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EFFECTS OF HEATING ON PROTEIN DENATURATION, WATER DISTRIBUTION AND TEXTURE OF SEA CUCUMBER, *CUCUMARIA FRONDOSA*

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ABSTRACT

The objective of this study was to explore optimized heating processing of sea cucumber, *Cucumaria frondosa* by means of investigating the effect of boiling temperature (40, 60, 80, 100℃), boiling time (15, 30, 45, 60, 120min) and steaming time (15, 30, 45, 60, 120min) on the protein denaturation, texture and water distribution changes of sea cucumber. Results showed that the TCA-soluble nitrogen, SDS-Page and LF-NMR parameters indicated that boiling at 60℃ and 80℃ could cause obvious proteins denaturation. The denaturation of proteins led to changes in water holding capacity, texture properties and enzyme activity. To take all the index into account, 45min is concluded to be sufficient for cooking of *Cucumaria frondosa*.

Keywords: Cucumaria frondosa, protein denaturation, texture, water distribution

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Contents

LIST OF FIGURES

1 INTRODUCTION

Sea cucumber is an invertebrate animal belonging to the phylum Echinodermata. There are about 1100 species of sea cucumber around the world, but only 40 of them are edible (Zhu, Wu, & Zhou, 2015). The major edible part of sea cucumber is the body wall. Most Chinese people are fond of sea cucumber because it contains a high quantity of collagen, and is low cholesterol and abundant physiological active substances for nutrition and health functions. Especially, the chemicals triterpene glycosides (Careaga, Bueno, & Muniain, 2009) and acid mucopolysaccharides (Su, Lou, & Chang, 2003) which have shown inhibiting effects on the growth and metastasis process of tumour cells.

In China, the production of sea cucumber was increased from 75.7 thousand tons to 204.4 thousand tons in the decade from 2006 to 2016 (Zhang L. X., 2017). In 2010, its total production value reached 3 billion USD. Interestingly, in 2012 the *Stichopus Japonicus* sea cucumber became the most valuable aquatic species in China (Xu & Li, 2013). Despite rapid development in aquaculture and fishing, the demand for this product still exceeds the supply, which offers opportunity for import of non-native species to China.

The dendrochirotic sea cucumber *Cucumaria frondosa*, which is an inhabitant of the North Atlantic Ocean, is the most common species of sea cucumber in Icelandic waters. The fishery for this species has been well-established since 1988 (Nelson, Macdonald, & Robinson, 2012). According to FAO statistics, the production quantity of *C. frondosa* is given in Figure 1. During 2008 to 2015, it varied greatly, and the yearly yield of 2015 was 1411 tons. More than 98% of the exported product was gutted and frozen, and traded as raw materials. However, the total value decreased from 2.5 million to 1.5 million USD from 2011 to 2013 (Figure 2). One of the main reasons for this trend is inappropriate handling and freezing of the sea cucumber when transported from Iceland to China, which can result in autolysis of *C. frondosa* in the shipping container.

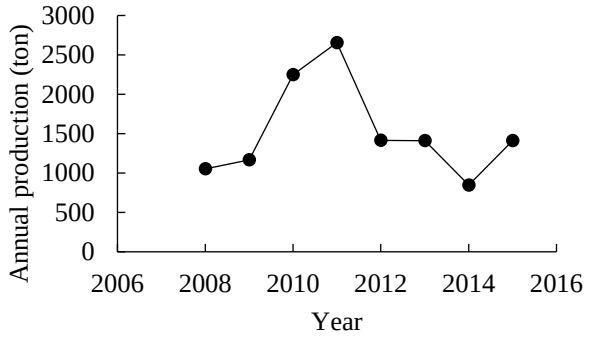


Figure 1. Production of *C. frondosa* in Iceland. (FAO database)

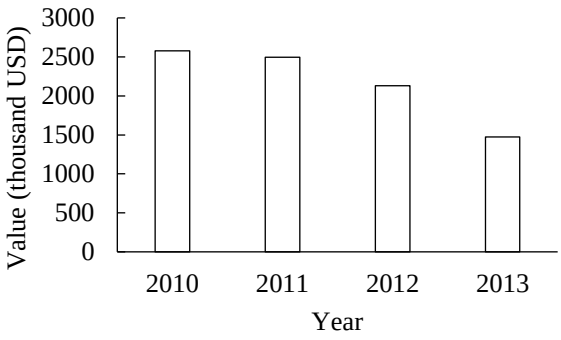


Figure 2.Value of *C. frondosa* in Iceland. (FAO database)

C. frondosa species are not only a valuable food source consisting of a high quantity of nutrients (Zhong, Khan, & Shahidi, 2007), but also have medicine potential benefit for health functions, such as antimicrobial, antioxidant, anti-cancer, and anti-inflammatory functions (Hu, et al., 2014).

At the present the Chinese market is far from saturation for sea cucumber, luckily, in Iceland, there is a supply of sea cucumber species that might be used to fill this demand gap.

There are three main processing methods for sea cucumber in China, namely drying, salting and soft canning (ready-to-eat products) (Xu & Li, 2013). Heat-induced protein denaturation and degradation which are extremely common in sea cucumber processing has a remarkable influence on the quality of product. To optimize the heating treatment, the heating effects of various heat treatments on the protein and texture changes of *C. frondosa* were analysed in this project.

1.1 Objectives

The aim of this project was to explore optimized heating treatment of Icelandic sea cucumber, *Cucumaria frondosa*. The effects of boiling temperature (40, 60, 80, 100℃), boiling time (15, 30, 45, 60, 120 min) and steaming time (15, 30, 45, 60, 120 min) on the protein denaturation, texture and water distribution changes of sea cucumber were investigated to provide reference for proper heat processing. Moreover, to analyse the correlation of protein degradation, Nuclear Magnetic Resonance (NMR) relaxometry and Texture Profile Analysis (TPA) parameters were analysed to provide more insights into the relationship between physicochemical properties and structure changes of *C. frondosa*. The result of this study could be valuable for Icelandic sea cucumber pre-treatment, and at the same time, provide suggestions to producers of *C. frondosa* both in Iceland and in China.

2 LITERATURE REVIEW

2.1 Processing methods of sea cucumber

2.1.1 Drying

Drying is the dominant preservation method of sea cucumber in China, and about 90% of the materials are processed into dried product (Duan, Wang, Ren, & Zhu, 2012). Currently, freeze drying (FD) and traditional solar radiation drying are regarded as the main methods of drying sea cucumber. Solar drying is economical but time-consuming and only makes inferior products with uncontrollable sanitary conditions. To date FD yields the best quality of dried sea cucumber which is convenient for storage and transportation. The product is furthermore delicate and smooth after rehydration, and the FD process preserves the most heat-sensitive nutrient components (Zhang, Zhang , Mu, & Liu , 2012). However, long drying times and high energy consumption cannot be avoided in FD of sea cucumber. Some other new technologies are also used for sea cucumber drying, such as a combination of freeze drying and microwave vacuum drying (Zhang, Zhang , Mu, & Liu , 2012), far infrared radiation drying and hot air drying (Moon, Kim, Chung, Pan, & Yoon, 2014), as well as combined electrohydrodynamic and freeze vacuum drying (Bai, Yang, & Huang, 2012). Most of these studies mentioned above were carried on *Stichopus japonicus* species, while no research was reported on the drying processes of *C. frondosa*. There is therefore a knowledge gap existing in relation to the processing ability of *C. frondosa*.

2.1.2 Salting

Salting is an ancient treatment for sea cucumber that is still used and to which many modern consumers are accustomed. For sea cucumber salting, the raw materials are gutted, cleaned, boiled, and soaked in salt water or mixed with salt grain (Hou, Wang, & Zhang, 2015). Then the materials are blanketed with charcoal powder and dried by sunshine. The salting process aims at dehydration and antisepsis, which represses the water activity, and prolongs the shelf life. The charcoal powder is used after salting because of its effects on air isolation and moisture-proofing and leads to long term preservation of salted sea cucumber. However, modern research shows that the nutrients in salted sea cucumber decreases significantly during processing when compared with those in the raw materials (Chen, 2009).

2.1.3 Soft canning

Rehydration and desalination are necessary for both dried and salted sea cucumber products, while the reprocessing is time consuming, and requires skilled cooking techniques, which is inconvenient for modern people. So, ready-to-eat sea cucumber, in a form of soft canned product, has gained popularity in recent years.

In most of the soft canning processes, the gutted and cleaned sea cucumber are sterilized by means of superheated steam or ultrahigh pressure. Modified atmosphere packaging is used for gas replacement, and packaging ensures that the taste, colour, texture, and appearance of the product do not change during storage. Research has been conducted to compare the texture changes of a ready-to-eat product compared to raw and heated sea cucumber. Results showed that the structure, and rheological characteristics of the instant sea cucumber changed greatly during the processing duration, and the sensory evaluation indicated that it had better quality

than the heated samples because of its higher flexibility and tenderness (Tang, Xue, Xu, He, & Zhang, 2007).

Overall, the practicality of the three processing methods of sea cucumber can be compared as follows.

- The drying technology is simple, and most nutrients, and physical properties of the product can be preserved, especially by freeze vacuum drying. These properties also result in the largest market share for sea cucumber products in China (Nong & Cao, 2017).
- Although salting is a traditional process which may lead to a serious loss of nutrients, and necessitates the desalination and rehydration the product before cooking, which has led to decreasing its popularity for modern consumers.
- Soft canned products are then more convenient for consumers and are therefore currently quite popular. However, some of the bioactive substances may be destroyed or degraded after superheating or ultrahigh pressure treatments, so there are lots of problems to be solved.

2.2 Heating effect on quality of sea cucumber

2.2.1 Heating effect on protein degradation

Research has shown the effect of heating temperature and time on the protein changes in sea cucumber. In a study by Chen (2009), the heating effects during a salting process on the quality changes of sea cucumber were studied, and protein, collagen, and fat content was observed to decrease with prolonged heating time. Furthermore, as the heating time increased, the muscle became tighter, indicating protein denaturation. Bi *et al*. (2016) assessed the structural changes of sea cucumber collagen, and demonstrated significant changes of the collagen structure and loss of water capacity when heated at 80℃ for 15 min, while no significant changes were observed when heated at 40℃ for 120 min. Dong (2010) studied the collagen changes during heating from 60℃ to 100℃. The results indicated that the triple helical structure of collagen was broken in the heating process.

2.2.2 Effect of protein degradation on texture change and water distribution

The following studies show correlations between protein denaturation and changes in texture and water distribution characteristics.

Chen (2009) showed that the heating time could increase the protein degradation, and the rheological parameter changed relatively at the same time during salting of sea cucumber. Zhu et al. (2017) furthermore showed that certain thawing methods applied on *Dosidicus gigas* accelerate protein oxidation and decrease in water holding capacity. Dong (2010) also showed that in the heating process, collagen fiber of sea cucumber became thinner, swelled, aggregated, and dissolved, and the time needed for the aggregation process decreased with heating temperature. The study also showed that after heating for a relatively long time at a high temperature, the shearing force and hardness was reduced, and the tissue of the sea cucumber became inapplicable to further processing. The studies of Chang *et al*. (2011) comparing two different heating methods, water bath and microwave oven heating of beef, revealed that the content of total collagen and soluble collagen varied with the heating method, temperature, and time, which in turn also affected on the texture profile analysis parameters.

Low field nuclear magnetic resonance (LF-NMR) is an effective tool to reflect the water distribution in muscle of aquatic products. The NMR relaxation curves indicate the content and changes of restricted water, immobilized water and free water. Stability and degradation regulation of sea cucumber collagen under high pressure treatment were researched, and it was found that the collagen deterioration was largely generated by the collagen fiber degradation, the conversion from restricted water to more free water, and the protein denaturation (Peng, Hou, Li, Zhang, & Xue, 2015). Gudjónsdóttir, Arason and Rustad (2011) studied the changes in water distribution and protein denaturation of salted and rehydrated cod fillets by applying low field NMR. Significant correlations were observed between NMR relaxation time and protein content, TCA-soluble protein, water holding capacity and water activity.

