View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by The International Islamic University Malaysia Repository

ISBN 978-81-933894-1-6
5th International Conference on Chemical, Agricultural, Biological and Environmental Sciences (CAFES-17)
Kyoto (Japan) April 18-19, 2017

Extraction Optimization and Characterization of Collagen from Chicken (*Gallus gallus domesticus*) Feet

Nur Dhiya Mokhtar[1], Widya Abdul Wahab[1], Nurul Ashima Hamdan[1], Hazrina Abdul Hadi[2], Muhamad Shafiq Abu Hassan[1] and Noraslinda Muhamad Bunnori[1]

[1]Kulliyyah of Science, International Islamic University Malaysia, Pahang, Malaysia

[2]Kulliyyah of Pharmacy, International Islamic University Malaysia, Pahang, Malaysia

***Abstract*:** *Previous studies have proved that chicken (Gallus gallus domesticus) feet can be one of the alternative sources of collagen. Besides of less risk of diseases and can be accepted by all religions, chicken feet also have high potential as a low cost of collagen source. Method of collagen extraction also plays an important role to obtain high percentage of end yield and the quality of collagen extracted. Optimization of collagen extraction was investigated by using three different soaking methods; 0.5 mol/L acetic acid with 0.1% bromelain, 0.5 mol/L acetic acid with 0.1% papain and 5% lactic acid for 36 hours. Various properties such as percentage of end yield, concentration of protein, pH, swelling percentage and SDS-PAGE patterns of collagen from chicken feet were evaluated in order to determine the best method of extraction and potential of bromelain to improve collagen extraction. No significant different ($P > 0.05$) have been recorded between all three methods used in this research which indicated that all treatments were not affected the pH, swelling percentage, percentage of end yield and concentration of extracted collagen. However, lactic acid method was showed the highest percentage of end yield and concentration with reading of 30.04% and 11.66 mg/ml respectively. Type I collagen has been determined as major component of collagen from chicken feet based on the result of SDS-PAGE. It also proven that bromelain has same potential as papain in order to improve protein digestion.*

Keywords: *Chicken feet, collagen, lactic acid, papain, bromelain, SDS Page.*

1. Introduction

Collagen has been widely used in many industries around the world including Malaysia. Food, pharmaceutical, cosmetic, biomedical and nutraceutical industries are such common industries which used collagen in various applications (Munasinghe et al., 2014; Schmidt et al., 2016). Usually, collagen is used in form of gelatin and collagen peptide or also known as collagen hydrolysate (Aberoumand, 2012). These forms of collagen were applied in the process of making soft and hard capsule shells, tablet coating, serum and also as injection for health benefits (Afifi et al., 2014).

Porcine and bovine are the main sources of collagen following by marine sources such as various species of fish, sponge, jellyfish, squid and also seaweed (Jeevithan et al., 2014; Silvipriya et al., 2015). However, because some allergic cases have been reported among consumers who have allergic reaction to marine and bovine based products (Nik Aisyah et al., 2014), study on other alternatives of the collagen source are imperative.

At the same times, the pandemic outbreak of diseases such as bovine spongiform encephalopathy (BSE) or commonly known as mad cow disease, foot and mouth disease (FMD) have resulted in anxiety among consumers (Wibawa et al., 2015) and created awareness regarding the sources of collagen to be taken (Kiruthiga et al., 2013). Thus, other potential and low cost of collagen sources such as poultry feet (chicken and duck), bones and skin have risen to replace the mammalian and marine sources (Huda et al., 2013). Poultry as an

alternative source of collagen showed that collagen composition of chicken feet was two-times larger than the commercial gelatin and the result of analysis showed no difference between chicken feet gelatin and meat gelatin at 95% level of significance (Almeida et al., 2013).

In this research, method of extraction by using bromelain enzyme was tested and compared to the method that using papain and lactic acid. Bromelain present in large amount in fruit, leaves and stems of varieties of pineapples. It is known as proteolytic enzyme or protease as protein-digestion enzyme and usually used as meat tenderizer. Bromelain catalyze the breakdown of protein into amino acid building block by hydrolysis reaction (Martins et al., 2014). In this reaction, the peptide bonds are broken with the insertion of water components at the end chain. As it has ability to speed up the digestion of protein, bromelain might have potential similar as papain to be used in collagen extraction method and might produce higher end yield.

