www.thaiagj.org Thai Journal of Agricultural Science 2011, 44(5) : 354-360

Extraction of Collagen from Hen Eggshell Membrane by Using Organic Acids

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Abstract

The procedure of membrane separation and collagen extraction, presented in the membrane, were investigated. Membranes were separated from the shell wall in a tank (15 L) of water fitted with a mechanical stirrer. Membrane separation was enhanced by addition of the elutant, EDTA at 5% w v^{-1}. Optimum weight of eggshells was 500 g 15 L^{-1} aqueous solution, with maximum yielding of the membrane about 8% of eggshells. Collagen was extracted from the separated membranes with the addition of either of two organic acids, 0.5 M acetic or 0.5 M citric acid. Highest collagen yields of 507 and 495 mg $100g^{-1}$ dry sample were obtained when acetic and citric acids relative to membrane weight were added at a ratio of 1:8. Thermal solubility of collagen at 40 ºC was about 14.7 and 18.0 mg 100 g^{-1} dry sample for water and 0.45 M NaCl solution, respectively. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the collagen from eggshell membranes yielded type 1 collagen with $\alpha 1$ and $\alpha 2$ fibrils.

Keywords: collagen, hen eggshell membrane, organic acid

Introduction

Raw collagen is a biochemical product now increasing demand for the production of skin graft and tissue replacement products, dental implants, and cornea repair. Purified collagen is now selling for up to $1,000 per gram (Abdullah, 2003). The skins of cattle and pig, as well as their bone, are the main sources of collagen used in food, pharmaceutical, cosmetic and leather industries (Bailey and Light 1989; Cavallaro, et al., 1994; Ogawa et al., 2004). The outbreak of mad cow diseases has resulted in anxiety among users of cattle gelatin, due to the not fully confirmed hypothesis, that the infective agent can be transferred from animals into human beings. Additionally, the collagen obtained from pig's bones cannot be used as a component of some foods for religious reasons. Therefore, there is a strong need to develop alternative collagen sources such as fish offal, bone, scales, fins as well as skins (Sadowska et al., 2003; Nalinanon et al., 2007; Sadowiska and Skierka, 2007).

Hen eggs provide a useful amount of complete protein at the cheapest price when compare with meat, poultry and fish; thus eggs are one of the important food that are consumed throughout the world. Besides, eggs have some which are very useful for food modification, such as gel production and emulsifying agent. Therefore, eggs are widely used as a raw material for making many different types of food such as ice-cream, deep-fat fried food, bakery products and mayonnaise. In Thailand, hen-eggs were produced 9,424 million units or 471,200 tons of each day, generating 47,120 tons of waste eggshells (Office of Agricultural Economic of Thailand, 2009). The shells from industrial companies were used as a source of calcium in animal feed. However, the shells were still over supplied for this case. Companies, in USA, are paying up to $ 100,000 per year to dispose of this waste in landfills that are quickly

reaching capacity (Abdullah, 2003). Mac Neil (2001) reported that eggshell had 8% (w w^{-1}) shell membrane and the membrane contained 10% (w w^{-1}) collagen. Collagens from hen-eggshell membrane are type I, V and X (Candish and Scougall, 1969; Wong, et al., 1984; Arias et al., 1991).

There are three methods for collagen extraction. Saito et al (2002) extracted collagen from sea cucumber by using enzyme pepsin at 4 °C for 2 days. The result indicated that collagen was extracted up to 70%, however, the enzyme was too expensive for commercial production. Davison (1991) extracted collagen from rat-tail tendons by using organic amine, 0.1 M methylenediamine hydrochloride, at 4 °C for overnight. During the reaction, thiol reagent (mercaptoethanol) was added to increase collagen yield and periodically homogenized. The result indicated that collagen was extracted marked type I, III and V. Nevertheless, the chemicals were expensive and the procedure of extraction was too complicate. Moreover, almost of proteolytic enzyme, such as pepsin trypsin and papain, and ethylenediamine were non-specific for collagen extraction. These enzymes removed only the telopeptide (non-helical ends) of collagen resulted in changed collagen properties and solubilized non-collagen protein (Cliche, et al; 2003; Sadowska and Skierka, 2007). Subsequently Sadowska and Skierka (2007) indicated that the best solvents for solubilized collgen fibrils were acetic and lactic acid. However, only α_1, α_2, and β chains were observed in the electrophoretogram. Sadowska et al. (2003) extracted collagen from cod skin by using organic acid as acetic and citric acids. The extractability of collagen depends both on the concentration of acids and the ratio of skin to acid. The largest percentage of total content of collagen about 85% could be extracted by either 0.5 M acetic or 1.5 M citric acid, at 4 °C for 24 h; using the ratio of skin to acetic and citric acids at 1:40 and 1:20 (w v^{-1}), respectively.

