

## **Effect of ethanol used in a degreasing process on Bali cattle bones on the physicochemical properties of extracted collagen**

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### **Abstract**

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This study aimed to evaluate the effect of the degreasing process, using cattle bones as raw materials on the quantity and quality of collagen extracted. Raw materials in this study were scapula bones (*os scapula*) of Bali cattle. Degreasing treatments included various concentrations of ethanol solutions, with two processing times. Data were analyzed by ANOVA; treatment differences were further tested by the Duncan Multiple Range Test where the significant effect was found in the ANOVA. The results showed that application of ethanol with a concentration of 60% and the time process of 1 day produced better physicochemical properties of the collagen extract, however. Functional properties of products collagen extract from the overall combined treatment showed identical results.

*Keywords:* Bali cattle bones; collagen extract; degreasing; fat; raw material

### **Introduction**

Bones are animal by-products that have a very high benefit and economic value (Jeong et al., 2013). Residual products of slaughter are important to further process and use as a source of income. The problem is that until now, the results of follow-up products are still often regarded as

waste. Bone has a potential as a source of collagen extracts as raw materials for food supplements.

In Indonesia, meat production from Bali cattle produces a great deal of bone waste. Development of uses for Bali cattle bones is important and of interest, because the characteristics of Bali cattle bones are allegedly different from the bones of other cattle breeds. Husbandry differences may account for

some of the bone difference: Bali cattle are often allowed to roam freely while other breeds in other countries are kept largely penned. Bali cattle have less body fat than other cattle, which is thus, allegedly associated with the lower fat content of the bones. Heretofore, bones of Bali cattle are not used, so any product that can be derived has the potential for considerable economic value.

The fat content of the bones can be a problem in producing a quality collagen extract; fat can be expected to hinder the process of extraction of collagen. One of the problems to be solved in order to improve the quality of collagen extracts, therefore, is improving the degreasing process. The degreasing process serves to dissolve some of the fat that deposited on the surface of the bone (Ockerman & Hansen, 2000), which may decrease the ability of the dissolution process of collagen in the extraction process.

The use of polar solvents can lower the fat content of the bones. The main advantage of using ethanol as compared to other solvents are that ethanol has a higher polarity, so it is easy to dissolve resins, fats, oils, fatty acids, carbohydrates and other organic compounds (Zhou et al., 2013). This study aimed to evaluate the effect of ethanol solvents on degreasing process stages on the properties of the collagen extract using bones from Bali cattle as raw materials.

## Materials and Methods

### Materials

The study was using bones from shoulder blades (*os scapula*) of Bali cattle as a raw material, from slaughtered males, approximately 2.5–3 years old. Pure ethanol ( $C_2H_5OH$ ) (Merck) was used to produce the various degreasing solutions. The study was a Completely Randomized Design (CRD) factorial design, where the first factor was three concentrations of ethanol (60%, 70%, and 80%) (v/v) and the second factor was the degreasing time process (1 day and 2 days). Results were analyzed by analysis of variance (ANOVA). Effects that were significant then were tested further via treatment comparisons by Duncan Multiple Range Test (DMRT) (Steel and Torrie, 1991).

### Production process of collagen

Dilutions were prepared using the dilution formula  $V_1M_1 = V_2M_2$  (AOAC, 2005). A number of 5 kg bone samples was prepared. Samples were cut into small bone pieces measuring 1-2 cm<sup>2</sup>, and weighing approximately 300 g. Degreasing process was done by soaking the bone samples in a 60%, 70%, or 80% (v/v) ethanol solution for either one day or two days. The bones were then cleaned with water to remove any traces of ethanol. Next, the bones were demineralized using

an acid solution ( $H_2SO_4$ , 1 M) for 48 hours. The samples were then neutralized with 20%  $Ca(OH)_2$  (w/v) solution.

