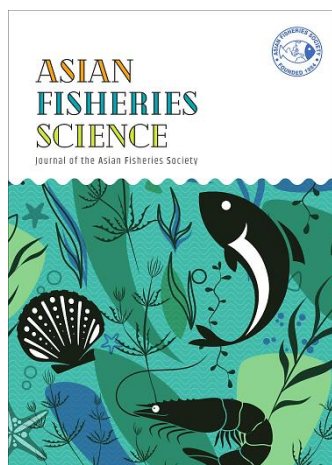




Isolation of Hyaluronic Acid from Yellowfin Tuna *Thunnus albacares* (Bonnaterre, 1788) Eyeball

ANGELECA SUMOGOD¹, LODA NACIONAL¹, MAE GRACE NILLOS², ENCARNACION EMILIA YAP^{1,*}

¹Institute of Fish Processing Technology, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo 5023, The Philippines

²Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo 5023, The Philippines

*E-mail: esyap@up.edu.ph | Received: 13/07/2019; Accepted: 04/03/2020

©Asian Fisheries Society

ISSN: 0116-6514

E-ISSN: 2073-3720

<https://doi.org/10.33997/j.afs.2020.33.1.001>

Abstract

Hyaluronic acid (HA) is commonly extracted from terrestrial and bacterial sources. However, due to the risk associated with animal and bacterial derived contaminants and the laborious production that tend to compromise the quality of HA, research studies have recently shifted to the exploration of HA in marine resources and the enhancement of the protocols for its production. In this study, isolation of HA from yellowfin tuna *Thunnus albacares* (Bonnaterre, 1788) eyeball was carried out through standardisation of the different steps in HA isolation, including tissue extraction, enzymatic hydrolysis, precipitation with cetylpyridinium chloride (CPC) - sodium chloride (NaCl) solution, filtration and diafiltration, precipitation in alkaline hydroalcoholic solution, and lyophilisation. Results revealed that the highest HA yield and purity were observed in the indirect treatment wherein the raw material was pre-treated with acetone, formaldehyde, and sodium acetate solution prior to water extraction and incubation of the mixture for 24 h. Enzymatic hydrolysis revealed that 6 h was the maximum tissue hydrolysis period. Results further showed that the optimum conditions for HA isolation were through the use of 3 % CPC:3M NaCl concentration for recovery and fractionation and a 1:3 mL.mL⁻¹ supernatant:ethanol ratio for alcoholic precipitation. The data gathered from this study could help maximise the benefits of the tuna processing waste in the country and may open a new opportunity for more cost-effective production of a valuable bioactive compound from a natural source.

Keywords: *Thunnus albacares*, hyaluronic acid, waste utilisation

Introduction

Hyaluronic acid (HA), the only unsulfated glycosaminoglycan, is formed by repeating disaccharide units of N-acetyl-D-glucosamine and glucuronic acid linked alternately by (β-1, 3) and (β-1, 4) glycosidic bonds (Saranraj and Naidu, 2013). The production of this polymer is gaining growing interest due to its applications in cosmetic, biotechnological, pharmacological, and medical sectors (Chen et al., 2014; Yang et al., 2015). However, most of the commercially available HA has so far been extracted from either higher vertebrates or bacterial sources that often point to the issues related to the risk of contaminations with proteins, nucleic acids, and heavy metals, and interspecies viral infection (Saranraj and Naidu, 2013). Due to the increasing demand for HA and abovementioned problems associated with the existing

HA sources, attempts have been made on the isolation of this polymer from aquatic resources like yellowfin tuna *Thunnus albacares* (Bonnaterre, 1788) (Mizuno et al., 1991), bigeye tuna *Thunnus obesus* (Lowe, 1839) (Amagai et al., 2009), swordfish *Xiphias gladius* Linnaeus, 1758 and shark *Prionace glauca* (Linnaeus, 1758) (Murado et al., 2012), and stingray *Aetobatus narinari* (Euphrasen, 1790) (Sadhasivam et al., 2013). Interestingly, in fishes, the only apparent source of HA is the vitreous humour (VH) (Vazquez et al., 2013) since this tissue source has lesser interferences as compared to other sources, having a composition by weight of ~ 99 % water and 0.9 % salts with the remaining 0.1 % divided to protein and polysaccharide components (Bishop, 2000).

Aside from problems on HA sources, research is being conducted on the application of this polymer to

different fields without enough regards on the quality enhancement and optimisation of the existing production process (Murado et al., 2012). Factors such as the purity and yield are affected by the contaminants extracted from the tissue along with the HA. It has been reported that the larger the amount of proteins and biological interferences present in the extracts, the more complex, costly, and laborious the production steps will become, and these may lead to low yield of the final product (Amagai et al., 2009). Therefore, an attempt was made in the present investigation to isolate HA from yellowfin tuna eyeball. Optimum yield and purity for the isolation of HA were assessed quantitatively using several factors such as the method of extraction, incubation period, hydrolysis period, sodium chloride (NaCl) concentration, and ethanol volumes.

Materials and Methods

Sample collection and preparation

Two hundred pieces of frozen yellowfin tuna eyeballs (64.11 ± 24.40 g) were obtained from Philippine Cinmic Industrial Company, Barangay Tambler, General Santos City, South Cotabato ($6^{\circ}03'28''$ N $125^{\circ}09'54''$ E). Upon arrival, the eyeballs were cleaned, washed with 1 % CPC solution to disinfect the samples, and frozen until needed (Balazs et al., 1987).

Tissue extraction

The tuna eyeball was subjected to two treatments. In the direct treatment (T1), the eyeball was cut and homogenised for 2 min using a tissue homogeniser (HumanLab, Korea) to facilitate complete deconstruction of the humour. The homogenised sample was filtrated using a fine nylon mesh and mixed with deionised water for extraction. For the indirect treatment (T2), the eyeball was pretreated with acetone (99.9 %), formaldehyde (37 %), and sodium acetate solution (1.0:0.1:0.05, pH 7.0-7.5) for 4 h at room temperature. The mixture was decanted while retaining the eyeball. The eyeball was washed with acetone (1:0.5 mL.mL⁻¹) and dried at room temperature for 24 h. The VH was manually dissected, homogenised, and extracted with deionised water.

