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Critical study on the extraction of collagen from eggshell membrane by enzymatic hydrolysis reaction

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ABSTRACT

Eggshells and their membranes are classified as refuse, which is costly to be disposed of in landfills. But many people do not realise that they can actually offer valuable collagen, which is obtained through the enzymatic hydrolysis process. This study aims to address the effects of enzyme type, enzyme-to-membrane ratio and water-to-material ratio on collagen extraction rate. It is found that the processing condition with a pepsin-to-membrane ratio of 0.5 and a water-to-material ratio of 1:200 can achieve an extraction rate of 2.44%. This research has created an opportunity to turn eggshell membrane waste into one of the potential sources of extracting collagen, relieving the pressure on waste treatment.

KEYWORDS Collagen; eggshell membrane; enzymatic hydrolysis reaction; pepsin; extraction
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Received 3 March 2022

1. Introduction

Easy to prepare and digest, eggs are deemed a staple food and a great source of complete proteins, vitamins and minerals. Besides, scientific evidence has proven that eggs are a preventive food for chronic and infectious diseases. With the growth of population and the development of the food processing industry, the utilisation of eggs and their products has greatly increased across the globe. According to research statistics, egg production has dramatically expanded by more than 150% in Asia in the past 30 years (Bunyaratavej and Wang, 2001). Although the increased consumption of eggs leads to an improved quality of life, the visible side-effects of the increased quantity of eggshells and the resulting environmental issues have attracted attention.

Eggshells, which represent about 11%-15% of an egg's weight, are the main waste in the processing of egg products such as liquid egg white and egg yolk (Damen et al., 2006). An eggshell comprises two parts, the outer shell and the eggshell membrane. The outer shell, which is composed of approximately 90% calcium carbonate, is supposed to be one of the most favourable natural calcium sources to produce healthy, absorbable and balanced calcium for the healthcare industry due to the presence of trace minerals, such as magnesium, iron, molybdenum, zinc, etc. (Flynn, 2003). Meanwhile, the eggshell membrane can be obtained from the eggshell in a variety of ways, including manual detachment, microwave treatment (Hussain, 2010), acid-reductant reaction (Torres-Mansilla and Delgado-Mejía, 2017; Ponkham et al., 2011), dissolved air flotation (Yoo et al., 2009), cavitation in the steam environment (Vlad, 2009), etc. Among all these processes, separation by acid-reductant reaction allows high recovery of both the outer shell and eggshell membrane. Eggshell membrane, located between the egg white and the outer shell, is another valuable eggshell component and features various ingredients, including amino acids, hyaluronic acid, chondroitin sulphate, collagen, etc. Collagen, accounting for 10% of the eggshell membrane, is an important type of protein, which presents as a fibrous material in the human body to connect and support bodily tissues, such as skin, bone, tendons, muscles, cartilage and other internal organs. It is widely used in medicine, biochemical, pharmaceutical, food and cosmetics industries. Around 25 types of collagens can be found in the human body, with three of them (collagen type I, V and X) extractable from eggshell membrane (Torres-Mansilla and Delgado-Mejía, 2017). Biochemical, cytotoxicity and genotoxicity tests have shown that collagens extracted from eggshell membrane are safe for consumption without causing any autoimmune or allergic reactions (Ruff et al., 2012).

Various collagen extraction processes, such as hot water extraction, alkaline extraction and enzymatic hydrolysis (Hussain,2010; Park et al., 2016, Pasarin and Rovinaru, 2019, Yoo et al., 2009), have drawn the attention of academic and industrial communities over the past decades. Among all of these processes, enzymatic hydrolysis reaction is the most promising since it does not affect the structure and activity of the polypeptides. This paper thus focuses on studying the effects of the critical processing factors, namely enzyme type, enzyme-to-membrane ratio and water-to-material ratio, on the extraction rate for maximising the yield.

*The first author who was at the age of 35 or below on the closing date of submission to the HKIE Outstanding Paper Award for Young Engineers / Researchers 2022.

