American Journal of Animal and Veterinary Sciences

Original Research Paper

Potency of Pepsin Soluble Collagen from Indonesian Local Goat Skin as an Antioxidant

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Article history
Received: 08-05-2021
Revised: 04-07-2021
Accepted: 06-07-2021

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Abstract: Pepsin Soluble Collagen (PSC) hydrolyses is derived from the collagen extraction process from Indonesia local goat skin (Kacang) was carried out in some previous studies. This study is considering the first research used the collagen from the goat skin as an antioxidant compound. The main purpose of study were to investigate antioxidant activity of PSC hydrolysis after pepsin treatment. The experiment was applied enzymatic treatment using pepsin as 0.1 U unit/g per g collagen for various (0, 30, 60, 90 and 120 min) hydrolysis time under 37°C to determine the degree of hydrolysis. Collagen solubility, protein molecular weight and value of radical scavenging activities were observed. The result of the study showed that the highest of degree of hydrolysis of PSC from goat skin found at 90 min incubation (20.05±0.76%). The highest collagen solubility of PSC was hydrolyzed for 30 min with the value 2.59 mg/mL. PSC before hydrolysis has molecular weight of 57.82 - 162.06 kDa and after hydrolysis using pepsin and incubation at 37ºC for 120 min, the molecular weight decreased into 9.09 - 46.75 kDa. The concentration of 500 ppm of PSC after hydrolysis for 90 min has 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity 59.35% with IC_{50} 198.59 ppm. This study indicated that hydrolysis time influenced solubility, molecular weight and radical scavenging activity of PSC hydrolysate from Indonesia local goat skin.

Keywords: Antioxidant Properties, Molecular Weight, Pepsin Soluble Collagen, Protein Hydrolysates

Introduction

Antioxidants are substances that could be used to reduce uncontrolled tissue or cell of the production of free radicals in the body. The production of free radicals that attack macromolecules was cause the production of free radicals to increase. Naturally, ROS in human body has function to fight microbial infection, but when it overproduces, it can be dangerous (e.g., attack protein, DNA, lipid membranes and other bioactive macromolecules in the body) (Yu, 1994; Krapfenbauer *et al*., 2003). It may lead to dangerous diseases for examples is diabetes mellitus, cancer and cardiovascular disease, Alzheimer's disease and neurodegenerative diseases. ROS can protect by utilizing antioxidants to fight free radicals. Antioxidant naturally can be found in the animal tissues. Human skin has endogenous enzymatic (catalase, superoxide dismutase, glutathione peroxidase) and non-enzymatic antioxidants (glutathione, vitamin C and E and coenzyme Q10).

Consumption of exogenous antioxidants is very essential to human body to avoid overproduction of ROS during oxidative metabolism. Exogeneous antioxidants consist of synthetic antioxidants and natural antioxidants. The dangers of lipid oxidation in foods can be reduced by using synthetic antioxidants. Consumption of synthetic antioxidants in human body has negative effect to health. So, it is recommended to consume natural antioxidants. (Moure *et al*., 2002; Pihlanto, 2006; Vercruysse *et al*., 2009). Natural antioxidants can be divided enzymatic antioxidants and non enzymatic antioxidants. In food systems for using natural antioxidants there are a lot of restrictions, examples low solubility, low shelf life, packing and handling is difficult, losses due to environmental stresses and during digestion of instability in various conditions (Fang and Bhandari, 2010). Collagen

is one of the sources natural antioxidants, but it has structure is very complicated. The hydrolysis enzymatic was effective step for divided structure of collagen to peptides which can be used as for antioxidants sources.

Attention researches about antioxidant peptides are extracted from natural proteins from fishes, livestock and poultry has increased in last decade. The materials used to prepare potential *antioxidant* peptides are animal tissues which are rich of protein. Antioxidant peptides was found from enzymatic hydrolysates processing were jellyfish (Zhuang *et al.*, 2009), chicken muscle (Centenaro *et al*., 2014), milk casein (Suetsuna *et al*., 2000), mackerel (Wu *et al*., 2003), wheat protein (Zhu *et al*., 2006) and porcine myofibrillar protein (Saiga *et al*., 2003). 33% of protein component in human body is collagen. Amino acids in collagens is hydrophobic amino acid which has higher antioxidant activities than other proteins (Mendis *et al*., 2005). The lipid peroxidation inhibitors, free radical scavengers and cultures of transaction metal ion can be protected using antioxidant collagen (Alemán *et al*., 2011). Oyster (Crassostrea gigas) are reported containing antioxidant peptides with stronger inhibitory activity to attack polyunsaturated fatty acid (Qian *et al*., 2008).

