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Research article

Yield and Properties of Collagen from Nile Tilapia (*Oreochromis niloticus*) Scales: Effects of Ultrasonic Pretreatment on Pepsin-Aided Extraction

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Abstract

The yield and characteristics of collagen from Nile tilapia scale pretreated with ultrasound at 40 kHz for 2 h before pepsin-aided extraction for 12-72 h (US collagen) were investigated compared to collagen from the scales without ultrasonic pretreatment (non-US collagen). Both collagens' yields increased with prolonged extraction time (P<0.05). Nevertheless, the yield of ultrasound-pretreated collagen (2.20-4.31%) was approximately 2 times greater than that of collagen without ultrasonic pretreatment (1.06-2.03%) (P<0.05). The amino acid compositions of both collagens were comparable, consisting mainly of glycine, alanine, proline, and hydroxyproline (329-330, 115,119, 114-116, 85 residues per 1000 residues, respectively), and both were classified as type I collagen. Moreover, the thermal transition temperatures (39.38-39.43°C) and enthalpy (0.55 J/g) were comparable between both collagens (P>0.05). Analysis of the FTIR spectra indicated that the ultrasonication pretreatment of the scale before the collagen did not alter the triple-helical structure of the collagen. Therefore, pretreatment of the Nile tilapia scales with ultrasonication before the pepsin-aided process could increase yield without significantly affecting the characteristics and triple-helical structure of the collagen.

Keywords: collagen; ultrasound; extraction; fish scale; yield

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1. Introduction

Nile tilapia is a popular freshwater fish in Thailand due to its white meat and affordable price (Kittiphattanabawon et al., 2019). In 2023, Thailand produced approximately 256,484 tons of Nile tilapia, of which 66% was processed as tilapia fillets (Boon-Ek, 2025). During the processing, approximately 2% of the tilapia fillets is the scales, which are discarded as waste with low market value (Kittiphattanabawon et al., 2019). Due to the scales having collagen as a major component that has a low undesirable fish odor and flavor (Huang et al., 2016), the scales represent a potential source for collagen extraction. However, the fish scale structure has a two-layer structure, an outer layer and inner layer. The outer layer is composed of hydroxyapatite ($Ca_5(PO_4)_3OH$) crystals and randomly oriented fibers, whereas the inner layer consists of a plywood structure with collagen fibers (Feng et al., 2020). This hierarchical arrangement makes the scales resistant to extraction solvent and pepsin penetration into the scale matrix during extraction. Consequently, collagen extraction from the scale matrix is rendered ineffective, diminishing collagen yield. According to previous research, using a conventional method, collagen extraction from Nile tilapia scales demonstrated a low yield (1.48%) (Kittiphattanabawon et al., 2019). Although pepsin was used for hydrolysis, the crosslinked component at the telopeptide region of collagen should be readily solubilized and easily extracted, the yield was still low due to the complicated structure of the scales. High-intensity ultrasound is an emerging potential processing that can enhance mass transfer via the cavitation effect, which is a phenomenon where bubbles form and collapse in a liquid generated by compression and rarefaction during ultrasonication (Baite et al., 2021; Mondal et al., 2021). The cavitation effect results in a porous scale matrix, enhancing solvent and pepsin penetration and scale matrix swelling and softening, making collagen more easily solubilized from the matrix and increasing mass transfer. Recent applications of ultrasound technology have demonstrated success in the extraction of collagen from various fish species, including seabass scales (Bavisetty et al., 2024), golden carp skin (Ali et al., 2018), sharpnose stingray skin (Shaik et al., 2021), and bighead carp scales (Tu et al., 2015). Nonetheless, the literature reveals a paucity of studies focusing on collagen extraction from Nile tilapia scales utilizing a synergistic approach combining ultrasonic assistance with a pepsin-aided process. Hence, this research aimed to extract collagen from Nile tilapia scales pretreated with ultrasound before the pepsin-aided process and to evaluate both yield and the characteristics of the collagen obtained compared to those obtained without ultrasonic pretreatment.

