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Effects of acetic acid on efficiency of collagen extraction from jellyfish *Rhopilema hispdium* (Vanhöffen, 1888)

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Abstract
Collagen is an extremely important fibrous protein in the body. It is the main structural material of all tissues: Skin, bones, ligaments, tendons, cartilage. The source of traditional collagen production is mainly from the skin and bones of animals. However, marine animals are currently a promising alternative source of materials with low risk of disease transmission, no religious barriers to consumption, abundant raw materials, and high-efficiency extraction. In present study, the effects of several factors: Acetic acid concentration, the ratio of jellyfish and acetic acid solution (w:v), as well as the time of extraction on efficiency of collagen extraction process from jellyfish (*Rhopilema hispidum* Vanhoffen, 1888) were investigated. The results showed that the collagen which was extracted from jellyfish in 0.1 M acetic acid, with the ratio 1:3 between the jellyfish and acetic acid solution (w:v) in 5-day extraction had high extraction efficiency. Extracted collagen in present study was mainly type I collagen, consisting of 3 polypeptide chains: β chain (~ 250 kDa), α1 chain (~ 40 kDa) and α2 chain (~ 100 kDa).

Keywords: *Rhopilema hispidum*, collagen, jellyfish, extraction.

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INTRODUCTION

Collagen is the most abundant protein in vertebrates making up approximately 20–30% of total protein [1, 2]. Collagen is a major structural material of all connective tissues: Skin, bones, ligaments, tendons, and cartilage, as well as the interstitial tissues of all parenchymal organs [3]. All collagens have a triple helical structure composed of three polypeptide chains (a chains) with a repeated sequence of three amino acids, glycine-X-Y, in which X and Y are mostly proline and hydroxyproline [3]. Currently, collagen and its denatured form (gelatin) have been widely used in the food, pharmaceutical, biotechnology, biomedical and cosmetic industries [4]. Traditional raw materials in the collagen production are mainly from the skin and bones of terrestrial animals such as cows and pigs [5]. However, use of porcine and bovine collagen poses the risk of transmitting diseases such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), and foot and mouth disease (FMD) [4, 6]. In addition, for religious reasons, Muslims and Hindus do not consume products from hedgehog or cows. Therefore, collagen from marine organisms is a promising alternative, because of a low risk of disease transmission, no religious barriers to consumption, abundant raw materials, and higher extracting efficiency compared to other raw materials [7]. In the world, research on isolation and extraction of collagen from jellyfish has been of interest since 2000 [8, 9]. Recently, studies have shown that collagen derivatives from jellyfish were effective in preventing and curing rheumatoid arthritis, osteoarthritis and osteoporosis, high blood pressure and anti-fatigue effects [10]. However, there has not been any published result on collagen extraction from jellyfish in Vietnam so far.

On the other hand, there were abundant and diverse jellyfish resources in Vietnam with 128 recorded species, in which four species had high economic value, consisting white jellyfish (*Rhopilema hispidum*), red jellyfish (*Rhopilema esculentum*) and two other species (*Crambione mastigophora* and *Lobonema smithii)*. The estimated reserve was mainly from white jellyfish with 986,880 tons, which are distributed in shallow water along the coast of the Gulf of Tonkin, with depth < 20 m, salinity < 31.0‰ and temperature of surface seawater < 26^{o}C. The average density of white jellyfish in the western coastal area of the Gulf of Tonkin reached 2,218 individuals per square meter (38.1 tons/km^2) and in the Central Coast was 506 individuals/km^2 (5.8 tons/km^2) [11].

Therefore, this paper provided initial results on acetic acid's effect on collagen extraction efficiency from white jellyfish (*Rhopilema hispidum*), contributing to establishing the collagen extraction process from jellyfish in Vietnam.

MATERIALS AND METHODS

Materials

Jellyfish were collected directly from fishing boats in Do Son - Hai Phong area in March 2019. Jellyfish were washed, refrigerated, and brought immediately to the laboratory for analysis. The species was identified based on references such as Mayer (1910); Kramp (1961), Omori et al., (2001), Nishikawa et al., (2008), Thu et al., (2009) [12–17].

Methods

Characteristics of the jellyfish material

Some characteristics of jellyfish material were determined, consisting moisture content, protein content, lipid content, ash content, and carbohydrate content.

Moisture (g/100 g) was determined according to TCVN 3700-90; the sample was dried at 105–110^{o}C, then determined by weight method [18].