Previous studies reported that Pepsin Soluble Collagen (PSC) extracted from Indonesia Local Goat (Kacang) has highest yield collagen, but potential collagen has not been studied further. Pepsin enzyme could cleave covalent cross links of skin collagen via aldehyde group condensation in telopeptide regions. Furthermore, pepsin is able to cleave the network cross links among collagen molecules, which will increase collagen solubility so that collagen could be extracted properly (Zhang *et al*., 2009). Collagen soluble peptides hydrolyzed from bovine bone collagen has ability of scavenging activity free radical is significant (Fardet and Rock, 2018). The study from Wang *et al*. (2016) is reported that PSC can be regulate of body defense system because can be protection the balance of ROS. PSC hydrolysate from Indonesia Local Goat local goat skin should be investigated for its antioxidant effect by enzymatic treatment. The purpose of study were hydrolysis enzymatic treatment using pepsin by 0.1 units/gcollagen with various hydrolysis times (0, 30, 60, 90 and 120 min) at 37°C and determine the degree of hydrolysis, collagen solubility, protein molecular weight and value of radical scavenging activities.

Materials and Methods

Materials

PSC from Indonesia local goat skin (Kacang), pepsin from porcine gastric mucosa purchased from Merck (Germany), 1,1-Diphenyl-2 Picrylhydrazyl (DPPH), methanol, sodium hydroxide purchased from Merck Kga (Germany), Trichloroacetic Acid (TCA) and Bovine Serum Albumin (BSA).

Methods

Collagen Hydrolysis

Collagen hydrolysis method based on Li *et al*. (2013) with slight modification. The best from Pepsin Soluble Collagen (PSC) with the amount of yield was 51.20% in the previous study was hydrolyzed using pepsin. One gram PSC samples are dissolved to 100 mL with using buffer pH 2.0 and incubated at 37°C for 15 min. Furthermore, 0.1 U pepsin was added to sample. The samples then incubation for 0, 30, 60, 90 and 120 min. The reaction stop by soaking into boiling water for 5 min then cooling and than netralisation with 1 M NaOH and centrifuged at 1,000 g for 15 min. Some of supernatant containing collagen peptides is frozen (collagen peptides) and some were dried with freeze drying. The collagen peptides were used to analysis of the Degree of Hydrolysis (DH), while the freeze dried collagen peptide were used for antioxidant analysis.

Degree of Hydrolysis (DH)

Degree of hydrolysis PSC is determined by the method based on Silvestre *et al.* (2013) with slight modification by TCA 20% precipitation to produce 10% dissolved protein fraction and 10% insoluble fraction. 500 µL of freeze dried PSC is thawing at room temperature, then 500 µL TCA 20% added to the sample and then it was homogenizing and incubation at 4°C for 30 min. The solutions is centrifuge at 3000 g for 20 min. The soluble protein and total protein is analysis using the method of Kresge *et al.* (2005).

BSA as a standard and degree of hydrolysis was calculated as follows:

$$DH(\%) = \frac{10\%\ soloble\ protein of\ TCA}{total\ protein} \times 100$$

Collagen Solubility

The collagen solubility is measured using Montero et al. (1991) method with slight modification. Collagen (3 mg/mL concentration) was dissolved with 0.5 m acetic acid, then 8 mL was taken and the pH was adjusted to neutral (pH 7). It was stirred and centrifuge at 10,000 g at 4°C for 30 min. Protein concentration is measured with Kresge *et al.* (2005) method. Sample was analyzed using One Way ANOVA with 5 replicates.

SDS-Page Analysis

Protein molecular weight was analyzed using SDS-PAGE electrophoresis with 5 replicates. SDS-PAGE is determined following the method of Laemmli (1970) with 7.5% separating gels and 5% stacking gels. Gel staining was performed with coomassie brilliant blue and distained with methanol: Acetic acid (2:1). Standard molecular mixture marker (protein marker) by Sigma was used to identify the separated protein bands.