2. Materials and Methods

2.1 Chemicals

All the chemicals supplied were of analytical quality, sourced from Bio-Rad Laboratories (Hercules, CA, USA), Sigma Chemical Co. (St. Louis, MO, USA), and Merck KGaA, (Darmstadt, Germany). These chemicals were used for protein pattern analysis, collagen extraction, and standard protein markers, respectively.

2.2 Preparation of tilapia scale

Tilapia (*Oreochromis niloticus*) scales, each weighing 0.4 to 0.6 kg, were procured from a local market in Phatthalung, Thailand. The removal of scales from the skin was conducted utilizing a knife, followed by a thorough washing process with cold water, maintaining

temperatures between 2-10°C. Subsequent to processing, the scales were allocated into polyethylene bags at an approximate weight of 100 g per bag and were stored at -20°C for a duration not exceeding 3 months. The moisture content of the scales determined by the AOAC method (AOAC, 2023) was 76.21%. Before extraction, the packed frozen scales were defrosted under continuous flow of tap water until their core temperature of the scales reached 8-10°C.

2.3 Pretreatment of Nile tilapia scales

2.3.1 Removing of non-collagenous proteins and mineral

The prepared scales were subjected to a non-collagenous protein and a mineral removal process was conducted at 2-4°C (Kittiphattanabawon et al., 2019). The scales were treated with NaOH and EDTA with continuous stirring at 200 rpm to remove non-collagenous proteins and minerals, respectively. The details of both pretreatment processes are shown in Table 1.

Table 1. Conditions of non-collagenous protein removal and demineralization processes

Pretreatment process	Pretreatment Solutions	Solid-to-Solution Ratio (w/v)	Pretreatment Time (min)	Washing Process after Pretreatment
Non-collagenous protein removal	0.1 M NaOH	1:10	2 h, repeating the process three times	Rinsed with cold water until the pH of the wash water stabilized at neutral or slightly basic levels.
Demineralization	0.5 M EDTA-2Na (pH 7.4)	1:10	8 h, repeating the process three times	Rinsed with cold water for 30 min, repeating the process twice.

2.3.2 Ultrasonic pretreatment

The demineralized scales were immersed in 0.5 M acetic acid at a solid-to-solvent ratio of 1:15 (w/v) and placed in an ultrasonic bath (model S06H, Zealway, Delaware, USA) for sonication at 40 kHz for 2 h. To maintain the temperature at 4°C, ice was added in the ultrasonic bath throughout ultrasonication. Then, the mixture was subjected to collagen extraction by a pepsin-aided process.

2.4 Extraction of collagen from Nile tilapia scales by pepsin-aided process

Collagen was isolated from tilapia scales using the protocol established by Kittiphattanabawon et al. (2019). The procedures were conducted at 2-4°C. Each mixture of pretreated scales, both with and without ultrasonic treatment, underwent collagen extraction using a pepsin-aided method. A porcine pepsin (80 units per g of pretreated scale) was integrated into each mixture. The proteolytic activity was evaluated using the

technique described by Nalinanon et al. (2008). The mixture was continuously stirred for periods of 12, 24, 36, 48, 60, and 72 h, followed by filtration through a double-layered cheesecloth. In order to precipitate the collagen from the filtrate, NaCl was gradually introduced until the concentration reached 2.6 M. Subsequently, tris(hydroxymethyl)aminomethane was incorporated into the solution to reach a final concentration of 0.05 M, and pH was adjusted to 7.5 by adding either 0.1 M HCl or 0.1 M NaOH. Each mixture was then centrifuged at 20,000 ×g and 4°C for 60 min to collect the precipitate. The resulting pellet was resuspended in a minimal volume of 0.5 M acetic acid and subjected to dialysis in 25 volumes of 0.1 M acetic acid for 12 h, followed by 48 h in distilled water. Finally, the dialysate was subjected to lyophilization. The resulting collagen from ultrasound-treated and untreated samples was labeled as "US collagen" and "non-US collagen," respectively.