Extraction was done in a ratio of 1:2 mL.mL⁻¹ of the sample and deionised water, pH 7.0-8.0 and incubation at room temperature for 24, 48, and 72 h, respectively. Prior to incubation, 10 mL of aliquot per treatment was pooled for the determination of total carbohydrates using the phenol-sulfuric acid assay (Dubois et al., 1956), uronic acid content using the carbazole assay (Bitter and Muir, 1962), and total soluble proteins using the Lowry assay (Lowry et al., 1951). Subsequently, the incubated samples were clarified by centrifugation (Centurion Scientific, UK) at 3000 ×g for 30 min, room temperature and the recovered viscous VH was assayed again for the

quantification of total carbohydrates, uronic acid, and total soluble protein contents (Murado et al., 2012). Yield and per cent purity were computed using the following formula:

$$\text{Yield} = \frac{\text{Uronic acid}}{\text{Weight of raw material}}$$

$$\text{Per cent purity} = \frac{\text{Uronic acid} \times 100}{\text{Total carbohydrates} + \text{Total soluble proteins}}$$

Enzymatic hydrolysis

A solution of papain in 50 mM tris-HCl buffer, pH 7.5 (20 mg.mL⁻¹) was added in the supernatant from the previous experiment (5 mL.mL⁻¹) and the mixture was incubated in a water bath (Premium, USA) at 60 °C for 6, 9, and 12 h. After hydrolysis, the sample was boiled for 10 min, centrifuged at 3000 ×g for 30 min, room temperature, and the recovered supernatant was analysed for the determination of the total carbohydrates, uronic acid, and total soluble protein contents (Da Rosa et al., 2007; Sadhasivam et al., 2013; Raghuraman, 2013).

Recovery and fractionation

The supernatant retrieved from the previous experiment was fractionated using 3 % CPC containing different sodium chloride (NaCl) concentrations (1, 2, and 3 M) in a ratio of 2:1 mL.mL⁻¹ of supernatant and was then left for 15 min at room temperature. The mixture was filtered in 0.2 µm polyethersulfone membrane filter, 2.0 µm (Sterlitech, USA) and was diafiltrated against 5 volumes of NaCl (1, 2, and 3 M) at 30 kDa molecular weight cut-off (MWCO), hydrophilic polyethersulfone (PESH), ultrafiltration membrane (Sterlitech, USA) (Prescott, 2003). Polycarbonate stirred cell was used as the filter holder in the filtration-diafiltration steps (Advantec, Japan). Samples were then analysed for the determination of total carbohydrates, uronic acid, and total soluble protein.

Alcoholic precipitation and lyophilisation

The solution in the previous experiment was precipitated with ethanol (99.89 %) (1:1, 1:2, and 1:3 mL.mL⁻¹) for 24 h at -19 ± 2 °C. Then, it was centrifuged at 6000 ×g for 15 min (room temperature) to recover the pellet. The pellet was washed with 80 % ethanol, centrifuged again (3000 ×g for 30 min, room temperature), dissolved in deionised water (50 mg.mL⁻¹), and recovered through freeze-drying (Eyela, Japan) (Da Rosa et al., 2007). The lyophilised sample was dissolved in deionised water (10 mg.mL⁻¹) for the quantifications of total carbohydrates, uronic acid, and total soluble proteins (Prescott, 2003).

Statistical analysis

Statistical analysis of the experimental data was made using analysis of variance (ANOVA) followed by Tukey's test ($P \leq 0.05$) using SPSS 20.

Results

Tissue extraction

The total carbohydrates, uronic acid, and total soluble proteins of the yellowfin tuna eyeball for direct and indirect treatments during tissue extraction at different incubation periods are presented in Figure 1.

Results revealed that the highest total carbohydrate was obtained in 0 h for both treatments (Fig. 1a). Also, incubation of the samples at 24 h or longer results to lower total carbohydrates but these values were not significantly different from each other. For the uronic acid content and total soluble proteins, results showed a significantly higher initial uronic acid in T1 than T2 (Fig. 1b and 1c) while erratic values were observed in both treatments as the incubation period progressed. For the yield and per cent purity, highest values were found at 24 h incubation period for the two treatments tested (Fig. 2).

Based on the results of the tissue extraction protocol, T2 incubated at 24 h was the more efficient treatment: incubation period combination among the treatments tested having a total carbohydrates, uronic acid, total soluble proteins, yield and per cent purity of 0.11 mg.mL^{-1} , 0.03 mg.mL^{-1} , 1.99 mg.mL^{-1} , $2.34 \text{ mg uronic acid.kg eyeball}^{-1}$, and 1.42% , respectively.

Enzymatic hydrolysis

The significant decline in the values of total carbohydrates and uronic acid content were noted after 6 h digestion time while no significant change in the total soluble proteins was detected as the digestion period progressed (Fig. 3). The highest values of yield and per cent purity were at 6 h digestion period (Fig. 4).

In summary, the results of the enzymatic hydrolysis revealed that 6 h was the optimum digestion period for the yellowfin tuna eyeballs. This condition has a total carbohydrate of 0.48 mg.mL^{-1} , uronic acid content of 0.10 mg.mL^{-1} , total soluble proteins of 3.80 mg.mL^{-1} , a yield of $4.78 \text{ mg uronic acid.kg eyeball}^{-1}$, and purity of 2.64% .

