Pressurized Hot Water Hydrolysis of Shrimp Shell and Chicken Feet for Value-added Compounds Production

by

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ABSTRACT

The interest in the valorization of animal by-products such as shrimp shell and chicken feet has been growing as a sustainable approach to produce valuable biopolymers such as chitosan and collagen, that is also in line with the sustainability concept. These biopolymers could find applications in the food industry to develop sustainable products or functional food ingredients. Acid and enzymatic treatments have been conventionally utilized to produce chitosan and collagen from animal by-products with some limitations as they are not eco-friendly, time consuming, and costly processes. Therefore, the main objective of this thesis research was to obtain chitosan-rich residue and collagen/collagen fragments from animal by-products using subcritical water assisted by ultrasound and pressurized hot water technology, respectively. In the first study, shrimp shell was deproteinized using subcritical water assisted by ultrasound. Then, the deproteinized shrimp shell was demineralized, bleached, and deacetylated to obtain chitosan-rich residue. The nitrogen and free amino acids contents of the hydrolysates, and the degree of deproteination, yield, degree of deacetylation, color, functional groups, crystallinity, and surface morphology of chitosan-rich residue were determined. Subcritical water assisted by ultrasound (1200 W) at 180ºC/50 bar/60 min resulted in the highest nitrogen content of 99.01 mg/g shrimp shell. However, the highest degree of deproteination (80.93%) and the highest free amino acid content (70.92 mg/g shrimp shell) were obtained by subcritical water assisted by ultrasound (1200 W) at 260°C/50 bar/60 min. At this condition, chitosan-rich residue with a yield of 10.56%, whiteness index of 60.42, degree of deacetylation of 64.27% with similar functional groups to the alkali treated sample and the commercial sample were obtained. It had a lower relative crystallinity (32.66%) compared to the alkali treated sample (50.64%) and the commercial chitosan (50.52%), indicating its better solubility. In the second study, pretreated chicken feet were processed with pressurized hot water

at 40-180°C and 50 bar for 10-60 min. The content of collagen/collagen fragments, degree of hydrolysis, amino acid profile, and molecular weight distribution were determined. The best condition to obtain collagen/collagen fragments of 152.04 mg/g chicken feet was 140°C/50 bar/10 min. At all conditions investigated, hydroxyproline, lysine and glycine were the main free amino acids in the hydrolysates. Increasing the temperature and time increased the degree of hydrolysis where a maximum of 11.64% was obtained at 180°C/50 bar/60 min. The results of molecular weight distribution indicated that collagen was the main component at temperatures of 40-140°C while at higher temperatures of 160°C and 180°C collagen was hydrolyzed to gelatin and collagen fragments, respectively. These results indicated that pressurized hot water technology is promising to valorize animal by-products towards production of valuable-added compounds.

Keywords: Chicken feet, chitosan, collagen, pressurize hot water, shrimp shell, sustainability.

PREFACE

This thesis is an original research work performed by Mashaer Matouri and has been written as per the guidelines provided by the Faculty of Graduate Studies and Research at University of Alberta. The concept of the research work in this thesis originated from my supervisor Dr. M.D.A. Saldaña. This thesis consists of 5 chapters, where Chapter 1 provides the introduction and objectives while Chapter 2 summarizes the literature review.

Chapter 3 of this thesis has been presented as "Matouri M. and Saldaña M.D.A. Effect of pressurized fluid assisted by ultrasound processing on hydrolysis of shrimp shell" at the 13th International Symposium on Supercritical Fluids in Montreal, Quebec, Canada in May 2022. I was responsible to design and perform the experiments, collect the data, and interpret the results, and write the manuscript. Dr. Saldaña provided the project idea, advice on the experimental design, helped in discussing the results, and provided the financial support.

In Chapter 4, Dr. Saldaña provided the research idea and contributed to the experimental design, data discussion and revisions of the thesis. I was responsible to the design and performing the experiments, data collection, analysis, and thesis writing. Dr. Fernando performed the SDS-PAGE analysis.

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ABBREVIATIONS

ABTS	2,2'-Azino-bis [3-Ethylbenzothiazoline-6-Sulfonic acid]
BSA	Bovine serum albumin
DH	Degree of hydrolysis
DDA	Degree of deacetylation
EDTA	Ethylene diamine tetra acetic acid
FTIR	Fourier transform infrared spectroscopy
FAO	Food and agricultural organization
HPLC	High performance liquid chromatography
M_W	Molecular weight
MALDI-TOF MS	Matrix assisted laser desorption/ionization time of flight mass spectrophotometry
NR	Not reported
OPA	o-Phthalaldehyde
PHW	Pressurized hot water
RC	Relative crystallinity
sCW	Subcritical water
SEM	Scanning electron microscope
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC-GPC	Size exclusion-gel permeation chromatography
UV/Vis	Ultraviolet/visible wavelength
VCEAC	Vitamin C equivalent antioxidant capacity

WS	Water solubility
XRD	X-ray diffraction

Chapter 1: Introduction

1.1. Rationale

Sustainability has become a critical challenge due to environmental concerns posed by food waste together with feeding the growing global population. Annually millions of tons of seafood and meat by-products are generated at different stages of food production worldwide. These by-products have valuable biopolymers such as polysaccharides and proteins, and nutrients like essential amino acids, and minerals. For example, bovine blood consists of 80.9% water, 17.3% protein, 0.23% lipid, 0.07% carbohydrate, and 0.62% minerals (Toldrá et al., 2012). Also, seafood by-products have a wide variety of nutrients and biomolecules. For example, shrimp shell has high protein (49%) and ash (27%) contents and low fat (4.5%) (Bruno et al., 2019) and carotenoid (0.015%) contents (Prameela et al., 2017).