2. Materials and methods

Raw eggshell waste was obtained from a pasteurised liquid egg manufacturer, Luen Tai Hong Kong Fresh Liquid Eggs Limited. Eggs were washed with water before being separated into egg white, egg yolk and eggshell with an egg breaking machine. Then, the collected eggshell waste was ground in a centrifugal mill to produce particles with diameters ranging from 2 to 5 mm.

2.1. Separation of eggshell membrane

In this study, an acid reduction reaction was used to separate eggshells and eggshell membranes. The eggshell waste collected from the centrifugal mill was washed with distilled water to remove the remaining egg liquid. Then, 10 g of eggshell was mixed with 1 mol/L acetic acid. The solution was stirred at 160 rpm until free of bubbles. Once the eggshell was completely dissolved, its membrane was collected as a filter residue.

2.2. Collagen extraction from eggshell membrane

Collagen was obtained by the enzymatic hydrolysis process. The filter residue eggshell membrane was soaked in 1 mol/L acetic acid. The pH value was then adjusted to the optimum value for papain (6.8), pepsin (2.5) and trypsin (8.5) activity (Cheison et al., 2011; Klein and Kirsch, 1969; Mat et al., 2018). In this study, a completely randomised design was used to study the effects of enzyme type, enzyme-to-membrane ratio and material-to-water ratio on the extraction rate of eggshell membranes. The complete randomised design scheme is presented in Table 1.

Table 1. Complete randomised design scheme.

Level	Factor		
	Enzyme Type	Enzyme-to-Membrane Ratio	Material-to-Water Ratio
1	Papain	0.25	1:100
2	Pepsin	0.5	1:200
3	Trypsin	1	1:300

2.3. Analysis of hydroxyproline and collagen

Hydroxyproline and hydroxylysine are the most common amino acids found in collagen and are rarely found in other proteins (Gauza-Włodarczyk et al., 2017). Therefore, the collagen concentration can be calculated by measuring the concentration of hydroxyproline (Dunphy, 1987). To determine the hydroxyproline concentration, the UV-Vis spectrophotometric method, which is based on the oxidation reaction of hydroxyproline and chloramine T, was used. Since the oxidation product can react with dimethylaminobenzaldehyde to form a fuchsia compound with a characteristic absorption peak at 550 nm, the concentration of hydroxyproline can be determined by measuring the peak intensity by UV-Vis spectrophotometry. The intensity of colour was measured in a 1.0 cm glass cuvette at a wavelength of λ = 550 nm. The concentration of hydroxyproline is defined by:

$$c_{hydroxyproline} = \frac{A_{testing} - A_{blank}}{A_{standard} - A_{blank}} \times c_{standard} \times \frac{V_{standard}}{V_{testing}}, \quad (1)$$

where $A_{testing}$, A_{blank} and $A_{standard}$ are the absorbance of the sample, blank solution and standard solution respectively. The $C_{standard}$ is the concentration of the standard solution. The $V_{standard}$ and $V_{testing}$ are the volume of the standard solution and the testing solution respectively.

The collagen extraction rate (η) is calculated by:

$$\eta(\%) = \frac{c_{hydroxyproline} \times V_{total} \times k}{m_{membrane}} \times 100\%, \quad (2)$$

where k is the hydroxyproline conversion factor. Based on the hydroxyproline assay kit, the value of k is 13.4. The $m_{membrane}$ is the membrane weight and V_{total} is the total volume of the solution.

3. Results and discussion

3.1. Effect of enzyme type on collagen extraction rate

Different enzymes, such as papain, pepsin, and trypsin, were utilised in the extraction process to evaluate the effect of enzyme type on the collagen extraction rate of eggshell membranes. Since the activity of biological enzymes is highly dependent on the hydrogen ion concentration, the pH value needs to be adjusted before the extraction process. The found optimum pH values of papain, pepsin and trypsin were 6.8, 2.5 and 8.5 respectively. The enzyme-to-membrane ratio and material-to-membrane ratio were set to 0.5 and 1:100. It can be found in Table 2 and Figure 1 that the pepsin extraction rate of the two groups were 1.70% and 1.69%, much higher than that of papain and trypsin. Compared to the use of papain, trypsin obtained more collagen but three times less than pepsin. Therefore, pepsin exhibits better performance in the collagen extraction process.