Mac Neil (2001) investigated the method to separate the shell membrane from hen eggshell by using their physical properties. The shell particles, being heavier than the membrane particles, settle down at the bottom of the tank while the membrane particles are relatively light and therefore to maintain suspended in the water of the tank. So the suspend membrane was removed from the shell. However, Arias et al (1991) indicated that after soaking the shell in 0.5M EDTA (tetrasodium salt) could improve the separating of membrane from shell. So it should have way to research how the shell membrane to be able to separate from the eggshell and study for the appropriate condition of collagen extraction from eggshell membrane, as the economic value added and the use of eggshell.

Materials and Methods

Egg shell waste was obtained from the process of pasteurized liquid egg of Bangkok Produced Agroindustry Co. Ltd., Thailand. The eggs were washed by using 50 °C water before passing through the egg breaking machine to separate egg white, egg yolk and egg shell. Liquid egg white and egg yolk was pasteurized while egg shell was ground by centrifugal mill, resulted 2-5 mm diameter particle size.

Separation of Egg Shell Membrane

Egg shell membrane was separated from egg shell by using 20 liters separating equipment as in Figure 1. The equipment composed of a stirring container and a separating container. The egg shell (300, 500, 700, 900 and 1,100 g) was mixed with 5 L of 5% EDTA solution and stirred at the speed of 50 rpm for 30 min by using stirrer paddles of the stirring tank. The heavier egg shell would be precipitated while the lighter membrane would be float and collected on a sieve of separating container. The membrane was then rinsed with distilled water.

Proximate Analysis

Total protein, fat and ash of the separated collagen were determined by the methods of AOAC (1990).

Figure 1 The separating equipment
(a) stirring container (b) separating container

Collagen Extraction From Egg Shell Membrane

Pretreatment of egg shell membrane

Pretreatment process was followed the method of Sadowska et al. (2003) to remove the impurity such as soluble non-collagen compound, lipid, pigment and off-flavor. In the first step, soluble non-collagen compounds were removed by cold water and salt solution. Egg shell membrane was blended with cold water (1:6 w v^{-1}) by using a blender at 4 °C for 3 min and filtered. The membrane was then mixed with 0.45 M NaCl (1:6 w v^{-1}) by using magnetic stirrer for 3 min. The retentate was homogenized with 0.45 M NaCl (1:6 w v^{-1}) at 6,000 rpm for 4 min. Subsequently, the retentated membrane was washed with distilled water (1:6 w v^{-1}) and centrifuged at 2,000 g, 4 °C for 30 min. The supernatant was analyzed hydroxyproline amino acid by using HPLC and total nitrogen by Kjeldahl method to evaluate the pretreatment loss. Finally, the precipitate was collected to remove lipid, pigment and off-flavor by stirring in the following chemicals: 0.2% (w v^{-1}) NaOH, 0.2% (w v^{-1}) H_2SO_4 and 0.7% (w v^{-1}) citric acid, respectively, at the ratio of precipitate to each solution 1:7 (w v^{-1}), for 4 min, washed with water to pH 7 and filtered. After that, the retentate was soaked in 10% (w v^{-1}) NaCl for 24 h at room temperature, filtered, bleached with alkaline hydrogen peroxide (1% v v^{-1}) H_2O_2 in 0.01 M NaOH) in the ratio of 1 to 6 (w v^{-1}) for 24 h at room temperature, neutralized and washed with distilled water.

Collagen extraction

Collagen was extracted from membranes, obtained from the pretreatment process, with either 0.5 M acetic acid or 0.5 M citric acid. Membranes were thoroughly mixed with either acid in one of four ratios (1:4, 1:6, 1:8 and 1:10 w v^{-1}), in a shaker water bath at 4 °C for 2 h. Mixtures were then removed, centrifuged for 4 min at 6,000 rpm, again mixed in the shaker bath (4 °C) for 24 h, homogenized for 2 min (6000 rpm, 4 °C) and finally centrifuged at 10,000g 10 °C for 20 min. The precipitate was extracted three times. The volume of each supernatant (soluble collagen) was determined the hydroxyproline amino acid following the method of Dunphy at al. (1987) and the type of collagen was determined following the method of Goncalves-Note et al. (2002). The supernatant of samples extracted with citric acid was also dialyzed in distilled water at 4 °C for 24 h before analysis.