2. Materials and Method

2.1. Materials

Chicken feet were obtained from local broiler supplier located at Pahang, Malaysia and the raw samples were stored in cold room at -20^{o}C. Material preparation was adopted from Munashinghe et al. (2014) with slight modifications.

2.2. Enzymatic Extraction Method

Papain and bromelain enzymes were used separately to isolate the collagen from chicken feet. All procedures were performed at 4^{o}C to avoid protein degradation. The ground samples were defatted under continuous stirring with 10 volumes of ethanol for 6 hours. The defatted samples then decalcified under stirring with 0.5 mol/L ethylenediaminetetraacetic acid-disodium (EDTA-2Na) for another 6 hours. In order to remove non-collagenous protein material from samples, the treated samples were soaked in 0.1 mol/L NaOH for next 6 hours. The residues were rinsed thoroughly with cold distilled water and then suspended in 0.5 mol/L acetic acid containing 0.1% (w/v) enzyme (papain/bromelain) for 24 hours. The samples then were centrifuged at 3,500 x g for 3 hours. Supernatants were collected and salted out by adding 0.75 M NaCl with overnight stirring. Salted out supernatants were centrifuged again for another 3 hours at 3,500 x g to obtained final solution which then dialyzed for 24 hours against distilled water by using dialysis bag with range of molecular weight cut off in between 12,000 to 14,000 Dalton. The final products were lyophilized in freeze dryer to obtain the powder form of collagen.

2.3. Acid Extraction Method

Acid extraction was done according to method obtained from Liu et al. (2001) and Huda et al. (2013) with slight modification. The grounded chicken feet were soaked for 36 hours at 4˚C by using 5% (w/v) lactic acid with sample to solution ratio. The treated samples of chicken feet suspended solution was homogenized by using Panasonic blender for 5 minutes and the sample was filtered with stainless steel filter to remove all bone residues. The filtrate was neutralized to pH 7 with 0.1 M NaOH and the centrifuged at 3500 x g for 45 minutes at 10˚C. The precipitates were collected and lyophilized by freeze dryer to obtain powder form of collagen.

2.4. pH, Swelling Percentage and End Yield of Extracted Collagen

At the end of the each treatment, the pH and swelling percentage of treated samples were determined to examine the relationship between extracting efficiency and factors which might influence the end result of extracted collagen. The pH value was determined by using pH meter (HANNA, HI 8314) and swelling percentage was calculated according to formula as follows (Lin et al., 2013): (Weight of dry collagen / Initial weight of sample treated) x 100%. The percentage of end yield crude collagen from each method was calculated by using the following formula which adopted from Hashim et al., 2014: (Weight of crude collagen / Weight of ground chicken feet) x 100%

2.5. Protein Concentration and SDS-PAGE

Protein quantification procedure was conducted based on Bradford Assay manual provided by Bio-Rad Laboratories Incorporation. Appropriate amount from each sample of collagen powder was dissolved in sample buffer. The stock standard solution was prepared with concentration 2.0 mg/ml of Bovine Serum Albumin (BSA) which dissolved in the same buffer used for samples. SDS-PAGE was used to separate the protein contents of collagen samples by following the method from Widyasari and Rawdkuen (2015) with slight modifications. The percentages used for resolving and stacking gel (Jongjareonrak et al., 2005) were 7.5% and 4% formulation respectively which were suggested to distinguish the molecular weight and types of collagen extracted from chicken feet. The gel was stained with Coomasie Blue R250 dissolved in acetic acid solution for one hour or overnight. The gel then was viewed using densitometer. All the data from experiments were analyzed by using Statistical Package Social Science (SPSS) version 20. The differences between means were evaluated by using Tukey's test.

3. Result and Discussion

3.1. pH, Swelling Percentage and Percentage of End Yield

The percentage of swollen chicken feet is depends on the pH of soaking solution. The sample of chicken feet was treated with acid solution is able to made porous and enhances the acid or enzyme digestion which then resulted on more molecular crosslinks between collagen and the other components can be weakened by disruption of the covalent bonding (Cheng et al., 2008). Thus enable the protein collagen to be extracted. Low pH of soaking solutions is able to increase the yield of collagen extraction. The pH values, swelling percentage and end yield percentage of the sample treated with different methods are shown in Table 1. The average swelling percentage of the samples for bromelain added into acetic acid method and lactic acid method were showed only small different which are 225.68% and 224.60% respectively. Method of acetic acid with papain recorded the lowest percentage among other methods. However, based on statistical analysis by one-way ANOVA and post-hoc test, all these three methods have showed no significant different. The different methods of collagen extraction were not affected the swelling percentage of the chicken feet.