3 MATERIALS AND METHODS

3.1 Experiment design

The experimental design of the study is described in Figure 3. Boiling and steaming of the sea cucumbers were applied in the project, since these are commonly used heating processes for the production of dried or instant sea cucumber products. Experiments of varying boiling temperature (40, 60, 80, 100℃), boiling time (15, 30, 45, 60, 120 min), and steaming time (15, 30, 45, 60, 120 min) were performed. A good quality sea cucumber product should be high in protein content with lower TCA-soluble nitrogen. It is better to have soft and elastic texture with higher water holding capacity, and lower in enzyme activity. The effects of these different heating treatments on the cooking yield, total nitrogen, TCA-soluble nitrogen, SDS-Page electrophoresis, texture, water content, water holding capacity, water distribution, and autolysis enzyme activity of *C. frondosa* were analysed. (Here boiling refers to heating in water – even though temperatures lower than 100°C is used.)

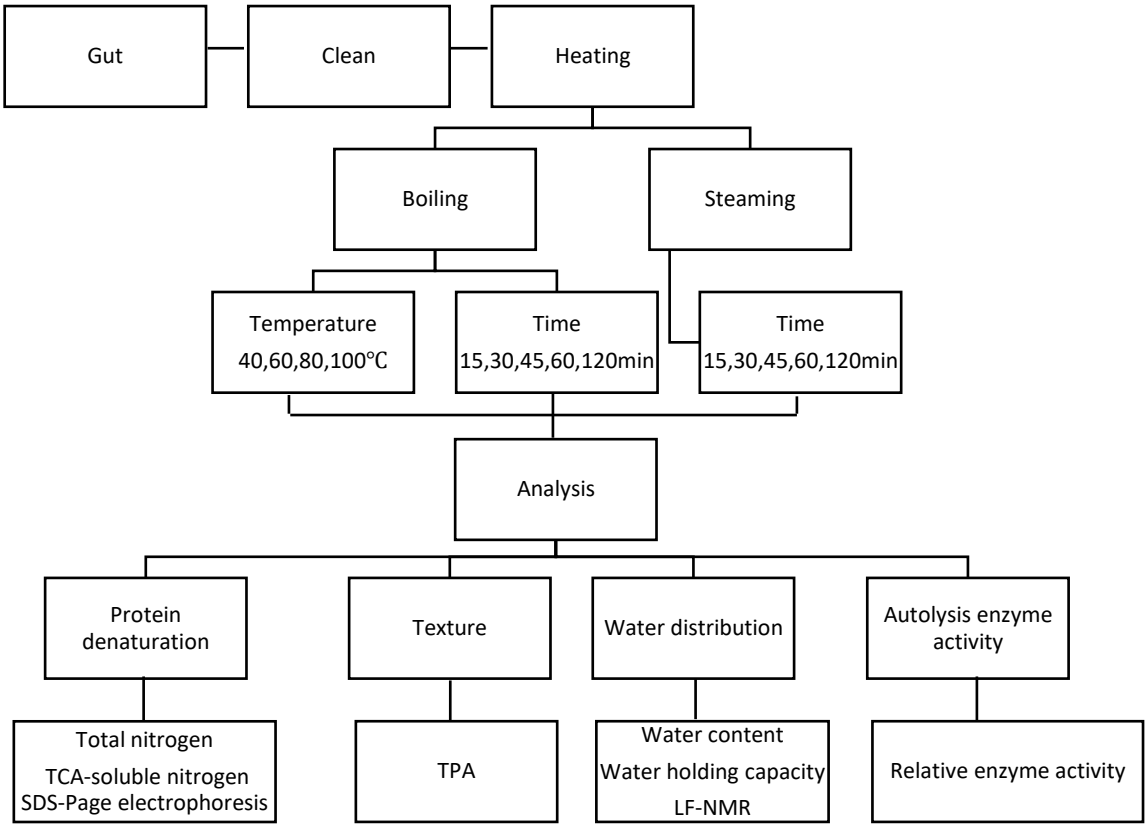


Figure 3. Experimental design diagram of the project

3.2 Raw material

The raw material was caught at the east coast of Iceland, out of Stöðvarfjörður in December 2017 by dredge (2.4m wide), which was dragged for maximum 25 minutes each time. Then the sea cucumber was bled and stored at -25℃.

Before processing, the raw material was thawed at 4°C for 20 h, and then gutted and the crown of the oral tentacles was carefully removed and cleaned with water, and placed on ice, before being objected to the boiling or steam treatments as shown in Figure 3.

3.3 Heat processing and sampling

An electro-thermostatic water bath and a steam oven was used for the boiling and steaming of the sea cucumbers, respectively. In the boiling groups, the samples were put into plastic bags and submerged in water. The boiling water and outside water were at same temperature and the weight ratio between samples and water were 1:2. In the steaming groups, samples were placed on trays, and the tendon part of the sea cucumber were put downwards. After cooking, the samples were placed in plastic bags and put on ice until analyzed.

3.4 Cooking yield

The cooking yield of the different heat treatments was calculated as the ratio between the weight before and after cooking.

3.5 Protein determination

3.5.1 Total protein content

The total nitrogen (TN) content was measured by the micro-Kjeldahl method (Mizuta, et al., 2013), and the total protein content was calculated by multiplying the total nitrogen content with a factor of 6.25.

3.5.2 TCA-soluble nitrogen

The protein fraction was precipitated with trichloroacetic acid (TCA). Approximately 20 g of minced sea cucumber were weighed accurately into a 250 mL flask, and 50 mL of 10% (w/w) TCA were added. The mixture was mixed together for 4 min in an Ultra Turrax mixer (T25, IKA, Labortechnik, Germany). This was followed by centrifugation at 4°C for 30 min at 2300 g. Then 10-12 g of the supernatant were used for the determination of TCA-soluble nitrogen by the Kjeldalh method (ISO 5983-1979).

The ratio of TCA-soluble nitrogen (TCA-N) to TN in the cooked samples and the raw materials were calculated to represent the extent of protein degradation during the heating process.

TCA-N is easy to dissolve into water, so the total (TCA-N) produced during heating was obtained by the following calculation, where **TN** stands for **total nitrogen**, **PN** for **protein nitrogen**, and the subscript **R** stands for the amount of these components in the **raw materials**, the subscript **S** for the component concentration in the **samples**, and the subscript **W** stands for **water**. The total nitrogen was viewed as a stable content.

$PN_S=TN_S\text{-}TCA\text{-}N_S$

$\text{Total TCA-N}= \text{TCA-N}_S + \text{TCA-N}_W = TN_R - PN_S$

3.5.3 Electrophoresis SDS-PAGE

Protein degradation was analysed with sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The samples were homogenized totally and the proteins in the samples were extracted with a protein extraction solution (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid disodium salt, 10 mM DTT, 1% Triton X- 100, 0.1% SDS) at a sample to extraction solution ratio of 1:3 (w/v) on ice for 20 min (Qi, et al., 2016). Then the extracts were centrifuged at 12,000 g for 15 min, at 4 °C. 30 μL of supernatants were added to 10 μL of sample buffer (277 mM Tris-HCl, pH 6.8, 44.4% glycerol, 4.4% LDS) separately. A Bradford colorimetric assay was adopted to obtain a protein concentration of 2 mg/mL. The diluted sample solution was boiled for 5 min, and 20 μL of the boiled solution were analysed with 10% polyacrylamide gel electrophoresis and a running buffer of Tris/ Tricine/ SDS using a Mini Protein system (Bio-Rad Laboratories).

The boiling and steaming liquid was also collected to analyse the protein lost from the sea cucumbers into the cooking liquid.

3.6 Texture analysis (TPA)

Samples were cut into 3 cm × 3 cm × 1 cm portions and analysed with a cylindrical probe P/50 (Texture analyser TA.XT Plus, Stable MicroSystems, London, UK). The pre-test speed, test speed and post-test speed were set as 1 mm/s, 0.5 mm/s and 1 mm/s, respectively. The deformation rate was set to 60% in agreement with the description of Bi *et al*. (2016).

3.7 Water distribution

3.7.1 LF-NMR proton distribution

Two small pieces were cut from the body wall, middle part and tendon, respectively from each sea cucumber sample. Each piece was placed in an individual NMR sampling tube, which were then inserted into the NMR instrument and analysed one by one. A Bruker Minispec mq-20 benchtop low field NMR instrument was used for the analysis. Transverse relaxation times were obtained with the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence, using an interpulse spacing of 100 μs, a 90° pulse of 13 μs and 180° pulse of 26 μs. The number of scans were set to 8 with a repetition delay of 10 s, and 8000 echoes were collected during each scan. The instrumental gain was furthermore set to 75 dB. All analyses were performed at the ambient temperature.

The obtained NMR relaxation data was maximum normalized prior to any further analysis. The data was then processed in two ways; firstly by principal component analysis (PCA) of the normalized relaxation data in Unscrambler (CAMO, Trondheim, Norway), and secondly by discrete relaxation fitting to a multi-exponential model with the LF-NMR toolbox for Matlab as described by Pedersen et al. (2002) to assess the proton population distribution of the samples.

3.7.2 Water content

The moisture content was measured by drying 5 g of minced sea cucumber samples in a ceramic bowl at 105 °C for 4 h. The moisture content was based on the weight changes before and after drying of 3 replicates for each sample (ISO 6496 1999).

3.7.3 Water holding capability (WHC)

Approximately 2 g of the samples were weighed precisely into a vial and centrifuged (Sorvall RC-5B, Dupoint Company, USA) at 210 g (1500 rpm) and temperatures in the range of 2–5°C for 5 min. The WHC (%) is calculated as the ratio of water in the sample after centrifugation to water in the sample before centrifugation. Results were presented as an average of three measurements (Gudjónsdóttir, Arason , & Rustad, 2011).

3.8 Autolysis enzyme activity

For measuring autolysis enzyme activity, the following procedure was used: (1) 6 g of minced sample were taken, and 30 mL of phosphate buffer solution were added (pH=7, preparation: phosphate-KH_2PO_4 0.24g+Na_2HPO_4 1.42g+NaCl 8g+KCl 0.2 g dissolved in 500 mL distilled water, adjust the pH with 1mol/L NaOH solution, and then diluted to 1000 mL with distilled water). The mixture was shaken for 40 sec, and let to stand at 4°C for 6 h. (2) The solution was centrifuged at 4000 rpm for 8 min. 3.0 mL of each enzyme solution sample were added into a new tube, 3.0 mL of distilled water was taken as blank control. (3) The tube was placed in a constant temperature water bath at 30°C which was preheated for 5 min, and then 2.0 mL of 0.5% casein solution were added to each of them. The temperature of the water bath was set at 30°C, and the samples were accurately timed for heating for 10 min, where after 2.0 mL of 10% trichloroacetic acid (TCA) solution were added immediately to stop the protein denaturation reaction. (4) The solution was then centrifuged at 4000 rpm for 8 min, and 1.0 mL of supernatant was taken from each tube and dropped into a new sample tube, then 0.5 mL of 0.55 mol/L Na_2CO_3 solution and 0.5 mL of Folin-Phenol reagent were added in every sample tubes. The solution was then shaken, and put into a 30°C water bath for 15 min. (5) The absorbance (OD) of the sample was measured by a UV spectrophotometer at 650 nm (Xia, et al., 2009).

The relative enzyme activity was calculated as the relative enzyme activity to the relative protein content in the sample compared to an untreated control sample as follows,

$$Relative\ enzyme\ activity = \frac{Activity_{sample}/Activity_{control}}{Protein\ content_{sample}/Protein\ content_{control}} \times 100\%$$

3.9 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using Microsoft Office Excel 2016 (Microsoft Inc., USA) and the IBM SPSS Statistics 22 software.

4 RESULTS

4.1 Heating effects on the cooking yield of *C. frondosa*

The cooking yield of *C. frondosa* after various heating treatments is shown in Figure 4. It was obvious that the cooking yield decreased with increasing heating temperature and time. However, no significant differences in product weight were observed between 40°C boiled sea cucumbers and the raw material. While increasing the boiling temperature from 40°C to 100°C, the yield rate dropped rapidly from 100.3% to 71.5%. When prolonged cooking times at 100°C were applied, significant effects of the boiling and steaming treatments arose. The yield rate levelled off after boiling of the sea cucumber for 45 min, while the yield declined steadily to 64.6% after steaming for 120 min. In general, yield rates of the steamed samples were 3.5% lower than the boiled samples.

