.89    | 116.14    |
| V              | 75           | 86.30                | 84.14                | 79.62     | 85.11     |
| VI             | 60           | 59.43                | 59.70                | 60.26     | 64.57     |
| VII            | 45           | 43.75                | 43.85                | 44.06     | 45.71     |
| VIII           | 35           | 34.43                | 34.43                | 34.51     | 31.26     |
| IX             | 25           | -                    | -                    | -         |
| X              | 20           | -                    | -                    | -         |
| XI             | 10           | -                    | -                    | -         |
| XII            | 5            | -                    | -                    | -         |

Some factors that can affect the appearance of protein bands in the gel include gel concentration, temperature, and pH. High temperatures due to heating can break down the structure of proteins so much that protein bands show smaller molecular weights. A pH that is too high or too low can cause a denaturation process, while a gel concentration that is too high causes the pores of the gel to get smaller, so that the bands that appear show a smaller molecular weight [12].

### 3.3. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrums of local goat bone collagen in the range of 4000-400 cm\(^{-1}\) were shown in Figure 2. The absorption of amide A is assigned to N-H stretching vibration in the wavenumber range 3.440-3.400 CM\(^{-1}\). The absorption of amide B assigned to asymmetrical stretch of CH\(_2\) were at wave number range 2.935-2.915 cm\(^{-1}\). The Amide I band, the characteristic frequencies ranging from 1.690-1.600 cm\(^{-1}\), was mainly associated with the stretching vibrations of carbonyl group. The characteristic frequencies ranging from 1.575-1.480 cm\(^{-1}\) names amide II represented N-H bending vibration to C-N stretching vibration. The Amide III ranging 1.301-1.229 cm\(^{-1}\) indicate C-N stretching vibration caused by wagging of CH\(_2\) from the glycine backbone and proline side chains.
Figure 2. Fourier transform spectroscopy of local goat bone collagen in different level of pepsin enzymatic hydrolysis: (a) 0.1%; (b) 0.3%; (c) 0.5%; and (d) 1%.
Table 2. Characteristic of collagen wavenumber range with wavenumber of local goat bone collagen in different level of pepsin enzymatic hydrolysis

| Amide   | Wavenumber range (cm⁻¹) | Local goat bone collagen wavenumber (cm⁻¹) | indicate               |
|---------|-------------------------|------------------------------------------|------------------------|
| Amide A | 3440-3400               | 3406,25; 3413,74; 3411,76; 3400,47         | Vibrasi stretching NH  |
| Amide B | 2935-2915               | 2917,96; 2916,61; 2915,92; 2915,96          | Asimetrikal stretching CH₂ vibrasi |
| Amide I | 1690-1600               | 1634,67; 1635,40; 1634,87; 1634,66          | stretching C=O         |
| Amide II| 1575-1480               | 1.552,79; 1.554,71; 1.554,11; 1.556,54       | CN stretching, NH bending |
| Amide III| 1301-1229              | 1.258,27; 1.252,15; 1.259,24; 1.258,92       | CN stretching, NH bending |

The samples has absorption in accordance with the absorption of collagen. It’s shows that the product not change into gelatin. The absorption in Amide I also indicate characteristic of collagen secondary chains like α-helix (1.658-1.654 cm⁻¹), β-sheet (1.642-1.624 cm⁻¹), β-turn (1.666, 1.672, 1.680, 1.688 cm⁻¹), dan random coil (1.648±2 cm⁻¹) [6]. Fourth of samples absorption shown β-sheet and β-turn.

3.4. Thermal stability

DSC thermograms of local goat bone collagen shown in Figure 3 and Table 3. The endothermic peaks, with denaturation temperatures (Td) of 57.4, 56.9, 57.09, and 56.72 °C, were observed for collagen change to gelatin phase, respectively. The result suggested that the intramolecular hydrogen bonds stabilising the triple helix structure of collagen might be disrupted and change to double or single helix chains [13].
Figure 3. Thermal analysis diagram of local goat bone collagen in different level of pepsin enzymatic hydrolysis: (a)0,1; (b)0,3; (c)0,5; and (d)1%.
Denaturation Thermal analysis (Td) shown gelation transition of collagen, then break up of hydrogen bonds and amorph polymer formed. Tmax indicate sample’s melting. The samples has varies Tmax. Thermal stability related with amino acid composition especially hydroxyproline effect on triple helix stability [14].

4. Conclusion
The conclusion of this research was collagen was isolated from local goat bone followed by pepsin. All the collagens isolated by different levels of pepsin enzymatic hydrolysis. While there were also differences in yields, FTIR spectra, and similar thermal stability. These results provide some basis for the large-scale production and further application of collagen from goat bone.

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\begin{table}
\centering
\caption{Characteristic of collagen thermal analysis of local goat bone collagen. in different level og pepsin enzymatic hydrolysis}
\begin{tabular}{|c|c|c|}
\hline
Level enzim pepsin (%) & Td (°C) & Tmax (°C) \\
\hline
0,1 & 57,4 & 128,2 \\
0,3 & 56,96 & 189,32 \\
0,5 & 57,09 & 131,35 \\
1,0 & 56,72 & 124,43 \\
\hline
\end{tabular}
\end{table}
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