TABLE I: ph , Swelling and End Yield Percentage.

Method	pH	Swelling %	% end yield
Acetic acid+Bromelain	2.23 ± 0.44	225.68 ± 28.47	12.80 ± 8.36
Acetic acid+Papain	2.25 ± 0.61	202.70 ± 20.19	12.63 ± 8.63
Lactic Acid	1.91 ± 0.28	224.60 ± 20.00	30.04 ± 15.88

According to Puspitasari et al. (2015), the low pH of the acid solution (> pH 2) that cause the high swelling percentage is able to influence the percentage of end yield of the extracted collagen. This condition causes the collagenous structure become loose due to high water absorption and the increase of water bonding ability which finally helps the collagen to be solubilized (Skierka & Sadowska, 2007). However, according Giménez and Montero (2001), low er pH value (< pH 2) of soaking solution might cause denaturation of collagen during the extraction process. Result showed lactic acid method recorded the lowest pH value of soaking solution produced the highest percentage of end yield (30.04%) and the extracted collagen did not undergo denaturation. Enzymatic extraction methods were showed very low percentage of end yield which are 12.80% and 12.63% respectively compared to lactic acid method.

This situation might due to the different of the extraction method and influenced the end yield and other properties of the collagen extracted (Tiago et al., 2014). For the enzymatic methods, the molecular structure of collagen content in the sample will be broken down and dissolved in acid solution after soaking process with aid of enzyme added (Cheng et al., 2009). The supernatant from both enzymatic methods were collected before proceed to the freeze dry step. The result of extracted collagen in low amount powder form was collected after the freeze dried due to the sublimation process under high vacuum to heat. The sublimation process causes the

supernatant that have high water contain and pre-freezed at -80^{o}C to be directly sublimed to vapour form without undergo liquid phase, leaving only solid, dried components of the original liquid (Nireesha et al., 2013). Compared to the lactic acid method, the precipitate was collected and was proceed to freeze dry. The amount of collagen powder was increase because of low water content in the precipitate compared to supernatant. Liu et al. (2001) has showed in his study which soaking duration is one of factors that can influence the yield of collagen extracted. However, the increased of soaking time only cause small changes in end yield percentage and this condition is depends on the types of acid solution used in extraction process (Skierka & Sadowska, 2013).

3.2. Protein Concentration

Concentration of protein from the extracted collagen is shown in Table 2. Data indicated that lactic acid extraction method was recorded the highest reading of concentration and method that using papain enzyme was recorded as the lowest yield. This result was proportionate to the result of percentage end yield which recorded the method of lactic acid was produced the highest yield. However, based on statistical analysis by one-way ANOVA, there was no significant different between all three methods which brought a conclusion that, different treatments had not significantly affect the protein concentration of extracted collagen.

TABLE 2: Protein Concentration of Extracted Collagen.

Method	Concentration (mg/ml)
Acetic acid+Bromelain	8.67 ± 2.37
Acetic acid+Papain	6.68 ± 1.34
Lactic Acid	11.66 ± 4.94

The concentration values obtained indicate as whole protein concentration not for pure collagen itself. The observation of all methods that had been chosen in this research had removed most of contaminants and uninterested protein components from the extracted collagen (Khiari et al., 2014) and remained the major protein components in the sample extracted which identified as collagen type I. This can be seen in the SDS-PAGE pattern of the samples' extracted.

3.3 Protein Separation by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the methods that used to characterize the purity of protein sample. By using this method, protein collagen and its fragments can be separated according to their molecular sizes. SDS-PAGE pattern in Fig. 1 showed band patterns of collagen extracted from chicken feet according to method used. The highest protein bands could be observed approximately at 260 kDa of molecular size which known as β-bands and two distinct α-bands (α_1 and α_2-bands) were observed at molecular sizes approximately 148 kDa and 135 kDa respectively. The SDS-PAGE patterns of collagen type I derived from chicken skin and rabbit in Fig. 2 were compared and showed the slightly similar pattern of protein bands from all three sources.

