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Unicorn Fish *(Naso reticulatus* Randall, 2001) Skin Collagens Prepared Using Two Pepsin Sources: An Assessment on Physicochemical Characteristics

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***Abstract*—Fish collagens have gained considerable attention from numerous researchers due to their attractive traits and are more acceptable from most religious beliefs. This paper aimed to extract and characterize collagens from the skins of unicorn fish (*N. reticulatus*) influenced by pepsin from porcine (UCP) and pepsin from bovine (UCB). The yield of the UCP sample (15.60%) was significantly higher (P<0.05) compared to the UCB (10.40%). In addition to this, the swelling value of two collagens showed significant differences (P<0.05), with a greater percentage obtained in UCP 9261.23%) rather than UCB (196.75%). Both UCP and UCB were classified as type I collagen owing to the existence of two alpha chains under SDS-polyacrylamide gel electrophoresis. Under infrared and ultraviolet-visible parameters, the triple helical structure of collagens prepared using pepsin from porcine and bovine was preserved, and it was comparable to previous findings in fish collagen literature. All samples showed a different thermostability value, the higher one observed in the UCP (43.63°C) compared to the UCB sample (35.25°C), and their variations of thermostability were in agreement with the hydroxyproline content of UCP (83.57 mg/g) and UCB (81.47 mg/g). The unicorn fish (*N. reticulatus*) skin may be used as a good source of collagen, mainly utilized for industrial perspective.**

***Keywords*— *N. reticulatus;* skin by-product; protease-aided process; characterizations.**

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I. INTRODUCTION

The unicorn fish, scientifically known as *Naso reticulatus*, belongs to the Acanthuridae family and is commonly referred to as a surgeonfish [1]. In the world's tropical and subtropical coral reefs, approximately 20 distinct species of *Naso* fish can be encountered [2]. The initial discovery of *Naso reticulatus* occurred in Taiwan and Indonesia [1]. As noted by Ringo in 2020, this unicorn fish is edible and is even a traditional dish among the Filipino community, known as baked unicorn fish. Unicorn fish can grow to a maximum length of approximately 49-57 cm, weighing around 2.3 kg. This size makes this fish suitable for frozen food processing, including products like fish balls and fillets. It is essential to mention that only fish meat is typically utilized, with other parts like skin, scales, fins, and bones going to waste. Consequently, by-products such as the bones and skin of the unicorn fish can be repurposed as valuable commodities like collagen and gelatin [3].

Collagen is a fibril protein obtained in the cells of multicellular organisms. It is a crucial structural component, contributing to the strength of skin, ligaments, tendons, and other organs. The typical collagen structure is maintained by a repeating sequence of amino acids (glycine, proline, and hydroxyproline) [4]. To date, around 29 different collagen types have been identified. Amongst them, type I accounts for more than 90% of the collagen in the human body, primarily sourced from the connective tissues of mammals. Type I

collagen has widespread applications in foods, cosmetics, nutraceuticals, and biomedicine [5].

However, using collagen derived from land animals can raise consumer concerns due to the potential presence of infectious diseases like mad cow disease and avian influenza [6]. Additionally, religious communities such as Islam and Judaism avoid pork and its derivates, while Hinduism abstains from beef and beef-based products. In response to these concerns, fish collagen has attracted considerable attention in recent years. Fish collagen is readily available and less susceptible to transmissible diseases and exhibits favorable biochemical properties after structural modification [7]. Numerous studies have documented the extraction of fish collagen from various fish species. For example, it has been extracted from the skin of lizardfish (*Saurida tumbil*) [8], [9], grouper (*Epinephelus fuscoguttatus*) [10], barracuda (*Sphyraena* sp.) [11] and Java barb fish (*Barbonymus gonionotus*) [12]. These sources have been thoroughly evaluated, and it is worth noting that collagen extracted from fish skin shows feasibility due to its higher yield compared to cartilage, scale, frame, and other body parts. Furthermore, fish skin collagen holds significant potential for developing novel collagen-based commodities, especially from a manufactured perspective.