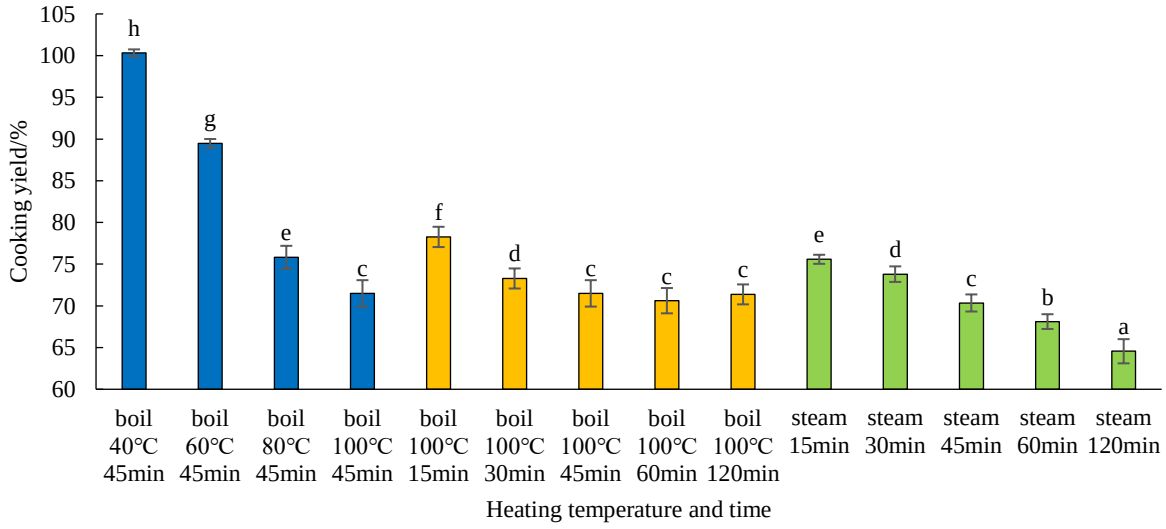


Figure 4. Cooking yield of *C. frondosa* after various heat treatments. Different letters (a,b,c,d,e,f,g,h) indicate significant differences between the treatments as determined via one-way ANOVA ($p < 0.05$). In specific, there is no significant difference between two groups with same letter.

4.2 Heating effects on the water of *C. frondosa*

4.2.1 Water content changes after different heating treatment

The water content of *C. frondosa* before and after the different heating temperatures and times is shown in Figure 5. The maximum water content was observed in the sea cucumber boiled at 40°C and 60°C for 45 min. The water content of the cooked (steamed and boiled) *C. frondosa* was significantly negatively correlated with the cooking temperature, but a positive correlation between the water content and time was observed when the sea cucumber had been boiled for 45 min or more at 100°C. This was believed to be due to rehydration of the sea cucumber body wall during boiling. However, the water content decreased gradually during the steaming period. A minimum water content (79.7%) was observed in the sea cucumbers steamed for 120 min, which was a significantly lower water content than in the other treatments ($p<0.05$).

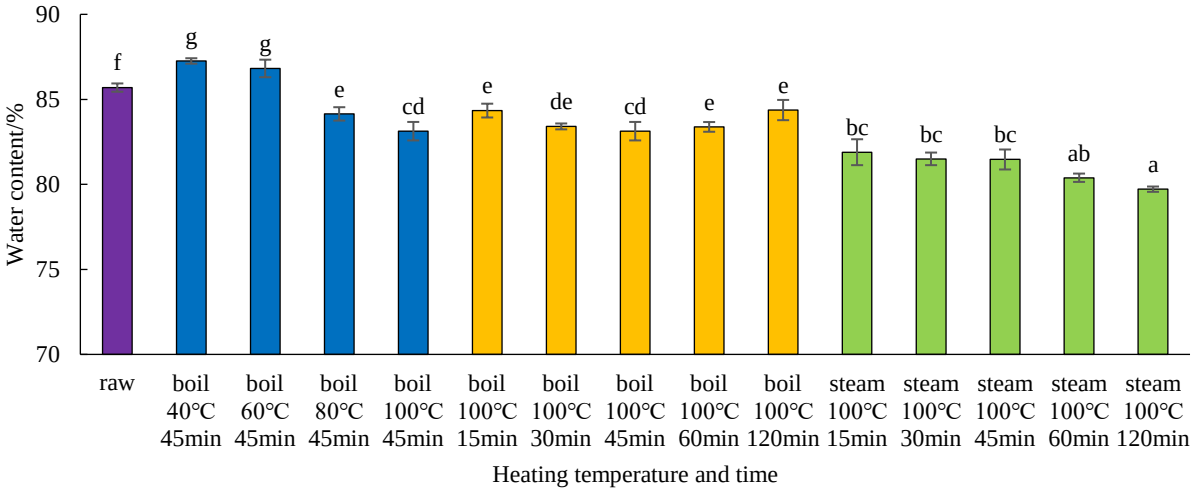


Figure 5. Water content of *C. frondosa* after various heat treatments. Different letters (a,b,c,d,e,f,g) indicate significant differences between treatments as determined via one-way ANOVA ($p < 0.05$).

4.2.2 Water holding capability changes after different heating treatment

The water holding capacity (WHC) of *C. frondosa* as affected by the different heating procedures is shown in Figure 6. Both of heating temperature and time exerted significant effects on WHC ($p<0.05$). A higher WHC of *C. frondosa* was obtained when shorter heating times and lower heating temperatures were applied. This indicated that protein degradation and microstructure change occurred when the boiling temperature exceeded 80°C, and the heating time exceeded 45 min at 100°C, which lowered the WHC significantly. This was especially evident in the boiled sea cucumbers, which showed significantly lower WHC ($p < 0.05$) than the steamed sea cucumber when heated at 100°C for 30 min or more.

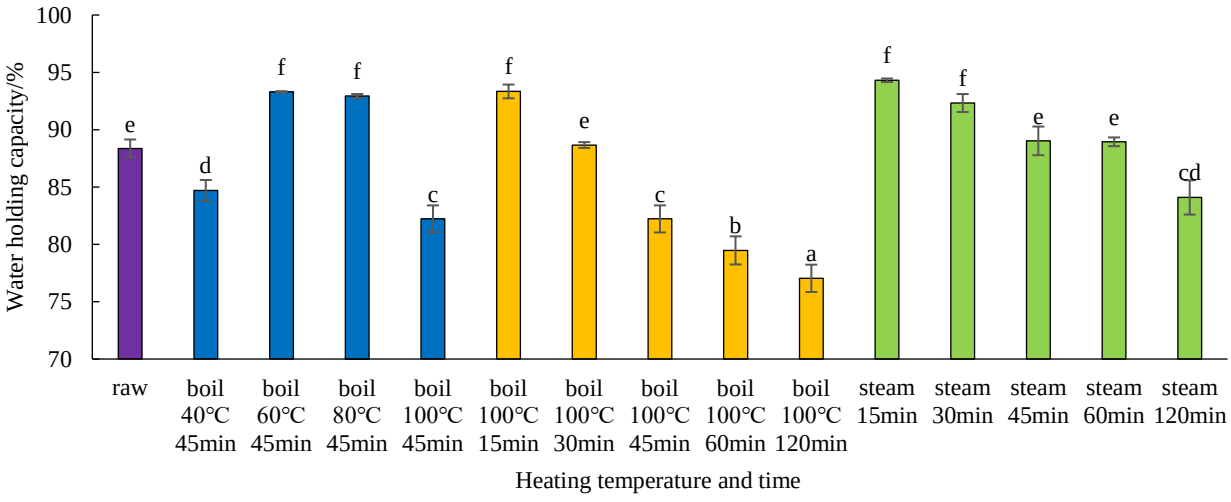


Figure 6. Water holding capacity of *C. frondosa* after various heat treatments. Different letters (a,b,c,d,e,f) indicate significant differences between treatments as determined via one-way ANOVA ($p < 0.05$).

4.3 Heating effects on the protein denaturation of *C. frondosa*

4.3.1 Crude protein content changes after different heating treatment

Figure 7 presents crude protein content of *C. frondosa* on a dry basis before and after cooking. Results showed that elevation in boiling temperatures led to a raise in protein content. This was due to the moisture release from the *C. frondosa* into the boil liquid while cooking. This caused a drop in both cooking yield and water content, which in turn lead to an increase in relative protein content of the samples. When the sea cucumbers were boiled and steamed at 100°C, the crude protein content increased for the first 30 min, followed by a decrease at prolonged heating. This could be caused by a joint effect of moisture and protein loss into the cooking liquid.

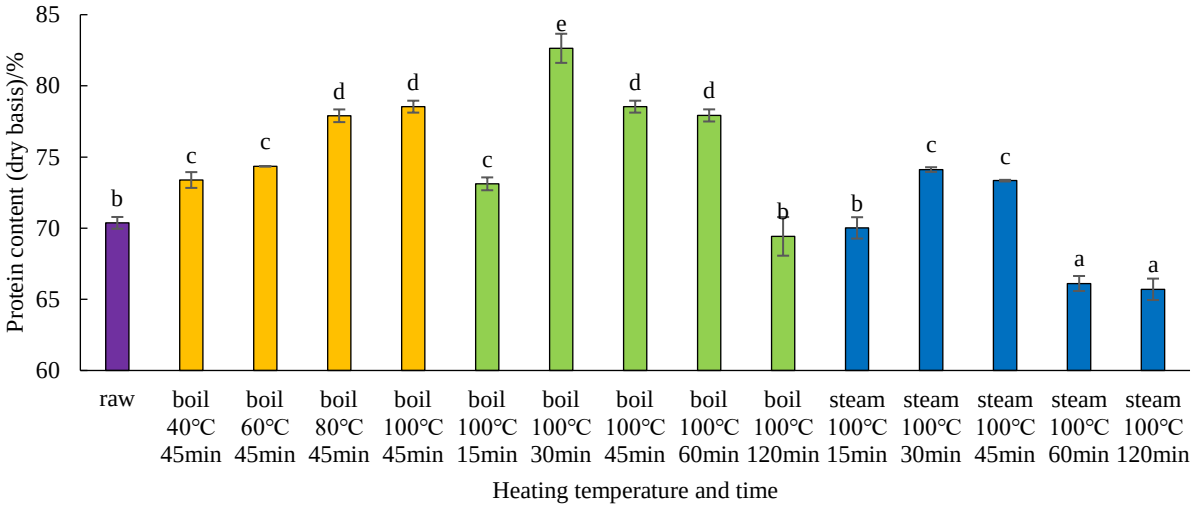


Figure 7. Protein content (dry basis) of *C. frondosa* after various heat treatments. Different letters (a,b,c,d,e) indicate significant differences between treatments as determined via one-way ANOVA ($p < 0.05$).

4.3.2 TCA-soluble nitrogen produced after different heating treatment

TCA-soluble nitrogen was formed during the different heating treatments as presented in Figure 8. TCA-soluble nitrogen includes amino acids and small peptides, and according to the results, did the content of TCA-soluble nitrogen grow with increased heating temperature and time in general. This is believed to be because TCA-soluble nitrogen can form during protein denaturation. When the mass transfer between the material and the cooking liquid was taken into account, was the TCA-soluble nitrogen content of the samples boiled at 60°C for 15 min significantly different from that of the raw material, which illustrated that protein degradation had already begun.

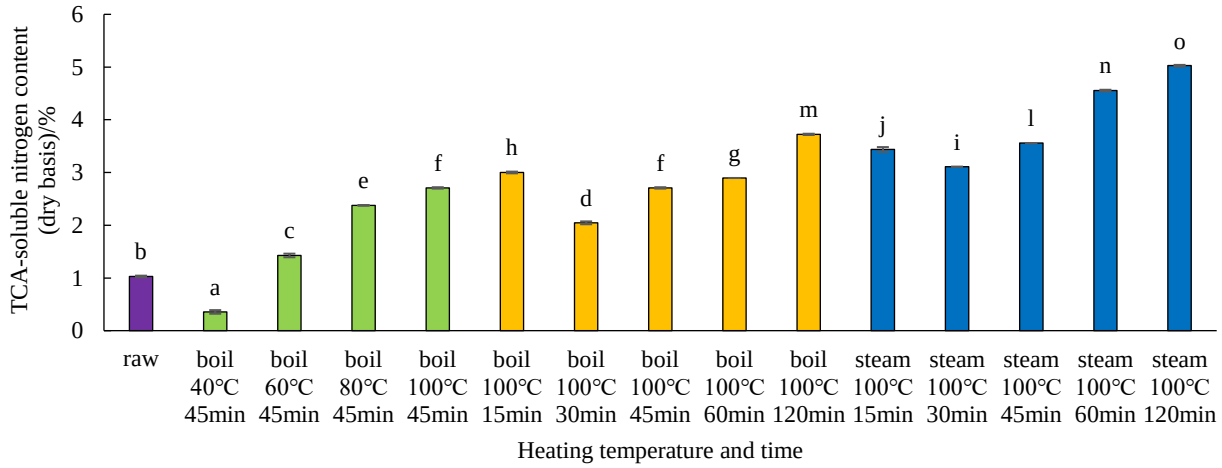


Figure 8. TCA-soluble protein content (dry basis) of *C. frondosa* after various heat treatments. Different letters (a,b,c,d,e,f,g,h,i,j,k,l,m,n,o) indicate significant differences between treatments as determined via one-way ANOVA ($p < 0.05$).