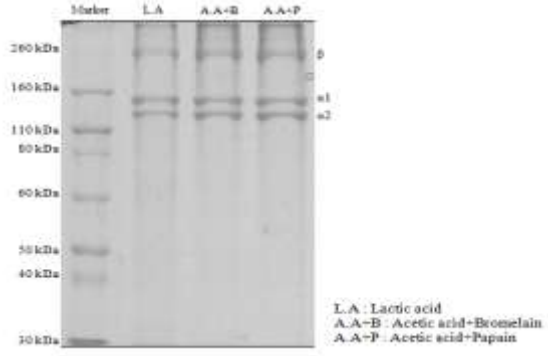

Fig. 1: Protein Bands Pattern of Collagen Type I Extracted from Chicken Feet.

According to Islam et al. (2015), collagen type I are heterotrimers which have two distinct α chains. The heterotrimers consist of two identical α_1-chains and one α_2-chain. Rabotyagova et al. (2011) have recorded the

migration of collagen type I was generated three bands on SDS-PAGE gel are monomeric α_1 and α_2, β-band and γ-band. The β-band is a dimer and represent for α_1 and α_2-chains together or two α_1-chains while γ-band represents three α-chains together. However, the γ-bands not appeared on the gel in Fig. 1 due to the denaturation of collagen by heat and the collagen is not in the triple helix conformation. Generally, protein collagen consists of three identical or non-identical polypeptides (Richard-Blum, 2015) that coil to each other into helices to form triple-helix tropocollagen (Parmar et al., 2015). Small difference in molecular weight due to the different amino acid sequences make the bands are slightly different between collagen from various species (Tiago et al., 2014).

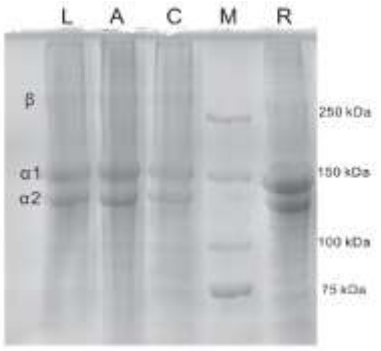

Fig. 2: SDS-PAGE of collagen type I derived from chicken skin with different treatment. L: Lactic acid-pepsin treatment; A: Acetic acid-pepsin treatment; C: Citric acid-pepsin treatment; M: High MW marker; R: Rabbit collagen type I (Chia et al., 2013)

Based on this result, it showed that protein fragment with lower molecular weight than α-chains for all three methods of extraction could not be observed. This indicates that other contaminants such as hemoglobin and enzyme have been removed from the extracted collagen (Brock et al., 2013). Furthermore, the results of SDS-PAGE also proved that structural integrity of the extracted collagen could be maintained throughout the extraction processes for each different method (Lin & Liu, 2006).

As conclusion, all three methods that were used in this research have not showed any statistically significant difference in pH, swelling percentage, percentage of end yield, and protein concentration. It showed the acid-bromelain extraction method is able to produce the percentage of end yield as much as acid-papain and lactic acid extraction methods. Thus, this proof that acid-bromelain can be one of alternative methods of collagen extraction besides of papain to replace the extraction method that using pepsin to avoid the uses of any porcine related product. Collagen extracted method by using 5% lactic acid for 36 hours was showed as the most optimum extraction method which has recorded the highest percentage of end yield and protein concentration among other methods with reading of 30.04% and 11.66 mg/ml respectively. Type I collagen have been proof as major component of collagen extracted from chicken feet based on the observation of SDS-PAGE pattern which consist of α_1 and α_2-chains.

4. Acknowledgements

This project was funded by Research Initiative Grant Scheme (RIGS15-103-0103) from Malaysian Ministry of Higher Education.

5. References

1) Aberoumand, A. (2012). Comparative Study between Different Methods of Collagen Extraction from Fish and its Properties. *World Applied Sciences Journal, 16*(3), 316–319.

2) Afifi, M., Halim, A., Mahyeddin, Mohd, Salleh, M., Mohd, Ariff, I., Nordin, Norhaslinda. (2014). Halal Pharmaceuticals: Legal, Shari'ah Issues and Fatwa of Drug, Gelatine And Alcohol Contribution/ Originality. *International Journal of Asian Social Science International Journal of Asian Social Science ISSN International Journal of Asian Social Science, 4*(412), 1176–1190.

