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Extraction of Glycosaminoglycans Containing Glucosamine and Chondroitin Sulfate from Chicken Claw Cartilage

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ABSTRACT

Chicken cartilage (claw) is a waste of chicken cuts which are widely available in Indonesia. Cartilage part of chicken claw becomes a potential source of chondroitin sulfate (CS) and glucosamine (GS). This aim of this study was to determine the most optimal extraction methods of CS and GS from cartilage of chicken claw. Various types of extraction methods used in this study were taken from the extraction by using boiling water (2 and 2.5 hours), acetic acid (7 and 17 hours), as well as proteolysis by papain (24 and 48 hours). Parameters observed include chemical characteristics of powdered cartilage of chicken claw as well as CS and GS levels in powdered cartilage of chicken claw extract. The results showed that the levels of CS and GS of chicken claw cartilage powder were 2.17% and 13%, respectively. The highest GS level was obtained from the extraction with water treatment for 2.5 hours (8.1%). The treatment and duration of extraction will significantly affect the number of GS which was produced. The highest content of CS was obtained from the extraction with the enzyme treatment for 48 hours (2.47%). The best treatment is the extraction with water treatment for 2.5 hours which were the extracts with GS levels of 8.1% and 2.03% CS was selected through the analysis of multiple attribute.

Keywords: Extraction; glucosamine; chondroitin sulfate; chicken cartilage claw.

INTRODUCTION

Chicken claw are regarded as wastes from chicken slaughter house and produced in large quantities every year. The total number of chicken claw in 2012 was estimated to have reached 1.29 billion pieces and was estimated that the number will continue to rise (Miwada, 2013). Cartilage contains of glycosaminoglycans which are full of benefits (Gupta et al., 2015). Glycosaminoglycan (GAG) is a major component of the extracellular matrix of a connective tissue. Several types of GAG between chondroitin sulfate and hyaluronic acid are regarded as polymer of glucosamine. Glucosamine is an amino monosaccharide whose function is to reduce damage to the joints. Chondroitin sulfate is a component that is composed of N-acetyl-galactosamine sulfate and glucuronic acid. The functions of chondroitin sulfate, are as joint health therapy, anti-inflammatory, arthritis, arteriosclerosis and cancer (Nakano et al., 2001).

Commodities which were once used as a source of glycosaminoglycans were a shark, beef, shrimp, and pork. Unfortunately, the application of such commodities experienced problems such as high cost of raw materials, allergy effect or not acceptable for consumption. Other sources that can be used are the cartilage of chicken claw. Chicken claw cartilage has been assumed to contain CS and GS, yet it remains debatable about the most appropriate method to maximize the

number of CS and GS extracted. Several methods will be analyzed in this study, which has been reported to be able to extract CS and GS of the tissues which are the extraction of water, acid and enzymatic. The objective of this study was to determine the best type of extraction for chicken claw cartilage sample.

MATERIALS AND METHODS

Materials which were used during the manufacture of raw materials such as powdered chicken claws were from chicken claws with a weight of 50±5 g each, obtained from semi modern market in Karangploso, Malang. Materials used in the extraction of chondroitin sulfate and glucosamine powder chicken claws were included pH 7 distilled water, glacial acetic acid (Merck) with purity PA, Na_2HPO_4, NH_2PO_4, papain enzyme activity shaped crystal with 1.5-10 units/mg solids (Sigma), EDTA and tyrosine standards. The ingredients used in the testing of raw materials and the extraction were the chemical purity PA, such as NaOH (Merck), concentrated sulfuric acid (Merck), HCl 37%, boric acid, kjedahl tablet, petroleum ether, standard glucurunolactone (Sigma), sodium borates (Bio-world), *Carbazole* (Sigma) and *glucosamine assay kit* (Megazyme).

Ingredient Preparations.

Raw materials (chicken claws) were freshly obtained from the Karangploso-Batu market and transported to the laboratory in a cold condition. Chicken claws were then cleaned from nails and dirt using clean water. Once cleaned, the chicken claws were boiled for 5 min and the cartilage was separated. Cartilage obtained was dried for 24 hours in cabinet dryer at 65°C and pulverized to pass the 20 mesh sieve (Khan et al., 2013).

