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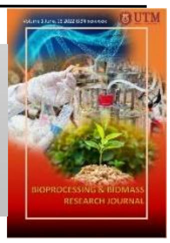

Research Article

# Hyaluronic Acid Extraction from Eggshell Membrane with Papain and Bromelain

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**ABSTRACT**

Eggshell membrane is a natural source of glycosaminoglycan such as hyaluronic acid (HA), a therapeutic compound with various cosmetic and medical applications. HA is produced commercially using either animal-based extraction or genetically modified bacterial fermentation. Extraction of HA from eggshell membranes is a sustainable alternative for HA production which may also greatly reduce food wastes globally. The objective of this research was to determine the yield of HA extracted from eggshell membrane using a combination of papain and bromelain enzymes. The optimum condition for the hyaluronic acid (HA) extraction from eggshell membrane was studied using different ratio of enzyme mixtures at different temperatures and pH. The yield of HA extracted was determined by using carbazole method. Based on the one factor at a time (OFAT) study, the ratio of 1:2 papain to bromelain, pH 8 at temperature of 60℃ were found to be the ideal processing condition for HA extractions. Further study of simultaneous optimization of enzyme ratio, pH and temperature together can be conducted using response surface methodology to understand the relationships and interaction between the variables on the HA yield.



## INTRODUCTION

Annually, around 65.5 million metric tons of eggshells are produced over the world, and worldwide eggshells output is expected to reach around 90 million tons by 2030 (Jambeck et al., 2015). According to Environmental Protection Agency, eggshell waste is the 15th largest food industry waste pollutants. Almost all eggshells are considered as wastes and thrown into landfills with little or no pre-treatment (Gao & Xu, 2012). Thus, it contributes to pollution by producing odors from microbial activities (Mignardi et al., 2020). The utilization of eggshells has the potential to reduce environment impact by diverting the eggshell wastes from landfill.

Currently, hyaluronic acid (HA) is produced commercially using either animal-based extraction or genetically modified bacterial fermentation (Putri et al., 2020). HA can be extracted from various animal tissues such as human umbilical cords, rooster combs, bovine vitreous humor, and bovine synovial fluid (Liu et al., 2011). Even though production through animal-based tissues remains the primary source of large-scale HA production, other production pathway has been studied to get better yield of HA with lower cost and simpler process since there are several limitations of the existing process. Animal extraction has practical and mechanical concerns in terms of cost and safety due to the grinding method and multiple repetitions of use of acid and organic solvents. One of the problems is that HA from animal tissues may remain bonded to hyaluronidase-specific binding cellular proteins (Fraser et al., 1997). Hyaluronidase is undesirable because it may cause the blockage of immunological response. Besides, during the extraction process, the transmission of infectious diseases in the form of nucleic acids, prions and viruses may occur (Shiedlin et al., 2004). In addition, this production

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pathway is costly and time-consuming, requiring extensive labour and specialized facilities to handle operations ranging from animal extraction to HA purification. Therefore, it is preferable to produce HA without using animal cells to reduce the possibility of contamination and production cost. On the other hand, bacterial fermentation requires high-cost infrastructure set up. With the extraction from eggshell membrane, which is a waste stream in the poultry sector, this might reduce the raw materials cost for the HA production.

HA can be recovered from a variety of biological wastes, including eggshell wastes. A number of research works have been conducted on enzymatic hydrolysis extraction from eggshell membrane using a single enzyme. However, there is a lack of study on the suitable extraction condition using mixture of papain and bromelain in enzymatic hydrolysis of eggshell membrane. Therefore, the objective of this study was to determine the suitable enzyme ratio, pH, and temperature for the extraction the HA from the eggshell membrane using a mixture of papain and bromelain.

## MATERIALS AND METHOD

### Sample preparation

Eggshell wastes were collected from a layer poultry farm in Johor, Malaysia. The eggshell membranes (ESM) were separated manually from the eggshell and washed with distilled water to remove impurities. Next, the ESM was dried using an oven at 35 ℃. The dried ESM was then ground into powder and sieved through 220 nm mesh sieves. The ESM powder was stored at -18 ℃ freezer until use.