3) Almeida, P. F. De, Calarge, F. A., and Santana J. C. C. (2013). Production of a Product Similar to Gelatin from Chicken Feet Collagen. *Eng. Agric., Jaboticabal, 33*(6), 1289-1300.
https://doi.org/10.1590/s0100-69162013000600021

4) Brock, F., Geoghegan, V., Thomas, B., Jurkschat, K., and Higham, T. F. G. (2013). Analysis of Bone "Collagen" Extraction Products For Radiocarbon Dating. *Radiocarbon*, *55*(2), 445–463.
https://doi.org/10.1017/S0033822200057581

5) Cheng, F., Hsu, F., Chang, H., Lin, L., and Sakata, R. (2009). Effect of different acids on the extraction of pepsin-solubilised collagen containing melanin from silky fowl feet. *Food Chemistry*, *113*(2), 563–567.
https://doi.org/10.1016/j.foodchem.2008.08.043

6) Chia, W. L., Loughran, M., Tsung, Y. S., and Shuo, W. T. (2013). Evaluation Of Convenient Extraction Of Chicken Skin Collagen Using Organic Acid And Pepsin Combination. *Journal China Social Animal Science, 42*(1), 27–38.

7) Giménez, B., and Montero, M. P. (2011). Food Hydrocolloids Functional and bioactive properties of collagen and gelatin from alternative sources : A review. *Food Hydrocolloids*, *25*(8), 1813–1827.
https://doi.org/10.1016/j.foodhyd.2011.02.007

8) Hashim, P., Ridzwan, M. S. M., & Bakar, J. (2014). Isolation and Characterization of Collagen from Chicken Feet. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, *8*(3), 147–151.

9) Huda, N., Seow, E. K., Normawati, M. N., and Aisyah, N. M. N. (2013). Preliminary Study on Physicochemical Properties of Duck Feet Collagen. *International Food Research Journal*, *12*(10), 615–621.
https://doi.org/10.3923/ijps.2013.615.621

10) Islam, Z., Islam, M. M., Saha, S., Jahangir, C. A., Basak, B., Islam, M. N., Islam M. S., Paul S., and Khalekuzzaman M. (2015). Identification and Computational Analysis of Chicken Alpha-1 Collagen Sequences. *International Journal of Scientific and Engineering Research*, *6*(1), 217–221.
https://doi.org/10.14299/ijser.2015.01.014

11) Jeevithan, E., Bao, B., Bu, Y., Zhou, Y., Zhao, Q., and Wu, W. (2014). Type II Collagen and Gelatin from Silvertip Shark (*Carcharhinus albimarginatus*) Cartilage: Isolation, Purification, Physicochemical and Antioxidant Properties. *Marine Drugs*, *12*, 3852–3873.
https://doi.org/10.3390/md12073852

12) Jongjareonrak, A., Benjakul, S., and Visessanguan, W. (2015). Food Chemistry Isolation and Characterisation of Acid and Pepsin-Solubilised Collagens from the Skin of Brownstripe Red Snapper (*Lutjanus vitta*). *Food Chemistry*, *93*, 475–484.
https://doi.org/10.1016/j.foodchem.2004.10.026

13) Khiari, Z., Ndagijimana, M., and Betti, M. (2014). Low Molecular Weight Bioactive Peptides Derived from the Enzymatic Hydrolysis of Collagen after Isoelectric Solubilization/Precipitation Process of Turkey By-products. *Poultry Science*, *93*, 1-16.
https://doi.org/10.3382/ps.2014-03953

14) Kiruthiga, N., Sujithra, S., & Kumaresan, R. (2013). Extraction and Characerisation of Type I Collagen From Broiler. *International Journal of Institutional Pharmacy and Life Sciences*, *3*(6), 53–60.

15) Lin, C., Loughran, M., and Tsai, T. (2013). Evaluation of Convenient Extraction Of Chicken Skin Collagen Using Organic Acid and Pepsin Combination. *J. Chin. Soc. Anim. Sic.*, *42*(1), 27–38.