Powdered cartilage was analyzed for levels of water, fat, crude protein, and ash as described by AOAC method, Ca concentration using AAS method, as well as levels of CS and GS using HPLC. Chicken claw cartilage powder was used as raw material for the extraction of CS and GS. The condition of extraction was conducted with water at 100°C for 2 and 2.5 h; pH 4.5 at 37°C for 7 and 17 hours and the enzyme papain at 65°C for 24 and 48 hours.

Water Extractions (modification of Shin et al., 2005).

10 g samples were extracted using 10 times volume of distilled water at 100°C for 2 and 2.5 hours. The mixture was then centrifuged at 4,500 rpm for 30 minute to separate supernatant, which later was dried at 50°C.

Acid Extractions (modification of Nakano et al., 2012).

10 g samples were incubated 10 times the volume of distilled water pH 4.5 at 37°C for 7 and 17 hours. pH of the solution was set at 4.5 by using glacial acetic acid at the beginning until the extraction was completed. The mixture was then centrifuged at 4,500 rpm for 30 min to separate supernatant. Supernatant obtained was then dried at 50°C.

Enzymatic Extractions *(*modification of Ganjanagoochor, 2007).

10 g of powdered cartilage was incubated at pH 7-phosphate buffer (containing EDTA) as much as 10 times the volume of the sample. The mixture then added papain as much as 4 mg/g of powdered cartilage and was incubated at 65°C for 24 and 48 hours. After incubation was completed, the mixture was centrifuged at 13,000 rpm for 30 min. Supernatant obtained then dried at 50°C.

Raw Extract of CS and GS Analysis.

Each extraction yield of levels of CS and GS were analyzed. GS levels were measured using *glucosamine assay kit* (Megazyme)

based protocols that have been provided by the manufacturer. CS levels were analyzed by analysis using *Carbazole* reagent (Bitter and Muir, 1962). Each sample was taken and diluted to a concentration of 1,000 ppm with distilled water. In the test tube, prepared 5 ml solution of sodium tetra borate in concentrated sulfuric acid (0.025 M) which had been cooled. Samples and standards glucuronic acid (1 ml) were gently mixed in a test tube in cold conditions. Tubes were then boiled at 100°C for 10 min and were cooled at room temperature. A total of 0.2 ml Carbazole 0.125% which was dissolved in absolute ethanol was added to the tube and was boiled for 15 min. The samples were then cooled at room temperature. The absorbance of the sample was measured at a wavelength of 530 nm. It is suggested that the calculations of glucoronate acid levels should be conducted with standard curve and chondroitin sulfate with the formula:

Chondroitin sulfate levels (%) = glucuronic acid content (%) x 2,593.

Data Analysis.

The study design was conducted by applying Random Design Nested (Nested Design). Each sample was treated with different extraction time and was repeated 4 times. Analysis of variance (ANOVA) was conducted with SPSS software. Once the real effect was found, further test by using BNT 5% must be pursued. Selection of the best treatments was done by using multiple attributes.

RESULT AND DISCUSSION

Raw Material Analysis.

Results of analysis from the raw material (powdered chicken claw cartilage) are presented in Table 1. Based on the data obtained, it can be seen that the largest component of powdered chicken claw cartilage is protein. Calcium levels which are measured in powdered cartilage chicken claw is also very small, which leads to the conclusion that the process of separation of the cartilage to bone is quite effective.

The reading of the sample results is conducted by looking at the peak recorded on the chromatogram. In the GS parameter, the two peaks which appear are identified as the standard glucosamine. The total amount of glucosamine is as much as 13%. In contrast to glucosamine, the result of CS chromatography shows that there is only one peak which can be identified, but for the type of chondroitin on the chromatography remains uncertain. The researchers assume that the possible types of chondroitin contained in chicken claw cartilage is chondroitin-4-sulfate and chondroitin-6-sulfate, in which, Lauder (2000) noted that both types of chondroitin in the cartilage of the trachea contains the most dominant content. Number of chondroitin in this study which was the raw material (chicken claw cartilage) was as much as 2.17%.