Extracting collagen from fish skin is pivotal in acquiring this valuable protein. Currently, various techniques are employed, encompassing acid, base, salt, and enzyme treatments [13]. Among these methods, extraction using acid and enzymes is preferred due to its capacity to yield more significant quantities of collagen. Enzymes offer a higher degree of specificity in their reactions and have fewer detrimental effects on collagen proteins. Notably, pepsin-based extraction is recognized for producing more collagen since pepsin can break telopeptide bonds from both sides of the triple helical structure [9]. Moreover, the enzymatic approach generates less waste and reduces processing time. Enzymatic hydrolysis is the primary choice for breaking down fish skin while preserving its nutritional value. This method is particularly favored in the food and pharmaceutical industries as it leaves no residual organic solvents or harmful chemicals in the final product [14].

Transforming unicorn fish skin by-product into collagen is a good strategy, and a little information related to the unicorn fish collagen has been published, especially using two different pepsin sources. Therefore, our research purposed to isolate and compare the physicochemical properties of unicorn fish (*N. reticulatus*) skin collagen prepared using different pepsins derived from bovine and porcine.

II. Materials and Method

A. Sample Preparation

Unicorn fish (N. reticulatus) was bought from a Kota Kinabalu, Malaysia fish market. After shipping, fish were rinsed and skinned manually. The fish skins were then subjected to cutting into suitable sizes (1.5 × 1.5 cm^2) with a sterile scissor and put in a polyethylene bag. The prepared fish skins were subsequently kept in a freezer at –20 °C for experimentation.

B. Extraction of Pepsin Soluble Collagen (PSC)

The extraction of PSC from unicorn fish (*N. reticulatus*) was performed using the approach described by Jaziri et al. [9] with slight adjustments. The prepared fish skins were allowed to be immersed in an alkaline solution for six h with continuous stirring. The pre-treated fish skin was then rinsed with chilled distilled water until pH reached 7.0. Next, the fish skins were dissolved in a butyl alcohol (10%) solution for 24 hours to remove the lipids of the skin samples. The pre-treated skins were then washed three times with a clod distilled water. After pre-treatment, the unicorn fish skins were extracted in acidic condition using an acetic acid (0.5 M) solution with pepsin enzymes (*i.e.*, bovine and porcine). The extraction process was carried out for 48 hours with continuous stirring. The treated samples were subsequently filtered and precipitated in sodium chloride solution with the addition of Tris-HCl. The sediments were then subjected to centrifuging at high speed for 15 min. The pellet was resuspended with an acid solution and dialyzed for 72 h. After dialysis, the dialysates were placed into small polyethylene containers and then lyophilized using a freeze-dryer. The dried collagen extracted with pepsin from porcine was recognized as UCP, while that extracted from bovine was known as UCB.

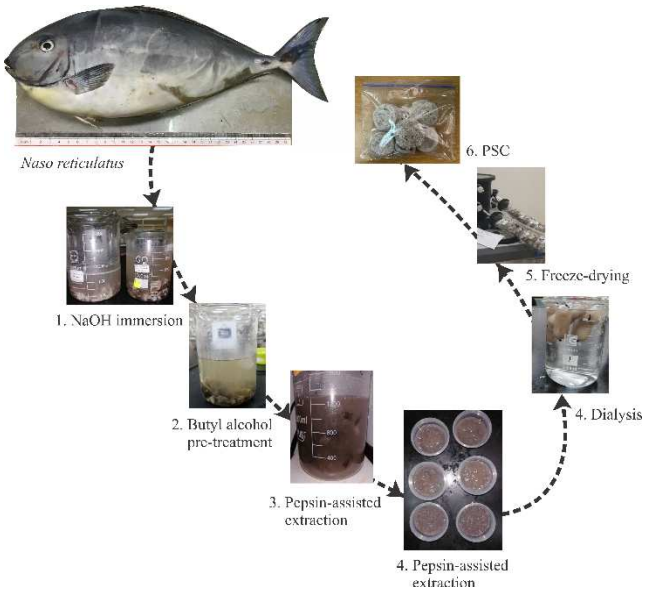

Fig. 1 The extraction process of PSC from unicorn fish (*N. reticulatus*) skin.