Besides, there is an increasing interest in the use of natural and sustainable ingredients in food production due to the consumer rejection on the use of synthetic compounds as food ingredients. Also, the demand for functional foods has been growing. Currently, valorization of food by-products is a promising solution not only to minimize food waste, and mitigate environmental problems associated with disposal of by-products, but also to produce biopolymers and biomolecules, and re-utilize them as valuable raw materials to produce high value-added products (Saldaña et al., 2015). In this regard, polysaccharides such as chitin and chitosan and the structural fibrous protein like collagen and their respective hydrolysates, including peptides, can be recovered from seafood and meat by-products, and re-used in food, cosmetic, and pharmaceutical industries.

Crustacean family comprises the largest proportion of the seafood industry worldwide (Ambigaipalan & Shahidi, 2017). Among this family, the interest in shrimp consumption has been

increasing noticeably during the past two decades due to its flavor and nutritional value (Ambigaipalan & Shahidi, 2017). In 2020, around 5 million tons of shrimp were harvested worldwide, and this amount is forecasted to reach 7.28 million tons by 2025, with a growth rate of 6.1% per year (Nirmal et al., 2020). During shrimp processing, generated solid waste, including shells and heads, accounts for 45-48% of the shrimp weight that is considered inedible shrimp by-products (Ambigaipalan & Shahidi, 2017). While shrimp shells could serve as a potential source of valuable compounds such as chitin, chitosan, essential amino acids, astaxanthin, polyunsaturated fatty acids, and α-tocopherol (Nirmal et al., 2020), just a minor portion of shrimp processing by-products are used as animal feed and fertilizers, the rest are dumped in landfills, causing serious environmental pollution (Bruno et al., 2019).

The major component of the crustacean's shell like shrimp is chitin that is a long amino-polysaccharide polymer chain that contains N-acetylglucosamine units connected by β-1,4-glycoside bonds (Irastorza et al., 2021; Arbia et al., 2013). Chitosan, β-(1–4)-2- amino-2-deoxy-β-D-glucan, is a positively charged amino polysaccharide, which is obtained by partial deacetylation of chitin. Chitin and chitosan have been reported as an antimicrobial, antioxidant, anti-proliferative and immunity-enhancing active compounds (Nouri et al., 2016). Chitin and chitosan have been explored for encapsulation of flavoring agents and in food formulations as thickening, stabilizing, and antimicrobial agents (Bruno et al., 2019).

According to the report from Food and Agricultural Organization (FAO), the global chicken meat production was 137 million tonnes in 2020. Where, United States was the leading chicken meet producer (20.5 million tonnes) followed by China (15 million tonnes) and Brazil (13.7 million tonnes). In Canada, 1.3 million tonnes chicken were produced in Canada in 2020, where 125 tonnes of the chicken were produced in Alberta (Statistics Canada). Feather, blood,

bones, tendons, feet, skin, and liver are produced as by-products during poultry processing. The consideration of these by-products as edible or inedible depends on the culture of the consumers. In Asian, South African, Jamaican, Peruvian, and Philippines cuisine, chicken feet are consumed as traditional meal and street food (Potti & Fahad, 2017), while in other countries like Brazil chicken feet are considered as an inedible by-product (Santana et al., 2020). A small amount of this by-product is used for animal feed production and unfortunately the rest is landfilled or incinerated (Mrázek et al., 2019). While chicken feet contain 17.42-21.58% protein mainly collagen, and 3.9-12.04% fat. This by-product is a potential source to produce nutritional compounds (Liu et al., 2001; Potti & Fahad, 2017; Dhakal et al., 2018). Thus, utilization of such valuable by-products is a potential solution to the environmental concerns.

Collagen is a helical protein that consists of three α-chains which are twisted together and stabilized by covalent crosslinks and intra-/inter chain hydrogen bonds (Cao et al., 2020). Each α-chain has a molecular weight of 100 kDa (Potti & Fahad, 2017; León-López et al., 2019), and is composed of a repeating tripeptide of glycine-proline-hydroxyproline. Collagen is a macromolecule with molecular weight bigger than 250 kDa (Irastorza et al., 2021). Collagen could be converted into gelatin by thermal treatment, resulting in partial cleavage of covalent and hydrogen bonds and eventually a helix to coil transition (Gómez-Guillén et al., 2011). Gelatin is a protein with molecular weight of 15-250 kDa (Irastorza et al., 2021). The structural and rheological properties of collagen make it an excellent biopolymer for food, pharmaceutical, and biomedical industries (Ahmed et al., 2020). Gelatin not only is used as stabilizer, thickener, and texturizer in food products but also is extensively used in encapsulation, and in bio-ink for 3D printing (Cao et al., 2020; Irastorza et al., 2021). It was reported that the collagen market reached US$ 4.2 billion in 2018 globally, where estimated to incraese up to US$ 6.6 billion by 2025 (Ahmed et al., 2020).

