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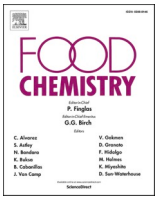
Food Chemistry 393 (2022) 133426

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Resource efficient collagen extraction from common starfish with the aid of high shear mechanical homogenization and ultrasound

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ARTICLE INFO

Keywords:
Collagen
Starfish
Pretreatment
Homogenization
Ultrasound

ABSTRACT

Processes currently used for collagen extraction are complicated requiring a great deal of time and chemicals. Here, high shear mechanical homogenization (HSMH) and ultrasound (US) were integrated in the pretreatment step of collagen extraction from common starfish to reduce chemical use and time consumption. Effects of the assistant technologies on yield, structural integrity and functionality of collagen were also investigated. HSMH reduced the deproteinization time from 6 h to 5 min and its required amount of alkali 4 times, compared with classic methods. HSMH + US reduced the demineralization time from 24 h to 12 h and improved its efficiency in extraction of minerals. Collagen extraction with HSMH and HSMH + US resulted in similar yield as the classic method and did not affect triple helical structural integrity, polypeptide pattern, thermal stability or fibril-formation capacity of the collagens. Altogether, HSMH and US can effectively improve resource efficiency during collagen extraction without imposing negative effect on collagen quality.

1. Introduction

Collagen is the major structural protein found in the animal body accounting for 25–35% of the total protein (Tun, Naing, & Thaw, 2012). It is found as structural element of skin, bone, cartilage and other body parts in vertebrates. In invertebrates such as starfish, collagen is found mainly in the body walls and cuticles (Silvipriya et al., 2015). Collagen is a large molecule having a triple helical structure with each polypeptide chain mostly containing repeating units of glycine, proline and hydroxyproline (Ricard-Blum, Ruggiero, & Rest, 2005). In common starfish (*Asterias rubens*) collagen is found as dense meshwork surrounding the ossicles in the arm. It has been reported that the structure of collagen from starfish is similar to type I (Tan et al., 2013, Lee et al., 2009). The protein patterns have been found similar to collagen from deep-sea redfish and carp but different from that of calf skin collagen (Tan et al., 2013).

Collagen is extensively utilized in cosmetic, pharmaceutical, biomedical and food industries (Rastian et al., 2018; Avila Rodríguez, Rodríguez Barroso, & Sanchez, 2018). Collagen from bovine or porcine sources are the most commonly used ones commercially. But the collagen from mammalian sources has concerns regarding its environmental sustainability, the transmissible diseases associated with the sources and religious constraints (Jafari et al., 2020). Hence, there is a lot of interest in collagen from alternative resources, and especially in the marine ones because of its wide acceptability. There are large volumes of underutilized marine resources which can be exploited as raw materials for collagen extraction. Starfish is one such underutilized marine resource which is not commercially used as food or for any other purposes and sometimes cause environmental problems. *Asterias* spp. is considered as a large problem by shellfish producers and one of the most devastating invasive species (Agüera, Saurel, Møller, Fitridge & Petersen, 2021). Within this genus, the common starfish (*Asterias rubens*) causes serious biological and environmental problems in northern Europe and creates hassle e.g., for the mussel farming industry. Currently, they are discarded when they get harvested along with the mussels, despite their contents of interesting biomolecules like collagen. Extraction of collagen from starfish has previously been reported for some other species such as crown-of-thorns starfish (*Acanthaster planci*) (Tan et al., 2013), *Asterias amurensis* (Lee et al., 2009) and *Asterias pectinifera* (Qi et al., 2017). Recently, collagen peptides have also been derived from the starfish *Asterias pectinifera* (Han, Won, Yang, & Kim, 2021). However, to the best of our knowledge, there is no study exploring common starfish for collagen extraction.

Currently, the most common processes for extraction of collagen from aquatic resources use acetic acid or a combination of acetic acid and pepsin. Both methods are very complicated, challenging and tedious

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https://doi.org/10.1016/j.foodchem.2022.133426
Received 8 February 2022; Received in revised form 29 May 2022; Accepted 6 June 2022
Available online 9 June 2022

(5–8 days). Further, they require large amount of chemicals and still result in low yield. All these drawbacks have made the collagen extraction from aquatic resources very inefficient, costly and unsustainable (Pal & Suresh, 2016). In case of starfish, 2–3 pretreatment steps are also necessary to remove non-collagenous proteins (NCP), fat and minerals requiring large amount of chemicals, which makes the process even more complicated. Some studies have targeted assistant technologies such as high shear mechanical homogenization (HSMH) and ultrasound (US) for increasing the collagen yield (Ali, Kishimura, & Benjakul, 2018; Tan & Chang, 2018; Petcharat, Benjakul, Karnjanapratum & Nalinanon, 2021). However, all these studies have focused on the main collagen extraction step to improve its yield, and there are, to the best of our knowledge, no study involving the application of these technologies in the pretreatment step of collagen extraction.

The present study was aimed to optimize the pretreatment step used in collagen extraction from common starfish to reduce the time and the amount of chemicals used. Two innovative assistant technologies; HSMH and US were integrated into the pretreatment step to improve the process, and their possible effects on the collagen extraction yield and its structural and functional properties were also thoroughly investigated.

2. Material and method

2.1. Materials

Fresh common starfish (*A. rubens*) were collected from local mussel farmers one hour north of Gothenburg, Sweden. The starfish were covered with ice and transported to the lab. They were then washed with cold water upon arrival in the lab, packed and stored at −80 °C until use.

2.2. Chemicals

All chemicals and reagents were of analytical grade. Pepsin, acetic acid, ethylenediaminetetraacetic (EDTA)-2Na, tris (hydroxymethyl) aminomethane, sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME), glycerol, and bovine serum albumin were purchased from Sigma-Aldrich Corp. (USA). Sodium hydroxide, hydrochloric acid and sodium chloride were provided by Scharlo (Scharlo Co., Spain).

2.3. Collagen extraction

All the processes described below were carried out at 4 °C to avoid the denaturation of collagen.

2.3.1. Optimization of pretreatment by HSMH

For the optimization of deproteinization and demineralization pretreatment steps, homogenization speed and raw material to alkaline ratio were considered.

Starfish (SF) sample (50 g) thawed under running cold water were cut into pieces of 0.5 to 1 cm and were mixed with 0.1 M NaOH solution at 1:20 ratio (w/v) and homogenization were carried out using a Siverson homogenizer (L5M, Silverson, MA, USA) at 4000 and 8000 rpm separately for 2.5 mins. An ice bath was used during the homogenization to keep the temperature of the sample at 4 °C. The mixture was then stirred for 6 h at 4 °C. Samples were taken at 0, 1, 2, 4 and 6 h, centrifuged at 2000 × g for 2 min and protein level in the supernatant was analyzed by Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951) and considered as total amount of removed non-collagenous proteins.

To evaluate the impact of the homogenization speed on demineralization time, the deproteinized starfish samples were mixed with 0.5 M EDTA-2Na solution at 1:15 ratio (w/v) and kept for 48 h at 4 °C under stirring. Samples were taken at 0, 6, 12, 24 and 48 h and subjected to centrifugation at 5000 × g for 5 min. Then, magnesium analysis of the supernatants was carried out using atomic absorption spectroscopy (AAS) 240FS AA (Agilent Technologies, Australia). A control sample without homogenization was also run for comparison.