16) Lin, Y. K., and Liu, D. C. (2006). Effect of Pepsin Digestion at Different Temperatures and Times on Properties of Telopeptide-poor Collagen from Bird Feet. *Food Chemistry*, *94*(4), 621-625.
https://doi.org/10.1016/j.foodchem.2004.12.007

17) Liu, D. C., Lin, Y. K., & Chen, M. T. (2001). Optimum Condition of Extracting Collagen from Chicken Feet and its Characetristics. *Asian-Australasian Journal of Animal Sciences*.
https://doi.org/10.5713/ajas.2001.1638

18) Martins, B. C., Rescolino, R., Coelho, D. F., Zanchetta, B., Tambourgi, E. B. and Silveira, E. (2014). Characterization of Bromelain from Ananas Comosus Agroindustrial Residues Purified by Ethanol Factional Precipitation. *Chemical Engineering Transection*, *37*, 781–786.

19) Munasinghe, K. A., Schwarz, J. G., & Nyame, A. K. (2014). Chicken Collagen from Law Market Value By-Products as an Alternate Source. *Journal of Food Processing*, *2014*, 1–6.
https://doi.org/10.1155/2014/298295

20) Nik Aisyah, N. ., Huda, N., Azhar, M., and Fazilah, A. (2014). Poultry as an Alternative Source of Gelatin. *Health and the Environment Journal*, *5*(1), 37–49.

21) Nireesha, G. R., Divya, L., Sowmya, C., Venkateshan, N., Babu, M. N., and Lavakumar, V. (2013). Lyophilization/Freeze Drying-An Review. *International Journal of Novel Trends in Pharmaceutical Sciences*, *3*(4), 87–98.

22) Parmar, A. S., Xu, F., Pike, D. H., Belure, S. V, Hasan, N. F., Drzewiecki, K. E., Shreiber, D. I., and Nanda, V. (2015). Metal Stabilization of Collagen and de Novo Designed Mimetic Peptides. *Biochemistry 54.* 4987-4997.

https://doi.org/10.1021/acs.biochem.5b00502

23) Puspitasari, D. A. P., Bintoro, V. P., and Setiani, B. E. (2013). The Soaking Effect On Different Hydrocloride Acid Level and Soaking Time on pH, Swelling Percentage And Collagen Yield Of Chicken Shank Bone. *Journal Indonesian Tropical Animal Agriculture*, *38*(2), 98-102.

https://doi.org/10.14710/jitaa.38.2.98-102

24) Rabotyagova, O. S., Cebe, P., and Kaplan, D. L. (2008). Collagen Structural Hierarchy and Susceptibility to Degradation by Ultraviolet Radiation. *Mater Science Engineering C Mater Biological Application*, *28*(8), 1420-1429.

https://doi.org/10.1016/j.msec.2008.03.012

25) Schmidt, M. M., Dornelles, R. C. P., Mello, R. O., Kubota, E. H., Mazutti, M. A., Kempka, A. P. and Demiate, I. M. (2016). Collagen Extraction Process. *International Food Research Journal, 23(3),* 913-922.

26) Silvipriya, K. S., Kumar, K. K., Bhat, A. R., Kumar, B. D., and John, A. (2015). Collagen : Animal Sources and Biomedical Application. *Journal of Applied Pharmaceutical Science*, *5*(03), 123–127.

https://doi.org/10.7324/JAPS.2015.50322

27) Skierka, E. & Sadowska, M. (2007). The Influence of Different Acids and Pepsin on The Extractability of Collagen from The Skin of Baltic Cod (*Gadus morhua*), *Food Chemistry, 105*, 1302–1306.

https://doi.org/10.1016/j.foodchem.2007.04.030

28) Tiago, H. S., Joana, M. S., Ana, L. P. M., Alberta, D., Yves, B. and Rui, L. R. (2014). Marine Origin Collagens and Its Potential Applications. *Marine Drugs*, *12*, 5881–5901

https://doi.org/10.3390/md12125881.

29) Wibawa, S. F., Retnoningrum, D. S., and Suhartono, M. T. (2015). Acid Soluble Collagen from Skin of Common Carp (*Cyprinus carpio* L), Red Snapper (*Lutjanus sp*.) and Milkfish (*Chanos chanos*). *World Applied Science Journal*, *33*(6), 990–995.

30) Widyasari, R. and Rawdkuen, S. (2015). Journal of Food Science and Agricultural Technology Gelatin From Chicken Feet : Papain-Assisted Extraction, Characterization and Its Application. *Journal of Food Science and Agricultural Technology*, *1*(1), 136–143.