C. Swelling and Yield Determination

The swelling was determined in percent according to the method described by previous work [15]. After dissolving in the alkaline solution during pre-treatment, the fish skin was allowed to stand for approximately 15 min. Subsequently, the fish skin was weighed, and the data was recorded. In terms of collagen yield, we applied the formulation reported by Oslan et al. [16]

D. Hydroxyproline Content

The hydroxyproline of UCP and UCB was referred to in Bergman and Loxley's procedure [17]. The collagen was allowed to hydrolysis in an HCl solution at 110°C overnight. Then, the hydrolysate was adjusted at a pH of 7.0, and subsequently, 2 mL of the sample was transferred into a test tube, and added 4 mL of isopropanol. After that, around 2 mL

of freshly prepared oxidant solution was pipetted into the tube and allowed to stand for 5 min. The prepared Ehrlich's reagent was then mixed and gently pipetted. The mixed solution was incubated at 60°C for 25 min and then cooled at room temperature. The absorbance of the sample was texted using a spectrophotometer (558 nm).

E. Color Attributes Determination

The color attributes of all PSC samples extracted from unicorn fish skins (*N. reticulatus*) were measured following the approach reported from a previous study [18], employing a colorimeter tool to assess the color parameter values. The color parameters considered in our present work included *L**, *a**, and *b**.

F. Protein Profile Determination

Both UCP and UCB were carried out to SDS-polyacrylamide gel electrophoresis to obtain the molecular weight (MW) and protein profile. This analysis was carried out using a Mini PROTEAN electrophoresis apparatus. The UCP, UCB, and standard collagen (2.5 mg) were dissolved in sodium dodecyl sulfate (5%) solution to prepare the sample. The mixed solution was then heated in a water bath for one hour. After heating, the mixture was rotated at 8,500× g for 4 min to eliminate insoluble components. Next, 15 µL supernatants were pipetted into a small tube and mixed with a 15 µL sample buffer. The mixture was reheated for 3 min and then pipetted onto an acrylamide gel containing a 4% stacking gel and a 7.5% resolving gel. Electrophoresis was set up for around 90 min. After that, the gel was stained in a Coomassie blue solution. The stained gel was then destained using a prepared destaining solution. The results of UCP and UCB were then compared to a standard protein marker. All procedures in this experiment were referred to the established SDS-PAGE methods [19].

G. Ultraviolet-visible (UV-vis) Absorption Analysis

UV-vis spectrophotometer was used to examine the absorption spectra of PSC samples from the skin of unicorn fish (*N. reticulatus*). About five milligrams of lyophilized collagens were dissolved in an acetic solution. The solution was rotated at 8,500× g for 10 min. After centrifugation, the solubilized sample was pipetted into a quartz cell and scanned under wavelengths of 400–200 nm [20].

H. Infrared Spectra Evaluation

The infrared spectra of both UCP and UCB were analyzed using an FTIR spectrometer. Briefly, approximately 20 mg of lyophilized samples were transferred onto the crystal cell of a FTIR instrument. Each spectrum of UCP and UCB was scanned at a 2 cm^{-1} resolution across a wavenumber range from 4000 to 800 cm^{-1}, with an average of 32 scans. After scanning, all spectral data were measured using FTIR software developed by Agilent Microlab [9].

I. Thermal Stability Test

The thermal stability of all collagens from the skin of unicorn fish (*N. reticulatus*) was carried out using a DSC apparatus. The procedure used in this study was adopted from Matmaroh et al. [21]. The UCP and UCB samples were immersed in deionized water for 48 h in a chiller. After incubation, around eight milligrams of samples were precisely weighed, placed in an appropriate pan, and directly sealed. Next, the sealed pan was put into a DSC cell holder and heated, initiating from 25°C to 50°C at 1°C per minute. After that, the data were collected, composed of the maximum transition temperature (T_{max}).