To find optimum raw material to alkaline solution ratio during the deproteinization step, the starfish samples were separately mixed with alkaline solution at ratios 1:5, 1:10 and 1:20 and homogenized at the optimum speed obtained by the previous trial which involved the highest ratio (1:20) and kept for stirring for 6 h. Alkaline solution change was carried after 3 h. Samples were collected every 30 mins, and protein estimation was carried out as explained above.

2.3.2. Optimization of pretreatment by US

To evaluate the impact of US on the deproteinization and demineralization efficiency and time, US (UIP 1000hdT, Hielscher, Ultrasound Technology, Germany) was applied after homogenizing the mixture of starfish and alkaline solution. Based on pre-trials (data not shown), an US amplitude of 75 % for 10 mins with 10/20 s pulses was selected and applied to the sample. The sample was kept in the ice bath and stirred using a magnetic stirrer (RCT Classic, IKA, Brazil) for maintaining the temperature at 4 °C. Samples were collected every 5 mins for 30 mins inthe deproteinization step. During demineralization, samples were collected at 0, 10, 60, 120, 240 and 720 mins. The effect of US on deproteinization and demineralization was studied by analyzing the protein and mineral content of the samples collected at different time intervals, and data was compared with samples subjected to only homogenization as explained above.

2.3.3. Collagen isolation using the conventional method

Collagen extraction from common starfish by the conventional method was carried out according to the method of Matmaroh, Benjakul, Prodpran, Encarnacion, and Kishimura (2011) with slight modification. Thawed starfish (250 g) were cut into small pieces of 0.5 to 1 cm and soaked in 0.1 N NaOH with SF to alkaline ratio of 1:20 (w/v). The mixture was stirred using an overhead stirrer (RW20, IKA, Brazil) for 6 h at 4 °C with a change of the alkaline solution at 3 h. After the alkaline treatment, the starfish samples were mixed with cold water in a 1:10 ratio (w/v) and the pH was adjusted to 7.4 using 6 N HCl. Then dewatering of the sample was carried out by centrifuging at 5000 × g for 5 mins at 4 °C. Thereafter, the sample was demineralized by mixing with 0.5 M EDTA 2-Na solution with a sample to solution ratio of 1:15 (w/v) and stirred in bottles with stirrers (Bioprocess Control AB, Sweden) for 48 h at 4 °C with a change into fresh EDTA solution at 24 h. The demineralized sample was washed with cold water to remove the residual EDTA. Then, the sample was dewatered by centrifuging at 5000 × g for 5 min.

The demineralized sample was subjected to extraction by mixing with 0.5 M acetic acid (sample to acid ratio of 1:15 (w/v)) and 1% pepsin (w/w) separately, and stirred for 48 h at 4 °C. Then, the sample was centrifuged at 10,000 × g for 20 min at 4 °C using a high-speed refrigerated centrifuge (Thermo Scientific, USA) to remove the undissolved matter. The obtained supernatant was then salted out using NaCl with final concentration reaching to 2.5 M in the presence of 0.05 M tris (hydroxymethyl) aminomethane. The precipitate was centrifuged at 15000 × g for 30 min at 4 °C. The pellet was resuspended in a minimum volume of 0.5 M acetic acid and dialysed using a dialysis membrane (Spectra/Por 6, CA, USA) with molecular weight cut off of 1 kDa, in 20 volumes of 0.1 M acetic acid for 48 h and subsequently in 20 volumes of distilled water for another 24 h. The dialyzed matter was dried using a freeze-dryer (model CoolSafe 55 ScanLaf A/S, Lynge, Denmark) for 72 h. The freeze-dried collagen sample was named starfish collagen (SFC) used for various analysis.

2.3.4. Collagen isolation with the aid of HSMH and US pretreatment

The frozen starfish samples were thawed, cut into small pieces, and subjected to deproteinization and demineralization according to the optimum conditions found in sections 2.3.1. and 2.3.2.

For collagen extraction with HSMH, the sample was mixed with alkaline solution (0.1 M NaOH) in a 1:10 ratio (w/v). Then, the mixture

was subjected to homogenization at 4000 rpm for 2.5 min with the Silverson homogenizer and centrifuged at 2000 × g for 2 min at 4 °C.

For collagen isolation with a combination of HSMH and US, the sample was mixed with alkaline solution in a 1:10 ratio (w/v) and homogenized at 4000 rpm for 2.5 min and then subjected to US treatment at 75 % amplitude for 10 min with pulse 10/20 s. The sample were kept in the ice bath during HSMH and US treatment to maintain the temperature of the sample not exceeding 4 °C. The US treated mixtures were also centrifuged at similar conditions. The precipitates from both the samples were collected and mixed with cold water and the pH was adjusted to 7.4. Then the samples were subjected to demineralization as mentioned in the conventional pretreatment method (2.3.1). However, the demineralization time was reduced to 24 h with a change into fresh solution at 12 h. The deproteinized and demineralized samples were then subjected to extraction using 0.5 M acetic acid, salting out, dialysis and drying as explained for conventional collagen. The freeze-dried collagen samples produced with HSMH and US were called HSFC (HSMH pretreated starfish collagen) and USFC (US pretreated starfish collagen) respectively and were used for various analyses. All extraction experiments were run at least in duplicate.

2.4. Characterization of collagen

2.4.1. Yield of collagen

The yield of collagen from starfish was calculated based on weight of initial dry starfish raw material according to following formula:

$$\text{Yield}(\%) = (\text{Weight of freeze} - \text{dried collagen})/(\text{Weight of initial dry starfish}) \times 100$$

2.4.2. UV–visible spectroscopy of collagen

UV–visible spectrum of SFC, HSFC and USFC was evaluated according to the method explained by Duan et al. (2009). Freeze-dried collagen samples were dissolved in 0.5 M acetic acid (0.5 g/L) and its UV–visible spectrum was recorded from 190 to 450 nm using a spectrophotometer (Cary 60 UV–vis, Agilent technologies, Santa Clara, USA).

2.4.3. Fourier transform infrared (FT-IR) spectroscopy of collagen

FT-IR spectroscopy of the starfish collagen samples was executed according to the method described by Chuaychan et al. (2015). Lyophilized collagen samples were placed onto the crystal cell of a Nicolet 6700 spectrophotometer (Thermo Scientific, MA, USA) and FT-IR spectra was obtained by scanning at a resolution of 4 cm^{-1} with measurement range from 4000 cm^{-1} to 400 cm^{-1}. All spectra were recorded in 32 scans at 25 °C.

2.4.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out according to the method of Laemmli (1970). Collagen samples prepared in 5 % SDS were mixed with the sample buffer (Bio-Rad, USA) at 1:2 ratio in the presence of 10 % β-ME to get the final protein concentration of 2 μg protein/μL. Then, 7.5 μL from each sample and 5 μL of marker (10–250 kDa, Bio- Rad, USA) were loaded onto a 7.5 % precast mini linear gel (Bio-Rad, USA). After being separated by electrophoresis at a constant current of 50 mA per gel, using a Mini Protein II unit (Bio-Rad, USA), the proteins were stained with and de-stained. Finally, an image of the gel was taken in Bio GelDoc Go Imaging system (Bio-Rad, USA).

2.4.5. Amino acid composition of collagen

Amino acid composition of the collagens was analyzed based on the method of Özcan and Şenyuva (2006) with some modifications. Freeze-dried collagen samples (10 mg) were mixed with 4 mL of 6 N HCl and hydrolyzed at 110 °C for 24 h. Hydrolyzed collagen samples were diluted using 0.2 M Acetic acid and automatically injected to LC/MS (Agilent 1100 HPLC, Waldbron, Germany) in replicates and compared against amino acid standards. Tryptophan and cysteine were not recovered with this method.