4.3.3 Electrophoresis SDS-PAGE after different heating treatment

In order to identify the underlying mechanisms of protein degradation, proteins were extracted from the cooked samples and analysed by electrophoresis SDS-PAGE, as presented in Figures 9 and 10.

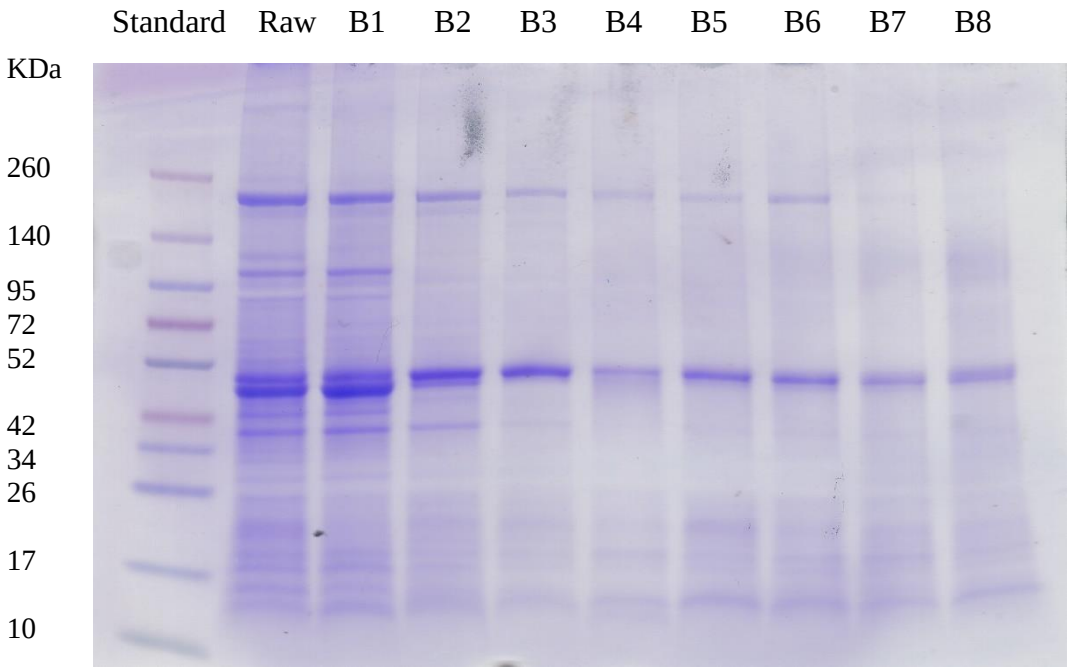


Figure 9. Protein degradation analysed by SDS-PAGE boiled *C. frondosa*. B1: boiled at 40℃ for 45 min; B2: boiled at 60℃ for 45 min; B3: boiled at 80℃ for 45 min; B4: boiled at 100℃ for 15 min; B5: boiled at 100℃ for 30 min; B6: boiled at 100℃ for 45 min; B7: boiled at 100℃ for 60 min; B8: boiled at 100℃ for 120 min.

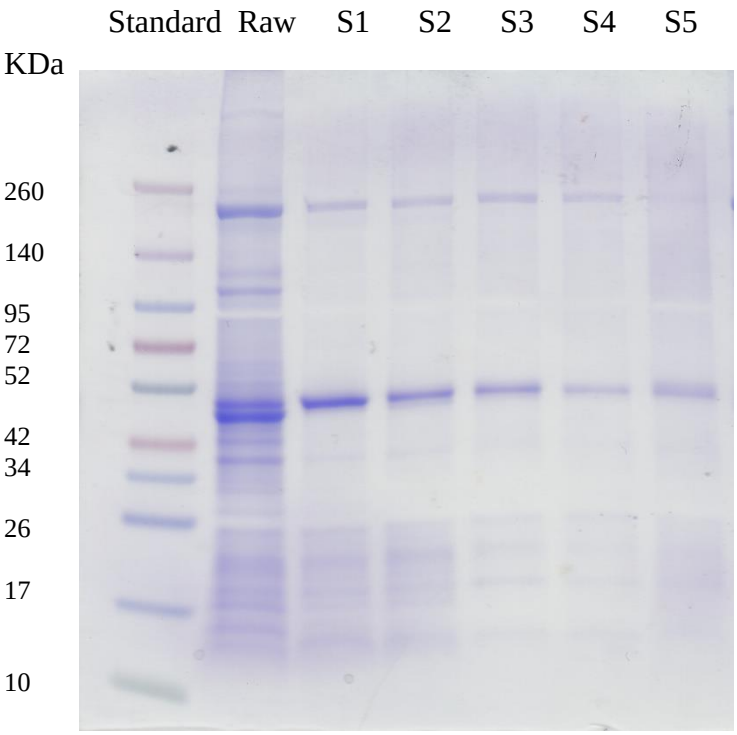


Figure 10. Protein degradation analysed by SDS-PAGE steamed *C. frondosa*. S1: steamed at 100℃ for 15 min; S2: steamed at 100℃ for 30 min; S3: steamed at 100℃ for 45 min; S4: steamed at 100℃ for 60 min; S5: steamed at 100℃ for 120 min; BL: boiled liquid collected from boil at 100℃ for 60 min; SL: steamed liquid collected from steam at 100℃ for 60 min.

The SDS-PAGE pattern revealed three major proteins with molecular weights (Mw) of 200 kDa (Band I), 52 kDa (Band II), and 44 kDa (Band III), which might be associated with the two same structural proteins as can also be observed in *Stichopus japonicus*: i.e. major yolk protein (MYP), and actin (Qi, et al., 2016). Overall analysis revealed that, although MYP seemed relatively intact, except after being boiled or steamed for 120 min, degradation of actin occurred already at heating temperatures of 60°C.

The cooking liquids were also collected and analysed, on one hand, the dry material in liquids were calculated to be 2.5-3.5%，this means about 3% of the raw material go into cooking liquid. On the other hand, we could see clearly what protein was released into cooking liquids and degrade during cooking.

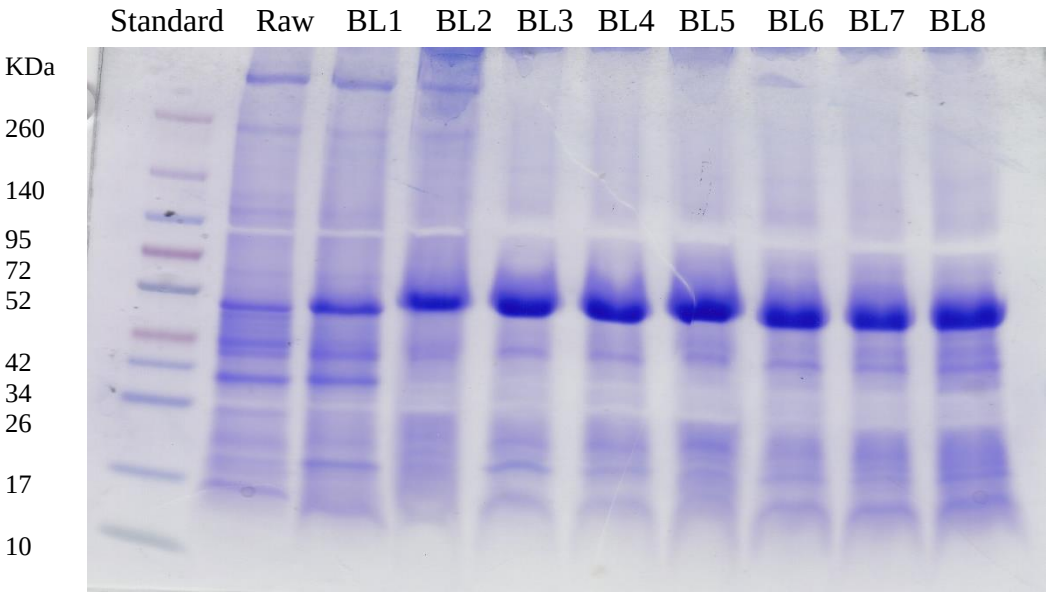


Figure 11.Boiling liquid analysed by SDS-PAGE. BL1: boiled at 40℃ for 45 min; BL2: boiled at 60℃ for 45 min; BL3: boiled at 80℃ for 45 min; BL4: boiled at 100℃ for 15 min; BL5: boiled at 100℃ for 30 min; BL6: boiled at 100℃ for 45 min; BL7: boiled at 100℃ for 60 min; BL8: boiled at 100℃ for 120 min.

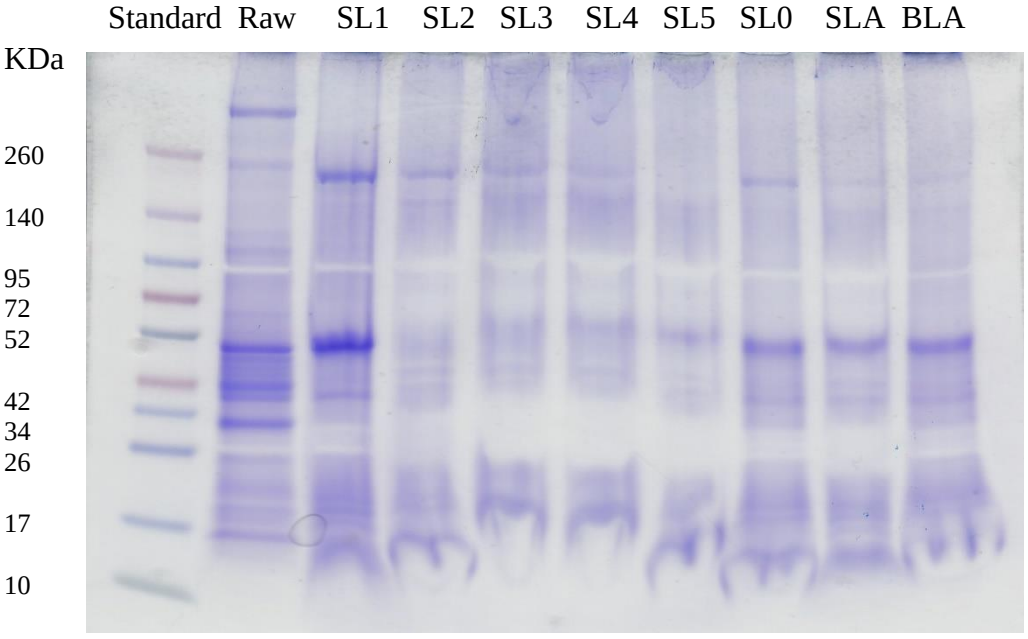


Figure 12. Steaming liquid analysed by SDS-PAGE. SL1: steamed liquid at 100℃ from 0-15 min; SL2: steamed liquid at 100℃ from 15-30 min; SL3: steamed liquid at 100℃ from 30-45 min; SL4: steamed liquid at 100℃ from 45-60 min; SL5: steamed liquid at 100℃ from 60-120 min; SL0: steamed liquid at 100℃ from 0-60 min; SLA: steamed liquid at 100℃ from 0-120 min; BLA: boiled liquid at 100℃ from 0-120min.

4.4 Heating effects on the water distribution in *C. frondosa*

The water distribution and characteristics were studied by low field NMR relaxometry. All relaxation data was maximum normalized prior to any further data analysis. A Principal Component Analysis (PCA) was then performed on the normalized relaxation data (Figure 13) to assess the variety in water characteristics between the samples.