A solution of  $H_2SO_4$  0.5 M was used as a material demineralization (removal of mineral components) for 48 hours with a ration of bone :  $H_2SO_4$  solution of 1:1.5. It was then neutralized with 10%  $Ca(OH)_2$  solution for 24 hours with a ratio of bone :  $Ca(OH)_2$  solution of 1:1.5. A total of 300 g sample of the bone was used and then four types of extract solution were used with a ratio of bone: extract solution (1:1.5) (v/v). Extracted bone samples were stratified for 48 hours (stage 1: 24 hours, temperature 55-60°C) resulted in fraction 1 and stage 2: 24 hours, temperature 65-70°C resulted in fraction 2. Fractions 1 and 2 were combined and then filtered using a flannel cloth to produce a filtrate. The filtrate was then dried in an oven at a temperature of 55-60°C for 48 hours to obtain collagen. The collagen was pulverized in a blender to further evaluate its properties.

### Parameters of study

The percent yields (%) was assessed by the equation  $Y(\%) = a/b \times 100\%$ , where a = weight of the end products, and b = weight of raw materials (Gimenez et al., 2005b). Collagen extracts solutions were prepared at a concentration of 6.67% w/v (i.e., 6.67 g collagen extract to which distilled water was added to 100 ml prior to heating at a temperature of  $\pm 60^\circ C$  until the extract was completely dissolved. The solution was then put into a container with a diameter of 5 cm and a height of 6 cm, and then refrigerated at a temperature of 5°C for 16-18 hours. Gel strength (*g Bloom*) was measured with a Universal Testing Machine (GY-4) equipped with a Teflon plunger cylinder (diameter 13 mm) (Arnesen & Gildberg, 2002). The samples, within the container, were placed at the bottom of the plunger ( $d = 13$  mm) for further testing processes. The strength test was repeated three times for each sample. The readings of the maximum forces on the plunger were  $F_{max}$  gelled samples ( $N/cm^2$ ). The plunger speed was penetrated into gel into 10 mm/min with the depth of 4 mm. Gel strength (*g Bloom*) =  $20 + 2.86 \cdot 10^{-3} \cdot D$ , where  $D = (F/G) \times 980$ ;  $F$  = maximum forces ( $N/cm^2$ ); and  $G$  = constant (0.07).

The viscosity (cP) of the sample was measured with a Brookville viscometer (Arnesen & Gildberg, 2002). Collagen solutions were prepared at a concentration of 6.67% w/v (6.67 g added with distilled water to 100 ml) prior to heating at a temperature of  $\pm 60^\circ C$  until the collagen particles were completely dissolved. Each collagen extract solution was poured into a bowl and then the viscosity measured. The measurement of the value of collagen extracts was done at 28°C. The result of the measurement was recorded in units of centipoise (cP).

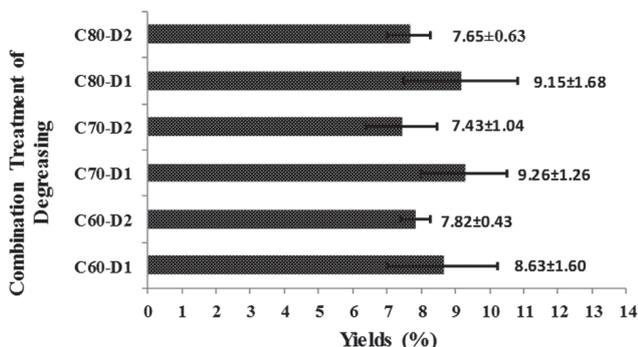
Fat content (%) (Fc) was determined by the Soxhlet method (AOAC, 2005). A total of 2 g of the sample was wrapped in filter paper and put into the Soxhlet flask. Prior to this, the flask was dried in an oven at 105°C for 2 hours and then cooled in a desiccator for 30 minutes. Petroleum ether solution was then put into Soxhlet flask containing the samples, which were then refluxed using a water bath for  $\pm 3$  hours. The flasks were then incubated in a reflux oven for  $\pm 1$  hour at 105°C and cooled in a desiccator for further weighing. Fat content was calculated using the formula:  $Fc = Wf/Ws \times 100\%$ , where  $Wf$  = weight of fat (g); and  $Ws$  = weight of samples (g)  $\times 100\%$ .