2.5 Yield of collagen from tilapia scales

The collagen yield was determined by measuring the hydroxyproline content in the material using a spectrophotometric method (Bergman & Loxley, 1963) and was calculated using the following equation.

$$\text{Yield (\%)} = \frac{\text{Hyp content in collagen } \left(\frac{\text{mg}}{\text{g}}\text{collagen}\right) \text{x collagen obtained (g)}}{\text{Hyp content in scale } \left(\frac{\text{mg}}{\text{g}}\text{scale}\right) \text{x scale used for extraction (g)}} \times 100$$

2.6 Characterization of collagen from the Nile tilapia scales

2.6.1 Amino acid composition analysis

The amino acid composition of both US and non-US collagen samples was determined using an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan) after complete hydrolysis. The hydrolysis procedure followed the method described by Kittiphattanabawon et al. (2019).

2.6.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the protocol established by Laemmli (1970). The sample solubilized in 5% (w/v) SDS was heated in boiling water for 1 min and centrifuged to eliminate insoluble particles. It was then mixed with a sample buffer in a 1:1 (v/v) ratio. The prepared samples, along with the standard protein marker and type I collagen derived from calf skin were loaded onto a polyacrylamide gel with a 7.5% resolving gel and a 4% stacking gel. Electrophoresis was carried out at a constant current of 20 mA per gel. After completion, the gels were treated with a fixing solution for 30 min, stained with a staining solution for 1 h, and then subjected to a destaining process until the background became transparent.

2.6.3 Differential scanning calorimetry (DSC)

DSC was conducted using a differential scanning calorimeter (model DSC 7, Perkin Elmer, Norwalk, CT, USA). Indium thermogram was used for calibration. The sample preparation

and condition for analysis were detailed in our previously studied (Kittiphattanabawon et al., 2019). The peak of the DSC thermogram was used to estimate T_{max}, the maximum transition temperature, whereas the total denaturation enthalpy (ΔH) was obtained by integrating the area under the curve.

2.6.4 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of collagens were obtained with a Bruker model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) and analyzed with the OPUS 3.0 data collection software (Bruker, Ettlingen, Germany). The conditions for analysis are detailed in Kittiphattanabawon et al. (2019).

2.7 Statistical analysis

The experiments were conducted in triplicate, employing three distinct batches of samples. Differences between means were assessed utilizing a T-test at a 95% significance level (Steel & Torrie, 1980). The data are presented as means with standard deviation.

3. Results and Discussion

3.1 Yield

Figure 1 depicts the collagen yield from Nile tilapia scales, with comparisons made between scales pretreated with ultrasound (US collagen) and those not treated with ultrasound (non-US collagen) before pepsin-aided extraction, based on extraction time. After the pretreatment, the yield of the US collagen rose significantly with the extension of extraction time to 48 h ($P<0.05$), while the non-US collagen showed a modest increased in yield after 12 h of extraction. Ali et al. (2018) also found a similar result for golden carp skin collagen. The yield of collagen obtained using ultrasonication treatment (2.20-4.31%) was nearly double that of collagen without ultrasonic treatment (1.06-2.03%) ($P<0.05$). This finding aligns with the results for collagen extracted from seabass scales treated with ultrasonication, where the yield was 2.07 times greater than the conventional method (Bavisetty et al., 2024). The ultrasonic treatment enhances the penetration of solvent and pepsin into the cell membranes of the scales via the cavitation effect, leading to improved mass transfer and greater pepsin hydrolysis at the telopeptide region. The result aligns with findings from collagen extraction using an ultrasound-aided method on sharpnose stingray and golden carp scales (Ali et al., 2017; Shaik et al., 2021). The mechanisms involve the shear forces of microstreaming generated during bubble motion or abrupt changes in temperature and pressure resulting from bubble collapse caused by the cavitation effect as ultrasound moves through a liquid medium (Mondal et al., 2021). Consequently, the collagen is released more from the matrix of the scale during acid-pepsin extraction, as noticed by the higher yield found in US collagen compared with the yield of non-US collagen. The fish scales generally possess a structure bound with hydroxyapatite and a lot of crosslinked regions (Chen et al., 2022), resulting in low yield obtained in the non-US collagen. The results showed that the pretreatment of the scales with ultrasonication before collagen extraction by the pepsin-aided process could increase yield.