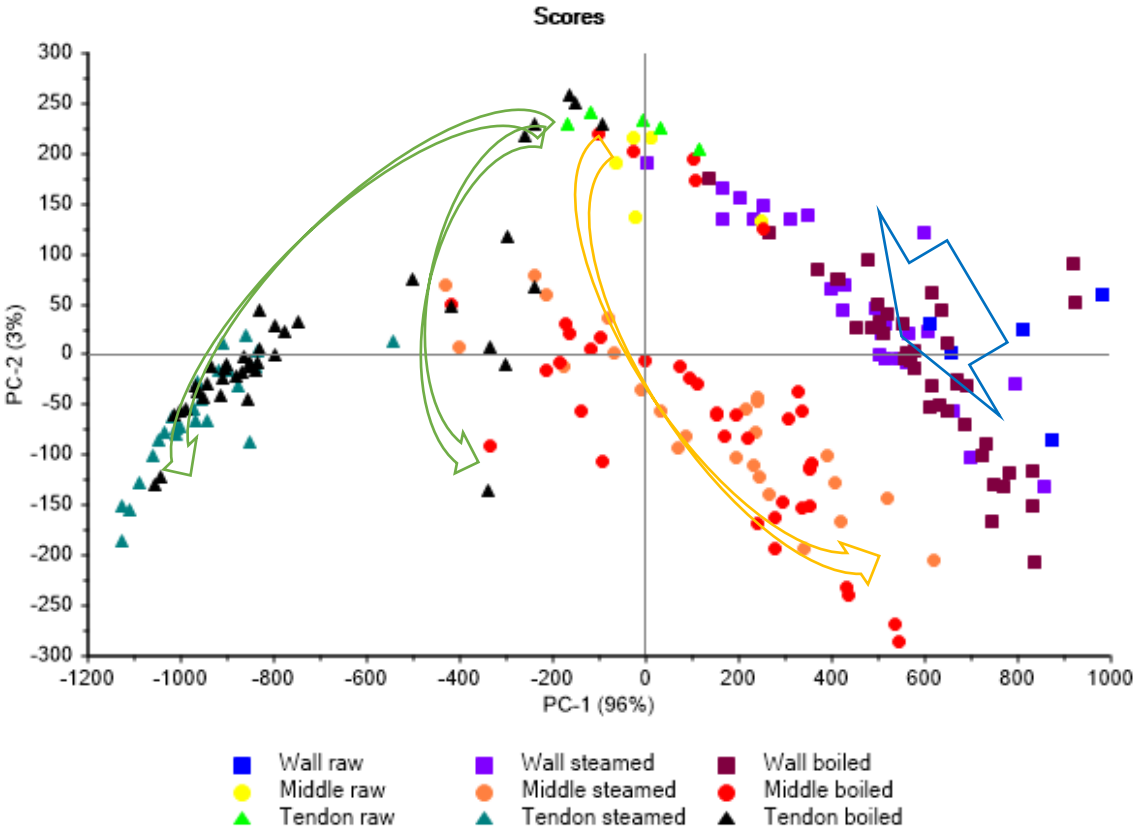


Figure 13. PCA of all LF-NMR samples in the main trial. The thick blue arrow indicates the overall effect of heating and heating time on the sea cucumber wall (boxes), the orange curved arrow the heating effect on the middle part of the sea cucumber (circles), and the green curved arrow indicated the overall effect of increased temperature and heating time on the tendon samples (triangles).

The first two principal components described 96% and 3% of the sample variation, respectively with regards to the LF-NMR response. The wide distribution of samples in the PCA indicated that the heating processes had quite different effects on the different parts of the sea cucumbers, and that a more detailed analysis was required of the relaxation behaviour of each part of the sea cucumbers. Therefore, discrete fittings of the relaxation data to a multi-exponential model were performed to gain a more detailed view of the characteristics and distribution of protons within the samples.

The relaxation time analysis indicted significant differences in the water distribution of the three sampling locations. Two to four proton populations were observed in the samples, including a fast relaxing component T_{21}, corresponding to water in close relation with macromolecules, such as proteins, an intermediate component T_{22} associated with intracellular water and entrapped water, a less restricted component T_{23}, mainly corresponding to extracellular water, and a small population of free water T_{24} in some of the samples. The changes in the relaxation times (T_{2i}, i=1-4) of these water populations, and their apparent ratios (A_{2i}, i=1-4) with steaming and boiling duration at 100°C, as well as the effects of different boiling/heating temperatures are presented in Figures 14-16.

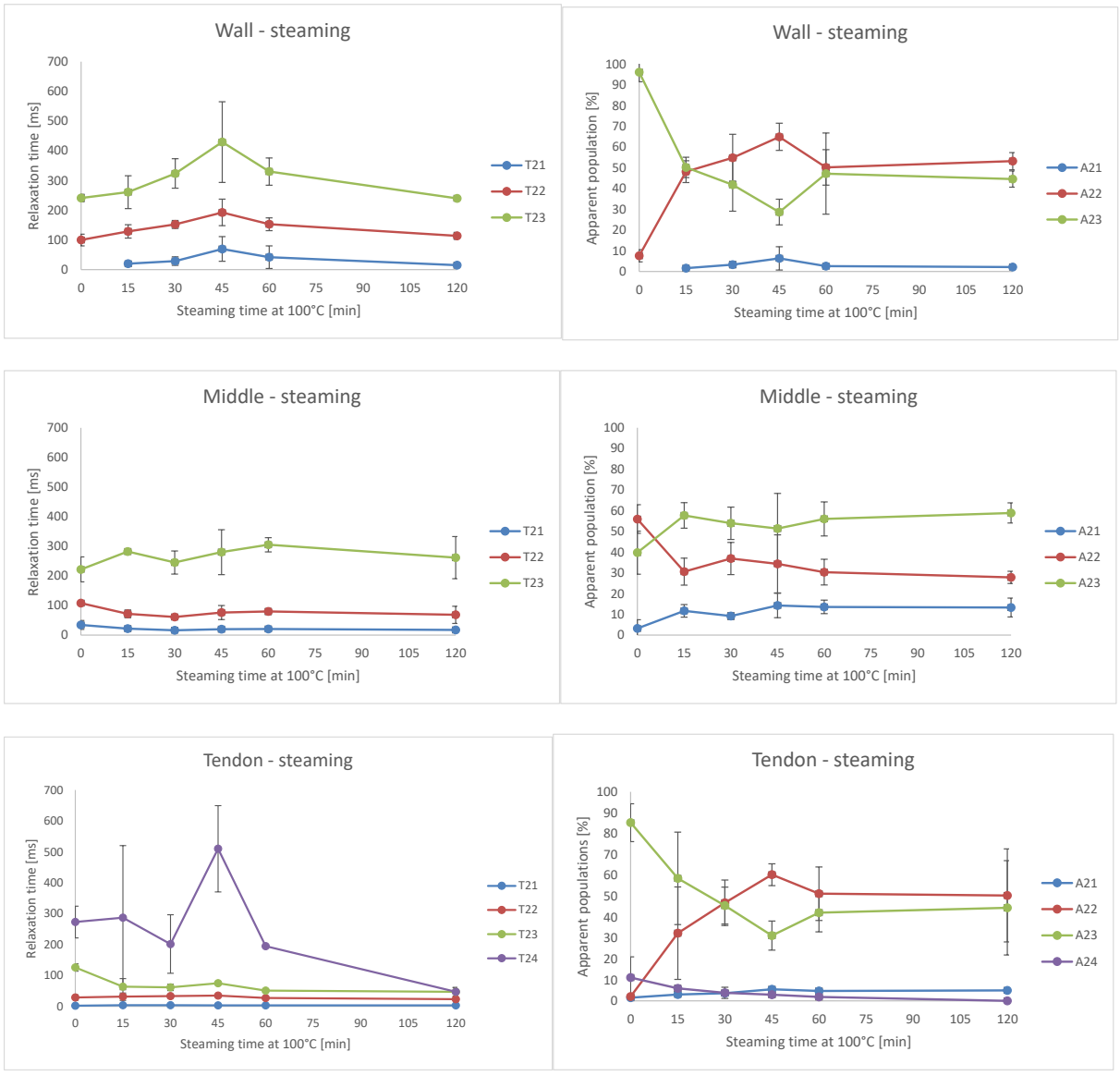
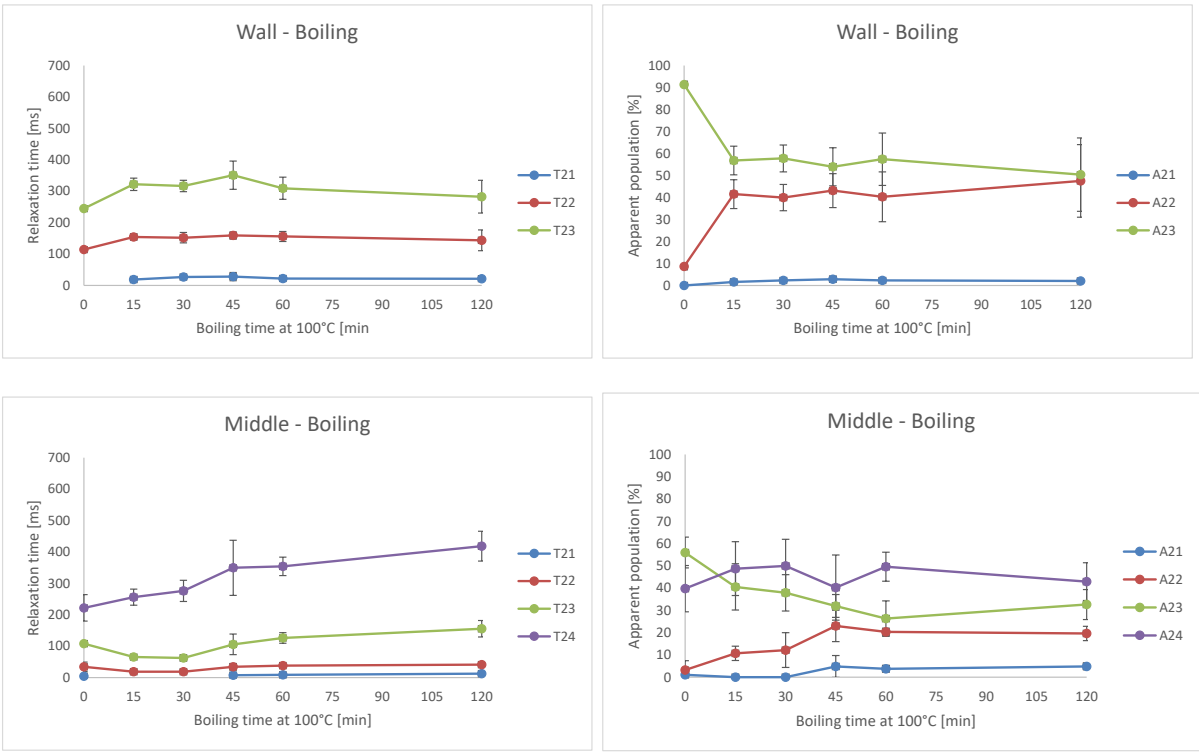


Figure 14. NMR relaxation time results (T21, T22, T23) and their apparent populations(A_{21}, A_{22}, A_{23}), indicating the heating effects on the *black wall* (top), *middle part* (middle) and *tendon part* (bottom) of the sea cucumber during *steaming at 100°C* for up to 120 min.

The NMR relaxation parameters were relatively stable in the middle part, except for a clear exchange of water between the two intermediate populations in the first 15 min of steaming, towards less restriction of the water ($A_{22} \rightarrow A_{23}$). This behaviour was also observed even stronger in the body wall and the tendon parts, indicating that these parts were more vulnerable towards water loss and denaturation. Furthermore, a small fourth water population A_{24} with free water was observed in the tendon part only. However, this population decreased and disappeared with prolonged steaming. These results are in agreement with the decrease in total water content in the sea cucumber, but the NMR analysis indicated furthermore that this water was mainly lost from the least restricted water populations A_{23} and A_{24}, leading to a proportionally higher water content in the intracellular water (A_{22}) population instead as the steaming process was prolonged. This exchange of water within the A_{22} and A_{23} populations may also partially be because increased temperatures leads to higher mobility of molecules, including water. No significant changes were observed in the relaxation times in the middle part, indicating that the steaming did not denature the proteins, or other components, significantly in this part of the sea cucumber.

Analysis of the relaxation times indicated a peak in all relaxation times in the black body wall for the first 45 min of steaming, and a similar increase in the T_{24} relaxation time in the tendon part. The increase in relaxation times during the first 45 min of steaming can be related to protein denaturation in the sea cucumber body wall and tendons. The results further indicate that this protein denaturation has a particular effect on the less restricted water populations, and that the tendons are especially vulnerable towards this protein denaturation during steaming.