Collagen can be extracted from the animal by-products like skin, bones, and tendons. The use of beef and pork collagen is limited in halal and kosher products with the risk of bovine spongiform encephalopathy (Mrázek et al., 2019; Liu et al., 2001). Seafood sources especially fish by-products, including the skin, bone, scale, and viscera are considered as a safe, reliable, and alternative sources of collagen. However, fish collagen has lower thermal stability and weaker rheological properties compared to the bovine and porcine collagen, which limits its applications (Gómez-Guillén et al., 2011). Furthermore, its strong odor and the risk of affecting allergic consumers limits the use of fish collagen in food and pharmaceutical industries (Chakka et al., 2017). For this reason, poultry by-products have gained considerable attention as a potential source of collagen in the past decade.

A variety of conventional methods such as salt, alkaline, and acid treatments have been utilized for extraction of valuable biomolecules of the animal by-products and residues. However, these methods are inefficient in terms of long processing time (1 h-16 weeks), excessive use of chemicals such as hydrochloric acid and sodium hydroxide (concentrations of 30-98.6%), and low selectivity (Rombaut et al., 2014; Ziero et al., 2020). Moreover, government regulations on the use of toxic chemical solvents and the safety of the remaining solvent in the final products are getting stricter.

Conventionally, production of chitosan from crustacean shell involves three steps: (1) demineralization with acid solution (HCl); (2) deproteination using alkali solution (NaOH); (3) deacetylation using strong alkali treatment (NaOH) (Ifuku et al., 2009; Tolaimate et al., 2003; Al Sagheer et al., 2009; Aneesh et al., 2020). Al Hoqani et al. (2020) reported a yield of 4.7% of chitosan obtained from shrimp shell with conventional fractionation and deacetylation. However, the use of 0.25-1 M hydrochloric acid (Al Sagheer et al., 2009; Yen et al. 2009) is corrosive to the

equipment and requires chitosan purification and wastewater treatment. Organic acids such as 1.25 M acetic acid (Mahmoud et al., 2007), 0.2-0.8 M lactic acid (Mahmoud et al., 2007) and 0.5 M citric acid (Devi & Dhamodharan, 2018) have been used for demineralization. Citric acid with a concentration of 0.5 M resulted in 87.5% removal of calcium from shrimp shell (Ameh et al., 2014). The mineral content of shrimp shell decreased from 30.7% to 3.1% and 3.7% when treated with 1 M hydrochloric acid (1:50 w/v, 100°C, 1 h) and 0.8 M lactic acid (1:50 w/v, 100°C, 1 h), respectively (Mahmoud et al., 2007). In the study by Nouri et al. (2016), isolation of chitosan from shrimp shell involved deproteination with 2% sodium hydroxide (1:30 w/v, 80°C, 2 h), demineralization with 10% acetic acid (1:40 w/v, 50°C, 4 h), and deacetylation with 50% sodium hydroxide (ratio not reported) assisted by microwave with a power of 720 W for 20 s, where a yield of 19.47% of chitosan was achieved. Also, biotechnological approaches have been investigated for deproteination of shrimp shell. In the study by Younes et al. (2016), demineralization of shrimp shell using 0.5 M HCl at a ratio of 1:10 w/v at 4°C and stirring at 30 rpm for 30 min, then deproteinization with the use of two different crude enzymes *Bacillus mojavensis* A21 and *Scorpaena scrofa* in separate reactions (7.75 U/mg of A21 at 60°C for 6 h, pH 9.0 followed with 10 U/mg of *S. scrofa* crude proteases at pH 9.0 and 50°C for 3 h) resulted in 96% of deproteination. However, enzymatic treatment is a costly process because of utilizing enzymes and the main limitation of this method is the long processing times.

There are two main steps in the process of collagen isolation: (1) pre-treatment of raw material to remove non-collagenous compounds like fat and minerals (Gómez-Guillén et al., 2011), and (2) hydrolysis of collagen to break various inter/intra-molecular covalent crosslinks, ester bonds, and hydrogen bonds (Hong et al., 2019; Schmidt et al., 2016).

Traditionally, acid hydrolysis has been used to obtain collagen from chicken feet (Liu et al., 2001). In the study by Potti & Fahad (2017), acid hydrolysis (0.5 M acetic acid, 1:20 w/v, at room temperature for 48 h) of chicken feet resulted in collagen yield of 8.24%, while a collagen yield of 28.46% was obtained by acid hydrolysis (5% lactic acid, 1:8 w/v, 4°C, 36 h) from chicken feet (Liu et al., 2001). Also, enzymatic hydrolysis of chicken feet (1% w/v papain, 30°C, 28 h) resulted in 32.16% of collagen (Dhakal et al., 2018).