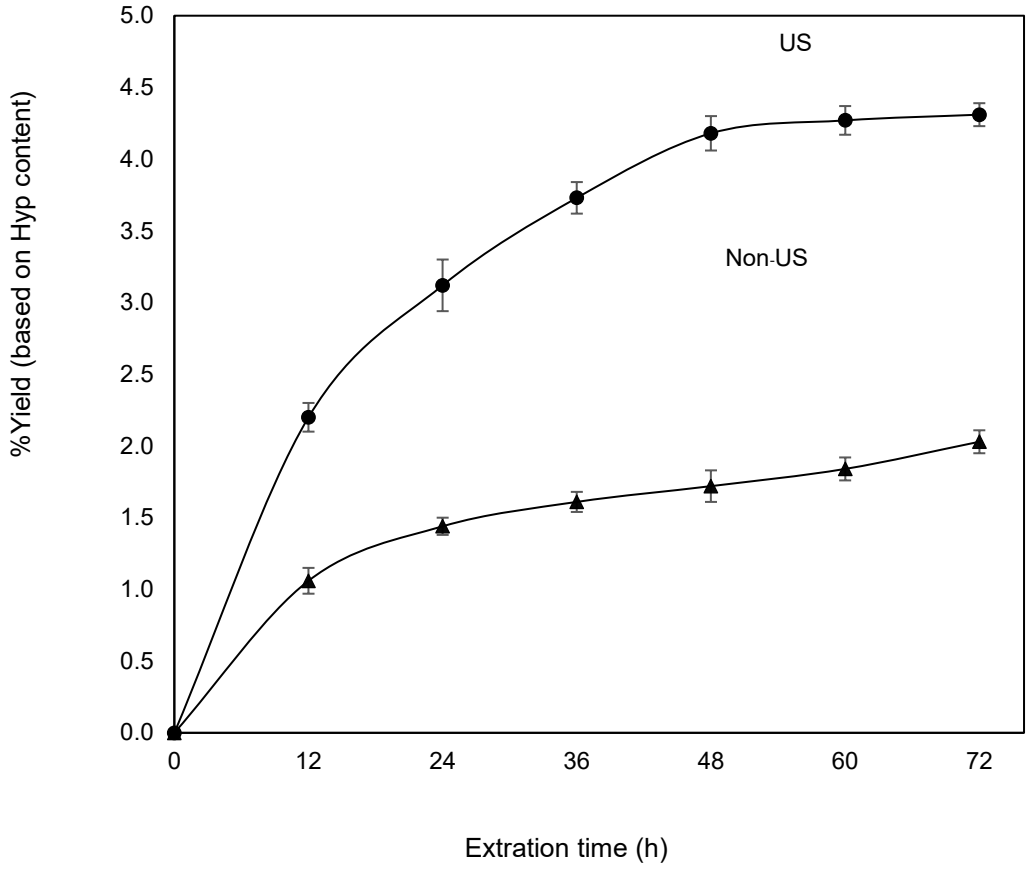

Figure 1. Yield of Nile tilapia collagen obtained from the scales pretreated with (US collagen) and without ultrasound (non-US collagen) before pepsin-aided extraction for different times

3.2 Amino acid compositions

The amino acid composition of both Nile tilapia collagen with and without ultrasonic pretreatment before pepsin-aided extraction for 72 h was similar (Table 2). Both collagens contained glycine of 329-330 residues/1000 residues, which was about one-third of the total amino acid residues, followed by alanine, proline, and hydroxyproline (115-119, 114-116, 85 residues/1000 residues, respectively) as the predominant amino acids. Additionally, they contained minor quantities of cysteine, histidine, hydroxylysine, methionine, and tyrosine (1-10 residues/1000 residues). The amino acid composition corresponded with type I collagen found in other fish scales (Ali et al., 2017; Bavisetty et al., 2024; Chen et al., 2016; Chinh et al., 2019; Li et al., 2018). In non-US and US collagens, the imino acid content was 201 and 199 residues/1000 residues, respectively. These values were higher and lower than collagen from cold water fish such as carp and bighead carp scales (Duan et al., 2009; Liu et al., 2012) and mammals (Li et al., 2013), respectively. The imino acid content is directly connected to their environmental habitat, influencing their