Similar effects of the heating duration were observed when the sea cucumbers were boiled (Figure 15). However, a smaller variation in the relaxation times was observed in the boiled samples, indicating less protein denaturation compared to the steamed samples. This refers particularly to the least restricted water populations T_{23} and T_{24} in the body wall, and tendons.



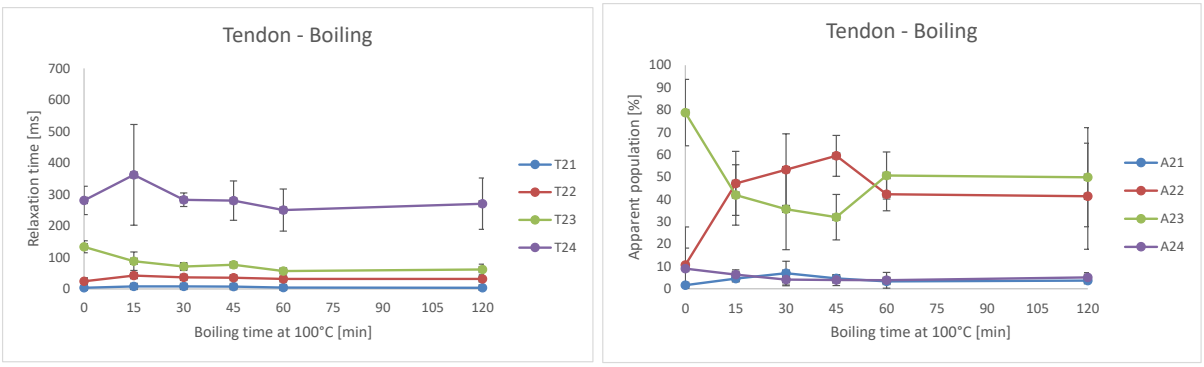


Figure 15. NMR relaxation time results (T21, T22, T23) and their apparent populations (A_{21}, A_{22}, A_{23}), indicating the heating effects on the *black wall* (top), *middle part* (middle) and *tendon part* (bottom) of the sea cucumber during *boiling at 100°C* for up to 120 min.

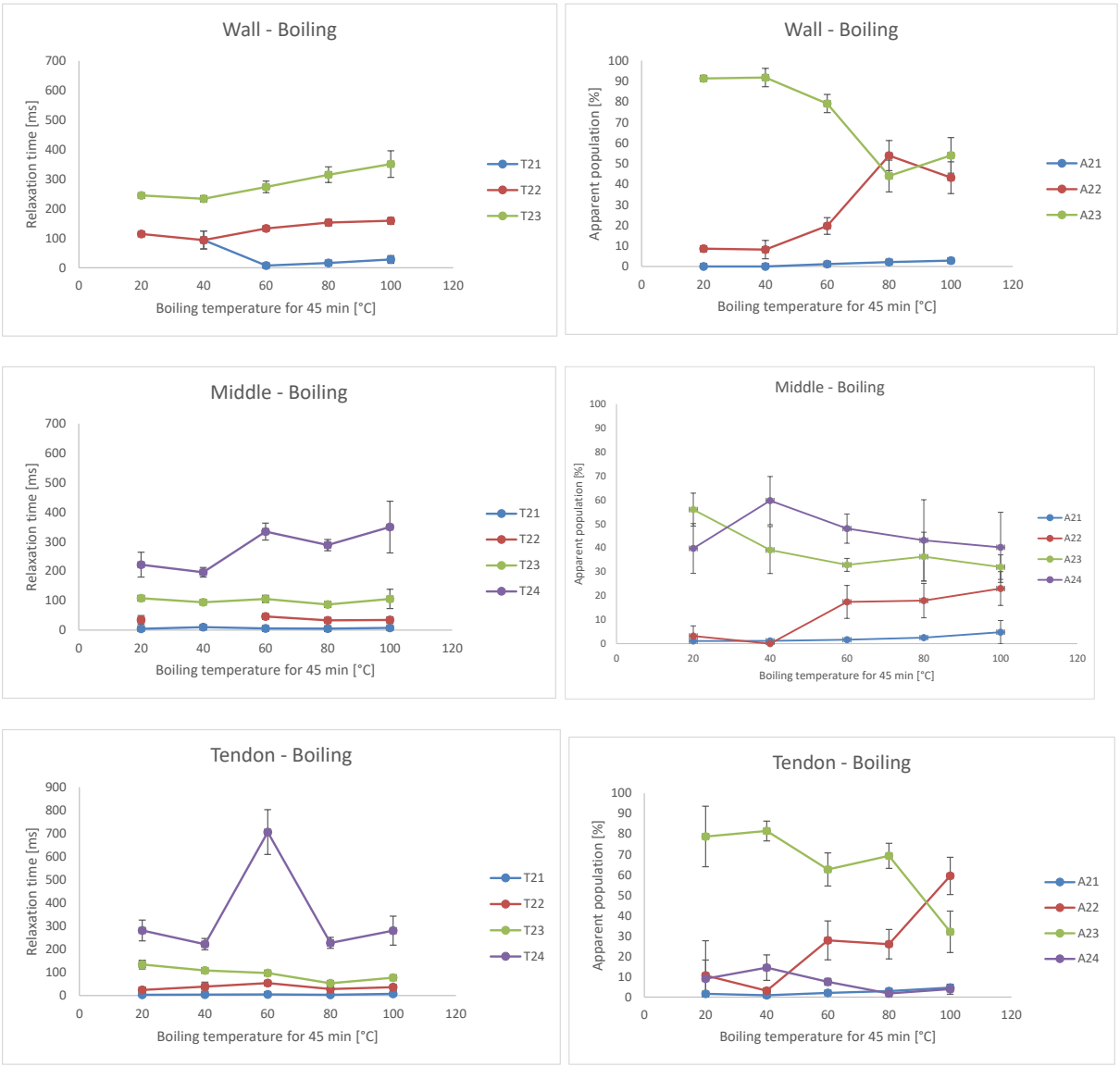
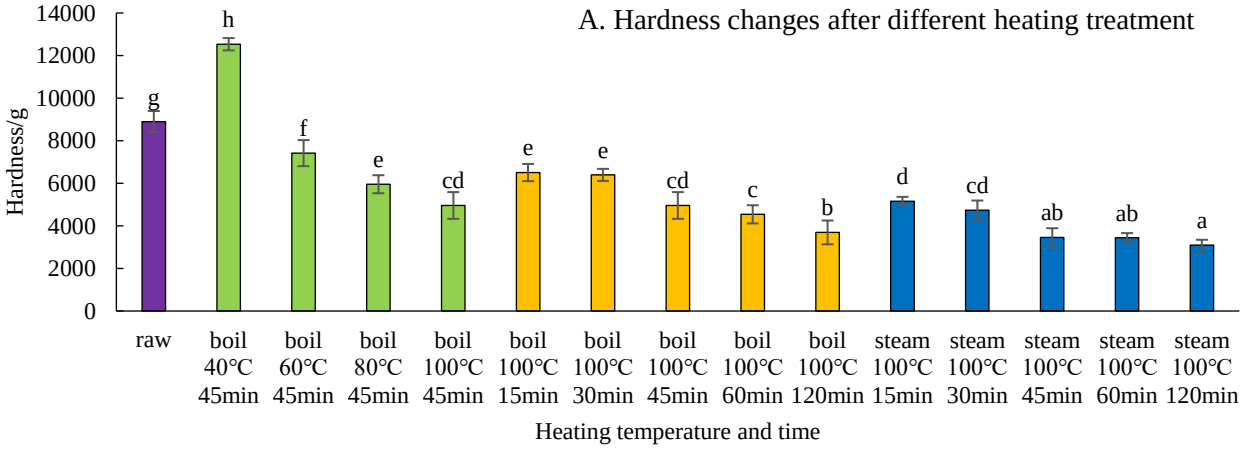
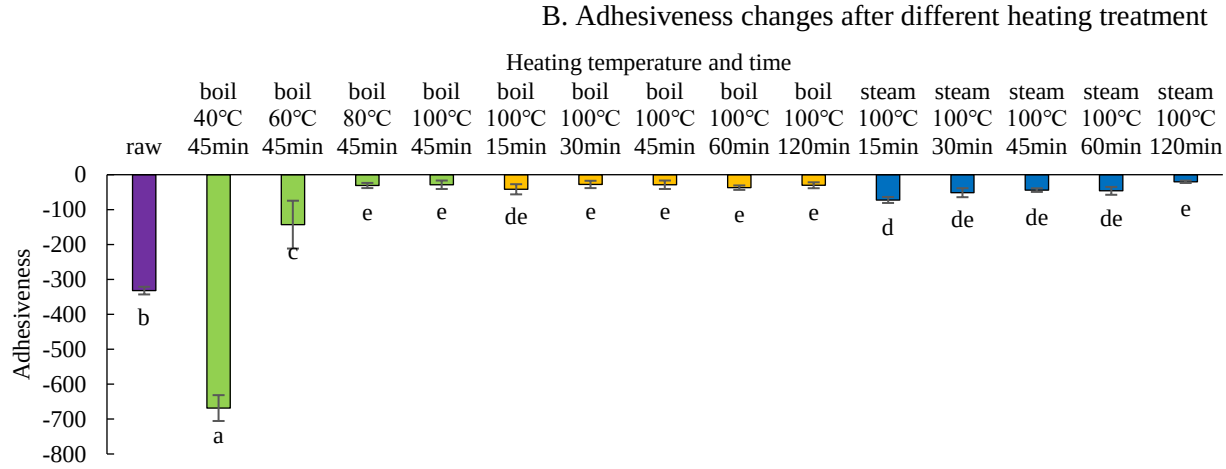


Figure 16. NMR relaxation time results (T21, T22, T23) and their apparent populations (A21, A22, A23), indicating the effects of increasing temperature on the *black wall* (top), *middle part* (middle) and *tendon part* (bottom) of the sea cucumber during *boiling for 45 min*.

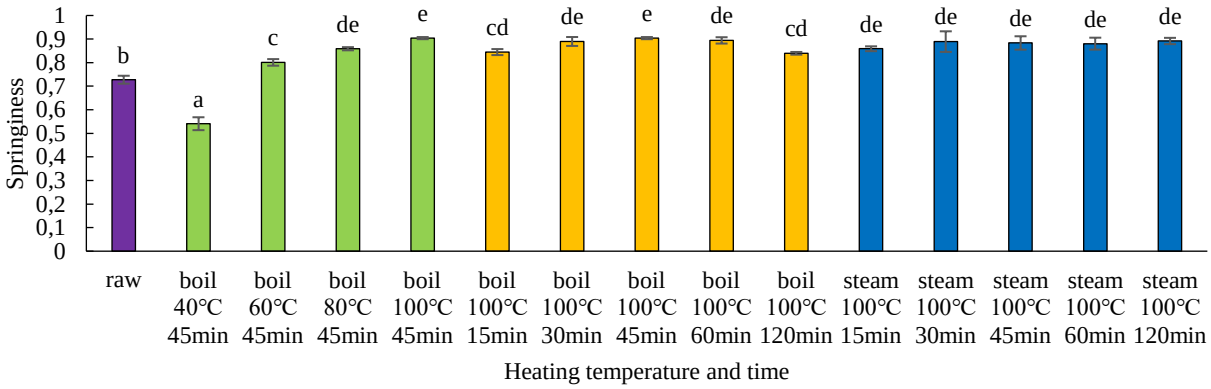
4.5 Heating effects on the texture of *C. frondosa*

Texture Profile Analysis (TPA) indicated a clear effect of varying the cooking temperature and time on the texture quality of the sea cucumber product (Figure 17). The raw *C. frondosa* was thick, firm and sticky, with a relatively high value of hardness and adhesiveness, while low in springiness, cohesiveness, chewiness, and resilience, which was quite similar to TPS results obtained on *Stichopus japonicus* (Bi, et al., 2016). After boiling at 40°C for 45 min, the hardness and adhesiveness in the sea cucumbers significantly rose. However, when the temperature was elevated to 60°C, the hardness and adhesiveness dropped, accompanied by significant increase in springiness, cohesiveness, chewiness, and resilience. The hardness, adhesiveness and chewiness decreased while resilience increased with the increase in heating temperature and time. Heat treatment on the dermic layer of sea cucumbers can cause changes in the cytoskeleton structure and muscle collagen which may lead to texture variation. Heat-induced shrinkage in the dermic layer resulted in the observed increase in toughness, while after a sufficient heating period, the dermic layer of *C. frondosa* became soft and ductile again. After boiling or steaming for 45 min at 100°C, the hardness and chewiness decreased significantly, while extended cooking times did not give rise to any additional obvious changes to the texture parameters. As a consequence, cooking at 100°C for 45 min was viewed to be sufficient for *C. frondosa* to obtain desirable texture.