Subcritical water (sCW) processing has shown to be a promising and eco-friendly technology to recover valuable compounds from animal by-products. sCW refers to a thermodynamic condition of water at temperature between 100°C and 374°C under sufficient pressure (below 221 bar) to maintain it in the liquid state. Earlier, Quitain et al. (2001) produced amino acids from shrimp shells by hydrothermal treatment at temperatures between 90℃ and 400℃. The highest yield (70 mg/g dry shrimp shell) of amino acids (mainly glycine and alanine) was obtained at 250℃ in 60 min.

Another green alternative to conventional methods is ultrasound for extraction and hydrolysis of biomolecules, which uses sound waves at frequency ranges of 20 kHz to 1000 kHz (Zou et al., 2019). The spread of ultrasound waves in a liquid medium causes cavitation (Silva & Saldaña, 2020). Explosion of microbubbles, generated by the cavitation, on the cell wall of the biomaterials accelerate the penetration of the solvent into the matrix by breaking down the particles and increasing the porosity of the cell walls (Sicaire et al., 2019; Silva et al., 2020). Ultrasound is considered a rapid, clean, and affordable method in the food industry (Bruno et al., 2019). Recently, Schmidt et al. (2021) investigated the effect of ultrasound pre-treatment (1:15 w/v water, 400 W, 24 kHz, 30 min) on the yield of enzymatic hydrolysis (1:2.5 w/w pepsin-protein, 48 h, 4°C) of collagen from chicken meat residue. Collagen yield increased from 10.8 to 15.11%. In the

study by Pezeshk et al. (2022), protein recovery yield from shrimp shell also increased from 82.3 to 95.31% with the assistance of ultrasonication. In their study, alkalin hydrolysis from shrimp shell was performed by homogenizing shrimp shell (8000 rpm, 2 min) with cold distilled water (1:5 w/v), pH was adjusted to 12.5 with 2 N NaOH then kept on ice for 20 min. For the ultrasound, the slurry was sonicated at 300 W and 20 kHz for 30 min.

Based on the above, for the first time, the effect of sCW assisted by ultrasound at different powers, temperatures, and times on protein hydrolysis of shrimp shell was investigated. Additionally, the effect of pressurized hot water on chicken feet hydrolysis to obtain collagen/collagen fragments was studied.

1.2. Hypothesis

It was hypothesized that:

- Ultrasound pre-treatment will effectively assist sCW to hydrolyze shrimp shell to increase the yields of protein and total amino acids while leaving a chitosan-rich residue with high degree of deproteination.
- Pressurized hot water will effectively hydrolyze chicken feet to obtain collagen/collagen fragments with higher yield than the conventional acid treatment.

1.3. Objectives

The main objective was to obtain chitosan-rich residue and collagen/collagen fragments from animal by-products using sCW assisted by ultrasound and pressurized hot water technology, respectively. The specific objectives were:

1) Evaluate the effect of temperature and time on the protein and total amino acids yields obtained by deproteination of shrimp shell using sCW assisted by ultrasound technology (Chapter 3).

2) Obtain chitosan-rich residue from deproteinized shrimp shell by demineralization with citric acid, bleaching with hydrogen peroxide, and deacetylation with sodium hydroxide solution and compare its physico-chemical and structural characteristics with the control sample obtained by alkali deproteination and the commercial chitosan (Chapter 3).
3) Evaluate the effect of temperature and time on the yield of collagen/collagen fragments obtained by hydrolysis of chicken feet using pressurized hot water technology (Chapter 4).
4) Evaluate the effect of temperature and time on the molecular weight distribution of chicken feet hydrolysates obtained by pressurized hot water technology (Chapter 4).

Chapter 2: Literature review

2.1. Subcritical water technology

Subcritical water refers to a thermodynamic condition for water at temperature between 100°C and 374°C under sufficient pressure (below 221 bar) to maintain it in the liquid state (Fig. 2.1). Subcritical water is a cheap, non-toxic, and environmentally friendly solvent for valorization of food by-products (Valdivieso Ramirez & Saldaña, 2014). At room temperature and atmospheric pressure, water acts as polar solvent due to its substantial hydrogen bonding but under subcritical condition, hydrogen bonds of water tend to break and that results in significant changes in the physico-chemical properties of water (Álvarez-Viñas et al., 2021; Bruner, 2009), including decrease in viscosity, surface tension, dielectric constant and increase in water diffusivity (Rincón et al., 2021). Thus, under subcritical conditions, water can interact with nonpolar compounds as an organic solvent due to the decrease in its dielectric constant from 78 at 25°C and 1.0 bar to 14.08 at 350°C and 200 bar (Ziero et al., 2020).

The increase in the ion-product constant of liquid water (K_w) from 10^{-14} mol^2L^{-2} at ambient condition to 10^{-11} mol^2L^{-2} at 300°C at subcritical condition increases the concentration of H_3O^+ and OH^- in the medium. As a result, the chemical activity of acidic or basic compounds increases (Ziero et al., 2020). Also, the density fluctuations of water within subcritical thermodynamic condition favor energy and mass transfer rate during the extraction/hydrolysis processes (Ziero et al., 2020).