Table 2. Amino acid compositions of Nile tilapia scale collagen with (US collagen) and without (non-US collagen) ultrasonic pretreatment before pepsin-aided extraction for 72 h (residues/1000 residues)

Amino acid	Non-US collagen	US collagen
Glycine	330	329
Alanine	119	115
Proline	116	114
Hydroxyproline	85	85
Glutamic acid/Glutamine	68	68
Arginine	50	48
Aspartic acid/Asparagine	43	44
Serine	39	40
Threonine	26	27
Lysine	25	24
Leucine	24	25
Valine	19	20
Phenylalanine	13	14
Isoleucine	11	13
Methionine	10	10
Hydroxylysine	8	8
Histidine	7	8
Tyrosine	5	5
Cysteine	1	1
Total	1000	1000
Imino acid (Pro+Hyp)	201	199

thermal transition temperature (Fujii et al., 2022). The ultrasonic treatment did not significantly affect the amino acid compositions, as evidenced by the lack of difference between the compositions of both collagens, indicating the ultrasonic treatment with power of 40 kHz for 2 h before pepsin-aided collagen extraction was not too harsh. Ali et al. (2018) also found that the ultrasonic treatment, when applied under the optimum condition, did not alter the amino acid composition of the collagen obtained.

3.3 SDS-PAGE patterns

SDS-PAGE patterns of Nile tilapia scale collagen treated with and without ultrasonic pretreatment before pepsin-aided extraction for 72 h are presented in Figure 2. Both US

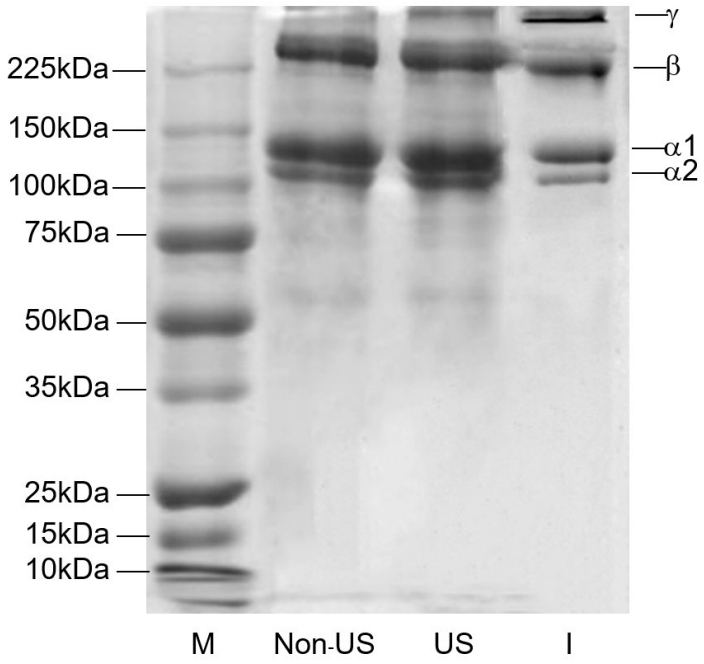

Figure 2. SDS-PAGE of Nile tilapia scale collagen with (US collagen) and without (non-US collagen) ultrasonic pretreatment before pepsin-aided extraction for 72 h compared with that of type I collagen from calf skin (I)

and non-US collagens presented α- and β-chains as the major components and had the α1/α2 chains ratio close to 2, indicated that both collagens were type I. The collagens from the scale of other fish species were also type I collagen (Liu et al., 2015; Moniruzzaman et al., 2019; Shalaby et al., 2020). The collagen obtained from the scales pretreated with ultrasound (US-collagen) retained intact α1- and α-2 chains without any noticeable degradation. It suggested that the ultrasonic treatment under the condition used could increase yield without significantly destroying the collagen structure. However, using an ultrasound-assisted process under harsh conditions that involved high intensity and/or long period for collagen extraction resulted in the degradation of α1- and α-2 chains (Kim et al., 2012).