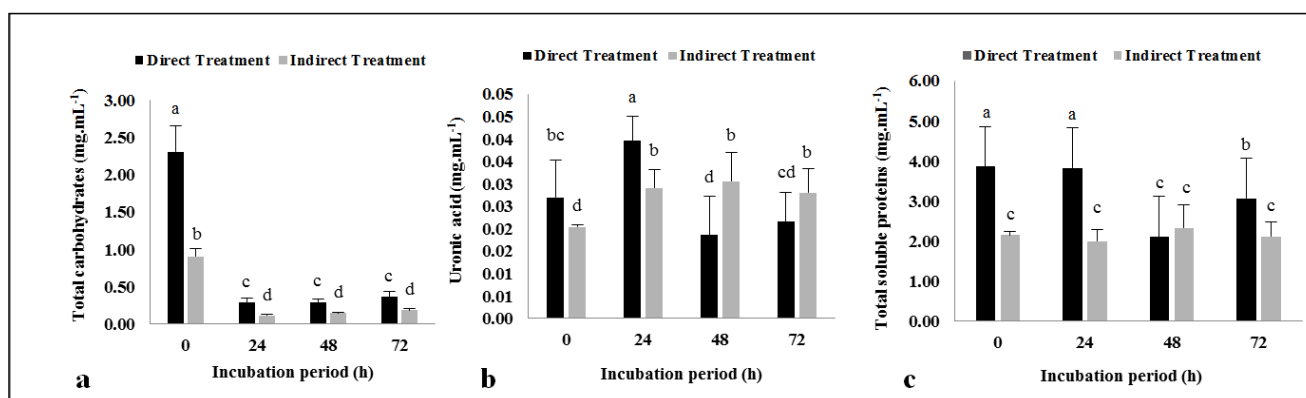


Fig. 1. Total carbohydrates (a), uronic acid (b), and total soluble proteins (c) of the yellowfin tuna *Thunnus albacares* eyeball for direct and indirect treatments during tissue extraction at different incubation periods. Data are mean $\pm$ SD of three determinations. Values with the same superscripts are not significantly different ($P < 0.05$).

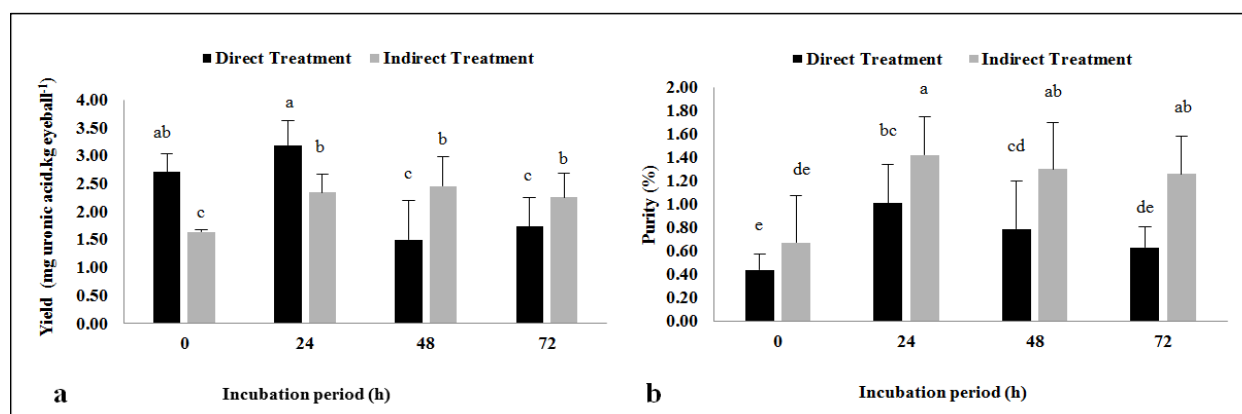


Fig. 2. Yield (a) and per cent purity (b) of the HA from yellowfin tuna *Thunnus albacares* eyeball for direct and indirect treatments during tissue extraction at different incubation periods. Data are mean $\pm$ SD of three determinations. Values with the same superscripts are not significantly different ($P < 0.05$).

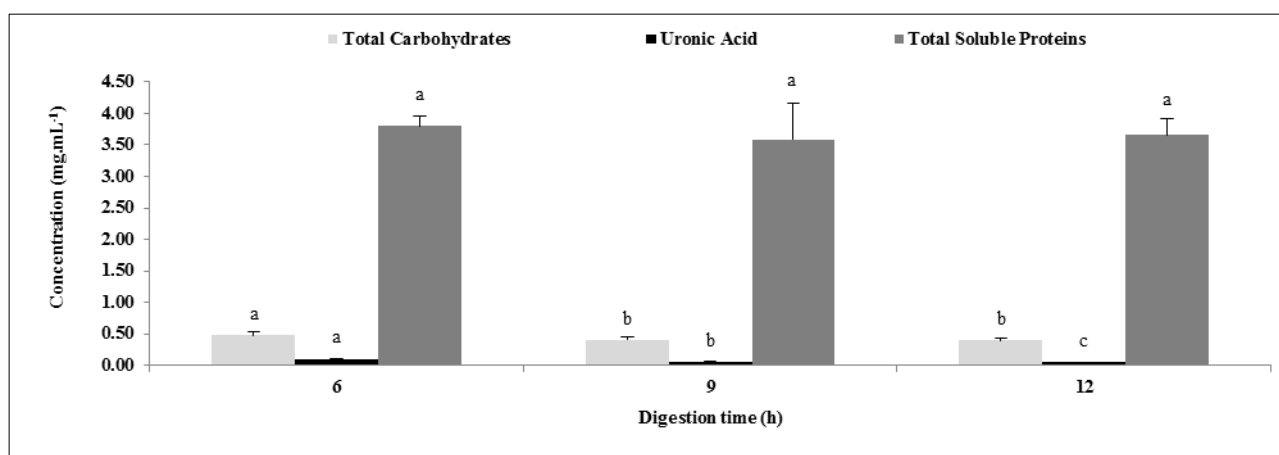


Fig. 3. Total carbohydrates, uronic acid, and total soluble proteins of the yellowfin tuna *Thunnus albacares* eyeball during enzymatic hydrolysis at different digestion periods. Data are mean $\pm$ SD of three determinations. Values with the same superscripts are not significantly different ($P < 0.05$).