DPPH Radical Scavenging

The DPPH radical scavenging activity of PSC hydrolysate was measured using the Razali *et al.* (2015) with slight modification. The mixture of sample solution (4.5 mg/mL) is dissolve with 0.5 m acetic acid and adjusted with 500 µL methanol and 125 µL 0.02% (w/v) of DPPH in 99.5% methanol. The mixture is stirred and incubated under light-tight conditions for 60 min. Positive control using Butil-Hidroksitoluena (BHT). Solution is measured using spectrophotometer at 517 nm and it each replicated to 3 times. The calculation of DPPH radical scavenging activity was calculated fas follows:

$$\text{Radical scavenging activity} = \left[\left({}_{A}blank - {}_{A}sample\right) / {}_{A}blank \times 100\right]$$

Where:
${}_{A}blank$ = Absorbance of the control
${}_{A}sample$ = Absorbance of the sample

Statistical Analysis

Degree of hydrolysis and total collagen solubility was analyzed using SPPS 16.0 with One Way Analysis of Variances (ANOVA) design with each 5 replicates. The activity of DPPH radical scavenging activities was analyzed using SPSS 16.0 with randomized complete design with 3 replicates. One-way Analysis of Variances (ANOVA) was used to determine differences between the hydrolysis time with the degree of hydrolysis, collagen solubility and DPPH radical scavenging activity for PSC sample. Data is presented as mean standard deviation. The statistical analysis was conducted using a statistic program (Stat-Soft, Rusia Russia).

Results

Collagen Hydrolysis

The Pepsin-Soluble Collagen (PSC) hydrolysate from a previous study. The collagen hydrolysis of the PSC process was carried out by soaking into buffer phosphate pH 2.0 and incubation at 37°C for 3 h. The PSC sample is dissolved with pepsin (0.1 U/g) with various hydrolysis time (0, 30, 60, 90 and 120 min) at 37°C and degree of hydrolysis was measured. Each treatment was soaked in boiling water for 5 min to inactivate enzyme. The sample was centrifuge at 6000 g for 5 min at 4°C.

Degree of Hydrolysis

Degree of Hydrolysis (DH) of Pepsin Soluble Collagen (PSC) is shown in Fig. 1. The time of hydrolysis in 90 min (20.05% ±0.76) and 120 min (19.05% ±0.44) have degree of hydrolysis higher than 0, 30, 60 min. The 90 and 120 min incubation had significant value to the 0, 30 and 60 min treatment. Study of Zhou *et al.* (2016) showed that DH of PSC from chicken feet is 24% at the first hour of hydrolysis.

Collagen Solubility

Collagen solubility of PSC from Indonesia local goat skin is measured with the method of (Kresge *et al*., 2005). Result of collagen solubility of the PSC was shown in Fig. 2. The collagen solubility of PSC of sample hydrolyzed at 30 and 60 min were of 2.59±0.57 mg/mL and 2.56±0.14 mg/mL significantly higher than samples hydrolyzed at 0, 90 and 120 min and incubation of 90 and 120 min were significantly lower compare sample on incubation of 30 and 60 min. The time of hydrolysis at 120 min (1.57±0.03 mg/mL) had collagen solubility significantly lower than sample on incubation of 0, 30 and 60 min but no significant than incubation at 90 min.

Electrophoresis Pattern of Collagen from Indonesian Local Goat Skin by SDS PAGE

Electrophoresis pattern of collagen from Indonesian local goat skin PSC was measured by SDS-PAGE and shown in Fig. 3. PSC was hydrolyzed using pepsin with different time of hydrolysis (0, 30, 60, 90, 120 min). Hydrolysis can cut protein bond into small peptides.

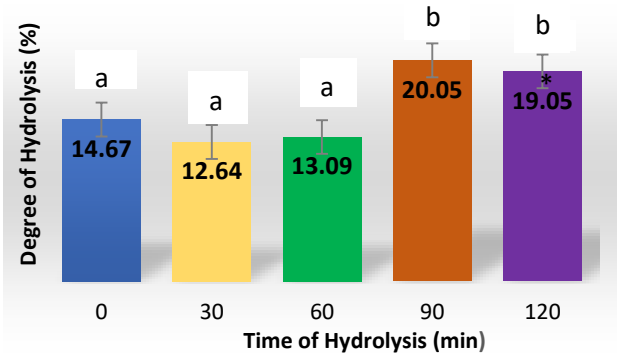

Fig. 1: Degree of hydrolysis of PSC with different time of hydrolysis. Values with the same letters indicated no significant difference ($P < 0.05$).