3.4 Thermal transition temperature and enthalpy

The thermogram of non-US and US collagens from tilapia scales as determined by differential scanning calorimetry is shown in Figure 3. No significant differences in thermal transition temperature (T_{max}) and enthalpy (ΔH) between both collagens were observed (P>0.05). Ali et al. (2018) reported that the ultrasound-assisted process for extracting collagen from the golden carp skin had no impact on T_{max} and ΔH of acid-soluble collagen but increased both T_{max} and ΔH of pepsin-soluble collagen. The T_{max} of tilapia scales was comparable to the scales of golden carp (37.67-37.83°C) (Ali et al., 2017) and seabass (38.17-39.32°C) (Chuaychan et al., 2015). As similar in T_{max} and ΔH, it suggested that the native collagen structure was not significantly damaged by the ultrasonic treatment.

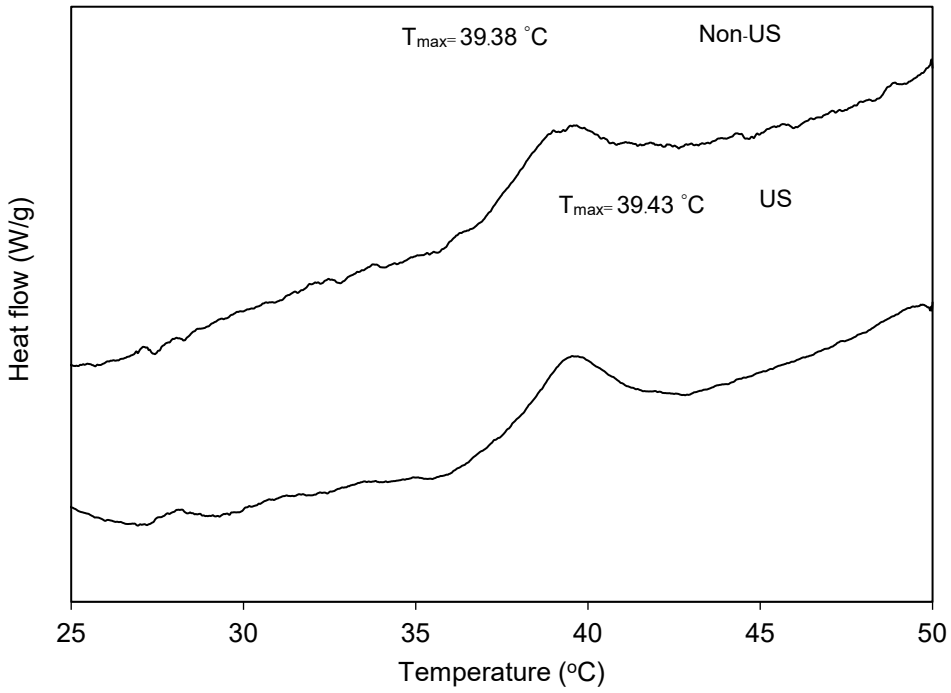

Figure 3. DSC thermogram of Nile tilapia scale collagen with (US collagen) and without (non-US collagen) ultrasonic pretreatment before pepsin-aided extraction for 72 h

3.5 FTIR spectra

FTIR spectra of tilapia scale collagen with and without ultrasonic pretreatment before pepsin-aided extraction for 72 h are shown in Figure 4. Predominant peaks in both collagens were identified in the amide regions, specifically amide A (3304-3307 cm^{-1}), amide B (2925-2926 cm^{-1}), amide I (1637-1639 cm^{-1}), amide II (1548-1549 cm^{-1}), and amide III (1239 cm^{-1}). FTIR spectra of fish scale collagen typically feature peaks corresponding to amide A, B, I, II, and III with approximately similar wave numbers for each peak (Ali et al., 2017; Bavisetty et al., 2024; Chen et al., 2016; Kittiphattanabawon et al., 2019). The amides A, B, I, II, and III are associated with N–H stretching or hydrogen bonding, CH_2 stretching, hydrogen bonding with COO^- or C=O stretching, N–H bending with C–N stretching, and C–N stretching or N–H deformation, respectively (Abe & Krimm, 1972; Doyle et al., 1975; Krimm & Bandekar, 1986; Payne & Veis, 1988; Muyonga et al., 2004; Chen et al., 2016). No significant difference was observed in the FTIR spectra between the non-ultrasound-treated (non-US) and ultrasound-treated (US) collagens (P>0.05). A crucial parameter for assessing the impact of ultrasonic treatment on collagen structure is the amide III/1454 cm^{-1} ratio being 1, indicating the presence of a triple helical structure in collagen (Plepis et al., 1996). The ratios of non-US and US collagens were 1.000 and 0.999, respectively. Ali et al. (2018) also found a ratio of 1 in golden carp skin collagen obtained from ultrasonication-assisted extraction. From the findings, it can be concluded that ultrasonic treatment does not alter the inherent collagen structure.