2.4.6. pH and salt solubility analysis

The pH and salt solubility of starfish collagens were determined according to the method of Jongjareonrak et al. (2005). Collagen solutions were prepared in 0.5 M with concentrations of 3 and 6 mg/mL. The pH of 8 mL of collagen solutions (3 mg/mL) were adjusted in range from 1 to 10 using 6 N NaOH or 6 N HCl. The volume of the sample solution was made up to 10 mL with distilled water preadjusted to same pH as collagen solutions. For salt solubility, 5 mL of collagen solutions (6 mg/mL) in 0.5 M acetic acid were mixed with 5 mL of cold NaCl in acetic acid of various concentrations, to obtain the final NaCl concentrations of 1, 2, 3, 4, 5 and 6 % (w/v). Both pH and salt solubility samples were stirred gently for 30 min at 4 °C and centrifuged at 10,000 × g for 30 min at 4 °C. The protein content of the supernatants was determined by Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951). The pH and salt solubility of collagen samples were calculated by comparing it to that obtained at the pH and salt concentration showing the highest solubility, respectively.

2.4.7. Determination of collagen fibril formation in vitro

Fibril formation of collagen from the starfish was performed according to the method of Bae et al. (2009). Collagen solutions were prepared dissolving freeze-dried collagens in1mM HCl solution (pH 3.0) to get a concentration of 0.3 % (w/v). The collagen solution was mixed with 0.1 M Na-phosphate buffer (pH 7.4) in 1:1 ratio (v/v), and the final pH of the solution was 7.25. Collagen fibril formation turns the transparent solution to turbid which was monitored at 21 ± 1 °C using a spectrophotometer (Cary 60 UV–vis, Agilent technologies, Santa Clara, USA) at a wavelength of 320 nm. This measure represents the speed of collagen fibril formation in a short time.

2.4.8. Measurement of degree of collagen fibril formation

Starfish collagen fibrils were formed for 24 h at 21 ± 1 °C, using the same conditions as described above. The fibrils formed were precipitated by centrifuging at 20,000 g for 20 min and the protein content of the supernatant was measured by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The degree of collagen fibril formation was defined as the percentage of the decrease in collagen concentration in the solution after the experiment, which means the percent of collagen molecules that formed fibrils.

2.4.9. Rheological analysis

Temperature sweep tests were performed on a Physica MCR300dynamic rheometer (Paar Physica) using a stainless steel cone/plate geometry (2°cone angle, 40 mm cone diameter) with the gap set at 0.21 mm. Collagen solutions were prepared at a concentration of 1.5 % (w/w) and subjected to dynamic temperature sweep tests. The tests were conducted within the linear viscoelastic range and at a constant strain of 1 % and constant frequency of 1 Hz. Collagen solution was heated from 10 to 30 °C at a rate of 0.5 °C/min to get the complex viscosity values which were used to determine collagen denaturation temperature.

2.5. Statistical analysis

All pre-treatments and extractions of collagen were carried out 2 times. Analysis of the extracted collagen was then done in duplicates and average values from these analyses were subjected to analysis of variance (ANOVA) to determine significant differences between pretreatments/extractions. Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie 1980). Data was regarded as significantly different when $p < 0.05$. Statistical analysis was performed using the Statistical Package for Social Science (IBM SPSS 28.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Optimization of pretreatment by HSMH and US

The starfish had moisture content of 70.92 % and the protein content of 37.46 % (based on dry matter). The deproteinization was carried out using HSMH at a raw material to alkaline ratio of 1:20, and was compared with the classic method (without HSMH). As can be seen in Fig. 1a, HSMH was very effective in improving the removal of NCP compared to the sample not subjected to HSMH. The highest removal of NCP was achieved almost right after the HSMH while at least 2 h of incubation was necessary to reach the maximum NCP removal using the classic method comprising only cutting sample into small pieces of 0.5 to 1 cm size. There was no significant difference between the removal of NCP using HSMH at 4000 and 8000 rpm during the 6 h of incubation. HSMH has earlier been used in the extraction of protein from different sources such as chicken muscle (Omana, Xu, Moayedi & Betti, 2010) and fish skin (Tan & Chang, 2018). The size reduction of the raw material and the uniform distribution of particles allows the solutions to interact efficiently with these, leading higher protein extraction. In addition, integrating HSMH at the deproteinization step had positive effect in the removal of minerals in the demineralization step as well (Fig. 1b). The mineral removal efficiency in the samples subjected to HSMH was significantly ($p < 0.05$) higher than that of the classic method. When using HSMH, the mineral removal reached the highest point at 12 h of incubation compared to 48 h in case of the classic method. This means that the incubation time during the demineralization could be reduced to 12 h with the aid of HSMH. As stated above, these improvements are reached because the HSMH leads to a substantial size reduction of the raw material during both deproteinization and demineralization compared with the classic method, which starts with chopping of samples into pieces of around 0.5 to 1 cm using a knife. The reduced particle size aids alkaline solution and EDTA to access more easily into the raw material and interact with NCP and minerals, respectively, which consequently speeds up the removal of the targeted components of the biomass. Based on the results, HSMH at 4000 rpm for 2.5 mins was selected for further trials.

In the next step, the possibility to reduce the raw material to alkaline solution ratio, the incubation time or introduction of a solution change after applying the HSMH at 4000 for 2.5 min was investigated. The results (Fig. 1c&d) showed that the aid of HSMH and a 1:5 solution ratio was effective in removing the NCP, but a solution change was still necessary to achieve a complete deproteinization. On the other hand, the total amount of NCP removed with one cycle of incubation at 1:10 ratio was significantly ($p < 0.05$) higher than that of 1:5 and 1:20. Thus, it was concluded that the 1:10 ratio was the best to apply for the removal of NCP and there was no need of solution change at this ratio. This setting resulted in 100 % reduction of the incubation time and 4 times reduction of the alkaline solution consumption compared with the classic method, which is normally based on 2 cycles of incubation at a 1:20 ratio.

The US treatment after the HSMH treatment of the raw material in alkaline solution showed a positive effect in deproteinization and demineralization. US at 75 % amplitude for 10 min at 10/20 s pulse was selected based on a pre-trial. Application of this US treatment slightly increased the removal of protein in the deproteinization step (Fig. 1e) and significantly ($p < 0.05$) increased removal of minerals in demineralization step (Fig. 1f). It could be noted that during deproteinization