Function group properties were determined using the KBr (potassium bromide) pellet method (Arnesen & Gildberg, 2002). Briefly, a total of 0.1-2.0% by weight of pulverized samples under infrared lamps together with KBr, pressed at a pressure of 8-20 tons/unit area to obtain the form of pellets, vacuumed. The pellets were then subjected to Spectrophotometry Fourier Transform Infrared (FTIR) (Shimadzu PC-8201) at wave numbers 4000–650  $\text{cm}^{-1}$  to determine of functional groups (Sastrohamidjojo, 1992).

## Results

### Yields

Comparison of the resulting collagen extracts yields on the implementation of different concentration and time process of degreasing process are presented in Fig. 1. The results of analysis of variance (ANOVA) of the data in Fig. 1 show that differences in the level of concentration and time process showed no significant effects. The results show that the solution of ethanol used as a degreasing agent was not able to influence the rate of formation of collagen extracts products.



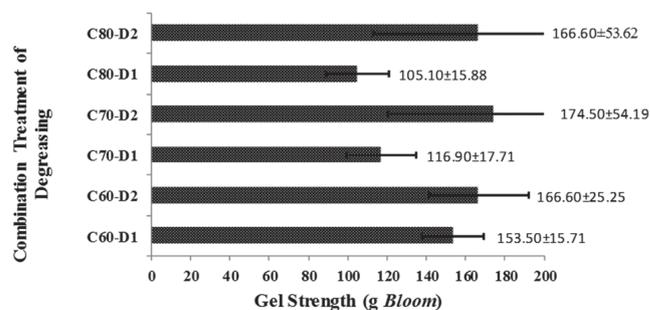
**Fig. 1. Yield (%) values of Bali cattle bones of extracted collagen with different concentrations of ethanol and time process**

$C_{60}$  = concentration of ethanol 60%;  $C_{70}$  = concentration of ethanol 70%;  $C_{80}$  = concentration of ethanol 80%;  $D_1$  = time process of 1 day;  $D_2$  = time process of 2 days

The yields are a parameter for determining the efficiency of the production process, essentially the amount of product produced from a number of raw materials processed (Giménez et al., 2005a). The production process used affects the value of the yields (Zhou & Regenstein, 2005). The acid administration would increase the  $H^+$  ions in solution, which in turn will accelerate the process of hydrolysis rate and tend to increase a number of collagen molecules converted and lead to increased yield (Zhou & Regenstein, 2005; Wahid, 2015). The yields resulted from a production process are greatly influenced by the extraction process (solvent) on the activity of the protein collagen (Kasankala et al., 2007). Increasing concentrations of solvents have affected on the collagen yield. The concentration has an enormous influence on the solubility of collagen into collagen. The increase in yield values associated with the amount of collagen that is converted and transformed into collagen (Zhou & Regenstein, 2005).

### Gel strength

Gel strength values are also influenced by the concentration of materials and processing time. Fig. 2 shows the difference in gel strength values of extract collagen treated with different combinations of concentration levels and processing times. The data in Fig. 2 show that levels of concentration and processing time did not significantly affect the gel strength values of the collagen extract.



**Fig. 2. Gel strength (g Bloom) values of Bali cattle bones of extracted collagen with different concentrations of ethanol and time process**

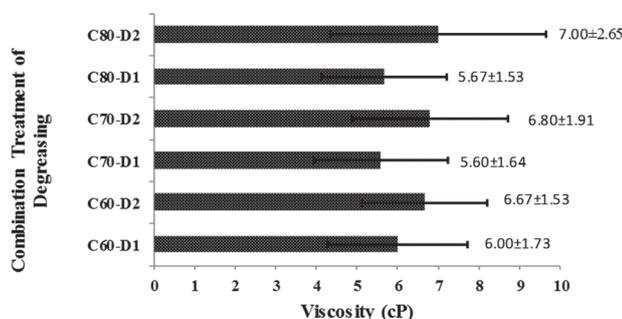
$C_{60}$  = concentration of ethanol 60%;  $C_{70}$  = concentration of ethanol 70%;  $C_{80}$  = concentration of ethanol 80%;  $D_1$  = time process of 1 day;  $D_2$  = time process of 2 days