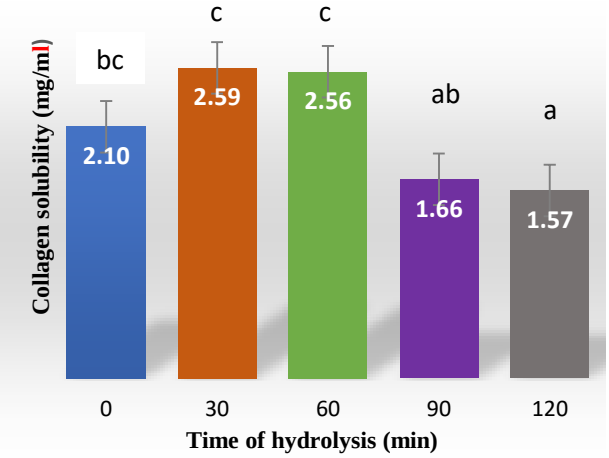

Fig. 2: Collagen solubility from Indonesian local goat skin with different time of hydrolysis. Values to differentiate letters indicated significant differences (P<0.05).

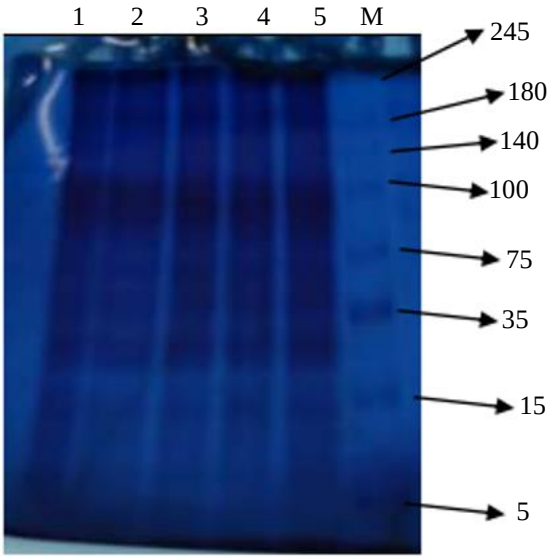

Fig. 3: SDS-PAGE pattern of collagen from local goat skin with different time hydrolysis. Ntes: Lane 1: 0 min; lane 2: 30 min; lane 3; 60 min; lane 4: 90 min; lane 5: 120 min, lane 6: Protein marker

Table 1: DPPH radical scavenging activity and IC_{50} of PSC from Indonesian local (Kacang) goat skin with 500 ppm concentration

Time of hydrolysis (min)	DPPH radical scavenging activity (%)	IC_{50} (ppm)
0	54.08±1.00[a]	262.28
30	56.63±1.68[ab]	208.39
60	56.88±1.97[b]	205.96
90	59.35±0.98[b]	198.01
120	58.53±1.32[b]	204.01

Values with the same letters indicated no significant difference (P<0.05)

The result of the study shown that PSC hydrolysis from 0 until 120 min had small molecular weight to α1 and α2 chain, that is 32.22 – 106.03 kDa (hydrolysis 0 min), 32.22 – 98.42 kDa (hydrolysis 30 min), 20.61 – 84.80 kDa (hydrolysis 60 min), 14.21 – 67.83 kDa (hydrolysis 90 min) and 9.09 - 46.75 kDa (hydrolysis 120 min). PSC prior to hydrolysis had molecular weight of 57.82 – 162.06 kDa and post-hydrolysis at 37ºC for 120 min showed slightly lower molecular weight of 9.09 – 46.75 kda.

DPPH Radical Scavenging Activity

The result study of DPPH radical scavenging activity of the PSC from Indonesian local goat skin is shown in Table (1) Study shown that PSC of hydrolysis time at 500 ppm concentration and 60, 90, 120 min had DPPH radical scavenging activity significantly higher than of control (0 min). The value of IC_{50} antioxidant activity it was strongly than the control.

Discussion

DH of PSC from Indonesian local goat skin and chicken feet have almost the same value. The activity of pepsin enzyme during hydrolysis process affect PSC molecules to show more exposed cleavage sites. The large number of peptides and amino acids dissolved in TCA caused an increase in DH and resulted in broken peptide bonds during the hydrolysis process (Haslaniza *et al*., 2010). The decreasing rate of DH of collagen be caused by inhibition of substrate hydrolysis process. Increasing rate of DH is caused by higher solubility of protein hydrolysate in water (Ovissipour *et al*., 2010). Total collagen solubility of PSC decreases rapidly at 60 min of hydrolysis. The protein solubility content is decreasing after 60 min of hydrolysis. This is probably caused by the breakdown of protein chain in the samples due to longer heating process causing protein total to decrease.