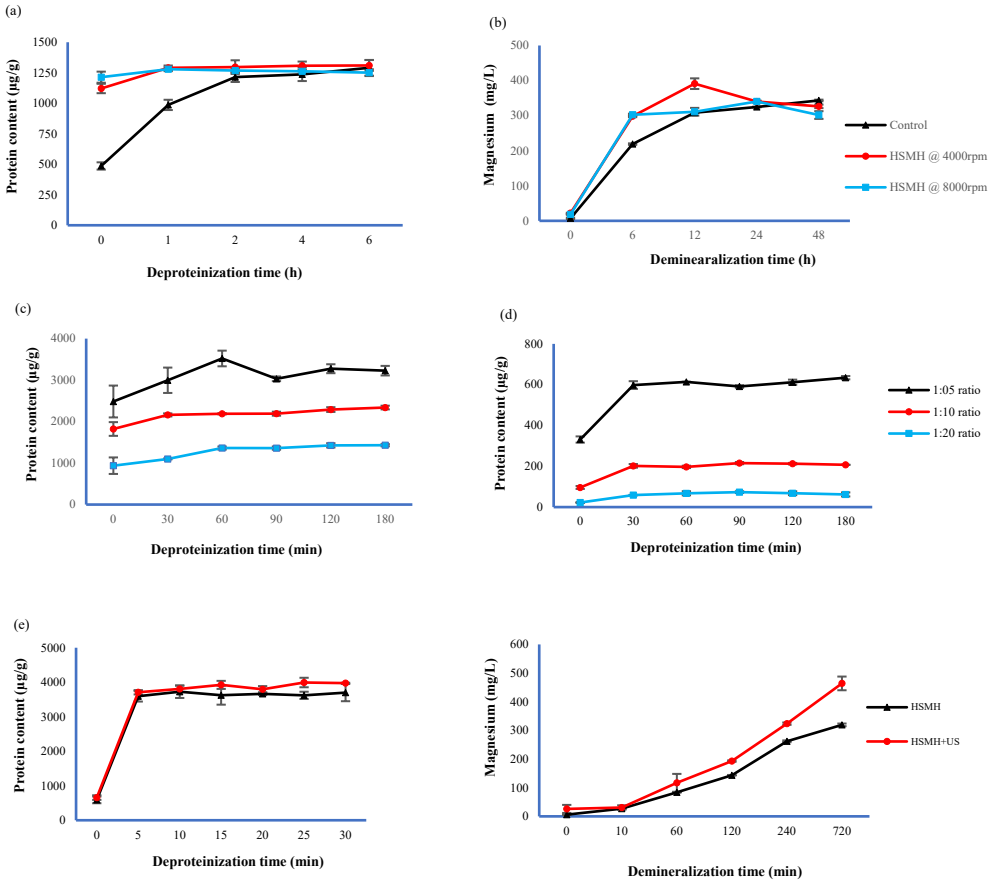

Fig. 1. Optimization of pretreatment by high shear mechanical homogenization (HSMH) and ultrasound (US). Deproteinization (a) and demineralization (b) at different homogenization speeds; c & d: deproteinization before and after solution change at different starfish to alkali ratios; e & f: deproteinization and demineralization during pretreatment by US.

with HSMH the removal of NCP peaked at 5 mins of incubation after homogenization. This indicated that during application of HSMH, the incubation time could be reduced from the above concluded 1 h down to 5 mins. There was a minute increase in the removal of NCP when US was applied compared to the HSMH alone. This could be due to the removal of most of NCP during the HSMH treatment, and very negligible amounts of NCP remained which were then removed by the application of US. Mineral removal was also increased with application of US in the deproteinization step after HSMH. The difference was little here, most likely due to the same reason as discussed for the deproteinization. The rate of mineral removal continued to increase until 12 h. Hence, there was still some minerals left to be removed even after 12 h. So, for the actual collagen extraction procedure, the demineralization was carried out for 24 h with one solution change at 12 h. The cavitation process during the US treatment most likely helped to open up tissues more, improving the removal of NCP and minerals. Based on the results, pretreatments were done with HSMH at 4000 rpm for 2.5 min and US treatment with 75% amplitude for 10 mins at 10/20 s pulse. The deproteinization time was hereby reduced from 6 h to 5 mins and the demineralization time from 48 h to 24 h with a solution change at 12 h. The amount of deproteinized sample after pretreatment by classic method (46.22 %) was higher than those obtained by pretreatment by HSMH (40.44 %) and HSMH + US (40.46 %). This indicated that even after reducing time and chemical use, the pretreatment using HSMH and US were more efficient in removing NCP than that from classic method. There was no significant difference between the amount demineralized samples after pretreatment from classic method (11.32 %), HSMH (10.02 %) and HSMH + US (11.44 %) indicating the reducing time and chemical use during application of HSMH and HSMH + US did not affect the yield. The characteristics of collagen produced by this method were subsequently compared with collagen extracted by the conventional method.

3.2. Collagen isolation yield

Yields of SFC, HSFC and USFC were 4.01 ± 1.65, 3.34 ± 0.78 and 3.37 ± 0.10 % w/w (dry weight, dw, basis) respectively. The protein content of SFC, HSFC and USFC were 80.30 ± 0.61, 81.47 ± 0.45 and 83.18 ± 1.74 % respectively which indicate the purity of collagen. There was no significant difference between the yield of collagen from the classic method and collagen extracted with the aid of HSMH and US in the pretreatment. This indicated that application of the HSMH and US during the pretreatment did not negatively affect the yield of collagen even though it decreased the pretreatment time and the amount of alkali used. In addition, the size reduction and mechanical energy used during the deproteinization step and demineralization did not result in the extra loss of collagen indicating no negative affect on its triple helical structure. Lee et al., (2009) reported a collagen yield of 5.8 % from the wet tissue of starfish *Asterias amurensis* when they used an extraction process taking more than 7 days, excluding the dialysis time. The yield of pepsin

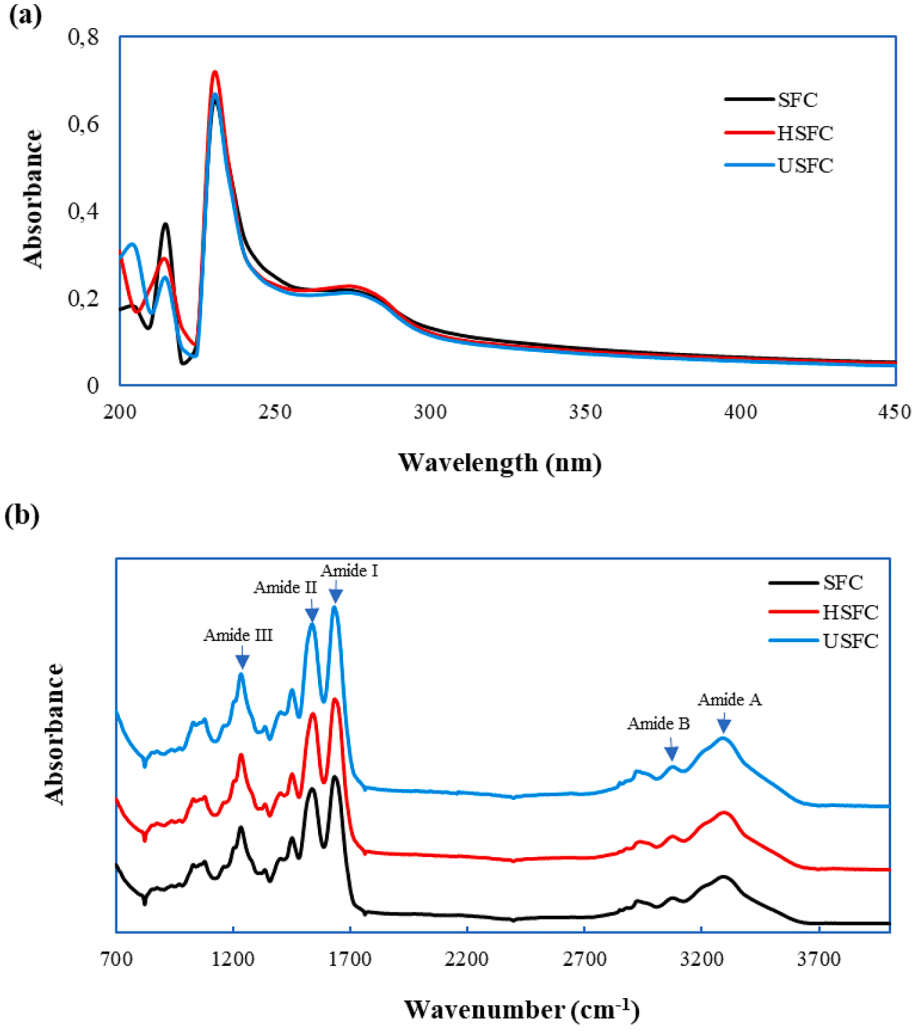

Fig. 2. UV–visible (a) and FTIR (b) spectra of collagen samples extracted from starfish SFC: Collagen extracted by classic method; HSFC: Collagen extracted with the aid of HSMH; USFC: Collagen extracted with the aid of HSMH and US.

soluble collagen from starfish (*Acanthaster planci*) was 2.29 % on a dw basis (Tan et al., 2013) when they used an extraction protocol taking more than 10 days. This can be compared to the 8 days of the classic collagen extraction method and 6 days in case of the new extraction method presented here, including HSMH and/or HSMH + US in the pretreatment steps. The yield in the present study was also comparatively higher than that of Tan et al. (2013) even though the extraction time was reduced. This indicated that HSMH and US can be effectively applied in the pretreatment step of collagen extraction to reduce the extraction time without negatively affecting the collagen yield.