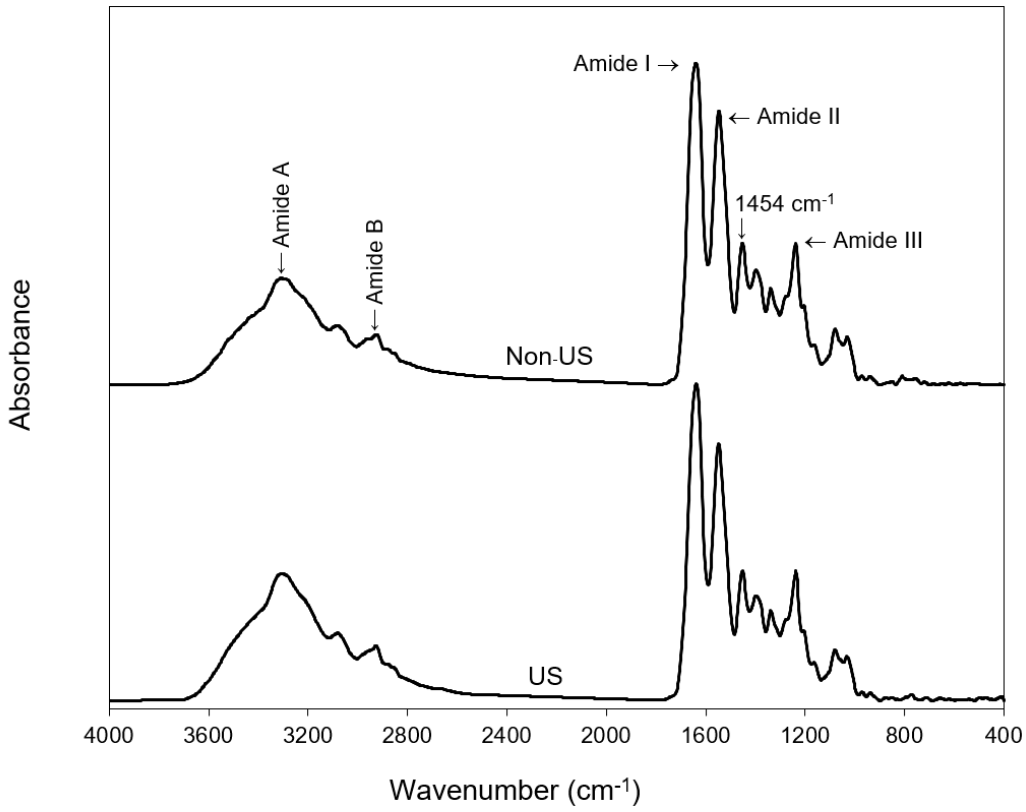

Figure 4. FTIR spectra of Nile tilapia scale collagen with (US collagen) and without (non-US collagen) ultrasonic pretreatment before pepsin-aided extraction for 72 h

4. Conclusions

The pretreatment of Nile tilapia scale with ultrasonication for 2 h before collagen extraction by pepsin-aided process (US collagen) could increase collagen yield by approximately 2 times greater than that obtained from conventional extraction (non-US collagen), with no significant effect on the amino acid composition and triple helical structure of the collagen. Although the ultrasonication can improve the extraction yield, the recovery yield is still low and necessitates further refinement in subsequent studies.

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6. Authors' Contributions

Phanat Kittiphattanabawon: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Hideki Kishimura: Resources. Soottawat Benjakul: Supervision, Resources. Wonnop Visessanguan: Supervision, Writing – review & editing.

7. Conflicts of Interest

No conflict of interest is declared.

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