Table 1. Analysis of Raw Material Powder Chicken Claw Cartilage

Parameter	Amount (%) ± SD
Dry yield	6.704±0.296
Water level	3.683±0,024
Fat level	19.426±2.173
Ash level	2.479±0.041
Protein level	68.767±2.677

Parameter	Amount (%) ± SD
Chondroitin sulfate	2.17
Glucosamine	13
Calcium	0.0581

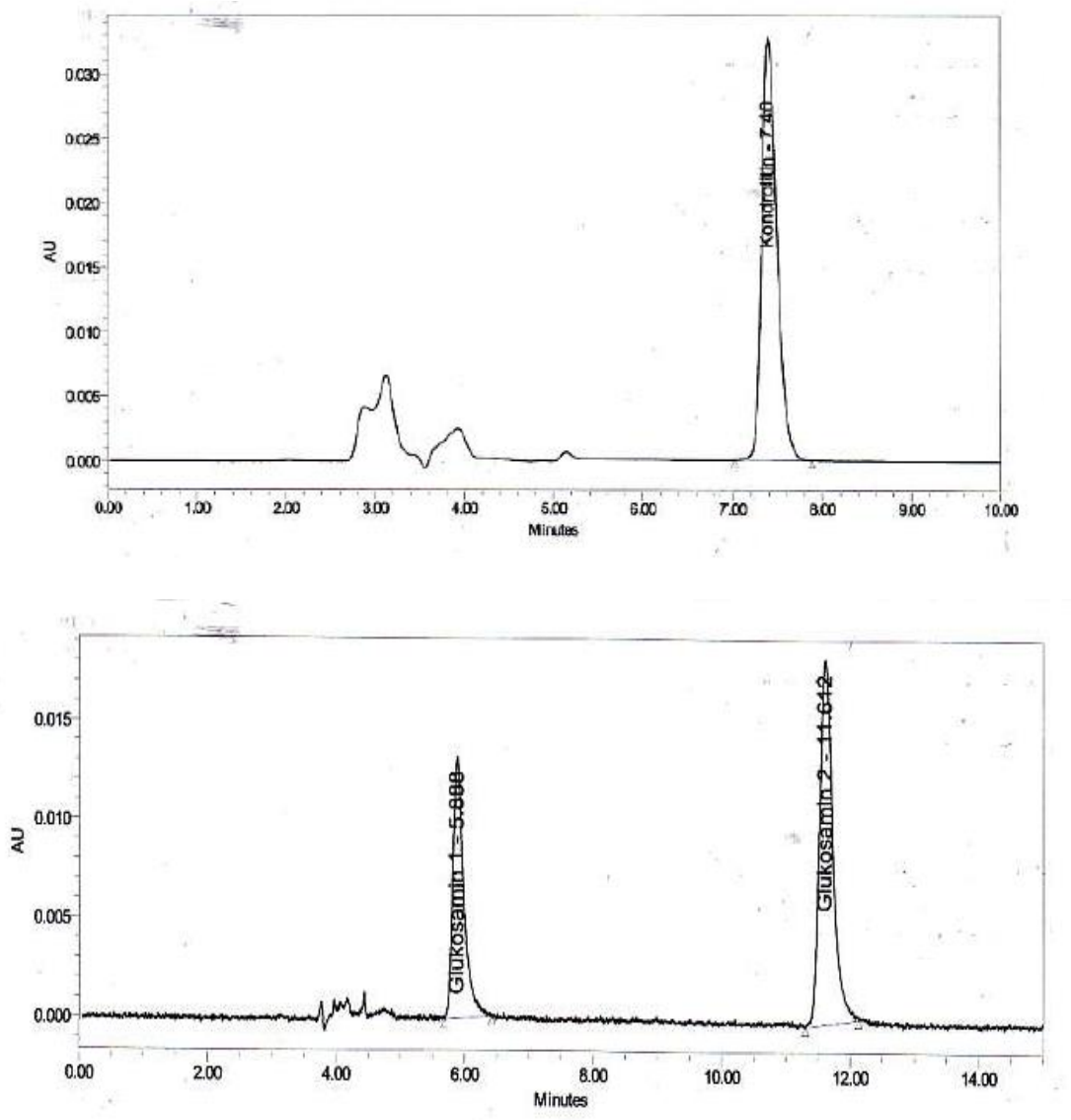

Figure 1. Chromatogram Test of CS (left) and GS (right) by applying HPLC.

The yield of chicken claw cartilage extract

Powdered cartilage has been extracted by each group by calculating the yield and by observing the characteristics. The yield of each extract is presented in Table 2.