According to Wu *et al*. (2015), longer hydrolysis time causes protein or protein chain to breakdown into smaller peptide fragment. The lower molecular weight of peptide fragments after hydrolysis was exist due to decreasing band intensity of the α-chain and crosslinked components. Hydrolysis process can affect collagen molecular weight to breakdown into smaller form (<75 kDa) because pepsin can cut telopeptide of a proteins and peptides (Jongjareonrak *et al*., 2005). Pepsin mechanism in protein structure can make the telopeptide to crosslink regions of the super triple helix of collagen without affecting the structure integrity (Kittiphattanabawon *et al*., 2010).

The value of IC_{50} is concentration hydrolysis sample that inhibit 50% of free radical DPPH. The longer the time of hydrolysis, the smaller IC_{50} value. The lower of IC_{50}, then the higher of free radical DPPH scavenging activity (Prior *et al*., 2005). Antioxidant properties are divided into 4 types. The IC_{50} < 50 ppm has strongest antioxidant properties, IC_{50} 50 – 100 ppm has strong antioxidant properties, IC_{50} 100 – 150 ppm has moderate antioxidant properties and the IC_{50} 150 – 200 ppm has low antioxidant properties (Molyneux, 2004). The low antioxidant activity of PSC is caused hydrolysis too long in the previous study so structure of amino acid have breakdown. The amino acids like tryptophan, methionine and cysteine can be destroyed because they are affected by temperature and pressure. (Villamil *et al*., 2017). The highest antioxidant activity of collagen peptides is of Trp, Tyr and Met (Davalos *et al*., 2004). DPPH scavenging activity is effected by pH, temperature and time for hydrolysis (Auwal *et al*., 2017).

The PSC from shark cartilage in study by Jeevithan *et al*. (2015) as 19.70% percent higher radical scavenging activity rate than PSC from Indonesian local goat skin. Antioxidant activity of collagen peptide was affected by many factors including composition, structure and hydrophobicity of peptide amino acids (Li *et al*., 2007), type of collagen hydrolyzing enzyme (Qian *et al*., 2008) that determines peptide size and sequence and peptide molecular weight (Hseu *et al*., 2008; Woo *et al*., 2008; Guillén *et al*., 2010; Giménez *et al*., 2009; Z. Li *et al*., 2013 and Wang *et al*., 2013). The ROS level can be balanced with using the antioxidant peptides to regulate body's antioxidant defense systems. The molecule structure of the partially collagen hydrolysis is a left-handed bundle of three peptides α-chain to form a right-handed triple helix collagen. The amino acids like Gly, Ala. Pro, Hyp, Glx and Asx are rich in collagen but but poor in Met. Cys, His and Tyr (Alemán *et al*., 2011). Trp, Tyr and Met amino acids had the highest antioxidant activity and were followed by Cys, His and Phe (Davalos *et al*., 2004). The antioxidant activity of peptides can increased lipid solubility because they are rich in hydrophobic amino acids (Kim *et al*., 2001). The reactive oxygen can be inactivated when the protein has significant antioxidant activity (Elias *et al*., 2006). The amino acid residues and their specific sequences can also affect antioxidant (Chen *et al*., 1996). The proton-donating amino acid residues that contain in collagen peptides also have antioxidant capacity. The collagen peptide derived from Alaska pollack skin contain 13 and 16 amino acid residues such as Gly residues on the C-terminus and Gly-Pro-Hyp with repeating motifs (Kim *et al*., 2001). The Gln-Gly-Ala-Arg amino acid residues contained in collagen hydrolysate peptides from porcine skin have the hightest antioxidant activity (Li *et al*., 2007).

Conclusion

The conclusion in this study is a hydrolysis time of the PSC from Indonesian local goat skin (Kacang) is strongly affected DH and solubility collagen. The longer hydrolyze time (120 min at 37°C) causes the solubility and molecular weight of protein to decreases. It also decreases antioxidant activities. PSC has low antioxidant properties because the IC_{50} >150 ppm.

Acknowledgement

Thank you much to Mr. Rifqi Okbah for the valuable English editing and suggestion. Dr. Nanung Agus Fitriyanto for the suggestion of SDS-PAGE experiment and also the protein profile characterization.

Funding Information

This research was partly funded by Ministry of Research, Technology and Higher Education of Indonesia through Directorate Research, Universitas Gadjah Mada.

Author's Contributions

Rina Wahyuningsih: Contributed to preparation sample, analysis sample and writing of the manuscript.

Rusman and Nurliyani: Contributed to preparing the research design and reviewing intellectual content significantly and critically.

Abdul Rohman: Contributed in assistance of the antioxidant analysis and also manuscript review.

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

Ethics

This article is original and contains unpublished material. The article has been read by all authors and approved by the corresponding author, so there are no ethical issues involved.

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