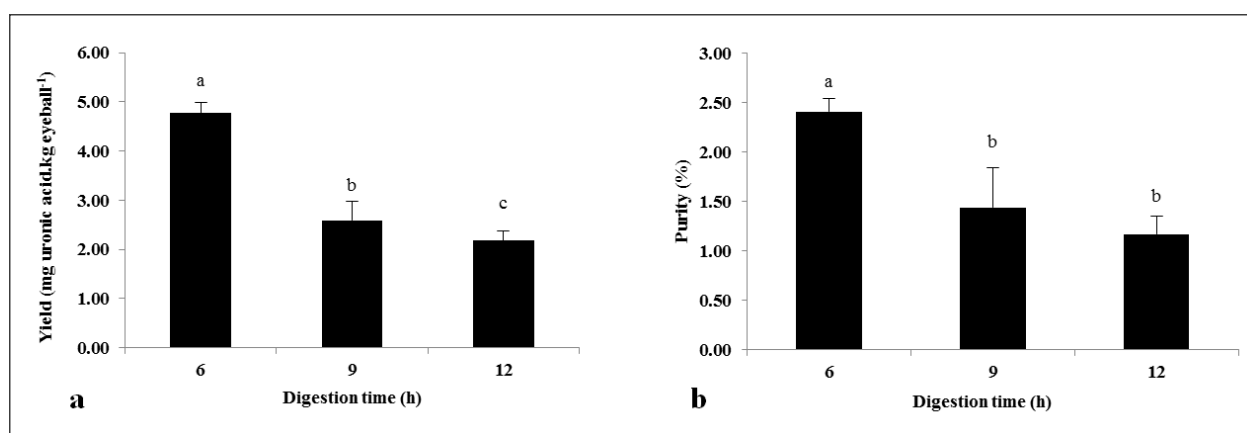


Fig. 4. Yield (a) and per cent purity (b) of the HA from yellowfin tuna *Thunnus albacares* eyeball during enzymatic hydrolysis at different digestion periods. Data are mean $\pm$ SD of three determinations. Values with the same superscripts are not significantly different ($P < 0.05$).

Recovery and fractionation

The CPC method together with the filtration-diafiltration was used in the recovery and fractionation steps. Results of the effects of the different NaCl concentrations on the recovery and fractionation of the digested supernatant containing HA revealed the highest peak at 3 % CPC containing 3 M NaCl both for the total carbohydrates and uronic acid content (Fig. 5). The total soluble proteins increased as the NaCl concentration was increased from 1 to 2 M but showed a decrease at a higher NaCl concentration of 3 M.

The 3 % CPC with 3M NaCl concentration obtained the highest yield and per cent purity among the samples treated with different NaCl concentrations (Fig. 6).

The more efficient condition for the recovery and fractionation of HA (3 % CPC:3 M NaCl) gave the following values: total carbohydrates at 0.02 mg.mL⁻¹; uronic acid at 0.01 mg.mL⁻¹; total soluble proteins at

0.14 mg.mL⁻¹; yield of 0.95 mg uronic acid.kg eyeball⁻¹; purity of 8.00 %.

Alcoholic precipitation and lyophilisation

Figure 7 reveals that the concentrations of total carbohydrates and uronic acid increase with the increasing volume of ethanol. Also, a significant decrease in the total soluble proteins was observed at 1:2 and 1:3 supernatant: ethanol ratios. For the yield and per cent purity, results range from 1.10–1.34 mg uronic acid.kg eyeball⁻¹ and 26.22 %–33.78 %, with the highest values observed at 1:3 supernatant:ethanol ratio (Fig. 8).

In summary, 1:3 supernatant:ethanol ratio, the efficient condition for the alcoholic precipitation and lyophilisation have a total carbohydrates, uronic acid, total soluble proteins, yield, and purity of 0.021 mg.mL⁻¹, 0.018 mg.mL⁻¹, 0.032 mg.mL⁻¹, 1.34 mg uronic acid.kg eyeball⁻¹, and 33.78 %, respectively.

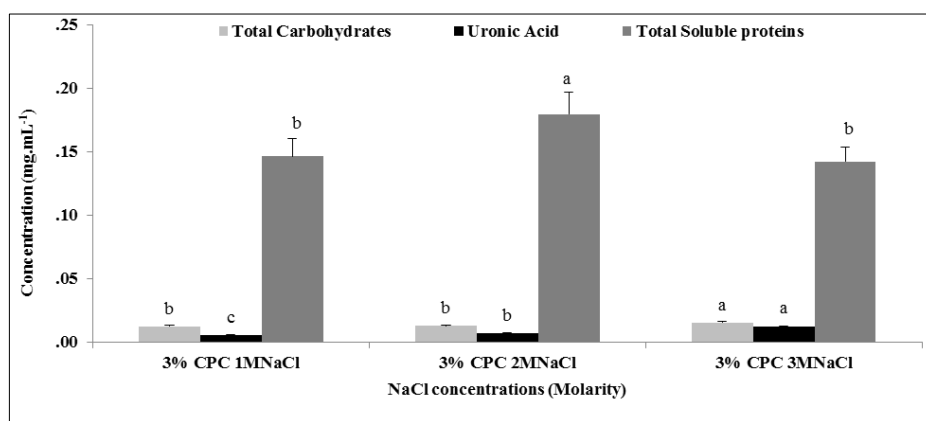


Fig. 5. Total carbohydrates, uronic acid, and total soluble proteins of the yellowfin tuna *Thunnus albacares* eyeball during fractionation at different NaCl concentrations. Data are mean ± SD of three determinations. Values with the same superscripts are not significantly different (P < 0.05).

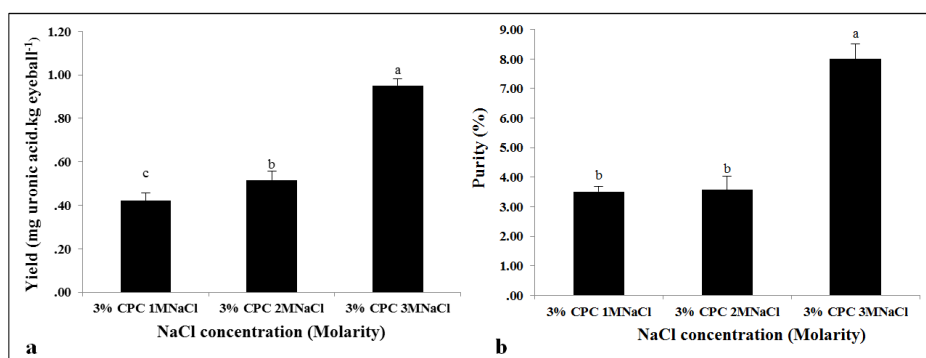


Fig. 6. Yield(a) and per cent purity (b) of the HA from yellowfin tuna *Thunnus albacares* eyeball during fractionation at different NaCl concentrations. Data are mean ± SD of three determinations. Values with the same superscripts are not significantly different (P < 0.05).