Table 2. The Yield of Chicken Claw Cartilage Extract

Treatment	Yield (%)± SD
Water for 2 hours	34.562±0.368
Water for 2.5 hours	38.438±0.438
Acid for 7 hours	15.866±0.150
Acid for 17 hours	12.032±0.133
Enzyme for 24 hours	94.188±0.224
Enzyme for 48 hours	92.377±0.358

Results from acid treatment extract after the centrifuge are yellowish in color, watery and are easily separated from the filtrate section. The results of acid extract after drying have such elastic characteristics (retractable), clear, and difficult to dissolve in neutral pH water. Based on a comparison with the weight of powdered cartilage, the data from acid extracts show that every 10 g of powdered cartilage is extracted with acid treatment of 7 hours, yielding 15.866% extracts.

Cartilage with an average weight of 10 g will produce acid treatment extract for 7 hours was 1,587 g. Powdered cartilage was extracted with acid treatment for 17 hours produces a yield of 12.032% extract in average. Cartilage powder weighing 10 g on average will produce 17 hours of acid treatment extract at about 1,203 g. On average, the result of acid treatment extract of 7 hours was proved more than that of 17 hours acid treatment extract, because the component was perfectly soluble yielding more solid extracts within the 7 hours extraction, compared to that with 17 hours extraction where it experiences the exhaustion causing not much solid extracts.

A result of extract from water treatment after the centrifuge was clear yellowish, watery and thickens which not immediately separated from the filtrate. Results of water extract after drying has characteristics which are dry, thin, elastic, transparent, and easily soluble in neutral pH water. Based on a comparison with the weight of powdered cartilage and a data of extract with water treatment, it is known that every 10 g of powdered cartilage which is extracted with water treatment for 2 hours on average will produce the extract yield of 34.562%. Powdered cartilage was extracted with water treatment 2.5 hours produces an average yield of 38.438% extract.

On average, the extracts which were produced with water treatment for 2.5 hours were more than that with 2 hours of water treatment. This is possibly because the extraction with 2 hours does not let the component to be maximally soluble, thus the solids derived are also less compared to the extraction of 2.5 hours, which gives the components a chance to be more soluble, thus more solid results are derived.

Results of extract from the enzyme treatment after the centrifuge were murky brownish-yellow color, more aqueous extracts among others and are very easily separated from the filtrate (as not much filtrate is produced). Results of enzyme extract after drying have characteristics of: dry, rather thick, inelastic, somewhat fatty, brownish-yellow color, and easily soluble in neutral pH water. Based on comparisons with heavy cartilage powder and data of

extract result from the enzyme treatment, it is known that every 10 g of powdered cartilage which was extracted by enzyme treatment for 24 hours produces an average yield of 94.188% extract. Powdered cartilage extracted with a 48 hours enzyme treatment produces an average yield of 92.377% extract.

On average, the result of extract from the enzyme treatment of 24 hours was more than the 48 hours of enzyme treatment. This is possibly because the 24 hours extraction time enable the components to dissolve maximally, thus the solids extract were more obtained. Meanwhile, the 48 hours extraction time shows a decrease, indicating a less soluble component to produce.

Levels of Glucosamine and Chondroitin Sulfate.

Based on the data in Table 3, it can be seen that the highest levels of glucosamine is a cartilage extract of chicken claw with water solvent of 2.5 hours. The result is then analyzed its variance confidence interval of 5% by using SPSS software.

Table 3. Levels of Glucosamine and Chondroitin Sulfate in Each Treatment Type.

Treatment	Glucosamine (%)	CS (%)± SD
Water for 2 hours	4,693±0,354 c	1,575±0,114
Water for 2.5 hours	8,112±0,946 e	2,035±0,149
Acid for 7 hours	2,627±0,129 a	0,563±0,195
Acid for 17 hours	7,151±0,647 d	0,517±0,054
Enzyme 24 hours	2,966±0,325 a	2,461±0,342
Enzyme 48 hours	3,681±0,494 ab	2,465±0,297

The result showed that the treatment type F count of 0.000 was smaller than 0.005 F table so it can be said that this type of treatment gives a real difference to the levels of glucosamine produced. F count with the duration of extraction time (0.000) was smaller than F table 0.05, so it can be stated that the type of treatment and the duration of extraction impacts on the levels of glucosamine produced.