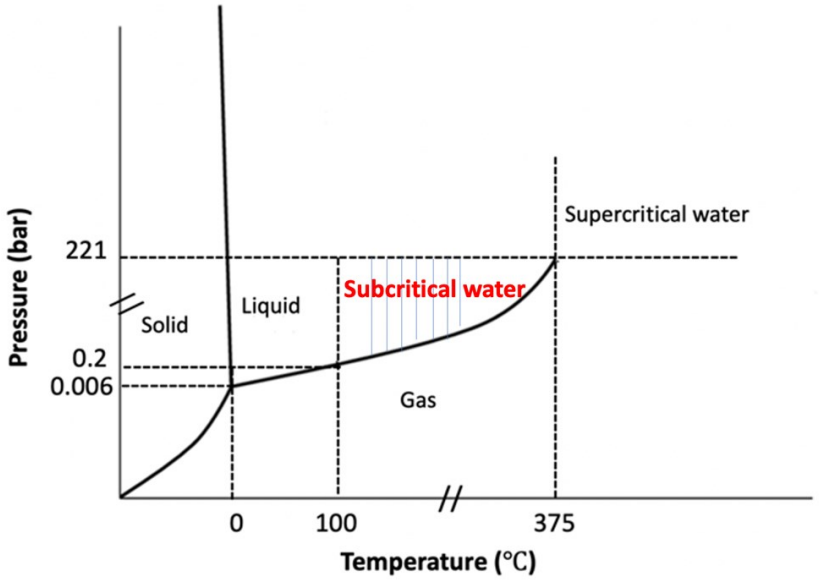

Figure 2.1. Water phase diagram.

The application of subcritical water has been explored in extraction of valuable compounds from food by-products such as pectin from grapefruit pomace (Valdivieso Ramirez & Saldaña, 2014), apple pomace and citrus peel (Wang et al., 2014), sugar and phenolic compounds from ginseng root (Zhang et al., 2018), barley husk (Sarkar et al., 2014) and defatted rice bran (Hata et al., 2008), lignan and carbohydrate from flaxseed meal (Ho et al., 2007), phenolic compound from potato peel (Singh & Saldaña, 2011), anthocyanins and total phenolics from cranberry pomace (Saldaña et al., 2021) and palm oil from palm kernel meal (Bustillo Maury et al., 2019). Also, its application in hydrolysis of protein has been explored in different animal and vegetable protein sources. The investigated process conditions subcritical water hydrolysis of protein-rich by-products are summarized in Table 2.1.

Subcritical water treatment of each biomass has its specific challenges for development and optimization of the process due to the dependence of the hydrolysis on the properties, composition, particle size, and cell wall matrix of biomass. According to the literature, 220-260°C is the optimum temperature for amino acid recovery by subcritical water hydrolysis. The highest

yield of crude protein and peptides were obtained at temperatures of 120-170°C and 170-220°C, respectively.

Overall, subcritical water is mainly affected by the temperature as the increase in the temperature changes the thermodynamic properties of water such as diffusivity, dielectric constant. Moreover, increasing the temperature accelerates the reaction. The time of the process depends on the target product and temperature, the higher temperature, the shorter time. Although, the increasing temperature decreases the reaction time, at higher temperatures undesired compounds such as Maillard reaction products could be generated (Rivas-Vela et al., 2021). Overend & Chornet (1987) introduced severity factor (ln R) to combine the effect of temperature and time to evaluate the severity of the hydrolysis. The severity factor is defined in eq. (2.1):

$$R = t \times e^{\frac{T-100}{14.75}} \tag{2.1}$$

where, t is hydrolysis time (min) and T (°C) is the hydrolysis temperature.

Pressure primarily is to keep water in liquid state at high temperatures. Pressure does not affect the hydrolysis reaction significantly (Lee et al., 2013; Rivas-Vela et al., 2021). Use of additives has been shown to be effective in modifying the hydrolysis reaction. Additives such as malic acid (Liu et al., 2022) and sodium bicarbonate (Espinoza & Morawicki, 2012) have benefited the subcritical water hydrolysis. Espinoza & Morawicki (2012) reported that the use of 1.24 M of sodium bicarbonate at 291°C and saturated vapor pressure for 28 min increased the degree of hydrolysis of whey protein hydrolysate by four-times compared to water only. Liu et al. (2022) also observed that use of 1 to 10% malic acid increased the protein recovery from 7.03 to 39.93% during hydrolysis of shrimp shell at 260°C and 50 bar for 40 min. Also, the yield of free amino acids increased from 49.11 to 140.11 mg/g shrimp shell at 260°C and 50 bar for 60 min. Particle size of the protein source is other variable that may influence the yield of hydrolysis, the lower

particle size, the more surface area that enhance the rate of mass transfer. Incorporation of a pre-treatment step such as ultrasonication could benefit the hydrolysis reaction by increasing the porosity on the cell wall of the protein sources due to the cavitation phenomena.

Table 2.1. Subcritical water hydrolysis of proteinaceous by-products.