3.3. Spectral properties of collagens

UV–visible spectral scanning of collagen samples is depicted in Fig. 2 (a). SFC, HSFC and USFC showed their highest peak at 230 nm, the same wavelength as the triple helical structure of collagen has a highest absorption peak (Pal, Nidheesh, & Suresh, 2015). Therefore, this is one of the simplest methods to evaluate the purity of native collagen. Similar results were reported for collagen isolated from skin of Medusa fish (*Centrolophus niger*) (Madhuri et al., 2019), from scale and bone of common carp (Duan et al., 2009), and from catla (*Catla catla*) (Pal, Nidheesh, & Suresh, 2015). The maximum absorption peak observed between 220 and 240 nm is associated to the presence of C═O, –COOH, and CO-NH_2 in the polypeptide chains of the recovered collagens (Abdollahi, Rezaei, Jafarpour, & Undeland, 2018). There was also a small absorption peak at 280 nm which can be related to the presence of very low amounts of the aromatic amino acids tyrosine and phenylalanine in the collagen (Cui et al., 2007). Thus, the UV spectral scan of the extracts from common starfish confirmed that the recovered proteins were collagen. They also revealed that non-collagenous impurities were efficiently removed during the pretreatment step with the aid of HSMH and US, thereby shortening the pre-treatment time and the amount of alkali.

FTIR spectra of SFC, HSFC and USFC are depicted in Fig. 2b. This spectral analysis exhibited the presence of characteristic peaks related to amide bands I, II, III, A, and B. The amide bands I, II and III of the starfish collagen extracts showed vibrations in the range of 1600–1700 cm^{-1}, 1500–1600 cm^{-1} and 1200–1300 cm^{-1}, respectively (Fontaine-Vive, Merzel, Johnson, & Kearley, 2009). Amide I, II and III band vibrations are typical for collagen (Benjakul et al., 2010), and it is well established that amide band I is related to the stretching vibration of C═O. The amide I bands of SFC, HSFC were found at a similar wavelength of 1633 cm^{-1} whereas amide band I of USFC was found at 1631 cm^{-1}. Lower wavenumber generally represents the formation of hydrogen bond between the N—H stretch, where the CO residue is responsible for stabilizing the triple helix structure (Zhao, Chi, Zhao & Wang, 2018). The amide II band represents the N—H bending. SFC and USFC showed lower wavenumber of 1537 cm^{-1} for the amide II band when compared to that of HSFC (1541 cm^{-1}). The shifting of amide I and II bands of collagen to lower wavenumber suggests more hydrogen bond formation in the triple helical structure. For the amide III band, all the collagens displayed the same wavenumber of 1234 cm^{-1}, indicating the involvement of hydrogen bonds in maintaining the native structure. Amide III is the combination of C—N stretching and N—H deformation, which involves in the complex intermolecular interactions in collagen (Sinthusamran, Benjakul, & Kishimura, 2013). The FTIR spectra retrieved from the collagens of this study were similar to those reported for sea cucumber (Li et al., 2020) and some fish skin collagens such as those from seabass and channel catfish (Liu, Li, & Guo, 2007; Sinthusamran, Benjakul, & Kishimura, 2013).

The absorption band of amide A is associated with the N—H stretching vibration and the amide band B is related to the asymmetrical stretching of CH_2. The absorption peak for amide A occurs in the range of 3400–3440 cm^{-1} (Ikoma et al., 2003). The amide A band of SFC, HSFC and USFC was observed at the wavenumbers of 3292, 3294 and 3292 cm^{-1}, respectively. The shift in the wavenumber to a lower frequency suggested that the NH group was involved in hydrogen bonding (Fontaine-Vive, Merzel, Johnson, & Kearley, 2009). The amide B band was found at the same wavenumber, 3078 cm^{-1} for all the three collagen samples. The intensity ratio between amide III and the 1450 cm^{-1} band determines the triple helical structure of collagen (Benjakul et al., 2010). In this study, the ratio between the intensity values of the amide III band and that found at 1450 cm^{-1} were about 1.1 for all the collagens, confirming that their native triple helical structure was retained, which is necessary for biomedical applications. The FT-IR spectra was similar to that of collagen from calf skin with different bands showing peaks in similar wavenumbers. But the peaks of different bands from spectra of calf skin were lower than that from starfish collagen (Saallah et al., 2021). The UV and FTIR spectral results thus indicated that the size reduction of the raw material by HSMH and the cavitation process created by US in the pretreatment step of collagen extraction did not affect the triple helical structure of the extracted collagen, and their structural integrity was well-preserved with these new methods.

3.4. Polypeptide patterns

Polypeptide patterns of all the three collagens extracted with the different methods showed that they were composed of α_1, α_2 and β-chains as the major components (Fig. 3). The ratio of α_1 to α_2 chains was about 2:1 in all the samples suggesting that the extracted collagens were type I collagen (Benjakul et al., 2010; Shoulders & Raines, 2009). The α_1, α_2 and β bands of all the collagen samples showed similar molecular weight (MW). In addition, the β-component of all collagen samples was composed of β_{11} and β_{12} chains. The β_{11} is a homodimer of α_1 chains whereas the β_{12} chain is the heterodimer of α_1 and α_2 chains (Tan et al., 2013). In general, the common starfish collagens contained low amounts of the β- and γ-components, reflecting lesser cross-linking. This could be interesting from an application perspective since the α chains are the major components contributing to the functional properties of collagens. The band intensity of β-chain from starfish collagen

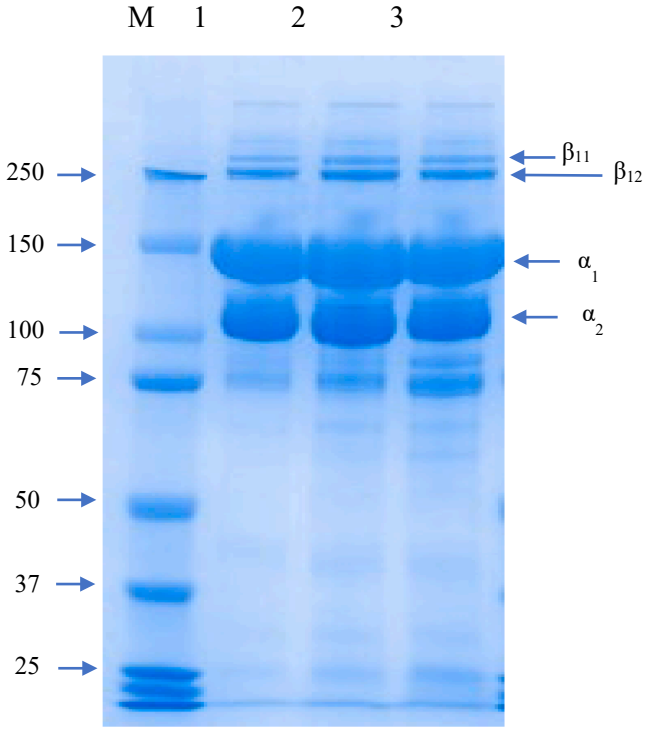

Fig. 3. Polypeptide patterns of collagen samples extracted from starfish, M: Marker; 1: Collagen extracted by the classic method; 2: Collagen extracted with the aid of HSMH; 3: Collagen extracted with the aid of HSMH and US.