Based on Table 3 it can be seen that the extract containing the highest number of chondroitin is from the chicken claw cartilage extract, using papain enzyme as a solvent extraction of 48 hours. In addition, the least extract containing chondroitin is from a chicken claw cartilage which used acid as a solvent extract with the extraction time for 17 hours. The result was then analyzed its variance confidence interval of 5% by using SPSS software. The result showed that the treatment type F count of 0.000 is smaller than 0.005 F table. Thus, it can be noted that this type of treatment gives a real difference to the levels of glucosamine which are produced. F count with the duration of extraction times (0.189) was greater than F table 0.05 which means that the duration of extraction times gave no significant effect on levels of chondroitin produced.

The type of treatment was difference quantitatively on both analysis of glucosamine and chondroitin. In general, the extraction mechanism of GAG component is the same, the only difference lies only on the number of measured compound. At extraction using acetic acid, the

temperature used was not too high (37°C); pH used was pH 4.5 with time of extraction for 7 hours and 17 hours. In these conditions the optimal conditions in which peptides from cartilage GAG obtained maximum results. The use of a pH of 4.5 for the solution of acetic acid at a low pH will produce a high yield. In general, the pH of 4.5 becomes the isoelectric point of the protein components of non-proteoglycans, so that at the time of extraction, non-proteoglycans protein will settle and will not be extractable (Puspawati et al., 2014). Extraction of proteoglycans will produce high yields with a temperature of 37°C.

Glycosaminoglycan is soluble in strong acid. The principle of a strong acid usage is regarded as a swelling method in the protein which can accelerate the next extraction (Cheng et al., 2008). Extraction which applied solvent (solid-liquid extraction) was a method of separation (isolation) from a component of a mixture by using a suitable solvent, in which the extracted substances was in the solid mixture (Cheng et al., 2008). The research proved that when the study was conducted, the swelling of the cartilage components (after being extracted) occurred, and gained more viscous soluble phase. The swelling causes the bond of protein component of proteoglycans increasingly tightens and weakens. Strong acids are also known to denature protein. Protein links between the hyaluronic acid and glycosaminoglycan, therefore, GAG component that is bound to proteins such as chondroitin and glucosamine can be easily separated. When proteins undergo denaturation, component of chondroitin sulfate and glucosamine will easily be detached from the bonds of protein.

The boiling water which was used to extract, causes proteoglycan components containing proteins will undergo denaturation. Denaturation can occur in the core protein in which the protein core connects each proteoglycans containing chondroitin, dermatan and keratan on hyaluronic acid will break up and apart. The release of these proteins results in the dissolution of proteins that bind to the hyaluronic acid, causing GAG split into more simple components such as chondroitin and glucosamine (Shin et al., 2005). GAG dissolved in water will then be measured to find out the levels of chondroitin sulfate and glucosamine.

Enzymes are proteins that function as catalysts for chemical reactions in biological systems, having high catalytic power. Enzymes are capable of improving the reaction speed of up to one million times faster than without enzyme reactions. Enzyme molecules also have a certain degree of specificity to the substrate of the catalyzed reaction. Papain is a proteolytic enzyme mixture contained in papaya latex, having proteolytic activity of at least 20 units/g preparation (Soda and Rudiana, 2013).

Protease is an enzyme that catalyzes peptide in the peptides, polypeptides and proteins by using the hydrolysis reaction into simpler molecules such as short-chain peptides and amino acids (Naiola and Widiastuti 2002). Many proteases catalyze the same reaction as the general chemical reactions and similar hydrolysis reaction is indicated as follows: Hydrolysis peptide bond is the addition-elimination reaction, in which the protease acts as nucleophiles or reacts to form one water molecule. In general, nucleophilic forms an intermediate tetrahedral with the carbonyl carbon atom of the peptide bond. One group of amines is released and removed from the active site, which was replaced simultaneously by one water molecule.

Papain deducts the bond if an amino acid sequence is as follows: (hydrophobic) -(Arg/las)-cut- (not val). Cutting mechanism of peptide bonds by papain is as follows:

1. Asparagine-175 orients the histidne imidiazole ring-159
2. Histidine-159 deprotonizes siteine-25
3. System-25 conducts nucleophilic attack on the carbonyl carbon of the peptide bond
4. Amino terminal of the peptide bond becomes free, and basil enzyme intermediates are formed.
5. Basil enzyme intermediates are then distilled by water, releasing the carboxyl terminal peptide. And so on.

Work of papain can be activated or inhibited. The compounds that can activate the work system of papain are: sulfides, sulfites, heavy metal chelator such as EDTA, and N-bromosuccinimide. Meanwhile, the compounds which are regarded as inhibitor of papain include PMSF, TLCK, TPCK, E-64, heavy metals, antipain, astatine and leupeptin.