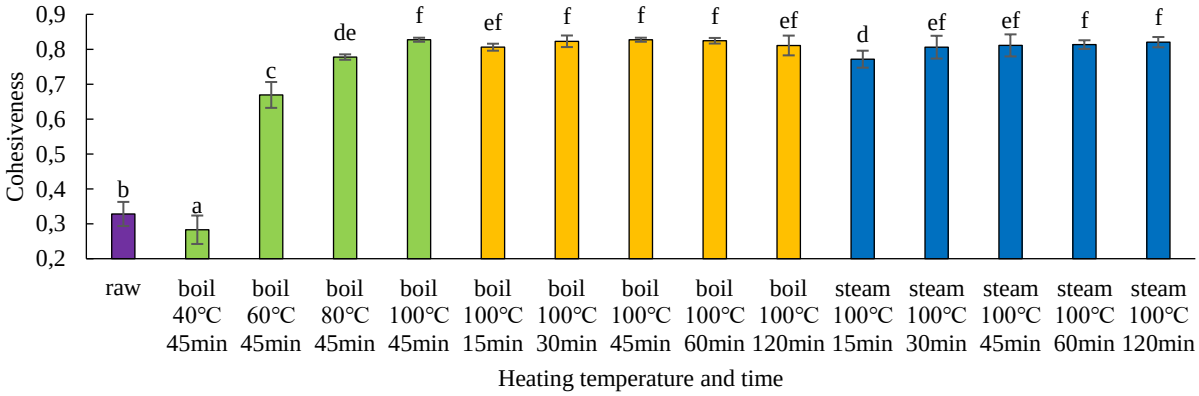




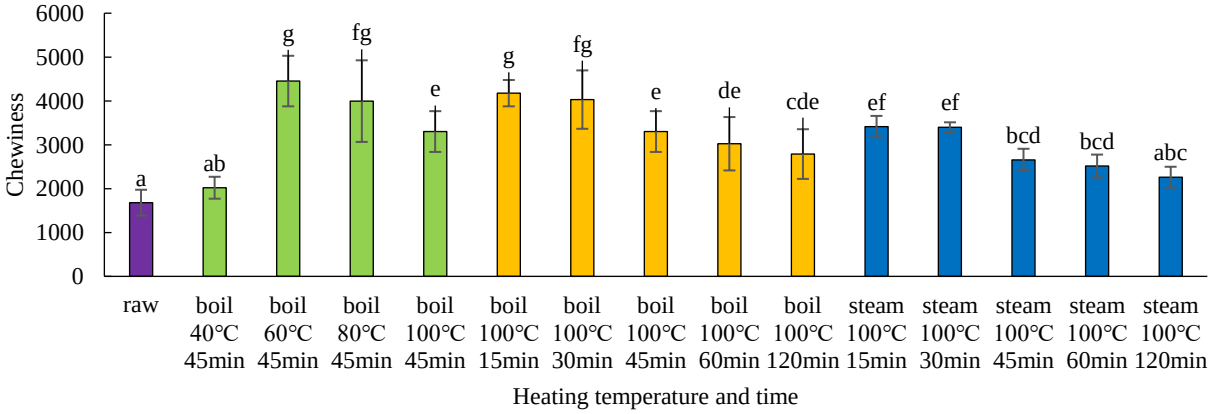
C. Springiness changes after different heating treatment



D. Cohesiveness changes after different heating treatment



E. Chewiness changes after different heating treatment



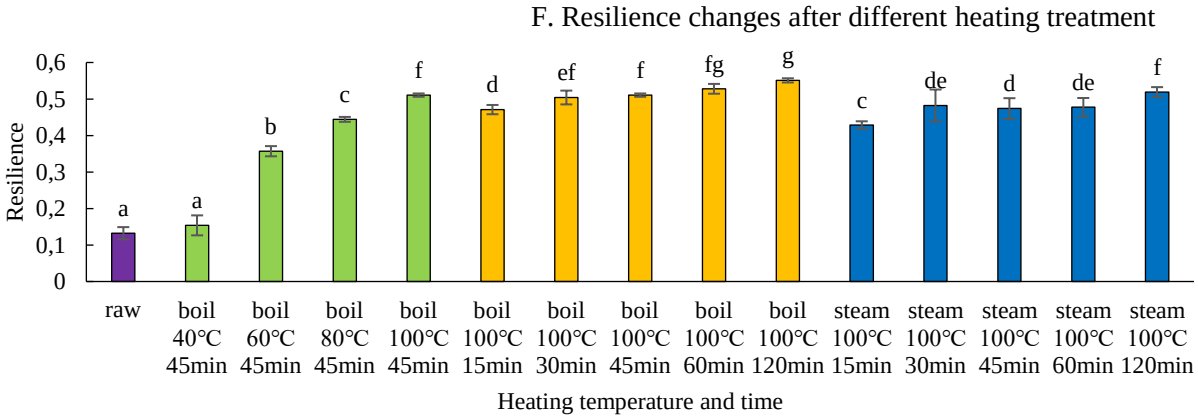


Figure 17. Texture of *C. frondosa* after various heat treatments. Different letters (a,b,c,d,e,f,g,h) indicate significant differences between treatments as determined via one-way ANOVA ($p < 0.05$).

4.6 Heating effects on the autolysis enzyme activity of *C. frondosa*

Enzyme denaturation is one of the most essential indexes for sea cucumber processing. The relative enzyme activity of the cooked samples is shown in Figure 18. Aparently, the autolysis enzyme of *C. frondosa* was of high stability, and only got inactived when subjected to high temperature and long heating periods. The minmum relative enzyme activity was 62%, which was obtained at 100°C boiled for 120 min, but this treatment differed significantly from other heating treatment ($p<0.05$) with regards to enzyme deactivation. In the steamed groups, longer heating treatment did not cause enzyme inactivation, when prolonging the steaming time from 15 min to 120 min, resulted in a relative enzyme activity in the range from 74 to 78%.

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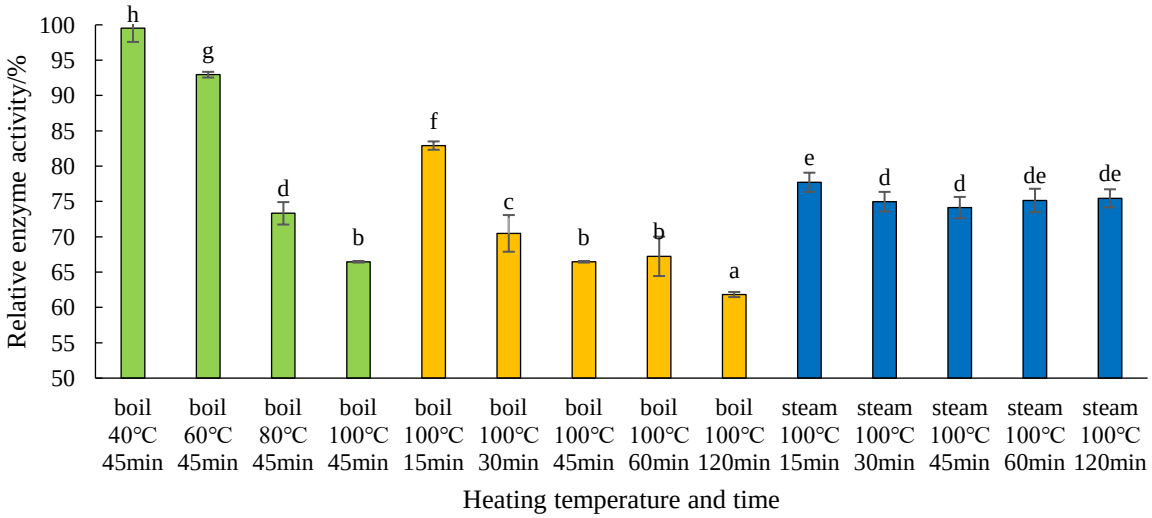

Figure 18. Relative enzyme activity of *C. frondosa* after various heat treatments. Different letters (a,b,c,d,e,f,g,h) indicate significant differences between treatments as determined via one-way ANOVA ($p < 0.05$).

4.7 Multivariate analysis

The effects of different heat treatments on parameters were taken into multivariate analysis, shown in Figure 19. Cooking at 40°C for 45min did not cause protein denaturation, so the test result was similar with those of the raw material. The steam treatment exerted similar effects with boiling for longer time. Cooking yield, hardness and water content of the raw and 40°C boiled samples were higher than other samples. With the increase in temperature, crude protein content, WHC grew higher. Then when prolong the heating duration, the springiness, adhesiveness, resilience and TCA-soluble nitrogen were increased.

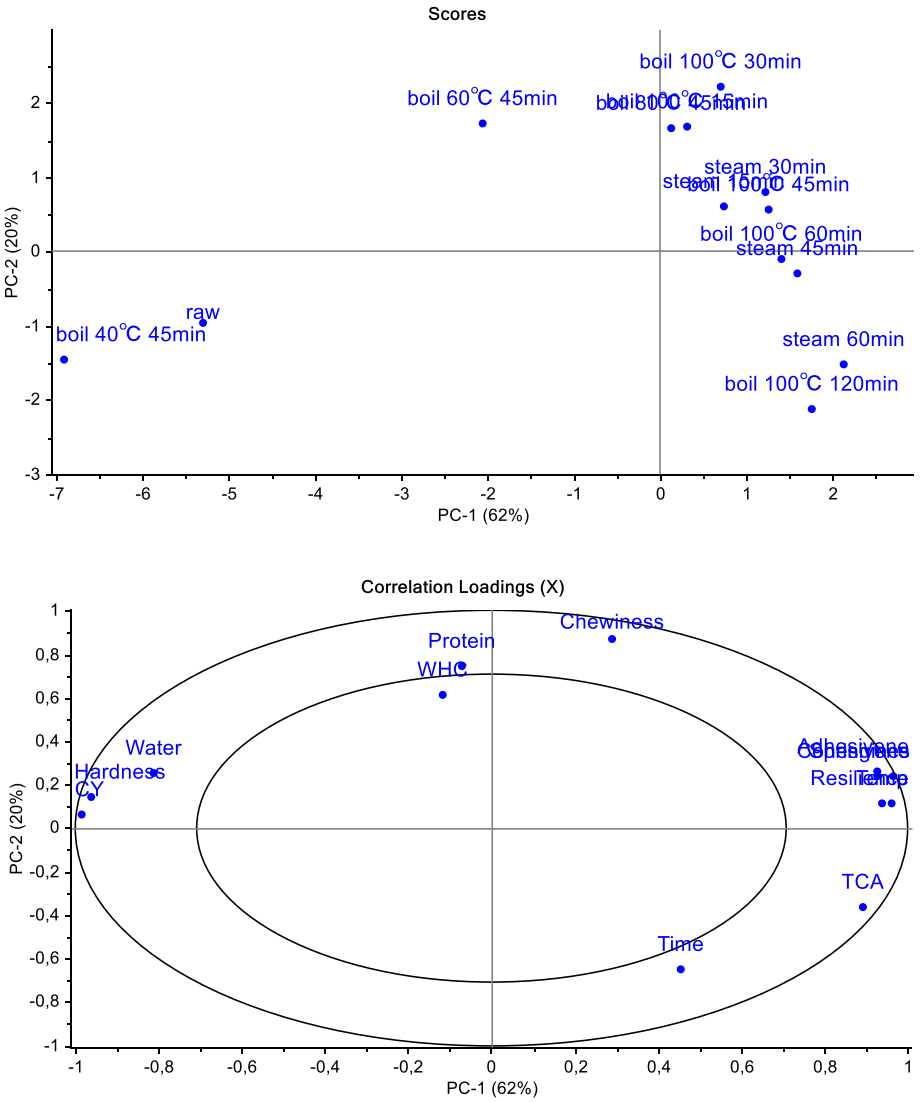

Figure 19. Principal Component Analysis of all variables, except NMR parameters.