By-product	Process Condition	Remarkable Finding	Reference
Animal source			
Porcine skin (weight not reported, skin sliced into 0.5 x 0.5 cm then blended and homogenized with water)	T= 300°C P= 80 bar t= 60 min Solid-solvent ratio= 1:2 w/w	- Crude protein content: 3.82%. - Hydrolysate with M_w less than 1 kDa. - Free amino acids content: 57.18 mM. - DH: not reported.	Jo et al. (2015)
Porcine placenta (weight not reported, placenta cut into 5 cm length)	T= 170°C P= 10 bar t= 30 min Solid-solvent ratio= 1:15 w/w Solvent: pure distilled water, 20% and 50% ethanol	- Highest free amino acids content: 8 mM using pure distilled water. - Highest crude protein content: 44% using 20% ethanol. - DH: not reported.	Park et al. (2015)
Porcine placenta (30 g, particle size not reported)	(1) T= 150,170, and 200°C P= 375 bar T= 0 min Solid-solvent ratio= NR (2) T= 170 °C P= 375 bar t= 0, 30, 60 min Solid-solvent ratio= NR	- Protein recovery increased from 58% to 71% by increasing temperature from 150°C to 170°C. - At 200°C, protein recovery decreased to 55%. - At 170°C, protein recovery increased to 77% by increasing holding time to 30 min and decreased to 48% at 60 min. - Free amino acids content increased approximately 5% by increasing holding time to 60 min and temperature to 200°C. - M_w decreased by increasing temperature and holding time from >20 kDa to 1.4-4.2 kDa. - Pressure had no effect on the hydrolysis of placenta. - Temperature and time had no effect on amino acid profile of the extracts. - DH: not reported.	Lee et al. (2013)

Table 2.1. Continue.

By-product	Process Condition	Remarkable Finding	Reference
Chicken intestines (weight and particle size not reported)	T= 260°C P= NR t= 28 min Solid-solvent ratio= 1:10 w/v H_2SO_4 concentration= 0.02%	- Free amino acid content: 11.49%. - DH: not reported.	Zhu et al. (2010)
Atlantic cod frame (60 g, particle size of 5 mm)	Flow rate= 10 mL/min T= 90, 140, 190, and 250°C P= 100 bar t= 30 min	- 100% protein recovery at 250°C. - M_w decreased from 1500 kDa to <4 kDa by increasing temperature to 250°C. - Temperature had no effect on amino acid profile of the extracts. - DH: not reported.	Melgosa et al. (2021)
De-oiled tuna skin and collagen extracted from the skin (3 g skin, 0.75 g collagen, particle size not reported)	T= 150-300 °C P= 50-100 bar t= 5 min Solid-solvent ratio: 1:200 w/v for collagen and 1:50 w/v for de-oiled skin	- Highest DH for skin (14.47%) and collagen (15.26%) at 250°C. - Highest ABTS radical scavenging activity at 280°C for skin (32.59 mg VCEAC/g hydrolysates) and collagen (50.68 mg VCEAC/g of hydrolysates). - Highest FRAP capacity at 280°C for skin (8.5 mg VCEAC/g hydrolysates) and collagen (23.82 mg VCEAC/g hydrolysates). - Highest antimicrobial activity at 280°C for skin (>0.5 cm inhibition zone of *Bacillus cereus* and *Staphylococcus aureus*) and collagen (>1.0 cm inhibition zone of *Bacillus cereus* and *Staphylococcus aureus*). - Highest structural amino acids at 220°C for collagen hydrolysates (2.68 mg/g). - Highest free amino acids at 250°C for collagen hydrolysates (0.57 mg/g). - DH: not reported.	Ahmed & Chun (2018)

Table 2.1. Continue.

By-product	Process Condition	Remarkable Finding	Reference
Squid muscle (6 g, particle size not reported)	T= 160-258°C P= 6-66 bar t= 3 min Solid-solvent ratio= 1:25 w/v	- Highest free amino acid yield (0.42%) at 250°C. - Highest structural amino acids yield (0.38%) at 220°C. - DH: not reported.	Asaduzzaman & Chun (2015)
Shrimp shell (9 g, particle size of 3.18 mm)	T= 260°C P= 53.6 bar t= 5 min Solid-solvent ratio= 9:100 w/w	- Protein hydrolysis yield: 14.07% shrimp shell. - DH: not reported.	Espíndola-Cortés et al. (2017)
Shrimp shell (2 g, particle size not reported)	T= 90-400°C P= 40 bar t= 5-60 min Solid-solvent ratio= 1:125 w/v dry basis	- Highest amino acid yield: 7% at 250°C in 60 min. - Increase temperature from 90 to 250°C increased the amount of glycine (0 to 28 mg/g) and alanine (4 to 17 mg/g). - All amino acids decomposed at 300°C in 30 min to organic acids (acetic acid) and ammonia. - DH: not reported.	Quitain et al. (2002)
Crab shell (100 g, particle size not reported)	T= 170°C P= NR t= 60 min Solid-solvent ratio= 1:15 w/v	- Amino acids were stable during hydrolysis at 170°C. - Yield not reported. - DH: not reported.	Hao et al. (2021)
Crab shell (0.2 g, particle size of 3 x 3 x 0.5 mm)	T= 300, 350, and 400°C P= NR t= 0.5-40 min Solid-solvent ratio= 1:15 w/w	- All proteins were hydrolysed to amino acids at 300°C for 30 min. - Yield: not reported. - DH: not reported.	Osada et al. (2015)
Comb pen shell (100 g, particle size not reported)	T= 120-220°C P= 30 bar t= 30 min Solid-solvent ratio= 1:30 w/v	- Highest protein content of 613.7 mg/g sample at 180°C - M_w decreased from 993.4 kDa to 1.3 kDa by increasing temperature to 220°C.	Chun et al. (2022)

Table 2.1. Continue.