### Enzyme-assisted extraction

The enzyme-assisted extraction was conducted according to previous studies by Ürgeová et al. (2016) and Coculová & Krajcovic (2018) with slight modification. Briefly, the sample solution was prepared by adding 0.5 g of dried eggshell membrane powder into 20 ml Britton-Robinson buffers solution (solid to liquid buffer ratio of 1:40). The sample solution was then pre-incubated at temperatures between 40 °C and 60 °C (**Table 1**) for 10 minutes before hydrolysis. After pre-incubation, the sample solution was treated with different ratios of food grade papain (100,000 U/g) and bromelain (200,000 U/g) (both obtained from Nanning Doing-Higher Bio-Tech Co. Ltd) (**Table 1**). The samples solutions were then incubated for 3 hours in an incubator shaker at 200 rpm to ensure homogenized protein digestion. Next, all the hydrolysed samples were heated up to 100ºC for 5 minutes to deactivate the enzymes. The hydrolysed samples were then cooled to room temperature and centrifuged at 5000 rpm for 20 minutes to remove impurities.

### Analysis of Hyaluronic Acid Using Carbazole Method

The content of HA in extracted samples were determined using the carbazole method reported by Cesaretti et al. (2003), where the uronic acid-bearing polyanions such as HA was hydrolysed, and the resulting uronic acid is colored with carbazole and the absorbance was measured.

Briefly, using a 96-well plate, 10 µl of extracted samples was diluted in 1 ml distilled water to obtain 10 mg/ml stock solution. 200 µl of a 25 mM sodium tetraborate in sulfuric acid solution was added. The plate was heated for 10 minutes at 100 °C. After cooling for 15 minutes at room temperature, 50 µl of 0.125% carbazole in absolute ethanol was added. The samples were then reheated at 100 ˚C for 15 minutes. The plate was cooled at room temperature for 15 minutes and the absorbance was read using a microplate reader (BioRad, Model 550) at 550 nm. Standard calibration curve for HA was prepared using sodium hyaluronate as HA standard. The yield of the hyaluronic acid was calculated by using the formula (Sumogod et al., 2019):

$$\text{Yield} = (\text{Uronic acid})/(\text{Weight of raw material})\times 100\%$$

**Table 1** Screening parameters and conditions for HA extraction from eggshell membranes (ESM) (n=3)

| Parameter | Conditions |
|---|---|
| **Ratio of enzyme in mixture (A)** | |
| Type of enzyme with ratio | Papain: Bromelain 1:1, 2:1, 1:2 |
| pH | 7 |
| Temperature | 60°C |
| Hydrolysis time | 3 Hours* |
| Enzyme to substrate concentration | 5g/g ESM* |
| **pH (B)** | |
| Type of enzyme | 1Papain: 2Bromelain (A) |
| pH | 6,7,8,9 |
| Temperature | 60°C |
| Hydrolysis time | 3 Hours* |
| Enzyme to substrate concentration | 5g/g ESM* |
| **Temperature (C)** | |
| Type of enzyme | 1Papain: 2Bromelain (A) |
| pH | 8 (B) |
| Temperature | 40°C, 50°C, 60°C |
| Hydrolysis time | 3 Hours* |
| Enzyme to substrate concentration | 5g/g ESM* |

Notes: A is selected ratio for pH and temperature parameter screening. B is selected pH condition for temperature parameter screening. C is selected temperature at the end of the OFAT study.
* Is the constant variable

### Statistical Analysis

All results were analysed with the student's t-test to determine the statistically significant difference between groups.

## RESULTS AND DISCUSSION

### Comparison of Enzyme Mixture in Different Ratios

As shown in **Table 2**, the papain to bromelain ratio of 1:2 gave the highest percentage yield of HA which is 6.7% whereas ratio of 1:1 and 2:1 yielded hyaluronic acid of 5.1% and 5.0%, respectively. Nevertheless, the t-test shows that there is no significant difference (P>0.05) among the groups.

Currently, there is a lack of research to investigate the use of enzyme mixture of papain and bromelain in extraction of HA from eggshell waste. However, there is report suggested the combination of synergic enzymes enhanced to breakdown microalgal cell wall and thus increase the algal oil extraction yield (Blanco-Llamero et al., 2021). This is supported by a study from Alparce et al. (2018) who reported HA extracted from eggshell membrane by digestion with papain (30000 USP-U/mg) at different temperatures of 20 °C, 40 °C, and 60 °C for 24 hours has yielded 0.15% (1.445 mg/g), 0.16% (1.55 mg/g), 0.55% (5.5 mg/g).