5 DISCUSSION

5.1 Heating effects on the protein denaturation of *C. frondosa*

Heat-induced protein denaturation is a phenomenon that involves transformation of a well-defined, folded structure of a protein, formed under physiological conditions, to an unfolded state under non-physiological conditions. To determine the procedure of proteins degrading of *C. frondosa*, different heating methods were adopted and effects of different heating temperature and duration were analysed. During the mildest heat treatment, namely boiling at 40°C for 45 min, the TCA-soluble nitrogen value was observed to be lower than in the raw material. That was because 40°C was not high enough to cause protein denaturation. When the temperature was increased, the result of TCA-soluble nitrogen, SDS-Page and LF-NMR revealed obvious protein degradation increasing with cooking temperature and duration, which led to continuous TCA-soluble nitrogen formation (**Error! Reference source not found.**). Bi *et al*. reported that the proteins of *Stichopus japonicus* denatured at 49.7°C, which is consistent with the result in this study (2016).

5.2 Effect of protein denaturation on the texture and water distribution changes of *C. frondosa*

Results showed the various influence of protein denaturation on the texture parameters and water distribution. Protein denaturation at 60°C caused a significant drop in cooking yield and rise in water holding capacity, which corresponded to the reports on *Stichopus japonicus, Apostichopus japonicus* and beef semitendinosus muscle (Chen, 2009; Zhang, *et al*., 2016; Chang, *et al*., 2011). The conclusions were similar that heating induced protein denaturation as well as muscle fiber coagulation and aggregation, which resulted in the increase of hardness, cohesiveness and chewiness. But when keep on increasing the temperature or prolonging the heating time, there was an obvious decline in WHC, hardness and the least restricted water populations.

When the sea cucumber was heated for a longer time at 100°C, mass transfer and heat transfer between the dermic material and heating medium were intensified. The increasing TCA-soluble nitrogen and higher number of small fractions in the SDS-PAGE gels of the boiled and steamed liquid also illustrated that protein degraded into peptides and amino acids in the sea cucumbers, and that the gelation tissue structure was weakened. This process resulted in a change in microstructure and properties of the *C. frondosa* dermic. The water content declined together with crude protein content, and both contributed to the cooking yield drop after long heat treatment. An enlarged interspace also indicated a significant reduction in water holding capacity, hardness and chewiness. Based on these results, cooking at 100°C for 45 min were sufficient for *C. frondosa*.

5.3 Comparison of boiling and steaming treatment on *C. frondosa*

Heating is inevitable in a sea cucumber production process. Boiling is most commonly used, while nowadays steamed instant sea cucumber products are gaining popularity. Furthermore, research on *Apostichopus japonicus* have shown that more nutrients remain in the product when steaming is applied instead of boiling (Hou Z. , 2015). In the present study, boiling and steaming experiments at 100°C were conducted on *C. frondos*a.

The cooking yield and water content of the boiled samples were higher than that of the steamed samples generally. The results indicated that there was more possibility for the boiled samples to take up water from the boiling liquid. However, during steam cooking, the surface of the sea cumber dried out. Absorption from the boiling liquid led to a higher free water proportion in the cooked sample, and significant lower water holding capacity.

Results showed that crude protein content is higher and TCA-soluble nitrogen is lower in boiled samples compared to steamed samples, which was different from earlier results obtained for *Apostichopus japonicus* (Hou Z. , 2015). There seemed to be a higher protein degradation level in the steamed samples. It is often assumed that heat transfer is better with water as a heating medium, rather than with air. However, water greatly facilitates thermal denaturation of proteins, and might therefore not be an optimal processing step for all products. It is worth to mention that in this experiment the dry basis content was used instead of wet basis to present a specific component content, which was not the case for the research of Hou (2015). That might explain some differences in results.

Result of the texture properties illustrated that the steamed samples were softer and more resilient after same cooking time (Figure 17. Texture of *C. frondosa* after various heat treatments. Different letters (a,b,c,d,e,f,g,h) indicate significant differences between treatments as determined via one-way ANOVA ($p < 0.05$). No significant differences were observed in adhesiveness, springiness or cohesiveness. Since a moderately soft and resilient texture is more popular among consumers, 45 min were viewed to be a sufficient heating time for *C. frondosa* cooking at 100°C.

Autolysis enzyme of *C. frondosa* was relatively stable. The activity was continuously declined during boiling process, while it kept stable when prolonged the steaming time from 30 min to 120 min. Compared with steaming treatment, boiling duration had a greater impact on enzyme inactivation. The enzyme inactivation pattern was consistent with that of *Apostichopus japonicus* (Hou Z. , 2015). Even when boiled at 100°C for 120 min, the relative enzyme activity was still as high as 61.8%. That is why the sterilization condition for canning product in Iceland could be 121°C for 2.5 min, while the Chinese producer prefer 20 min or even 30 min at 121°C. The heating time of the Chinese products is prolonged in order to inactivate enzyme rather than to kill bacteria.

6 CONCLUSION AND RECOMMENDATIONS

This study showed that boiling and steaming, as two major heating methods of sea cucumber processing, had significant effects on the water distribution, protein denaturation, and texture properties of the *C. frondosa* dermic.

The TCA-soluble nitrogen and LF-NMR parameters indicated that the protein denaturation was obvious when the temperature reached 60°C. The denaturation of proteins led to changes in water holding capacity, crude proteins, and TCA-soluble nitrogen content, and then resulted in different texture properties and enzyme activity. While taking all these variables into account, proper heating condition should be concluded as boiled or steamed at 100°C for no less than 45 min.

Analysis method of WHC, electrophoresis SDS-Page and relative enzyme activity were primarily proposed for *C. frondosa*.

Even though the SDS-Page results could explain the protein denaturation procedure to some extent, clear protein bands were not determined with precision. Therefore, to expound the protein denaturation mechanism, further research is needed on the determination of the degraded and stable proteins, and how they could cause changes in water distribution, and texture properties. Besides, the methods applied in the current study, differential scanning calorimetry (DSC) and scanning electronic microscopy (SEM) should be used to measure the denaturation temperature and the microstructure of *C. frondosa* further.

Quality of the raw material needs to be taken into consideration. Raw materials of low quality were used in the pre-trail. The *C. frondosa* was frozen directly after being caught and about 20% of the individuals were not totally intact, autolysis was obvious because of the holes on the dermic layer of the sea cucumber. Then in the second batch the raw materials were bled after being caught and then frozen. They were in relatively good quality when they arrived, and the individuals were much smaller in size than in the first batch. However, it also turned out that the samples had not been stored in proper way, because the autolysis had still taken place subtly and slowly. After one month of frozen storage at -25℃, 10% of the raw materials were observed to have holes on the dermic layer. The autolysis problem is always viewed as a bottleneck in sea cucumber production, and thus is further research needed to find the best way to store the raw material of *C. frondosa*.

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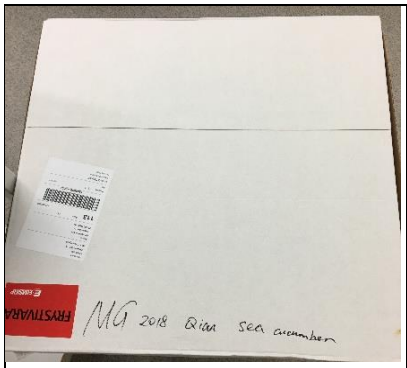
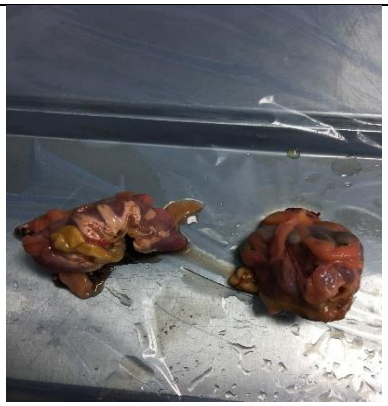
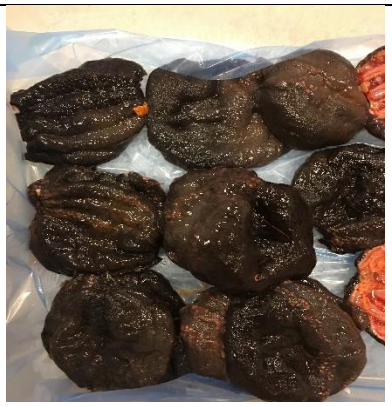
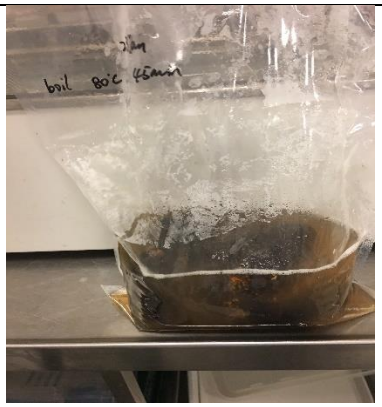
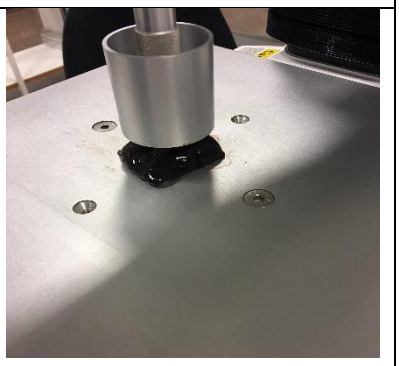
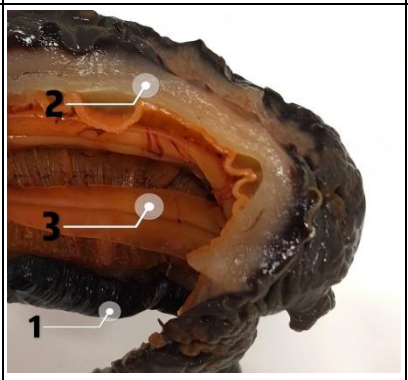
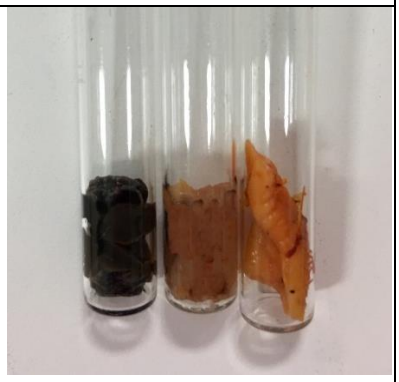
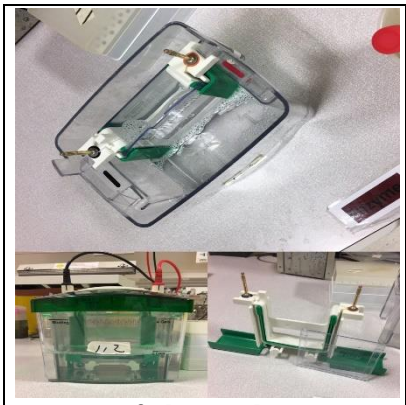
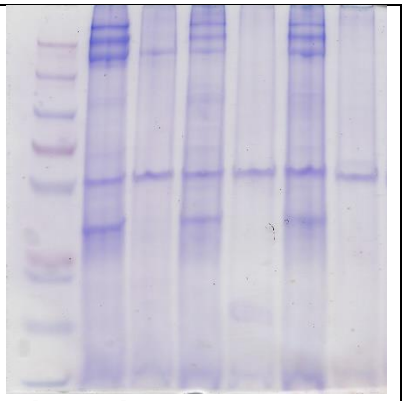
APPENDIX 1.

Experimental pictures

 Frozen sea cucumber was received by 27th Dec 2017	 Frozen block	 Frozen blocks were broken into small ones
 Raw material was thawed	 Crown of oral tentacles was removed	 Gutted sea cucumber dermis
 Cleaned sea cucumber (abdomen)	 Cleaned sea cucumber (backside)	 Samples were put into plastic bags and boiled in water bath

 Boiled samples (abdomen)	 Boiled samples (backside)	 Steamed samples (abdomen)
 Steamed samples (backside)	 Samples was cut into 3cm× 3cm for TPA test	 TPA test with P50 probe
 Samples after TPA test	 Black part; 2. Middle white part; 3. Orange tendon part for LF-NMR	 Three different parts were tested separately in LF-NMR

 Device of electrophoresis SDS-PAGE	 Protein was extracted and subjected to electrophoresis SDS-PAGE	 Electrophoresis SDS-PAGE 1: standard; 2,4,6: raw; 3;5;7: cooked samples