J. Statistical Analysis

The experimentation in this work was done in triplicate, and data were reflected as means with standard deviations. A one-way ANOVA and Duncan's multiple range were applied in SPSS Statistics (IBM Corp., Armonk, New York).

III. Results and Discussion

A. Swelling Rate and Yield of Collagens

Table 1. presents the swelling rate (in %) of UCP and UCB samples. The obtained results exhibited a higher swelling rate observed in UCP (261.23%) rather than that of UCB (196.75%), resulting in significant differences ($P<0.05$) of the collagen extractability, which UCP had greater yield compared to the UCB sample.

TABLE I
YIELD, COLOR ATTRIBUTES, HYDROXYPROLINE, AND UV-VIS SPECTRA OF PSC DERIVED FROM UNICORN FISH (*N. RICULATUS*) SKIN.

Parameters	Pepsin soluble collagen		
	UCP	**UCB**	**CFC**
Swelling (%)	261.23 ± 16.20[a]	196.75 ± 13.50[b]	-
Yield (%)	15.60 ± 6.70[a]	10.40 ± 4.24[b]	-
Hydroxyproline (mg/g)	83.57 ± 0.14[a]	81.47 ± 0.54[b]	-
Collagen content (mg/g)	643.50 ± 1.11[a]	627.48 ± 4.14[b]	-
L*	54.41 ± 0.05[c]	61.65 ± 1.40[b]	88.36 ± 0.01[a]
a*	4.67 ± 0.10[a]	4.76 ± 0.28[a]	-1.90 ± 0.02[b]
b*	7.96 ± 0.17[a]	7.76 ± 0.26[a]	4.77 ± 0.03[b]
UV-vis spectra	230.0 nm	232.9 nm	230.0 nm

UCP: Unicorn fish skin hydrolyzed with pepsin from porcine.
UCB: Unicorn fish skin hydrolyzed with pepsin from bovine.
CFC: Commercial fish collagen.

Cheng et al. [22] stated that the swelling process plays a vital role in collagen extractability because it can potentially disrupt the internal molecular configurations of proteins and stimulate collagen production by breaking non-covalent bonds. When a controlled-temperature solution, such as sodium hydroxide (NaOH), penetrates the skin's structure, it causes the skin to expand to two or three times its original size, resulting in the separation of non-permanent bonds both within and between molecules. NaOH is especially effective as a preliminary treatment for the skin because it induces significant swelling, which enhances collagen extraction by expediting the transfer of mass within the tissue framework, as emphasized in the study by Schmidt et al. [23]. All collagen treatments demonstrated a substantial increase in quantity (more than 250%), nearly five times the weight of the initial unicorn fish skin used. Regarding collagen extractability, our data were comparable to various pepsin-soluble collagens isolated from different fish species, including tilapia

(*Oreochromis niloticus*) (19.61%) [24], bigeye tuna (*Thunnus obesus*) (16.7%) [13], purple-spotted bigeye snapper (*P. tayenus*) (12.44%) [16], and golden pompano (*Trachinotus blochii*) (21.81%) [25].

B. Hydroxyproline Composition

Hydroxyproline serves as the primary constituent of the amino acid responsible for reinforcing the triple-helical arrangement of collagen. Given its nearly exclusive presence within collagen, the hydroxyproline content can be employed as a quantitative test for measuring collagen levels. The case of UCP and UCB, the hydroxyproline concentration (mg/g) was initially assessed. Then the total collagen content (mg/g) was calculated by multiplying the hydroxyproline measurement by a conversion factor of 7.7, by the procedure outlined in Kittiphattanabawon et al. [26]. As reported in Table 1, the hydroxyproline content of all extracted collagens from unicorn fish (N. reticulatus) skin varied, with a significantly higher ($P<0.05$) obtained in the UCP (83.57 mg/g) compared to the UCB (81.47 mg/g).