By-product	Process Condition	Remarkable Finding	Reference
Plant source			
Rapeseed cake (10 g, particle size of <1 mm)	T= 180-280°C P=12.3-74.5 bar t= 5-60 min Solid-solvent ratio= 1:10 w/v	- Highest amino acid yield (13.52%) was obtained at 215°C/30 bar and 36 min. - DH: not reported.	Pinkowska et al. (2014)
Flaxseed meal (2 g, particle size of 1.65 mm)	T= 130-190ºC P= 340 bar t= 180-420 min Solid-solvent ratio= 1:90, 1:150, 1:210 (w/v)	- Highest protein yield 22.5% meal at 160°C and 400 min. - DH: not reported.	Ho et al. (2007)
Soy meal (100 g, particle size not reported)	Enzyme pre-treatment: Enzyme: Protease M (51.5 AU/g) Enzyme-water ratio= 4:100 w/w T= 50°C t= 10-120 min Subcritical water treatment: T= 120°C P= NR t=20 min Solid-solvent ratio= 1:10 w/v	- Protein extraction yield: 59.3%. - Hydrolysates had higher neutral (Gly, Ser, Thr, Tyr, and Cys) and hydrophobic (Ala, Ile, Leu, Met, Phe, Val, and Pro) amino acids than soy meal. - DH: not reported.	Lu et al. (2016)
Rice bran (1 g, particle size not reported)	T= 100-220ºC P= 1-39.7 bar t= 5-30 min Solid-solvent ratio= 1:5 w/v	- Highest protein (21.9%) and amino acid contents (0.8%) at 200ºC/30 min. - Time had no effect on protein and amino acids contents. - Hydrolysate with the highest antioxidant activity was obtained at 200ºC/30 min. - DH: not reported.	Sereewatthanawut et al. (2008)

T: temperature, P: pressure, t: time, NR: not reported, M_w: molecular weight, ABTS: 2,2-´azino-bis [3-ethylbenzothiazoline-6-sulfonic acid], VCEAC: Vitamin C equivalent antioxidant capacity

Lu et al. (2016) reported that subcritical water treatment of soy meals (proximate composition not reported) at a solid-liquid ratio of 1:10 (w/v) and 120°C for 20 min (pressure not reported) significantly increased the protein extraction yield (59.3%) compared to the conventional alkaline extraction at pH 9.0 (ratio and extraction time not reported), (16.4%). Improvement in protein hydrolysis by subcritical water treatment has been explained by the disruption of large insoluble protein aggregates into smaller soluble protein and by the balance of hydrophobic and hydrophilic sites on proteins' molecular surface derived from protein unfolding and accompanying structural rearrangement at subcritical water conditions (Wang et al., 2011; Wang et al., 2012).

In other study, Pinkowska et al. (2014) optimized temperature (180°C/12.3 bar, 200°C/18.5 bar, 220°C/28.8 bar, 240°C/41.4 bar, 260°C/55.2 bar, and 280°C/74.5 bar) and duration (5, 10, 20, 30, 40, and 60 min) of subcritical water hydrolysis of protein from rapeseed cake with 34.98% initial protein. The highest amino acids yield (13.52%) was obtained at a solid-liquid ratio 1:10 (w/v) and 215°C/30 bar for 36 min. The degree of hydrolysis was not reported. However, Ahmed & Chun (2018) investigated the effect of temperature (120-300°C) on the degree of hydrolysis of de-oiled tuna skin at a pressure of 50 bar or just above the saturation vapor pressure, 280°C/80 bar and 300°C/100 bar for 5 min. The authors observed the minimum degree of hydrolysis (3.9%) at 120°C, which increased (14.47%) with increasing the temperature up to 250°C, after 250°C degree of hydrolysis decreased due to decomposition of the amino acids and conversion to organic acids (not identified and quantified). The maximum peptides (267.86 mg/100 g) and free amino acids (57.46 mg/100 g) contents were obtained at 220°C and 250°C, respectively. The sensitivity of amino acids to temperature was not the same, high molecular weight amino acids and more thermosensitive such as aspartic acid and serin decomposed faster than the lower molecular weight amino acids such as glycine and alanine (Ahmed & Chun, 2018).

In the study by Asaduzzaman & Chun (2015), squid muscle was hydrolyzed by subcritical water (160-258°C and 6-66 bar, 3 min). The highest yields of free and structural amino acids were 421.53 mg/100g and 380.58 mg/100g at 250°C and 220°C within 3 min, respectively. In their study, degree of hydrolysis was not analyzed. Esteban et al. (2010) hydrolyzed hog hair by subcritical water to recover amino acids with the highest yield of 325 mg/g protein at 250°C and a reaction time of 60 min. Which is much higher than the yield (4.22 mg/g) obtained by Asaduzzaman & Chun (2015) for squid muscle within 3 min at the same temperature, probably due to the prolonged time of hydrolysis as well as the difference in the raw materials.