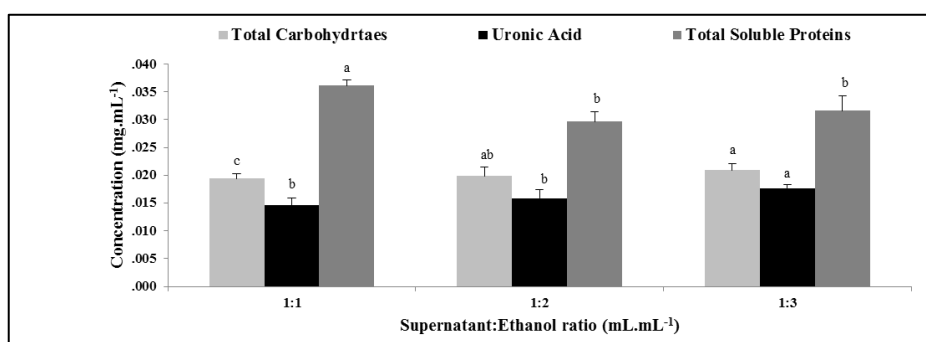


Fig. 7. Total carbohydrates, uronic acid, and total soluble proteins of the yellowfin tuna *Thunnus albacares* eyeball during alcoholic precipitation at different supernatant:ethanol ratios. Data are mean ± SD of three determinations. Values with the same superscripts are not significantly different (P < 0.05).

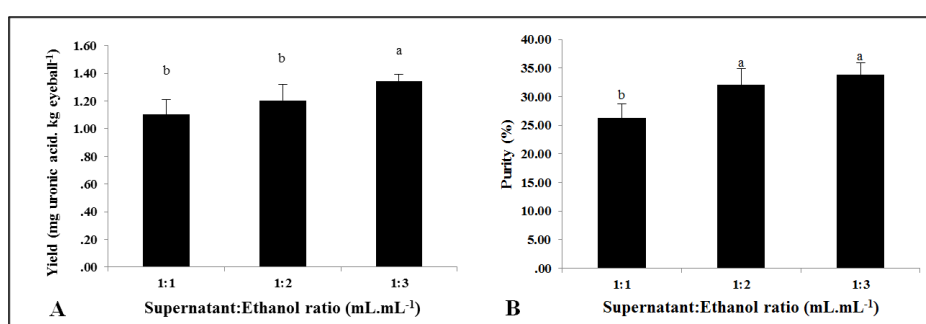


Fig. 8. Yield(A) and per cent purity (B) of the HA from yellowfin tuna *Thunnus albacares* eyeball during alcoholic precipitation at different supernatant:ethanol ratios. Data are mean ± SD of three determinations. Values with the same superscripts are not significantly different (P < 0.05).

Discussion

Tissue extraction

Results of different treatments and incubation periods reveal that the highest total carbohydrate was obtained in 0 h for both treatments and incubation of the samples beyond 24 h did not enhance the total

carbohydrates (Fig. 1a). The superior amount of the initial (0 h) total carbohydrates was presumably due to the blood embedded in the eyeball which leaks coincidentally with the vitreous humour (VH) containing the hyaluronic acid (HA). Dextrose, a common monosaccharide containing six carbons that are present in the blood presumably causes the overestimation of the total carbohydrates during phenol-sulfuric acid assay.

A significantly higher initial uronic acid was obtained in the sample extracted using the direct treatment (T1) than the sample extracted using the indirect treatment (T2). Also, erratic uronic acid values were obtained after incubation in both treatments tested (Fig. 1b). The higher initial uronic acid content of the extract in T1 was most likely due to the hydrophilic nature of the sugar units in the HA structure, making it highly soluble in water (Agerup, 2008). In contrast, the lower initial uronic acid values in T2 could be due to the insufficient amount of acetone and sodium acetate in the pretreatment mixture. The ketone and electrolyte function to reduce the solubility of HA during tissue pretreatment (Balazs et al., 1987). The erratic values observed in this experiment could be attributed to the degradation of HA by hyaluronidases and change in pH caused by fluctuating room temperature. Hyaluronidase degrade HA by breaking the glucosaminidic bond of the HA product and stimulating tissue absorption in the area (Kassir et al., 2011). Whereas, the rise in temperature that was associated with an increase in the molecular vibration and H^+ ion, decreased the tendency of forming hydrogen bond and thus, causing a reduction in the pH of the solution. The decrease in pH can negatively affect the HA concentration (Balazs et al., 1987). When the pH is lower than four or higher than 11, HA is degraded by hydrolysis (Maleki et al., 2008).

The total soluble protein obtained in T2 was statistically lower than T1, both for the initial and the incubated samples (Fig. 1c). Also, fluctuating protein values were observed with the progress of the incubation period. Lower protein values in T2 could be attributed to the aldehyde in the pretreatment solution which chemically modifies the primary structure of HA by reacting to the proteins in the aqueous media which then causes protein denaturation and immobilisation. As a result, the nitrogenous organic compound in the yellowfin tuna eyeball becomes insoluble in the subsequent aqueous extraction (Balazs et al., 1987). Whereas the higher amount of total soluble protein obtained in T1 could be due to the proteins that were extracted from the tissue along with the HA (Amagai et al., 2009). The observable fluctuating pattern of total soluble proteins in response to different incubation periods could be accounted to the denaturation-renaturation process. For instance, pH may decrease (acidic) or increase (alkaline) the solubility of protein (Nahar et al., 2017). Another factor that could be considered is the ability of the protein to undergo reversible denaturation or renaturation. The denatured proteins can reformulate hydrogen bonds between complementary single strand, making it likely to reform double helix structure again and therefore, renature (Wang et al., 2014).