Gel strength values are associated with hydrogen bonds between water molecules with free hydroxyl groups of the amino acid molecule groups (Arnesen & Gildberg, 2002; Bhat & Karim 2008). The use of ethanol did not affect the strength of the gel because the alcohol groups were not able to break the bonds between the collagen fibers. The use of

strong acids causes breaking of the chains of amino acids in the collagen protein molecule, so that the collagen will suffer damage to the structure of its hydrogen bond and triple helical structure, particularly in chain- $\alpha$ . This damage will affect the properties of gel strength of collagen extract (Giménez et al., 2005a; Kasankala et al., 2007).

### Viscosity

Viscosity values of collagen extract from the different concentration and processing time treatments are presented in Fig. 3. Based on the analysis of variance (ANOVA) data shown in Fig. 3, the difference in the level of concentration of ethanol and time process did not significantly affect ( $p>0.05$ ) the viscosity value of collagen extracts.



**Fig. 3. Viscosity (cP) values of Bali cattle bones of extracted collagen with different concentrations of ethanol and time process**

C<sub>60</sub> = concentration of ethanol 60%; C<sub>70</sub> = concentration of ethanol 70%; C<sub>80</sub> = concentration of ethanol 80%; D<sub>1</sub> = time process of 1 day; D<sub>2</sub> = time process of 2 days

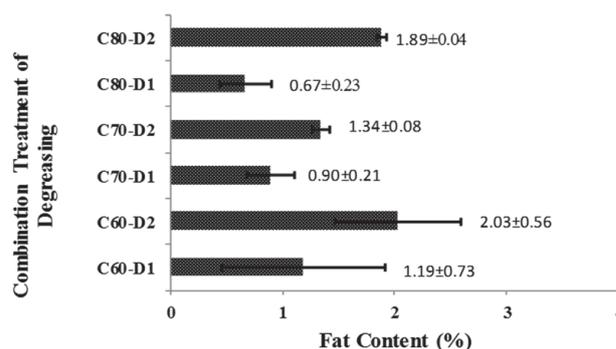
The process flow of a liquid was affected by its viscosity. This process in collagen occurs due to the adsorption process and the development of colloidal structures (Bhat & Karim, 2008). Viscosity values are associated with the arrangement of amino acids in the protein collagen. The different composition of amino acids of collagen structure will increase the viscosity of collagen (Giménez et al., 2005a). Similarly, the conversion rate of collagen will be highly dependent on the application of temperature, time and pH of raw materials (Muyonga et al., 2003; Schrieber & Gareis, 2007).

Viscosity values of collagen from the bones of Bali cattle are on average higher (5-7 cP) than collagen from red tilapia (*Oreochromis niloticus*) fish bones (1.78 cP) (Songchotikunpan et al., 2008) and also 3.20 cP (Jamilah & Harvinder, 2002). High viscosity values on Bali cattle bones seems to be caused by age differences. Bali cattle tend to have a relatively older age before slaughter, which thus allows the intermolecular and intermolecular bonds of

collagen to become more stable in the forming a triple helix conformation.

### Fat content

An overview of the fat content of collagen extracts from the various treatments is presented in Fig. 4. Based on the analysis of variance on the data shown in Fig. 4, the difference time process of degreasing showed significant effects on the fat content of the resultant collagen.