Analysis of Hydroxyproline and Collagen

The extracted collagen compound (1 ml) was hydrolyzed with 5 ml 6 N HCl at 116 °C for 16 hr and then flushed with nitrogen gas to evaporate acid. The dried sample was prepared to phenylthiocarbamyl (PTC) derivatives following the method of Koop et al. (1982). The sample was added with 3 μL 50% (v v^{-1}) ethanol and 7 μL of PTC mixture, (contained 90% (v v^{-1}) ethanol, triethylamine phenylisothiocyanate, 7:2:1 v $v^{-1}v^{-1}$), hold for 10 min at room temperature, and diluted with 1 ml mobile phase (contained acetonitrile:pure water:140 mM acetate buffer pH 6.3 at ratio 0.6:0.4:9.0 v $v^{-1}v^{-1}$, respectively).

The 10 μL diluted sample was injected into auto sample to determine hydroxyproline content by using HPLC (Model 717 Plus, Water Associate, MA, U.S.A.), following the method of Dumphy et al (1987). Analysis was performed by using 250 mm length x 4.6 mm diameter of

C_{18} Nova-Pak Column (Supelco, Bellefonte, PA, USA). A system comprised of the mobile phase, 1900 psi pressure, 1.5 ml min^{-1} flow rate and UV detector at 254 nm. The peak area of chromatogram was calculated as amino acid content, compared with hydroxyproline standard, and converted to collagen content, µg (µg of hydroxyproline content * 14.7, Dunphy at al., 1987).

Thermal Solubility of Collagen

Thermal solubility of collagen was investigated in the temperature range from 0 to 40 °C as the method of Montero and Borderias (1991) with some modification. The egg shell sample (2 mg) was suspended in water or 0.45 M NaCl solution (1:6, w v^{-1}) and incubated in a water bath for 24 h at 0, 5, 10, 15, 20, 25, 30, 35 and 40 °C. The samples were then centrifuged at 10,000 g for 30 min at 15 °C. Supernatant (1 ml) was analyzed as hydroxyproline content by HPLC and the result was then converted to collagen to determine the collagen solubility at various temperatures.

Type of Collagen Determination by SDS-PAGE

Standard collagen type I, from bovine achilles tendon, Sigma Chemical Co., St. Louis, Mo, USA, and extracted collagen samples were treated according to SDS page method.

Preparation of standard

The standard collagen type I was dissolved in 0.5 M acetic acid (3 mg ml^{-1}), dialyzed with buffer A (50 mM Tris-HCL, contained 0.2 M NaCl, 1 mM $CaCl_2$ and 0.02% v $v^{-1}$$NaN_3$, pH 7.4) and vacuum dried at 200 mbar 50°C for 5 h. The sample was dissolved with 100 µL 0.01M acetic acid, added with 50 µL 2% (v v^{-1}) β- mercaptoethanol and the dried at 110 °C for 1 min. Finally, the collected sample was stored at –20 °C for analysis.

Preparation of collagen sample

One milliliter of the extracted collagen was vacuum dried at 200 mbar 50 °C for 5 h. The dried sample was dissolved in 100 µL 0.01 M acetic acid and 50 µL 2% (v v^{-1}) β- mercaptoethanol, dried at 110 °C for 1 min and collected the sample at –20 °C for analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE were performed according to the method of Laemmli (1970). The stacking gel contained 5% polyacrylamide and the separating gel contained 12.5% polyacrylamide. The prepared sample was loaded per well and the migration was induced at a constant current 24 mA. for 90 min, or until the migration reached the end of the resolving gel. The gel were stained with Comassie blue R-250 for 30 min and destained with a solution of 10% glacial acetic, 40% methanol and 50% water. The type I collagen was used as a standard.

Statistical Analysis

The experimental data were analyzed statistically by analysis of variance, for statistical significance (p = 0.05) using Duncan's Multiple Range Test, SPSS for Window Version 17.0 (SPSS ID 5066789, permanent) and inferences were reported at the appropriate place.