For the yield and the per cent purity of the sample, the highest values were observed at 24 h incubation period for the two treatments tested (Fig. 2). Yield is affected by the uronic acid content. Since HA is highly

soluble in water, it is expected to have higher yield in T1 than T2. However, hyaluronidase have the optimum activity at 48 h incubation period (Sahoo et al., 2008), which could be the reason for the sudden decrease in the yield after 24 h incubation period. The incubation of the samples at room temperature with the absence of lower ketone and electrolyte favours the activity of hyaluronidase. Higher per cent purity in T2 could be attributed to the pretreatment and the consequent dissection of the VH in the yellowfin tuna eyeball which could have reduced the possible impurities (blood and proteins) in the extract and therefore, gave a higher per cent purity. Similarly, the proteins and blood components that were extracted together with the HA could have caused a decrease in per cent purity in T1, both for the initial and the incubated samples. The highest values that were observed in the two treatments after incubation illustrate that 24 h was the ideal incubation period for the extraction of HA in the VH of the yellowfin tuna eyeball. It further suggests that any extension beyond this period will no longer increase the yield and per cent purity of the extracted HA. This protocol for tissue extraction was more cost-effective and less laborious than the original patent for rooster comb which uses the same pretreatment prior to water extraction but took 72 h for the completion of the incubation period (Balazs et al., 1987).

Enzymatic hydrolysis

The significant decline in the values of total carbohydrates and uronic acid content was noted after 6 h digestion time (Fig. 3). In contrast, no significant change in the total soluble proteins was detected as the digestion period progressed. Thus, the changes brought by varying hydrolysis periods could be due to the breakage of core peptides of glycosaminoglycans (GAGs) which is attached to the disaccharides and the removal of the link proteins responsible for the binding of HA to other GAGs (Gandhi and Mancera, 2008). The exclusion of core peptides and the link proteins in the GAGs structure lead to the partial elimination of proteins, making it soluble and available for analysis. This reaction, in turn, increases the total carbohydrates and uronic acid of the sample. Also, since there was no significant decrease or increase in the concentration of the protein components at the end of hydrolysis period, it is assumed that almost all the peptide bonds in the extract containing HA that was available for hydrolysis were completely hydrolysed at 6 h digestion time. Thus, an extensive breakdown of core proteins could have led to a decrease in the concentration of the total carbohydrates and uronic acid (Lindahl et al., 1998). In the study on the extraction of GAGs from abalone, the reported optimum hydrolysis time was 10 h and, beyond this period, there would be a decrease in the amount of GAGs (Li et al., 2011). However, as shown in the results of this study, further increase in the digestion time beyond its optimum (6 h), during tissue hydrolysis,

could negatively affect the concentration of the HA being extracted. Some of the factors that could affect the duration of hydrolysis are the parts of the animal being tested and the amount of protein components in the tissue source. The harder the tissue is, the longer the required digestion period will be, and vice versa (Calatroni et al., 1969). Likewise, the higher the amount of protein constituents in the tissue, the slower the enzymatic hydrolysis is and the longer the digestion period will be.

The decrease in yield and per cent purity (Fig. 4) in response to the increasing hydrolysis period could be due to the reduction of the carbohydrates and the uronic acid components available for analysis. Since yield is dependent on the value of the uronic acid, the decrease in this parameter could negatively impact the yield. Also, the decrease in carbohydrates components at almost steady protein content concentration throughout the digestion periods could result in a decline of the per cent purity.

The results of the enzymatic hydrolysis revealed that the 6 h digestion period was fast and economical than the ones described in existing literature using the same raw material (Mizuno et al., 1991; Amagai et al., 2009). However, 6 h digestion period may be adjusted if other sources are to be used. In similar studies, digestion time of 24 h was used in the hydrolysis of the chicken crest (Da Rosa et al., 2007), chicken combs (Da Rosa et al., 2012), with the use of cysteine; and liver of marine stingray (Sadhasivam et al., 2013) with the use of papain. The results of the present study could be reasonable since those above-mentioned sources were hard tissues with higher amount of protein component and were therefore expected to have longer digestion period (Calatroni et al., 1969).

Recovery and fractionation

The 3 % CPC containing 3 M NaCl obtained the highest total carbohydrates and uronic acid content (Fig. 5). For the total soluble proteins, the values increased as the NaCl concentration increased from 1 to 2 M but showed a decrease at a higher NaCl concentration of 3 M. The increase in the total carbohydrates and uronic acid content at 3 M NaCl concentration could be attributed to the ability of the CPC to precipitate the carbohydrate components in the solution making it available for the analyses (Buzzega et al., 2010). Cetylpyridinium chloride is a cationic detergent that binds and precipitates anionic molecules which in the case of this study, are the HA and non-HA components present in the VH of the yellowfin tuna eyeball. This result is comparable to the observation that salt concentrations higher than 1.5 M in the retentate should be used for the optimal HA recovery (Murado et al., 2012). The increasing and the decreasing phases in the protein values could be due to the ability of the salt to denature proteins (Khalid et al., 2003). This result could be accounted to the

salting-in/salting-out phenomenon. For salting-in (increasing phase), the electrostatic interaction between proteins and NaCl prevents protein cohesion and precipitation. With this, the Na⁺ and Cl⁻ ions surround the proteins in the solution and in return allow its solubility. In this phenomenon, the solute-solvent interaction is stronger than the solute-solute interaction (Nahar et al., 2017). In the salting-out phase, there are more NaCl ions and less solvent to maintain protein solubility, and therefore, the solute-solute interaction is greater than solute-solvent interactions which lead to protein precipitation and decrease in solubility (Zidani et al., 2012). Also, salts could affect the protein-protein interaction wherein it introduces the Hoffmeister effects which effectively show the salt dependence of the different phase formation or precipitation of proteins (Chinchalikar et al., 2012).

Results indicate a higher yield and per cent purity at 3 % CPC with 3 M NaCl concentration (Fig. 6) which suggest that this ratio was the efficient CPC-NaCl combination for the recovery and fractionation of HA from yellowfin tuna eyeball.