Cartilage matrix is formed from glycosaminoglycans (GAGs) whose main component is chondroitin-4-sulfate and chondroitin-6-sulfate within the proteoglycans. Papain digests the cartilage which becomes GAGs binding to proteins. After separating the protein from the mixture, chondroitin sulfate can be separated by purification.

Determining the Best Treatment.

Determining the best treatment is performed in order to determine which treatment can simultaneously produce glucosamine and chondroitin with maximum results. Multiple attribute decision making method becomes a method that can be used to determine the most optimal treatment towards multiple attributes which were actual or subjective. The use of this method can determine an attribute, level, minimum and maximum criteria of the attributes specifically. The results of the treatment assignment can best be seen in Table 4.

Table 4. Results of Determining Best Treatment.

Treatment	Rank
Acid for 7 hours	6
Acid for 17 hours	5
Water for 2 hours	4
Water for 2.5 hours	1
Enzyme for 24 hours	3
Enzyme for 48 hours	2

CONCLUSION

From the six selected extractions, the treatment using water extraction at 100°C for 2.5 hours (150 min) is the best treatment. Extracts of chicken claw cartilage powder contains of 8.112% ±0.946 glucosamine and chondroitin sulfate 2.035% ±0.149.

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REFERENCES

Bitter, T and Muir, M. 1962. *A Modified Uranic Acid Carbazole Reaction.* Analytical Biochemistry. 4: 330-334.

Cheng, Fu-Yuang, Tien Chun Wan, Chao Wei Huang, Kana Tominaga, Liang Chuan Lin, Ryoichi Sakata. 2008 *The Effect of Chicken Leg Bone Extraction on Antioxidative Properties under Different Heating Condition*. Asian-

australian Journal Animal Science Vol. 21, No.12: 1815-1820. Taiwan

Gupta, V. K and Maria G. T. 2015. *Biotechnology of Bioactive Compounds Suorces and Applications.* First edition. John Wiley & Sons, Ltd. Published.London

Khan., H. M, Ashraf, M., Hashmi, A. S., Ahmad, M. D., Anjam, A. A. 2013. *Extraction and Biochemichal Characterization of Sulphate Glicosaminoglycans from Chicken Keel Cartilage*. Pak Vet Journal, 33 (4): 471-475.

Lauder, R. M., Huckerby, T. N., and Nieduszynski, I. A. 2000. *A finger-printing method for chondroitin/ dermatan sulfate and hyaluronan oligosaccharides.* Glycobiology, 10: 393-401.

Miwada, INS., dan Simpen. 2013. Kajian Waktu Curing Asam Asetat dan Rasio Kombinasi Kloroform-Etanol untuk Ekstraksi Protein Kulit Ceker. Majalah Ilmiah Peternakan. 16 (1): 28-31.

Naiola E. & Widhyastuti N. 2002. *Isolasi, seleksi dan optimasi produksi protease dari beberapa isolat bakteri*. Hayati. 6: 467-473.

Nakano T., Pietrasik, Z., Ozimek, L., and Betti, M. 2012. *Extraction, Isolation and Analysis Of Chondroitin Sulfate From Broiler Chicken Biomass*. Process Biochemistry, 47 (12): 1909-1918.

Nakano, T., N. Ikawa, L. Ozimek. 2001. *Extraction of Glycosaminoglycans from Chicken Eggshell.* Journal Poultry Science Association 80: 681-684.

Puspawati, Ni Made, I Nengah Simpen, Ni Luh Putu Suciptawati. 2014. *Karakterisasi sifat Fisiko Kimia Gelatin Halal yang Diekstrak dari Kulit Ayam Broiler Melalui Variasi Suhu*. Jurnal Kimia, 8 (1): 127-136.

Shin, S. C. S. J. You, B. K. An, C. W. Kang. 2005. *Study on Extraction of Mucopolysaccharide-Protein Containing Chondroitin Sulfate from Chicken Keel Cartilage*. Asian-Australian Journal Animal, 19 (4): 601-604.

Soda, F. N, and Rudiana Agustini. 2013. *Pengaruh Penambahan Ion Logam K + terhadap Aktivitas Enzim Papain.* UNESA Journal of Chemistry, 2 (2). 29-34.