Results and discussion

Amount of Membrane Separation and Chemical Components of the Membrane

By a preliminary experiment, the 5% EDTA solution assisted membrane separation from eggshell. As a result, the membrane separated about 6.5 g 100 g^{-1} eggshell after soaking in 5% EDTA solution for 30 min while the yield was only 5.0 g 100 g^{-1} eggshell by water soaking at the same time. Tung and Richad (1972) explained that disodium salt of EDTA solution could remove calcium, main component of eggshell, thus the eggshell membrane was separated from the shell. EDTA, prominence as a chelating agent, has ability to sequester di and tri-cationic ions such as Ca^{+2} and Fe^{+3}. After being bound by

EDTA, the Ca ions remained in the solution but exhibit diminished reactivity (Lewis, 1993). Thus, membrane was removed from the shell which contained 95% calcium carbonate (Fennema, 1985).

The amount of eggshell was significantly effect on percent yield of separated membrane ($p<0.05$). The 500 g eggshell weight gave the most enable membrane, with 7.5% yield (Table 1). While the increasing of eggshell from 700 to 1,100 g caused decrease membrane separation from 6.3 to 5.5%, respectively. This might be due to the exceeding of eggshell weight.

The chemical components of eggshell membrane were composed of 88.2% protein, 10.5% ash, 1.04% carbohydrate and 0.35% fat. As in the report of Candish and Scougall (1969) indicated that eggshell membrane mostly composed of protein and less in fat and carbohydrate.

Table 1 Yield of eggshell membrane at various amount of separated eggshell

Egg shell (g)	% Yield*
300	6.70 ± 0.01 [b]
500	7.53 ± 0.50 [a]
700	6.29 ± 0.25 [b]
900	5.56 ± 0.01 [c]
1100	5.45 ± 0.01 [c]

*Means average of three replications

[a, b..] Means with different letters are significantly different ($p<0.05$)

Acetic Acid and Citric Acid Extraction

Protein was loss only 0.11% from pretreatment step and hydroxyproline was not detected in the pretreatment solution. The extractability of collagen depends on the ratio of membrane to acid. Increasing of acetic and citric acid ratio could be able to increase in collagen extraction. With citric acid extraction, yield of collagen decreased with cycle of extraction (Figure 2). The extracted collagen was increased 4 times when the ratio of membrane to acetic acid increased from 1:4 to 1:8 ($p<0.05$, Figure 3). Compared with citric acid, extracted collagen increased 2 times at the same range ratio of membrane to acid. The largest percentage of the extracted collagen content was about 507 and 495 mg 100 g^{-1} dried sample by acetic and citric acid extraction, respectively. Yi et al. (2003) indicated that collagen protein was insoluble in water but the protein could soluble in common non toxic organic solvents, such as 3-mercaptopropionic acid in the presence of 10% acetic acid. This mainly caused by the presence of crosslinks of disulfide bonds. Sadowska et al. (2003) also reported that the increasing the ratio of fish (Baltic cod) skin to 0.5 M acetic acid from 1:10 to 1:40 could increase extractability of collagen in range 20 to 90%, respectively. However, collagen of the skin could limitedly dissolve in citric acid. Analysis type of extracted collagen from eggshell membrane by SDS-PAGE, demonstrated band pattern typical of type I collagen (Figure 4) which composed of α 1 and α 2 bands as reported in Wong et.al. (1984) and Yi et al. (2004).

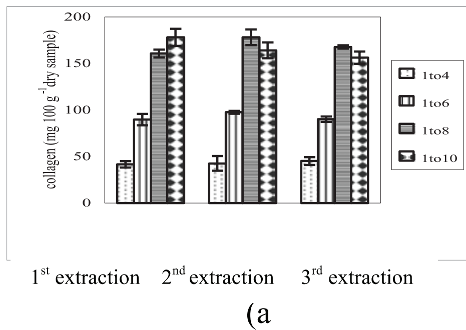
Thermal Solubility of Collagen

The thermal solubility of hen-eggshell membrane collagen depended on the medium solution (Figure 5). As a result, the membrane was heated in 0.45 M NaCl and water the solubility of collagen increased significantly at temperature above 20 ºC and 30 ºC, respectively. At 40 ºC, collagen solubilities were about 14.7 and 18.0 mg 100 g^{-1} dry sample in 0.45 M NaCl and water, respectively. Sadowska et al. (2003) reported that the suitable temperature for collagen extraction was less than 20 ºC to protect protein denaturation. In addition, Montero et al. (1995) investigated the water solubility of plaice skin collagen was increased from 4 to 90% at the temperature in range from10 ºC to 40 ºC, respectively.