Table 2. Collagen extraction rate for various types of enzymes.

	Eggshell (g)	Eggshell membrane (g)	Enzyme type	Enzyme weight (g)	DI water (ml)	HYG weight (μg)	Collagen (μg)	Extraction rate (%)
A-1	10.05	1.37	Papain	0.69	137.00	487.06	3634.75	0.26%
A-2	10.05	0.63	Papain	0.31	63.00	315.45	2354.12	0.38%
A-3	10.03	1.58	Pepsin	0.78	157.00	3595.01	26828.45	1.70%
A-4	10.02	1.24	Pepsin	0.62	124.00	2809.92	20969.54	1.69%
A-5	10.03	0.76	Trypsin	0.38	76.00	424.32	3166.57	0.42%
A-6	10.01	0.62	Trypsin	0.31	61.00	460.17	3434.08	0.56%

Table 3. Collagen extraction rate at various enzyme-to-membrane ratios.

	Eggshell (g)	Eggshell membrane (g)	Enzyme type	Enzyme weight (g)	DI water (ml)	HYG weight (μg)	Collagen (μg)	Extraction rate (%)
B-1	10.01	1.75	Pepsin	0.44	174.00	2214.71	16527.67	0.95%
B-2	10.02	1.24	Pepsin	0.62	124.00	2809.92	20969.54	1.69%
B-3	10.01	1.17	Pepsin	1.17	116.00	4391.00	32768.65	2.81%

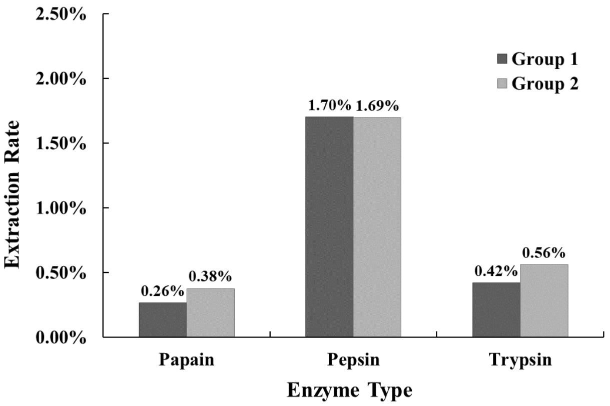

Figure 1. Effect of enzyme type on collagen extraction rate.

3.2. Effect of enzyme-to-membrane ratio on collagen extraction rate

The effect of the enzyme-to-membrane ratio on the collagen extraction rate is shown in Table 3 and Figure 2. Based on the results presented in Section 3.1, papain with best performance was used in the following experiments. The extraction process was carried out using different enzyme-to-membrane ratios: 0.25, 0.5 and 1. The other extraction parameters were set at a 1:100 material-to-water ratio. As shown in Figure 2, with the increasing enzyme-to-membrane ratio, the substrate hydrolysis reaction becomes more complete. The collagen extraction rate significantly improved from 0.95% to 1.69% with the enzyme-to-membrane ratio rising from 0.25 to 0.5. By increasing the ratio to 1.0, the collagen extraction rate increased slightly to 2.81% and the growth rate gradually decreased. This is because if a large amount of enzyme is added to the reaction chamber, the excess enzyme may coagulate, thereby reducing the extraction rate and inhibiting the hydrolysis reaction. Hence, the optimal enzyme-to-membrane ratio is 1.0 for producing collagen.

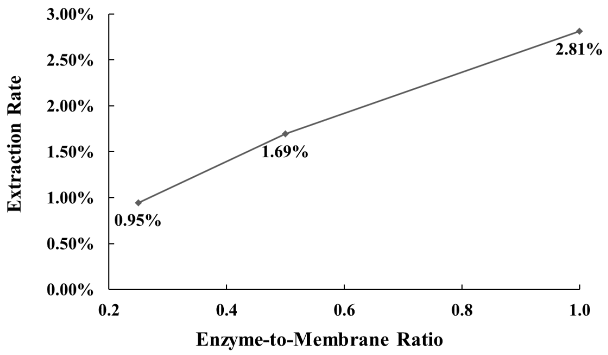

Figure 2. Effect of enzyme-to-membrane ratio on collagen extraction rate.