was lower than that from type I collagen of calf skin and collagen from skin of striped catfish. But the molecular weight of α_1 chain of starfish collagen was higher than those from type I collagen of calf skin and collagen from skin of striped catfish (Singh, Benjakul, Maqsood & Kishimura, 2011). The results were similar to the SDS-PAGE data reported for collagen from starfish *Asterias amurensis* (Lee et al., 2009) and pepsin soluble collagen from the body wall of crown-of-thorns starfish (*Acanthaster planci*) (Tan et al., 2013). The results demonstrated that the HSMH and US treatment did not affect the polypeptide pattern of the collagens, in terms of MW or intensity of each band. Kim, Kim, Kim, Park and Lee (2012) reported that the α_1 and α_2 chains of acid soluble collagen from seabass skin degenerated when the samples were subjected to US for a very long time (24 h). In this study, there was no difference in the intensity of α chains of SFC and USFC. This could be due to fact that here the samples were subjected to US treatment for a very short time. Ali, Benjakul, Prodpran, & Kishimura (2017) reported that US treatment with a higher amount of pepsin lead to lower intensities of the β and γ-chains. US loosens the matrix of the sample through the cavitation effect (Zou et al., 2017), which aids to the enhancement of pepsin activity during collagen extraction (Yu et al., 2014). However, there was no difference in the intensity of β- chains in SFC and USFC. This indicated that US did not affect the β- chains of starfish collagen, which could be due to the application of US in the pretreatment step rather than in the extraction step. There was no or very less amount of low molecular weight proteins noticed in the HSFC and USFC. Hence the HSMH and US did not affect negatively on the polypeptide structure of the extracted collagens.

3.5. pH and salt solubility

The effects of pH and NaCl concentrations on the solubility of the extracted collagens are shown in Fig. 4. SFC, HSFC and USFC had the highest solubility at pH 1. There was then a sharp decrease in the solubility abovepH4, and all the collagen samples had their lowest solubility at pH 5, indicating that this was the pI for the starfish collagen. The solubility of the collagen samples slightly increased at pH 6 and 7 and then remained steady until pH 10. This is mainly due to protein gaining a net negative or positive charge at pH above and below pI, respectively, leading to charge repulsion between the protein chains. This allows more water to interact with the charged proteins, increasing their solubility (Tan & Chang, 2018). The solubility of SFC, HSFC and USFC in the alkali pH ranges were lower compared to solubility at acid pH, which is typical of collagen (Foegeding et al., 1996). This result was similar to that of a previous study where the collagen from the body wall of starfish

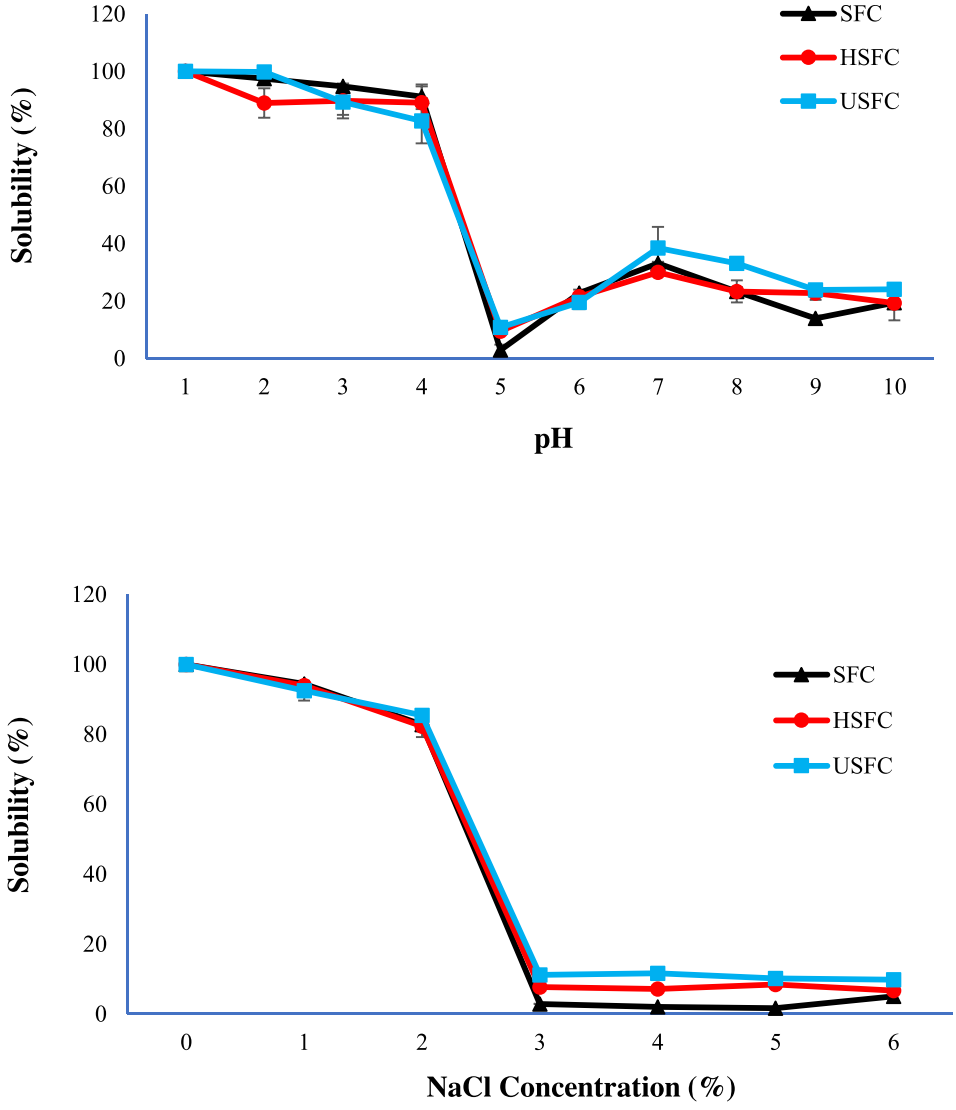

Fig. 4. pH (a) and salt (b) dependent solubility of collagen samples extracted from starfish, SFC: Collagen extracted by the classic method; HSFC: Collagen extracted with the aid of HSMH; USFC: Collagen extracted with the aid of HSMH and US.

(*Asterina pectinifera*) had its lowest solubility at pH 5 (Qi et al., 2017). In another study, the collagen from sea cucumber had its lowest solubility at pH 6 (Li et al., 2020). The pretreatments with HSMH or US had no effect on the pH-dependent solubility of collagen extracted from common starfish.