Our data were by other fish collagens, including miiuy croaker (*Miichthys miiuy*) (85 mg/g) [27], and bigeye tuna (*Thunnus obesus*) (82–87 mg/g) [13]. In addition, the collagen composition of UCP and UCB showed 643.50 mg/g and 627.28 mg/g, respectively. The Hyp (mg/g) and collagen (mg/g) levels observed in this study may be influenced by a range of factors, including the species, tissue formation, and components of the fish, in addition to the techniques used for extraction [8].

C. Color Parameters

Table 1. shows the color parameters of UCP and UCB compared to standard collagen. The obtained results were significant differences ($P<0.05$) on all parameters (L^*, a^*, and b^*) of collagens. UCB ($L^* = 61.65$) was lighter than UCP ($L^* = 54.41$), but all pepsin-soluble collagens were less light than the standard collagen. Our results were also comparable to those of various fish skin collagens, such as lizardfish (*S. tumbil*) ($L^* = 72.76$) [8] and seabass (*Lates calcalifer*) ($L^* = 44.76$) [28]. On the other hand, when compared snakehead (*Channa argus*) skin collagen pre-treated using an H2O2 solution ($L^* = 89.49$) [14], all mentioned collagens had lower L* value, suggesting that the addition of H2O2 solution during pre-treated treatment could enhance the lightness of collagen product. Sadowska et al. [29]stated a collagen product with a high lightness value is highly preferable for product development due to minimizing interference with the original product. Regarding the a^* and b^* values, all extracted collagens shared almost identically. These findings were also similar to the references above.

D. UV-vis Spectral Profile

Data of UV-vis absorption spectra from the UCP and UCB are presented in Table 1. All collagens had almost identical prominent peak spectra, representing around 230 nm. This result was stated by Chen et al. [30] who reported that fish collagen generally showed a maximum peak between 210 nm and 240 nm. Also, our present report aligned with some fish collagens, including puffer fish (*Lagocephalus inermis*) [31], and red drum (*Sciaenops ocellatus*) [32]. The spectral pattern found in the UCP, UPC, and standard collagen was related to functional groups of collagen structures consisting of amides, carboxyl, and carbonyl. This assessment indicates that polypeptide structures of PSC derived from the unicorn fish (*N. reticulatus*) skin were maintained.

E. Protein Pattern of UCP and UCB

The protein pattern of UCP and UCB was assessed through an SDS-PAGE analysis. As figured in Fig. 2, the electrophoretic patterns of all collagen samples were similar, representing two alpha chains, a β-chain, and a γ-chain, and each chain exhibited different molecular weights (MW). Benjakul et al. (2010) revealed that collagen with owning α1 and α2 in the electrophoretic gel was considered a type I. By this definition, the UCP and UCP were classified as a type I. This finding was also recognized in previous experiments on fish skin collagens, including lizardfish (*S. tumbil*) [8], bigeye tuna (*T. obesus*) [13], and purple-spotted bigeye (*P. tayenus*) [16]. Furthermore, the β- and γ-chains of all samples were comparable to numerous literatures of fish collagens [9], [33].

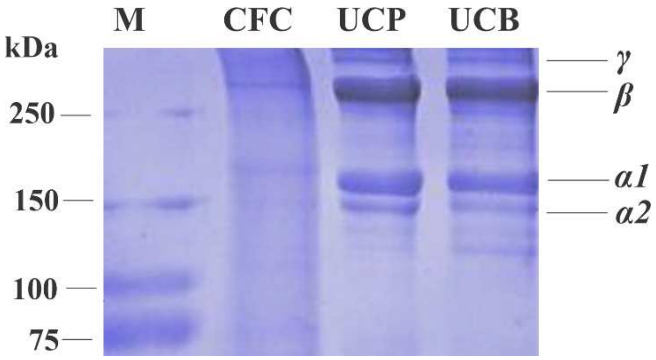

Fig. 2 Electrophoretic patterns of the pepsin soluble collagen from unicorn fish (*N. reticulatus*) skin. UCP: Unicorn fish skin hydrolyzed with pepsin from porcine; UCB: Unicorn fish skin hydrolyzed with pepsin from bovine; CFC: Commercial fish collagen.