3.3. Effect of material-to-water ratio on collagen extraction rate

The influences of the material-to-water ratio on extraction rate might be caused by the low solubility of collagen. In the extraction, the enzyme reacts with collagen to remove the non-helical ends of the collagen, thereby increasing its solubility. This results in a specific cleavage of the telopeptide region of the eggshell membrane collagen, which dissolves the collagen and allows it to diffuse out of the material. The material-to-water ratio was set to 1:100, 1:200 and 1:300 in this study. According to

Table 4. Collagen extraction rate at various material-to-water ratios.

	Eggshell (g)	Eggshell membrane (g)	Enzyme type	Enzyme weight (g)	DI water (ml)	HYG weight (µg)	Collagen (µg)	Extraction rate (%)
C-1	10.03	1.58	Pepsin	0.78	157.00	3595.01	26828.45	1.70%
C-2	10.01	1.26	Pepsin	0.63	252.00	4142.26	30912.36	2.44%
C-3	10.03	1.24	Pepsin	0.62	372.00	3260.74	24333.92	1.96%

the results presented in Section 3.2, the optimal enzyme-to-membrane ratio was 1.0, yet the process cost will increase with the enzyme-to-membrane ratio, which was thus set to 0.5 in the experiments. As shown in Table 4 and Figure 3, when the material-to-water ratio changed from 1:100 to 1:200, the collagen extraction rate rose from 1.69% to 2.44%. However, when the ratio reached 1:300, the collagen extraction rate fell to 1.96%. The reason for this phenomenon is that the hydrolysis reaction in the high-concentration system is incomplete, and the high-concentration collagen hinders the extraction process. Both the degree of hydrolysis reaction and the collagen extraction rate increase with a decrease of concentration. However, the membrane and enzyme concentrations are diluted with the addition of excess water, further resulting in a decrease in collagen extraction. Thus, the material-to-water ratio of 1:200 is deemed favourable in the collagen extraction process.

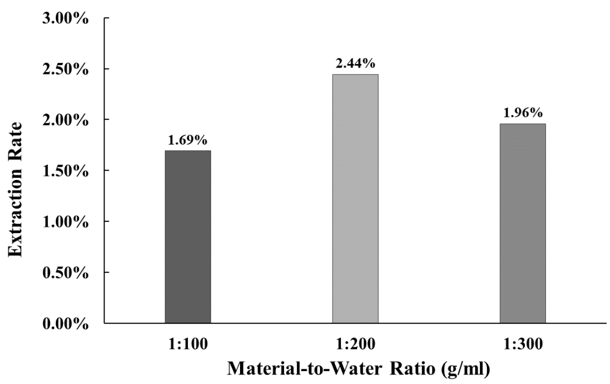

Figure 3. Effect of material-to-water ratio on collagen extraction rate.

4. Conclusions

This study showed that the enzyme type, enzyme-to-membrane ratio and material-to-water ratio critically affect the performance of the collagen extraction process. Enzymes such as pepsin, papain and trypsin can extract collagen from eggshell membranes through hydrolysis reactions. Pepsin exhibited a higher extraction rate than papain and trypsin during collagen extraction. The extraction rate of pepsin was 1.70%, which was three times that of papain and trypsin. The extraction rate can be improved with the increase of the enzyme-to-membrane ratio. A material-to-water ratio of 1:200 was deemed favourable in the collagen extraction process, which together with a pepsin-to-membrane ratio of 0.5 was found to be optimal for collagen extraction from the eggshell membrane, giving an extraction rate of 2.44%. The efficient extraction condition identified in this study suggests that eggshell membrane waste could be one of the potential sources for collagen, which is a valuable substance.

Acknowledgement

The work was supported by the Innovation and Technology Commission of the HKSAR Government under the R&D Cash Rebate Scheme (CRP/043/21).

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