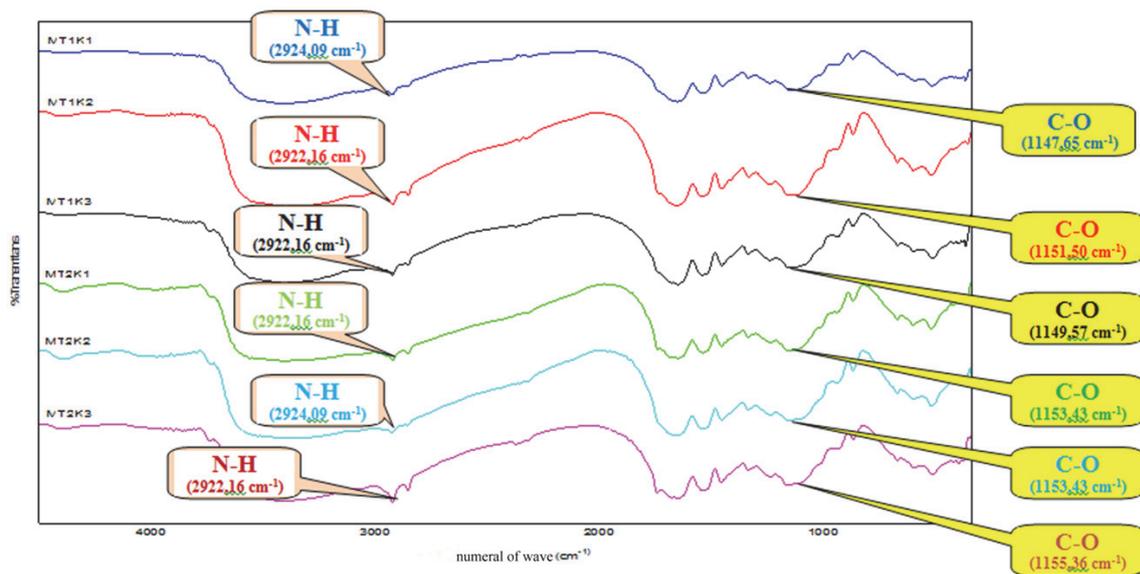
**Fig. 4. Fat content (%) values of Bali cattle bones of extracted collagen with different concentrations of ethanol and time process**

C<sub>60</sub> = concentration of ethanol 60%; C<sub>70</sub> = concentration of ethanol 70%; C<sub>80</sub> = concentration of ethanol 80%; D<sub>1</sub> = time process of 1 day; D<sub>2</sub> = time process of 2 days

The level of fat contained in a foods product directly relates to its quality (Samuel et al., 2014). Fat taste sensitivity has been negatively associated with dietary fat intake. Excess fat consumption has been linked to the development of obesity (Bolhuis et al., 2006). The fat requirements of collagen are <5% (Jobling & Jobling, 1983). Related to fat content collagen, test results of the collagen of Bali cattle bone shows average the fat content ranging (0.67-2.03%) is lower than the required standard.

### Functional properties

An overview of the functional groups of the collagen extracts is presented in Fig. 5. Results show that the product has a C-O functional group (diagnosed by absorption peaks from 1250 cm<sup>-1</sup> to 1000 cm<sup>-1</sup>); peaks are seen here at 1147.65 cm<sup>-1</sup>, 1151.50 cm<sup>-1</sup>, 1149.57 cm<sup>-1</sup>, 1153.43 cm<sup>-1</sup>, 1153.43 cm<sup>-1</sup> and 1155.36 cm<sup>-1</sup>. In addition to a C-O group, one collagen extract product also has an N-H group (amide) in the molecular chain, shown by peaks at 2924.09 cm<sup>-1</sup> and 2922.16 cm<sup>-1</sup>.



**Fig. 5. Functional groups of Bali cattle bones of extracted collagen with different concentrations of ethanol and time process**

$C_{60}$  = concentration of ethanol 60%;  $C_{70}$  = concentration of ethanol 70%;  $C_{80}$  = concentration of ethanol 80%;  $D_1$  = time process of 1 day;  $D_2$  = time process of 2 days

The existence of the group N-H (amide) is often an identifier that the product has a protein component. The presence of N-H group was detected by the same wave numbers indicate that the four have identical functional properties (Sastrohamidjojo, 1992), that the covalent bonds that make up a product will absorb different frequencies of electromagnetic radiation in the infrared region of the spectrum. If the peak spectra exactly the same two compounds in many respects the two compounds are identical.

## Conclusion

The different ethanol concentrations and time on stage degreasing process did not significantly affect the yields value, gel strength value, viscosity value, and fat content. Degreasing processes using an ethanol 60% (v/v) solution and 1 day of treatment was better than the other treatments. Overall, the treatment combinations extracted collagen with identical functional properties.