Conclusions

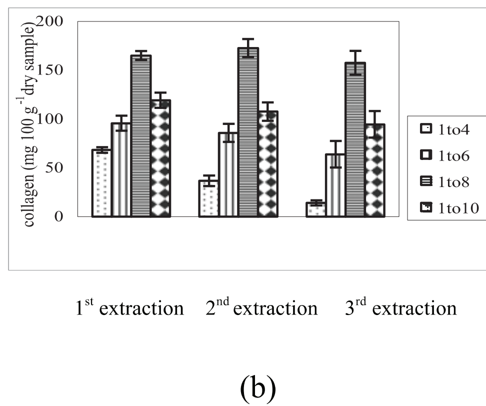
The maximum yielding of the membrane was 8% of eggshells by using the eggshells at 500 g per 15 L of 5% EDTA solution. The optimum ratio of the membrane to 0.5 M acetic acid for collagen extraction was 1:8 wt v^{-1}, with the highest collagen yields of 507 mg $100g^{-1}$ dry

sample and the extracted collagen was type I collagen.



1st extraction 2nd extraction 3rd extraction

(a



1st extraction 2nd extraction 3rd extraction

(b)

Figure 2 Extracted collagen by (a) 0.5 M acetic acid and (b) 0.5 M citric acid at various ratio of acid to membrane in each extraction

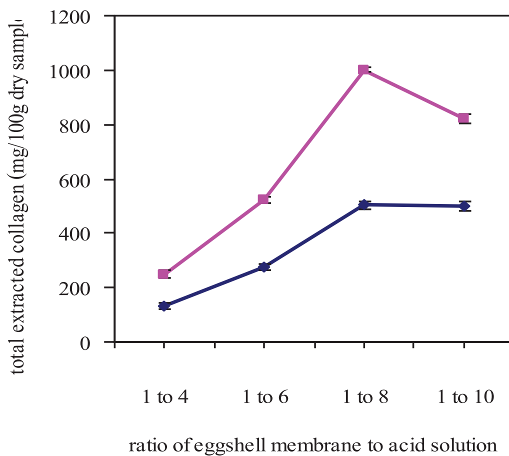


Figure 3 The extracted collagen from eggshell membrane by 0.5 M acetic acid (—◆—) and 0.5 M. citric acid (—■—)

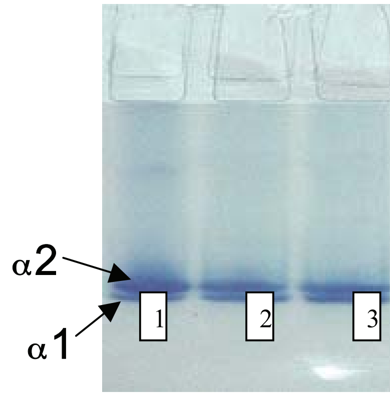


Figure 4 Protein bands from SDS-PAGE of eggshell membrane collagen. Lane 1 Standard collagen type I by acetic acid. Lane 2 Extracted collagen from egg shell membrane by acetic acid. Lane 3 Extracted collagen from egg shell membrane by citric acid.

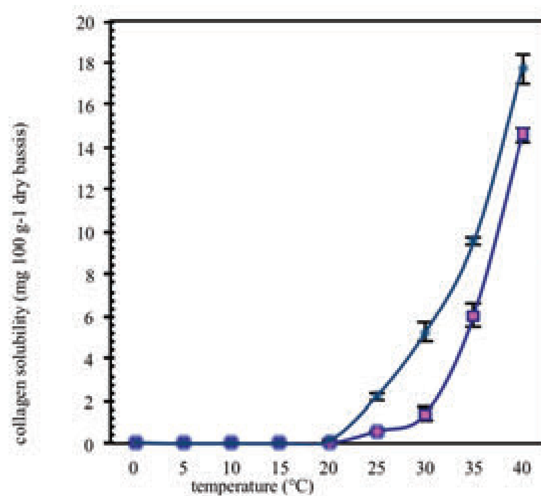


Figure 5 Solubility of eggshell membrane collagen at various temperature in 0.45 M NaCl (—◆—) and water (—■—)

Acknowledgements

The authors thank Thai Research Fund for financial support under industrial and research projects for undergraduate students. The authors also thank Professor F.W.H. (Bill) Beamish of Biological Science Department, Faculty of Science, Burapha University, Thailand for his assistance in English correction.

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This paper was originally presented at the International Conference on Agricalture and Agro-Industry 2010 (ICAA2010), November 19-20, 2010 Mae Fah Luang University, Chiang Rai, Thailand