Protein content (g/100 g) was determined according to TCVN 3705-90; the sample was distilled by Kjeldahl equipment to convert all forms of nitrogen (N-T) to NH_4^+, NH_4^+ is complex indophenol with sodium hypochlorite, phenol, and alkaline citrate in the presence of sodium nitroprusside as a catalyst. The concentration of NH_4^+ was measured by indophenol complexing spectroscopy. Protein content = N-T × 6.25 (consider protein containing 16% as nitrogen) [19].

The lipid content (g/100 g) was determined according to TCVN 3703-2009; the

sample was extracted by Soxhlet equipment, then the solvent was evaporated, dried at 100^{o}C and then the residue content was determined by weight method [20].

The ash content (g/100 g) was determined according to TCVN 5105-2009; the sample was heated with moisture at 500–600^{o}C and then determined by weight method [21].

Carbohydrate content (g/100 g) = 100 – *moisture – protein – lipid – ash*

Experimental design

Collagen from jellyfish was extracted according to the method of Nagai et al., (1999) [22] with some changes fitted with the experimental conditions, figure 1.

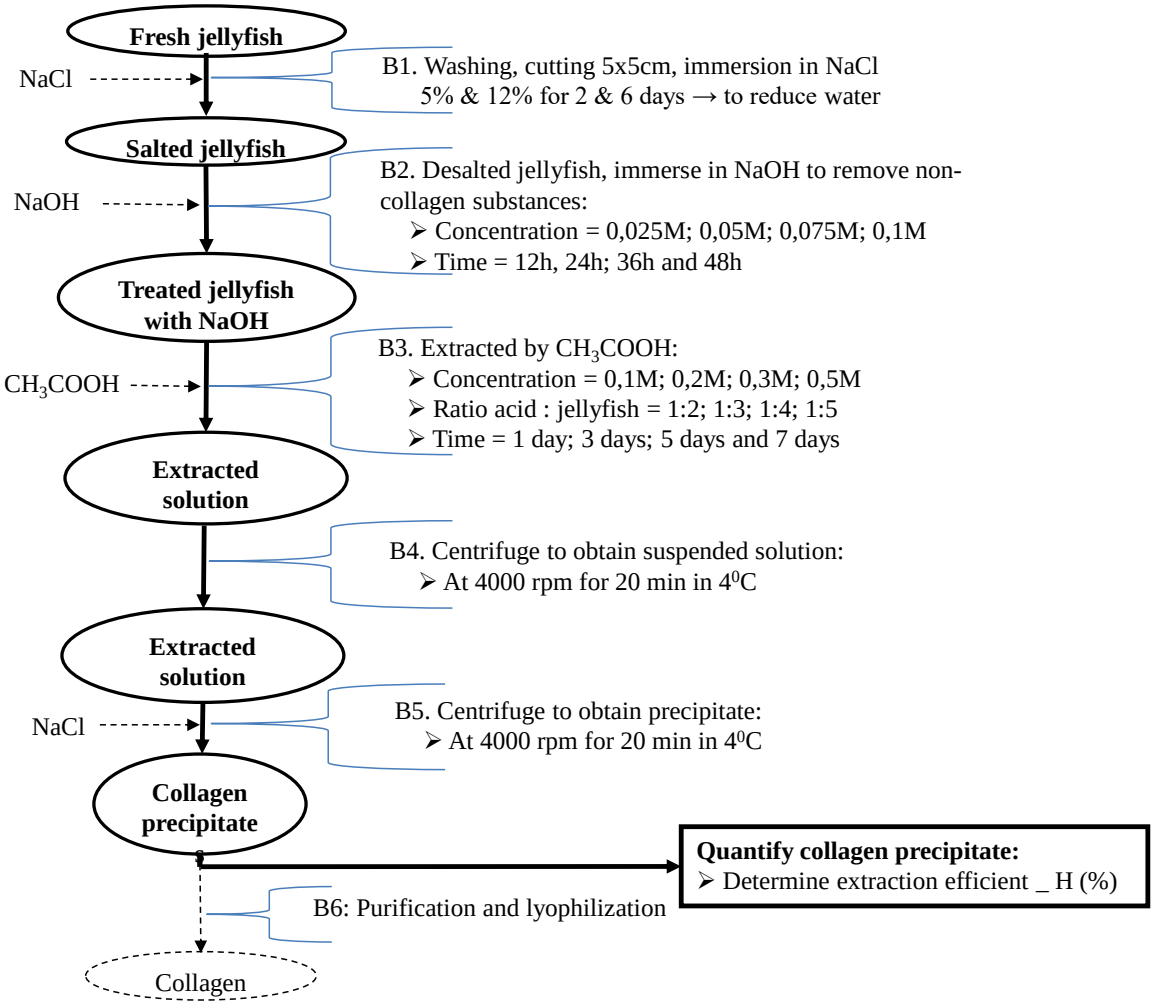

Figure 1. Diagram of research and experiment

The experiment to remove non-collagen substances by NaOH:

After removing viscosity, de-salting, and removing part of water, jellyfish was treated with NaOH to remove non-collagen substances at different NaOH concentrations: 0.025 M; 0.05 M; 0.075 M; 0.1 M and at different times: 12 hours; 24 hours; 36 hours and 48 hours.

The experiment to extract collagen by acid acetic:

After removal of non-collagen substances by NaOH, collagen was extracted from jellyfish sample by CH_3COOH with different experiments to optimize the extraction efficiency (H) as follows:

Investigation on the effect of acetic acid concentration (factor A) with experiments at different concentrations: A1 = 0.1 M; A2 = 0.2 M; A3 = 0.3 M; A4 = 0.5 M.

Investigation on the effect of the ratio of jellyfish mass to volume of acetic acid solution - w:v (factor B) with experiments at different ratios: B1 = 1:2; B2 = 1:3; B3 = 1:4; B4 = 1:5.

Investigation on the effect of the jellyfish extraction time (factor C) with experiments at different times: C1 = 1 day, C2 = 3 days, C3 = 5 days, C4 = 7 days.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970) [23] using a 8% resolving gel and a 4% stacking gel on vertical electrophoresis (omniPAGE WAVE Maxi System) from Cleaver Scientific in a buffer of 0.1% SDS, 0.025 M Tris and 0.192 glycine. The samples were then mixed with buffer at the sample buffer ratio of 1:1 (v/v) with and without b-mercaptoethanol and heated for 5 min at 95^{o}C. Each sample (20 µg protein) was loaded into a well and run at 80 V for 10 min, followed by 120 V for 1.5 h. Following electrophoresis, the gel was stained in a 0.05% (w/v) Coomassie brilliant blue R-250 solution in 15% (v/v) methanol and 5% (v/v) acetic acid. The molecular weight and composition of collagen protein were determined when compared with the standard protein (marker) whose size ranged from 75 kDa to 250 kDa.

Data analysis

All experiments were performed in triplicate, and data were presented as means ± SD. All experimental data were processed on Microsoft Excel software. A probability value of $P \leq 0.05$ was considered to be significant and analysis of variance (ANOVA) was performed to test for significant differences between experiments.

The f collagen extraction efficiency (H) was calculated by the following formula:

$$H_{collagen}(\%) = \frac{Weight\ of\ wet\ collagen}{Weight\ of\ treated\ jellyfish} \times 100\ \text{(gram)}$$

RESULTS AND DISCUSSION

Characteristics of jellyfish material

The characteristics of jellyfish materials were shown in table 1.

Table 1. The characteristics of jellyfish materials

No.	Ingredients	Content (% wet weight)
1	Moisture	97.100 ± 0.099
2	Lipid	0.150 ± 0.014
3	Protein	1.960 ± 0.015
4	Ash	0.790 ± 0.007
5	Carbohydrate	Not detected

The results in table 1 showed that jellyfish was composed mainly of water with proximately 97% content, followed by protein (proximately 2%), meanwhile fat (lipid), ash and carbohydrate content was very low. This result was also consistent with previous researches; jellyfish mainly consisted of water and protein [12].

Removing non-collagen substances by NaOH

De-salted jellyfish materials were experimented to remove non-collagen substances by NaOH at different concentrations (0.025 M; 0.05 M; 0.075 M; 0.1 M). The ratio of jellyfish:NaOH solution was 1:10 (w/v), experimental time was from 12 to 48 hours with stirring at the temperature from 5^{o}C to 10^{o}C to limit the growth of microorganisms and denatured collagen. Finally, the jellyfish materials were washed by distilled water and examined for the sensory properties; the results were shown in table 2.

The process of treating jellyfish with salt and NaOH solution is an essential step, NaOH not only removes non-collagen substances but also breaks down H-H bonds between 3 helix chains of collagen, to increases the solubility of collagen in solvents. The experimental results in table 2 showed that the NaOH concentration of 0.5 M and processing time of 36 hours gave the best efficiency in treating jellyfish samples with the characteristics: Yellowish and soft.