F. FTIR Spectra Analysis

Infrared (IR) of collagens isolated from the unicorn fish (*N. reticulatus*) skin is depicted in Fig. 3, and their detailed assignments are presented in Table 2. This analysis can be used to examine the triple helical structure of fish collagen, particularly at amide I, amide II, and amide III. As described by Benjakul et al. [34], using the following formula of $\Delta\nu(\nu_I - \nu_{II})$ where the difference in wavenumber (cm^{-1}) between amide I and amide II is below 100 cm^{-1}, indicating the triple helical structure of collagen is preserved. Our data showed that all samples had $\Delta\nu$ values less than 100 cm^{-1}, reflecting the triple helical structures of all collagens (UCP and UCB) did not break down during the treatment process. Another approach using the procedure from Doyle et al. [35], proposed the ratio of Amide III to the 1450 cm^{-1} band (AIII/A1450), and the results showed that the triple helical structures of UCP and UCB were still stable, as indicated by the absorption ratio values (~1.0). These findings agreed with some studies on fish skin collagens, including purple-spotted bigeye snapper (*P. tayenus*) [16], lizardfish (*S. tumbil*) [9], and sturgeon fish (*Huso huso*) [36].

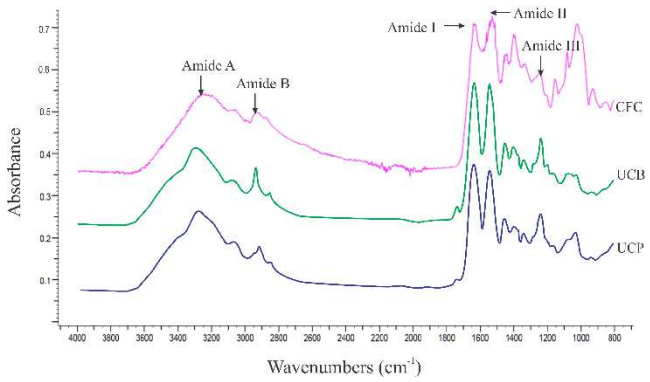

Fig. 3 FTIR spectra of the pepsin soluble collagen from unicorn fish (*N. reticulatus*) skin. UCP: Unicorn fish skin hydrolyzed with pepsin from porcine; UCB: Unicorn fish skin hydrolyzed with pepsin from bovine; CFC: Commercial fish collagen.

TABLE II
THE PEAK AREA AND DESCRIPTION OF PSC DERIVED FROM UNICORN FISH (*N. RETICULATUS*) SKIN

Peak	Type of collagen			Peak assignment
	UCP	UCB	CFC	
Amide A	3280.14 cm^{-1}	3285.73 cm^{-1}	3259.64 cm^{-1}	Stretching of N-H with hydrogen chain
Amide B	2926.03 cm^{-1}	2922.31 cm^{-1}	2931.62 cm^{-1}	Asymmetric stretching of CH_2
Amide I	1628.89 cm^{-1}	1628.89 cm^{-1}	1636.34 cm^{-1}	Stretching of C=O / hydrogen chain with COO-
Amide II	1541.29 cm^{-1}	1541.29 cm^{-1}	1522.66 cm^{-1}	N-H bond with stretching of C-N
Amide III	1235.64 cm^{-1}	1235.64 cm^{-1}	1241.23 cm^{-1}	N-H bond with C-H dan C-O stretching

UCP: Unicorn fish skin hydrolyzed with pepsin from porcine.
UCB: Unicorn fish skin hydrolyzed with pepsin from bovine.
CFC: Commercial fish collagen.