Alcoholic precipitation and lyophilisation

The total carbohydrates and uronic acid increased with the increasing volume of ethanol while there was a decrease in the total soluble proteins after alcoholic precipitation and lyophilisation (Fig. 7). The increase in total carbohydrates and uronic acid content with increasing ethanol volumes are comparable to the study which noted that precipitation, thus increased in the concentrations of total carbohydrates and uronic acid increased with increasing volumes of ethanol (Mariño et al., 2015). The decrease in the total soluble proteins could be attributed to the denaturation process. Exposure of the proteins with organic solvents can severely affect its structural properties (Grinberg et al., 1998). For instance, ethanol can act as a protein destabiliser for it can cause protein denaturation and can alter protein-protein interactions (Mousavi et al., 2008) thus allowing the transition in the proteins' secondary and tertiary structure (Dufour and Haertle, 1993). This effect is governed by the hydrophobicity of ethanol. Ethanol can attract the hydrogen ion in the protein thus leading to denaturation.

For the yield and per cent purity, the highest values were observed at 1:3 supernatant:ethanol ratio (Fig. 8). This could be due to the ability of the ethanol to precipitate the carbohydrates and denature the proteins in a given solution. It further implies that the alcoholic precipitation step further enhances the yield and purity of the HA. Several studies used the 1:3 supernatant:alcohol ratio for the precipitation of the GAGs. For example, in the *Streptococcus zooepidemicus* (Pan et al., 2015) and stingray *A. narinari* (Sadhasivam et al., 2013).

Conclusion

The source and the production protocols affect the yield and purity of the hyaluronic acid (HA). The results of the study showed that the efficient condition for the isolation of HA from yellowfin tuna eyeball were as follows: (i) pretreatment of the raw material with acetone, formaldehyde, and sodium acetate solution prior to water extraction and incubation at 24 h; (ii) a maximum of 6 h hydrolysis period; (iii) the use of 3 % CPC:3 M NaCl concentration for the efficient recovery and fractionation; and the use of 1:3 mL:mL⁻¹ supernatant:ethanol ratio for alcoholic precipitation.

The present study may bring new insight for more cost-effective isolation of HA from other potential sources while maximising the benefits of fish by-products utilisation. However, further studies are needed on the purification process and the bioactive potentials of the isolated HA to establish its functionality and commercial viability.

Acknowledgements

This work was supported by the Philippine Department of Science and Technology (DOST) - Accelerated Science and Technology Human Resources Development Program (ASTHRDP).

References

- Agerup, B., Wik, O. 2008. NASHA - The monograph. Q-Med AB, Uppsala, Sweden. pp. 9-23.
- Amagai, I., Tashiro, Y., Ogawa, H. 2009. Improvement of the extraction procedure for hyaluronan from fish eyeball and the molecular characterization. *Food Science and Technology* 75:805-810. <https://doi.org/10.1007/s12562-009-0092-2>
- Balazs, E.A., Lee, F., Leshchiner, A., Leshchiner, A. 1987. Chemically modified hyaluronic acid preparation and method of recovery thereof from animal tissue. United States Patent 4713448.
- Bishop, P.N. 2000. Structural macromolecules and supramolecular organization of the vitreous gel. *Progress in Retinal and Eye Research* 19:323-344. [https://doi.org/10.1016/S1350-9462\(99\)00016-6](https://doi.org/10.1016/S1350-9462(99)00016-6)
- Bitter, T. Muir, H.M. 1962. A modified uronic acid carbazole. *Analytical Biochemistry* 4:330-334. [https://doi.org/10.1016/0003-2697\(62\)90095-7](https://doi.org/10.1016/0003-2697(62)90095-7)
- Buzzega, D., Pederzoli, F., Maccari, F., Aslan, D., Turk, M., Volpi, N. 2010. Comparison of cetylpyridinium chloride and cetyltrimethylammonium bromide extractive procedures for quantification and characterization of human urinary glycosaminoglycans. *Clinical Chemistry and Laboratory Medicine* 48:1133-1139. <https://doi.org/10.1515/CCLM.2010.217>
- Calatroni, A., Donnelly, P., Ferrante, N. 1969. The glycosaminoglycans of human plasma. *The Journal of Clinical Investigation* 48:332-343. <https://doi.org/10.1172/JCI105989>
- Chen, B., Miller, R., Dhal, P. 2014. Hyaluronic acid-based drug conjugates: State-of-the-art and perspectives. *Journal of Biomedical Nanotechnology* 10:4-16. <https://doi.org/10.1166/jbnn.2014.1781>
- Chinchalikar, A., Aswal, V., Kohlbrecher, J., Wagh, A. 2012. Evolution of structure and interaction during aggregation of silica nanoparticles in aqueous electrolyte solution. *Chemical Physics Letters* 542:74-80. <https://doi.org/10.1016/j.cplett.2012.05.065>
- Da Rosa, C.S., Rotta, J., Barreto, P.L.M., Beirao, L.H. 2007. Extraction, quantification and molar mass determination of hyaluronic acid extracted from chicken crest. *Alimentos e Nutricao* 18:237-240.
- Da Rosa, C.S., Tovar, A.F., Mourao, P., Pereira, R., Barreto, P. 2012. Purification and characterization of hyaluronic acid from chicken combs. *Ciencia Rural* 42:1682-1687. <https://doi.org/10.1590/S0103-84782012005000056>
- Dubois, M., Gilles, K., Hamilton, J., Rebers, P., Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28:350-356. <https://doi.org/10.1021/ac60111a017>
- Dufour, E., Haertle, T. 1993. Temperature-induced folding changes of β lactoglobulin in hydromethanolic solutions. *International Journal of Biological Macromolecules* 15:293-297. [https://doi.org/10.1016/0141-8130\(93\)90029-L](https://doi.org/10.1016/0141-8130(93)90029-L)
- Gandhi, N., Mancera, R. 2008. The structure of glycosaminoglycans and their interactions with proteins. *Chemical Biology and Drug Design* 72:455-482. <https://doi.org/10.1111/j.1747-0285.2008.00741.x>
- Grinberg, V.Y., Grinberg, N., Burova, T., Dalgalarondo, J., Haertle, T. 1998. Ethanol-induced conformational transitions in holo-R-lactalbumin: Spectral and calorimetric studies. *Biopolymers* 46:253-265. [https://doi.org/10.1002/\(SICI\)1097-0282\(19981005\)46:4<253::AID-BIP7>3.0.CO;2-O](https://doi.org/10.1002/(SICI)1097-0282(19981005)46:4<253::AID-BIP7>3.0.CO;2-O)
- Kassir, R., Kolluru, A., Kassir, M. 2011. Extensive necrosis after injection of hyaluronic acid filler: Case report and review of the literature. *Journal of Cosmetic Dermatology* 10:224-231. <https://doi.org/10.1111/j.1473-2165.2011.00562.x>
- Khalid, E.K., Babiker, E.E., El Tinay, A.H. 2003. Solubility and functional properties of sesame seed proteins as influenced by pH and/or salt concentration. *Food Chemistry* 82:361-366. [https://doi.org/10.1016/S0308-8146\(02\)00555-1](https://doi.org/10.1016/S0308-8146(02)00555-1)
- Li, G., Chen, S., Wang, Y., Xue, Y., Chang, Y., Li, Z., Wang, J., Xue, C. 2011. A novel glycosaminoglycan-like polysaccharide from abalone *Haliotis discus hannai* Ino: Purification, structure identification and anticoagulant activity. *International Journal of Biological Macromolecules* 49:1160-1166. <https://doi.org/10.1016/j.ijbiomac.2011.09.017>
- Lindahl, U., Kusche-Gullberg, M., Kjellen, L. 1998. Regulated diversity of heparan sulfate. *Journal of Biological Chemistry* 273:24979-24982. <https://doi.org/10.1074/jbc.273.39.24979>
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 193:265-275.
- Maleki, A., Kjoniksen, A.L., Nystrom, B. 2008. Effect of pH on the behavior of hyaluronic acid in dilute and semidilute aqueous solutions. *Macromolecular Sympos* 274:131-140. <https://doi.org/10.1002/masy.200851418>
- Mariño, M., Freitas, S., Miranda, E. 2015. Ethanol precipitation of glycosyl hydrolases produced by *Trichoderma harzianum* P49p11. *Brazilian Journal of Chemical Engineering* 32:325-333. <https://doi.org/10.1590/0104-6632.20150322s00003268>
- Mizuno, H., Naomichi, I., Saito, T., Ogawa, H., Sawairi, M. 1991. Characterization of hyaluronic acid of yellowfin tuna eyeball. *Nippon Suisan Gakkaishi* 57:517-519. <https://doi.org/10.2331/suisan.57.517>
- Mousavi, S.H.A., Chobert, J.M., Bordbar, A.K., Haertle, T. 2008. Ethanol effect on the structure of β -Lactoglobulin B and its ligand binding. *Journal of Agricultural and Food Chemistry* 56:8680-8684. <https://doi.org/10.1021/jf801383m>