Table 2. Characteristics of jellyfish materials after removing non-collagen substances by NaOH

NaOH concentration / Time	0.025 M	0.05 M	0.075 M	0.1 M
12 h	Yellowish, tough	Yellowish, tough	Yellowish, tough	Yellowish, viscous
24 h	Yellowish	Yellowish, tough	Yellowish, hard	Yellowish, viscous
36 h	Yellowish	Yellowish, soft	Yellowish, slightly viscous	Yellowish, viscous
48 h	Yellowish	Yellowish, slightly viscous	Yellowish, slightly viscous	Yellowish, viscous

Extraction of collagen by acetic acid

The experimental results of assessment of the effect of acetic acid concentration, the ratio of jellyfish:acetic acid solution and extraction time on collagen yield efficiency were shown in figure 2.

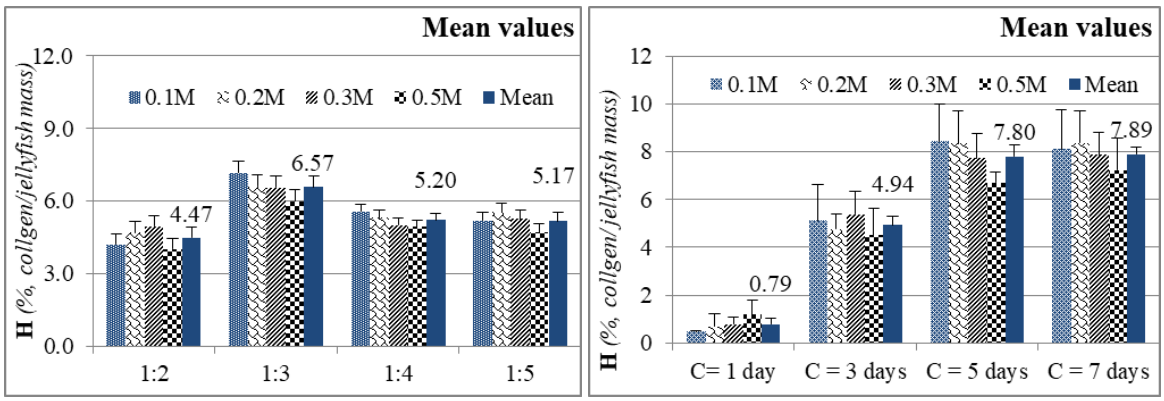

Figure 2. Effect of acetic acid concentration, the ratio of jellyfish:acetic acid solution (w:v) and extraction time on collagen yield efficiency

Figure 2 showed that collagen extraction efficiency which gradually increased over extraction time with 1 day, 3 days, 5 days, and 7 days was 0.79, 4.94, 7.80 and 7.89%, respectively. However, the extraction efficiency from fifth day to seventh day increased slightly (0.09%). In particular, the results of ANOVA test (table 3) also showed that the extraction efficiency in 5 days and 7 days was not significantly different ($P > 0.05$), while the extraction efficiency in 1 day and 3 days was significantly different from other experiments. Therefore, the 5-day extraction time should be selected for the collagen extraction from jellyfish.

The acetic acid solution concentration significantly affected collagen collection efficiency because collagen precipitates at the isoelectric point (pH = pI); when the collagen molecule is not electrically charged, without electrostatic repulsion it will be easy to coagulate or precipitate. When pH ≠ pI, collagen molecules which are electrically charged with the same sign would repel each other, cause challenge to precipitate. Therefore, it is imperative to find the right concentration of acetic acid in collagen extraction. The acetic acid concentrations were tested (figure 2), the variation of extraction efficiency tended not to be much. However, the extraction efficiency was highest in the experiment at the acetic acid concentration of 0.1 M and extraction time of 5 days (11.83%), although no significant difference was found between experiments

(ANOVA, $P > 0.05$). But, minimization of acidity in extraction is also consistent with the economic and environmental criteria. Thus, the 0.1 M acetic acid concentration should also be selected for the collagen extraction from jellyfish.

The results of experiments at four levels of the ratio between the jellyfish and the acetic acid solution (w:v) (figure 2) showed that the collagen extraction efficiency was the highest at the 1:3 ratio with the average efficiency ranging from 6% to 7%, especially the collagen extraction efficiency at extraction time 5 days and 7 days was 11.83 and 12.01%, respectively. On the other hand, the collagen extraction efficiency at the 1:3 ratio was also significantly different from other experiments (ANOVA, $P < 0.05$, table 3). Therefore, the ratio 1:3 between jellyfish and the acetic acid solution should also be selected for collagen extraction from jellyfish.