G. Thermostability Assessment

Fig. 4. presents the thermograms of extracted collagens from the unicorn fish (*N. reticulatus*) skin. The thermostability of UCP sample (T_{max} = 43.63°C) was greater than that of UCB (T_{max} = 35.25°C). The reason might be due to the content of hydroxyproline in the fish collagens, which play an essential role in the formation of pyrrolidine rings partially stabilized by hydrogen bonding through the hydroxyl group of hydroxyproline, as suggested by Benjakul et al. [34]. Furthermore, hydroxyproline has the capacity to enhance the stability of the triple helical structure through hydrogen bonding within the coiled-coil alpha chains, as elucidated by Bae et al. [37]. The mentioned data (Table 1) showed a high content of hydroxyproline. These findings also agreed with the experiments on fish collagens, including lizardfish (*S. tumbil*) (T_{max} = 40.24°C) [8], seabass (*Lates calcarifer*) skin (T_{max} = 39.32°C) [38], and loach (*Misgurnus anguillicaudatus*) skin (T_{max} = 36.03°C) [39].

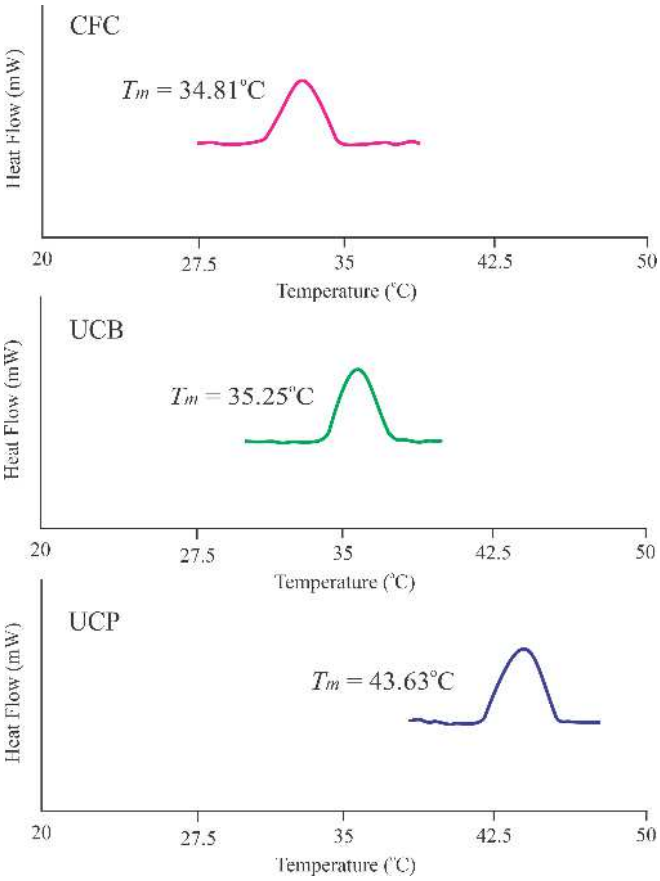

Fig. 4 Thermogram graph of the pepsin soluble collagen from unicorn fish (*N. reticulatus*) skin. UCP: Unicorn fish skin hydrolyzed with pepsin from porcine; UCB: Unicorn fish skin hydrolyzed with pepsin from bovine; CFC: Commercial fish collagen.

IV. CONCLUSION

The pepsin-soluble collagens derived from the skin of unicorn fish (*N. reticulatus*) have been isolated using two pepsin sources. UCP sample had a significantly higher yield (15.60%) than UCB (10.40%), which might be due to the swelling rate during the pre-treatment process. Both UCP and UCB were thought of as type I owing to the two alpha chains available after SDS-PAGE analysis, and the triple helical structures of those collagens were preserved, indicating no structural change during the extraction process. These analyses were proved using FTIR and UV-visible experiments. Interestingly, all collagens showed high thermal ability, with a higher observed in UCP compared to the UCB sample. This result could be influenced by the hydroxyproline content that plays a vital role in stabilizing the triple helical structure of fish collagen. Overall, the UCP sample may be electable in this study because it also has a high collagen extractability and thermostability.

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