- Murado, M.A., Montemayor, M.I., Cabo, M.L., Vasquez, J.A., Gonzales, M.P. 2012. Optimization of extraction and purification process of hyaluronic acid from fish eyeball. Food and Bioproducts Processing 90:491–498. <https://doi.org/10.1016/j.fbp.2011.11.002>
- Nahar, K., Zakaria, Z., Hasmin, U., Bari, F. 2017. Effect of pH and salt concentration on protein solubility of slaughtered and non-slaughtered broiler chicken meat. Sains Malaysiana 46:719–724. <https://doi.org/10.17576/jsm-2017-4605-06>
- Pan, N.C., Vignoli, J.A., Baldo, C., Pereira, H.C.B., Silva, R.S.S.F., Celligoi, M.A.C.P. 2015. Effect of fermentation conditions on the production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 39920. Acta Scientiarum 37:411–417. <https://doi.org/10.4025/actascibiols.v37i4.28176>
- Prescott, A. 2003. Method for purifying high molecular weight hyaluronic acid. United States Patent 6660853 B2.
- Raghuraman, H. 2013. Extraction of sulfated glycosaminoglycans from mackerel and herring fish waste. MS Thesis. Dalhousie University. pp. 51-108.
- Sadhasivam, G., Muthuvel, A., Pachaiyappan, A., Thangavel, B. 2013. Isolation and characterization of hyaluronic acid from the liver of marine stingray *Aetobatus narinari*. International Journal of Biological Macromolecules 54:84–89. <https://doi.org/10.1016/j.ijbiomac.2012.11.028>
- Sahoo, S., Panda, P.K., Mishra, S.R., Nayak, A., Dash, S.K., Ellaiah, P. 2008. Optimization of physical and nutritional parameters for hyaluronidase production by *Streptococcus mitis*. Indian Journal of Pharmaceutical Sciences 70:661–664. <https://doi.org/10.4103/0250-474X.45412>
- Saranraj, P., Naidu, M.A. 2013. Hyaluronic acid production and its applications: A review. International Journal of Pharmaceutical and Biological Archives 4:853–859.
- Vasquez, J.A., Rodriguez-Amado, I., Montemayor, M.I., Fraguas, J., Gonzalez, M.D., Murado, M.A. 2013. Chondroitin sulfate, hyaluronic acid and chitin/chitosan production using marine waste sources: characteristics, applications and eco-friendly processes: A review. Marine Drugs 11:747–774. <https://doi.org/10.3390/md11030747>
- Wang, X., Lim, H.J., Son, A. 2014. Characterization of denaturation and renaturation of DNA for DNA hybridization. Environmental Health and Toxicology 29:1–8. <https://doi.org/10.5620/eht.2014.29.e2014007>
- Yang, C., Wang, X., Yao, X., Zhang, Y., Wu, W., Jiang, X. 2015. Hyaluronic acid nanogels with enzyme sensitive cross-linking group for drug delivery. Journal of Controlled Release 205:206–217. <https://doi.org/10.1016/j.jconrel.2015.02.008>
- Zidani, S., Fahloul, D., Bacha, A. 2012. Effects of pH, NaCl, ethanol, and drying methods on the solubility of *Saccharomyces cerevisiae* proteins, CyTA-Journal of Food 10:42–47. <https://doi.org/10.1080/19476337.2010.543472>