Effect of different NaCl concentrations on the solubility of collagen in the three collagen samples was analyzed (Fig. 4b). One % and 2 % NaCl resulted in the highest solubility of the three collagens. At a NaCl concentration of 3 %, the solubility was greatly reduced, and similar solubility was observed at NaCl concentrations 3, 4, 5 and 6 %. This reflects a 'salting-out' phenomenon in which an increasing ionic strength results in protein precipitation since salt ions compete with the proteins for water, improving the interaction between protein chains (Tan & Chang, 2018). Also, hydrophobic-hydrophobic interaction reinforcement and interchain of polymerization result in 'salting out', leading to protein precipitation (Hukmi & Sarbon, 2018). The results were similar to the salt solubility of pepsin solubilized collagen from starfish *Asterias amurensis* which decreased greater than 3 % NaCl (Lee et al., 2009). The salt solubility of collagen from body wall of starfish (*Asterina pectinifera*) also decreased with the concentration of NaCl up to 4 % (Qi et al., 2017). Application of HSMH and US in the pretreatment step of the collagen extraction thus did not change the salt dependent solubility of the collagen extracted from common starfish, which means the collagen quality was not negatively affected by the modifications in the extraction process.

3.6. Amino acid composition of the collagens

The amino acid composition of the collagen samples from starfish recovered with the different methods is given in Table 1 (Supplementary). Glycine (Gly) was the major amino acid found in all the collagen samples followed by glutamic acid (Glu) and proline (Pro). This is due to the unique amino acid sequence of collagen where glycine appears at every third amino acid residue (Jongjareonrak et al., 2005). Amino acid sequence of collagen subunits is a repeat of Glycine-X- Y, where X is variable, but it is usually proline and Y is variable but normally is filled with hydroxyproline (Abdollahi et al., 2018). This result agreed with the glycine content of collagen from the body wall of crown-of-thorns starfish (*Acanthaster planci*) (Tan et al., 2013). The amino acid composition of the collagen samples was also similar to the collagen from starfish *Asterias amurensis* (Lee et al., 2009) and purple sea urchin (*Anthocidaris crassispina*) (Nagai & Suzuki, 1999). Glycine was also the

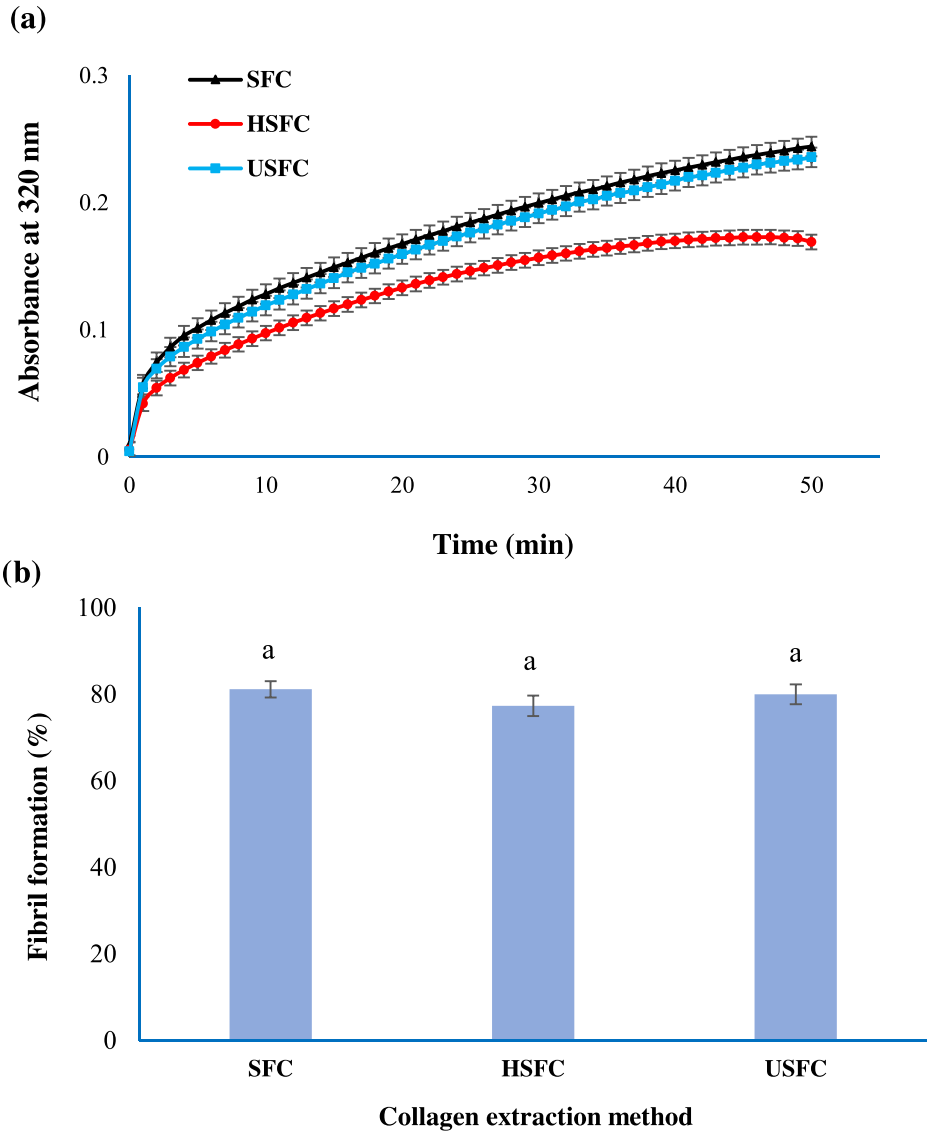

Fig. 5. In vitro progress of fibril formation (a) and degree of fibril formation (b) of collagen samples extracted from starfish, SFC: Collagen extracted by the classic method; HSFC: Collagen extracted with the aid of HSMH; USFC: Collagen extracted with the aid of HSMH and US.

major component of collagen peptides extracted from *Asterias pectinifera* (Han, Won, Yang, & Kim, 2021). The relatively high content of glycine and proline thus suggested that collagen was the major component of SFC, HSFC and USFC. The amino acids from starfish collagen were lower than that from fish skin (*Salmo salar*) except serine, aspargine and tyrosine (Gauza-Włodarczyk, Kubisz, & Włodarczyk, 2017). This is most likely because of the nature of collagen found in body-wall of starfish is much different from the one obtained from fish skin. Application of HSMH during the pretreatment step caused a significant ($p < 0.05$) reduction of some amino acids such as glycine, arginine, alanine, serin and proline in the extracted collagen which was further reduced with application of US during the pretreatment step. This could be due to the removal of amino acids in the telopeptide region of collagen by enhanced pepsin activity during the collagen extraction, resulting from the HSMH-derived size reduction and the cavitation process caused by US.

3.7. Collagen fibril formation in vitro and degree of fibril formation

The fibril-forming ability of starfish collagens was studied at 320 nm, and the results are represented in Fig. 5a. The turbidity increased rapidly as soon as the collagen solution was mixed with the buffer as evidenced by a higher increase rate in the absorbance at the initial stage of incubation. This suggested that the fibril formation started as soon as the solution was mixed. When the optimum temperature and pH is provided in the solution, the collagen molecules have the ability to spontaneously self-assemble. This is since all of the information needed for fibril formation is found in the collagen molecules themselves (Helseth & Veis, 1981). The fibril formation ability of collagen differs based on the raw material from which it has been extracted. For example, the collagen extracted from swim bladder of Bester sturgeon had higher fibril formation rate than that of collagen extracted from skin of the same fish and porcine collagen (Zhang et al., 2014). This indicated that all the collagens extracted from common starfish preserved their fibril formation capacity which is important for their biomedical applications. Collagen extracted with the classic method and with US had similar turbidity pattern over time, but the collagen extracted with the aid of HSMH (i.e. HSFC) showed lower turbidity compared with the two other samples, reflecting a lower rate of fibril formation and lower nucleation. US did not show any negative effect on the fibril formation of the extracted collagen, even though the starfish sample had been subjected to HSMH prior to the US treatment. This could also imply that the US treatment compensated for the effect of HSMH on the fibril formation as indicated by the higher absorbance for USFC compared to HSFC at 230 nm. Overall, it could be concluded that starfish type I collagen extracted with the different methods had a high ability of fibrillogenesis under the present experimental conditions.