Thus, the concentration of acetic acid 0.1 M, the ratio 1:3 (w:v) between the jellyfish and the acetic acid solution, and the extraction time of 5 days were suitable for collagen extraction from jellyfish.

Table 3. Results of one way ANOVA test between experiments (*P-value*)

Extraction time (day)				
P-value	C = 1 day	C = 3 days	C = 5 days	C = 7 days
C = 1 day		+	+	+
C = 3 days	**2.7E-06**		+	+
C = 5 days	**3.16E-06**	**0.000641547**		-
C = 7 days	**2.76E-07**	**7.55467E-05**	0.845355976	
The ratio of jellyfish:acetic acid (w:v)				
P-value	1:2	1:3	1:4	1:5
1:2		+	-	-
1:3	**0.000631**		+	+
1:4	0.031371	**0.002802476**		-
1:5	0.044921	**0.003260603**	0.901289334	

The molecular weight of collagen by SDS-PAGE

SDS-PAGE could be used to determine the composition, molecular weight of collagen, type of collagen, and the purity of product. The analysis results of the extracted collagen in present study was shown in figure 3.

The SDS-PAGE patterns in figure 3 showed that in the extracted collagen sample, there were 4 types of proteins with molecular weights of about 250 kDa, 140 kDa, and 100 kDa, which were β chain (band 250 kDa), α chain (band α1 - 140 kDa and α2 - 100 kDa) and γ chain (over 250 kDa), respectively. This result was also consistent and similar to some previously published results [24–26]. Moreover, SDS-PAGE results showed that the extracted collagen samples consisted of 3 chains (β, α1, α2), in which the molecular weight of α1, α2 chains was higher than that of β chain, this indicated that extracted collagen from jellyfish was type I collagen. Besides, several other studies also showed that marine invertebrates including the sea urchin (*Paracentrotus lividus*) [27], starfish (*Acanthaster planci*) [28] and squid (*Uroteuthis duvauceli*) [28, 29] consisted of type I collagen.

In addition, the results of SDS-PAGE also showed that there were still faint colored lines along the lands because the extracted collagen was not purified yet, no or few bands had weight less than 100 kDa. The SDS-PAGE results also indicated that the purity of the extracted collagens was relatively good.

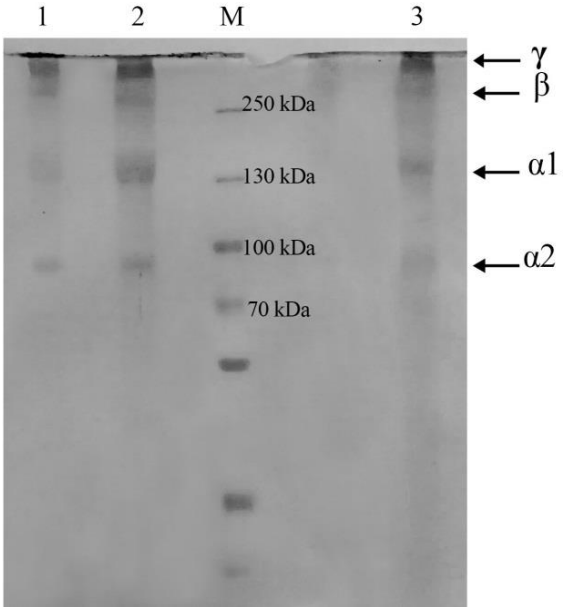

Figure 3. The electrophoretic patterns of collagen extracted from the jellyfish (*Rhopilema hispidum*): M - Molecular weight marker; 1, 2, 3 were extracted collagen samples

CONCLUSION

The jellyfish that was treated to remove non-collagen substances by NaOH at a concentration of 0.05 M with a processing time of 36 hours gave good results.

After treating non-collagen substances with NaOH, the jellyfish material which was extracted at 0.1 M acetic acid, with the ratio 1:3 between the jellyfish and acetic acid solution (w:v) in 5-day extraction had high collagen extraction efficiency.

Extracted collagen from jellyfish *Rhopilema hispidum* was a type I collagen, consisting of 3 polypeptide chains: β chain (~ 250 kDa), α1 chain (~ 40 kDa) and α2 chain (~ 100 kDa).

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