**Table 2** Yield of HA extracted from ESM using enzyme in mixture (n=3)

| Papain to Bromelain Ratio | Percentage yield of HA (%) |
|---|---|
| 1:1 | 5.192 ± 0.058 [a] |
| 2:1 | 4.971 ± 0.356 [a] |
| 1:2 | 6.692 ± 0.729 [a] |

Note: [a] The t-test shows no significant difference among the three groups ($p > 0.05$).

**Comparison of HA Extracted from ESM in Different pH**

The optimum pH range for papain is pH 3 to pH 9 while bromelain is at the range of pH 7 to pH 8 (Mamboya, 2012). Thus, the range of pH in this study was set from pH 6 to pH 9 to determine the optimum pH for HA extraction from eggshell membrane using mixture of enzyme papain and bromelain at the ratio of 1:2 (based on the results from the study on enzyme ratio). The percentage yield of HA extracted from ESM in different pH condition (6, 7, 8 and 9) were tabulated in **Table 3**. The yield of HA increases with the rising of pH from 3.2% to 4.4% with the highest percentage at pH 8 and it started to decrease at pH 9 to 4.1%.

**Table 3** Percentage yield of HA extracted from ESM in different pH condition (n=3)

| pH | Percentage yield of HA (%) |
|---|---|
| 6 | 3.156 ± 0.200 [a] |
| 7 | 3.404 ± 0.339 [a, c] |
| 8 | 4.401 ± 0.193 [b, c] |
| 9 | 4.161 ± 0.291 [a, c] |

Note: Different letters in the same column indicate significant differences according to the t-test ($p < 0.05$).

The HA yield at pH 8 is significantly higher than pH 6 ($p < 0.05$). This might be due to the effectiveness of the enzyme and the target substrate of the enzymes (Bhatia & Bhatia, 2018). In addition, variation in pH also resulted in change of polymer chain conformation (Tømmeraas & Melander, 2008). Other than that, HA is observed to be most stable at neutral pH and it is more labile at acidic than alkaline condition (Tokita & Okamoto, 1996; Khanmohammadi et al. 2014). A previous report has demonstrated that HA degrades at pH less than 4 and pH more than 11, and that there is almost no breakdown of the HA chains in the pH range of pH 4 to pH 11 (Maleki et al., 2007).

**Comparison of HA Extracted from ESM in Different Temperatures**

The optimum temperature for bromelain is around 50 °C and it will denature when exceed 60 °C. On the other hand, the optimal temperature for papain ranging from 30℃ to 70 °C (Martins et al., 2014). Therefore, the temperature range of 40 °C to 60 °C was studied. The results **(Table 4)** showed the yield of HA improved when temperature increased from 40 °C to 60 °C. However, no statistical difference was observed. As temperature increases, the amount of HA also increases. The amount of HA will continue to increase with the temperature until it reaches to a point where it is not fit for production of HA, thus it started to decrease in amount. The highest yield of total HA was obtained at the temperature of 60 °C at 4.457%. This might be due to the enzymatic reaction rate which is influenced by temperature in two different ways. Higher temperatures speed up the hydrolysis reaction, but they also speed up the enzyme's thermal inactivation (de Melo & Santana, 2019).

**Table 4** Percentage yield of HA extracted from ESM using enzyme in mixture in different temperature (n=3)

| Temperature (℃) | Percentage yield of HA (%) |
|---|---|
| 40 | 3.766 ± 0.210 [a] |
| 50 | 4.306 ± 0.418 [a] |
| 60 | 4.457 ± 0.505 [a] |

Note: [a] The t-test shows no significant difference among the three groups ($p > 0.05$).

It has been showed by previous studies that the highest incubation temperature that can be process the hydrolysis activity without denature for bromelain enzyme is 60 °C. At 70 °C, enzyme will begin to reduce its activity causing the decrease in total hyaluronic acid yield (Martins et al., 2014). This is supported by Ürgeová & Vulganová (2016), whereby at 60 °C, papain hydrolysed the HA from ESM with the highest production yield of 3.9% (39.02 mg/g DM). The yield at 50 °C was 34.5% lower than 60 °C and output of HA reduced by around 9.5% at 70 °C.

## CONCLUSION

Based on the present OFAT study, the ratio of 1:2 papain to bromelain, pH 8 and temperature of 60 °C were found to be the ideal processing condition for HA extraction. However, further study of simultaneous optimization of enzyme ratio, pH and temperature together using the response surface methodology may be conducted to understand the relationships between each variable on the HA yield.

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