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## References

- AOAC (2005). Official Methods of Analysis of AOAC International. 18th ed. Association of Official Analytical Chemists.
- Aarnesen, J. A., & Gildberg, A. (2002). Preparation and characterisation of gelatine from the skin of harp seal (*Phoca groenlandica*). *Bioresource Technology*, 82(2), 191-194.
- Bhat, R., & Karim, A. A. (2009). Ultraviolet irradiation improves gel strength of fish gelatin. *Food chemistry*, 113(4), 1160-1164.
- Bolhuis, D. P., Costanzo, A., Newman, L. P., & Keast, R. S. (2015). Salt promotes passive overconsumption of dietary fat in humans. *The Journal of Nutrition*, 146(4), 838-845.
- Giménez, B., Gómez-Guillén, M. C., & Montero, P. (2005a). The role of salt washing of fish skins in chemical and rheological properties of gelatin extracted. *Food Hydrocolloids*, 19(6), 951-957.
- Giménez, B., Gomez-Guillen, M. C., & Montero, P. (2005b). Storage of dried fish skins on quality characteristics of extracted gelatin. *Food Hydrocolloids*, 19(6), 958-963.
- Jamilah, B., & Harvinder, K. G. (2002). Properties of gelatins from skins of fish—black tilapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nilotica*). *Food Chemistry*, 77(1), 81-84.
- Jeong, H. S., Venkatesan, J., & Kim, S. K. (2013). Isolation and characterization of collagen from marine fish (*Thunnus obesus*). *Biotechnology and Bioprocess Engineering*, 18(6), 1185-1191.
- Jobling, A., & Jobling, C. A. (1983). Conversation of bone to edible product. In: D.A. Ledwar, A. J. Taylor, R. A. Lawrie (Ed.), *Upgrading Waste for Feeds and Food*. Butterworths, London.
- Kasankala, L. M., Xue, Y., Weilong, Y., Hong, S. D., & He, Q.

- (2007). Optimization of gelatine extraction from grass carp (*Catenopharyngodon idella*) fish skin by response surface methodology. *Bioresource Technology*, 98(17), 3338-3343.
- Muyonga, J. H., Cole, C. G. B., & Duodu, K. G.** (2004). Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*). *Food Chemistry*, 86(3), 325-332.
- Ockerman, H. W., & Hansen, C. L.** (2000). *Animal by-product processing & utilization*. CRC Press, USA.
- Samuel, L., Basch, C. H., Ethan, D., Hammond, R., & Chiazzese, K.** (2014). An analysis of sodium, total fat and saturated fat contents of packaged food products advertised in Bronx-based supermarket circulars. *Journal of Community Health*, 39(4), 775-782.
- Sastrohamidjojo, H.** (1992). Infrared Spectroscopy. *Liberty*, Yogyakarta. [In Indonesian]
- Schrieber, R., & Gareis, H.** (2007). *Collagene Handbook*. Wiley-VCH GmbH & Co, Weinheim.
- Songchotikunpan, P., Tattiyakul, J., & Supaphol, P.** (2008). Extraction and electrospinning of gelatin from fish skin. *International Journal of Biological Macromolecules*, 42(3), 247-255.
- Steel, R. G. D., & Torrie, J. H.** (1991). *Principle and procedure of statistics*. International Book Company, Tokyo.
- Wahid, A. M.** (2015). Influence of immersion and difference of ethanol concentration on value of rendement and physico-chemical properties of cowboy collagen. Bachelor thesis, Veterinary Faculty, Hasanuddin University, Makassar, Indonesia.
- Zhou, J., Lu, S., Wang, F. H., Wang, J. H., & Cai, C. J.** (2013). Research on degreasing method of the parts in aviation oxygen generating and charging equipment. In *Applied Mechanics and Materials* (Vol. 318, pp. 336-339). Trans Tech Publications.
- Zhou, P., & Regenstein, J. M.** (2005). Effects of alkaline and acid pretreatments on Alaska pollock skin gelatin extraction. *Journal of Food Science*, 70(6), c392-c396.

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