The degree of collagen fibril formation (Fig. 5b) was analyzed after 24 h of mixing of collagen solution with buffer. It ranged between 77 and 81 % for the starfish collagens, but there was no significant difference in the degree of fibril formation between the different collagen samples. The degree of fibril formation of starfish collagens was similar to that of pepsin soluble collagen extracted from rohu (*Labeo rohita*), and higher than those of acid soluble collagen extracted from catla (*Catla catla*) and rohu as well as pepsin soluble collagen extracted from catla (Pal, Nidheesh, & Suresh, 2015). However, the collagen extracted from skin and swim bladder of Bester sturgeon (*Huso huso* × *Acipenser ruthenus*) had a higher degree of fibril formation (greater than90 %) compared to that of the starfish collagens extracted in this study (Zhang et al., 2014). Overall, the results confirmed that the application of HSMH and US had no negative effect on the fibril formation capacity of the extracted collagens from starfish, thus, they have good functionality for different applications.

3.8. Rheological properties of collagens and their thermal denaturation

The change in complex viscosity of 1.5 % (w/v) solutions of the collagen samples recovered with the three methods is shown in Fig. 6. Regardless of the extraction method, the complex viscosity of the three samples decreased as the temperature increased. This is most likely due to a decrease in the resistance to the segment motion resulting from the increase in the energy for the heat motion of polypeptide chains (Zhang, Chen, Li, & Du, 2010). The initial viscosity of SFC was higher than that of HSFC and USFC. There was then a sudden decrease in the complex viscosity in the temperature range of 17 to 21 °C, reaching the lowest value at 21 °C. Thereafter, it became stable until 30 °C. This sudden decrease of η^* in magnitudes reflected the collapse of the collagen triple helix to a random coil, i.e., its denaturation (Yoshimura, Chonan, & Shirai, 1999). The denaturation temperature (T_d) under dynamic rheological measurement of collagen solution could be determined where the decrease of η^* reached 50 % of the initial value (Lai, Li, & Li, 2008). This result indicated that the denaturation temperature of all the collagen samples from starfish was around 18.5 °C. This means that application of HSMH and US in the pretreatment step had no effect on the denaturation temperature of the extracted collagens. Thermal denaturation of collagen from starfish *Asterias amurensis* was 24.7 °C as evidenced by reduction in the relative viscosity (Lee et al., 2009). Similar results were observed for the type I collagen derived from skin of largefin longbarbel catfish (*Mystus macropterus*) (Zhang, Chen, Li, & Du, 2010). The thermal stability of collagen mainly depends on its imino acid content, and also correlates with the body temperatures of the species it is extracted from, as well as their habitat temperatures (Matmaroh et al., 2011; Minh Thuy, Okazaki, & Osako, 2014; Pati, Adhikari, & Dhara, 2010). Collagen extracted from marine organisms living in cold water thus has a much lower denaturation temperature than collagen from warm water species (Jafari et al., 2020). The denaturation temperature found in this study for collagens from common starfish is close to those reported for other cold water resources such as skin from cod (14.5 °C) and salmon (19 °C) (Sun, Li, Song, Si & Hou, 2017; Yunoki, Suzuki & Takai, 2003).

4. Conclusions

Native collagen was efficiently extracted from common starfish and application of HSMH and US substantially reduced time and chemical requirement during the two pretreatment steps carried out to remove NCP and minerals. HSMH at 4000 rpm for 2.5 min reduced the required time for removal non-collagenous proteins from 6 h to 5 min compared with the classic method. The required amount of alkaline was reduced 4 times with application HSMH during the deproteinization step, compared with the classic method. A combination of HSMH and US reduced the demineralization time from 24 h to 12 h and improved its efficiency in terms of mineral removal. Collagen extraction yield from common starfish with the aid of HSMH and US was the same as with the conventional method. Application of HSMH and US did also not affect the triple helical structural integrity, polypeptide pattern, pH and salt solubility, as well as thermal stability of collagen extracted from starfish compared with the classic method. Thus, these processing steps could improve resource efficiency in terms of required amount of solvent and processing time without imposing negative effect on collagen yield and quality. In addition, starfish collagens extracted both with the new and conventional methods had good fibrillogenesis ability, which could be utilized in many medical and industrial applications. Finally, it could be concluded that common starfish could potentially be a promising source for extraction of native collagen.

CRediT authorship contribution statement

Naveen Kumar Vate: Data curation, Investigation, Methodology, Visualization, Writing – original draft. **Ingrid Undeland:** Writing – review & editing, Funding acquisition, Project administration. **Mehdi**

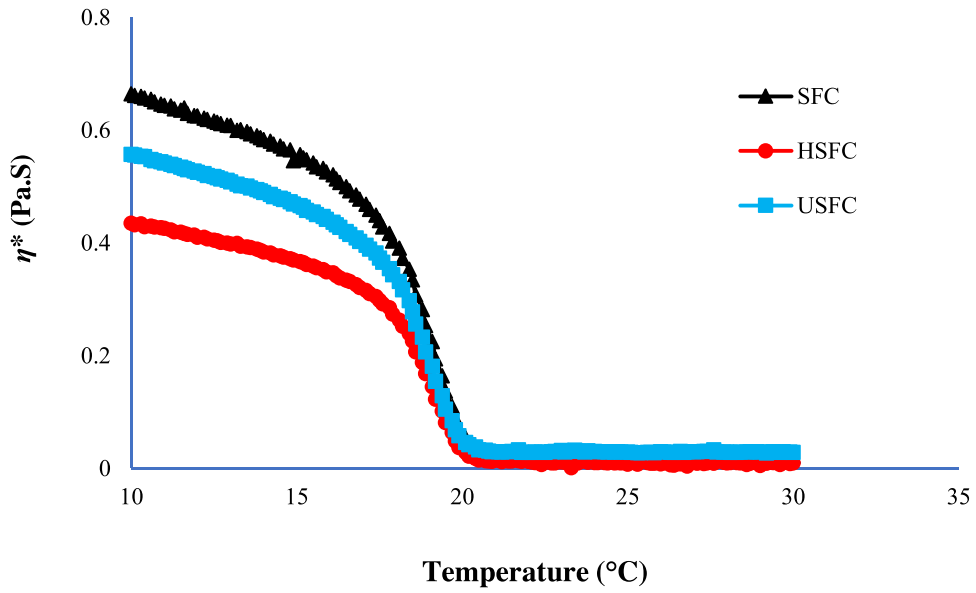

Fig. 6. Temperature sweep of collagens from starfish. SFC: Collagen extracted by the classic method; HSFC: Collagen extracted with the aid of HSMH; USFC: Collagen extracted with the aid of HSMH and US.

Abdollahi: Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors would like to thank Formas (Project # 2019-02362, Commercial exploitation of marine collagen and chitin from marine sources -BlueCC) and the ERA-NET COFUND BLUE BIOECONOMY 2019 for the funding. We would like to extend our gratitude to Chalmers University of Technology for providing research facilities and lab equipment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133426.

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