Melgosa et al. (2021) also hydrolysed 60 g Atlantic cod (*Gadus morhua*) frames using subcritical water at 90, 140, 190, 250°C and 100 bar with a flow rate of 10 mL/min for 30 min. In their study, the ratio of free to total amino acids increased from 2.6 to 22% by increasing the temperature from 90 to 250°C. The authors reported no significant changes in the amino acid profile of the hydrolysates obtained at different temperatures, also the amino acid profile was similar to the raw material. Similarly, in the study by Lee et al. (2013), the increase in temperature (150-200°C) and time (0-60 min) during subcritical hydrolysis of porcine placenta did not affect the amino acid profile of the hydrolysates. These results demonstrate the low selectivity of subcritical water for hydrolysis of amino acids.

Quintain et al. (2001) investigated protein hydrolysis from shrimp shell by subcritical water hydrolysis at 90, 200, 250, 300, and 400°C and 40 bar for 5-60 min with a sample-water ratio of 1:125 w/v. The authors obtained hydrolysates with the highest amino acid content (70 mg/g dry shrimp shell) at 250°C in 60 min. They also observed that by increasing temperature from 90 to 250°C, the amounts of glycine (from 0 to 28 mg/g shrimp shell) and alanine (from 4 to 17 mg/g shrimp shell) increased. The increase in the glycine and alanine contents was attributed to the

stability of these amino acids during hydrolysis at subcritical condition, as well as degradation of complex amino acids such as arginine and aspartic acid to form simple amino acids like glycine and alanine. Also, they observed that all amino acids were decomposed at 300°C in 30 min to organic acids such as acetic acid and ammonia by decarboxylation and deamination, respectively. The contents of each of these products were increased from 3 to 18 nmol/L when time increased from 90 to 300°C. The properties of amino acids are summarized in Table 2.2. The highest yield of peptides produced during subcritical water hydrolysis of pure α-globin (97%), β-globin (96%), and β-casein (100%) (range evaluated, 160-300ºC for 20 min) were obtained at 160ºC (Powell et al., 2016). Peptide bonds were observed to be stable at temperatures lower than 230°C (Rogalinski et al., 2005; Toor et al., 2011).

Table 2.2. The properties of amino acids at 25°C (Damodaran et al., 2007).

Category	Amino acid	Property
Aliphatic amino acid	Glycine	- Non-essential amino acid - Isoelectric point= 5.98 - M_w= 75.1 g/mol - WS= 249.9 g/L
	Alanine	- Non-essential amino acid - Isoelectric point= 6.00 - M_w= 89.1 g/mol - WS= 167.2 g/L
	Valine	- Essential amino acid - Isoelectric point= 5.96 - M_w= 117.2 g/mol - WS= 58.1 g/L
	Leucine	- Essential amino acid - Isoelectric point= 5.98 - M_w= 131.2 g/mol - WS= 21.7 g/L

Table 2.2. Continue.

Category	Amino acid	Property
Aliphatic amino acid	Isoleucine	- Essential amino acid - Isoelectric point= 6.02 - M_w= 131.2 g/mol - WS= 34.5 g/L
Aliphatic amino acid	Proline	- Non-essential amino acid - Isoelectric point= 6.30 - M_w= 115.1 g/mol - WS= 620 g/L
Aromatic amino acid	Phenylalanine	- Essential amino acid - Isoelectric point= 5.48 - M_w= 165.2 g/mol - WS= 27.6 g/L
	Tryptophan	- Essential amino acid - Isoelectric point= 5.89 - M_w= 204.2 g/mol - WS= 13.6 g/L
	Tyrosine	- Non-essential amino acid - Isoelectric point= 5.66 - M_w= 181.2 g/mol - WS= 0.4 g/L
Hydroxy amino acid	Serine	- Non-essential amino acid - Isoelectric point= 5.68 - M_w= 105.1 g/mol - WS= 422.0 g/L
	Threonine	- Essential amino acid - Isoelectric point= 5.68 - M_w= 119.1 g/mol - WS= 13.2 g/L
Acidic amino acid	Aspartic acid	- Non-essential amino acid - Isoelectric point= 2.77 - M_w= 133.1 g/mol - WS= 5.0 g/L
	Glutamic acid	- Non-essential amino acid - Isoelectric point= 3.22 - M_w= 147.1 g/mol - WS= 8.5 g/L

Table 2.2. Continue.

Category	Amino acid	Property
Basic amino acid	Histidine	- Essential amino acid - Isoelectric point= 7.59 - M_w= 155.2 g/mol - WS= 45.5 g/L
	Arginine	- Non-essential amino acid - Isoelectric point= 10.76 - M_w= 174.2 g/mol - WS= 855.6 g/L
	Lysine	- Essential amino acid - Isoelectric point= 9.74 - M_w= 146.2 g/mol - WS= 739.0 g/L
Sulfur amino acid	Cysteine	- Non-essential amino acid - Isoelectric point= 5.07 - M_w= 121.1 g/mol - WS= 0.1 g/L
	Methionine	- Essential amino acid - Isoelectric point= 5.74 - M_w= 149.2 g/mol - WS= 56.2 g/L
Amide amino acid	Asparagine	- Non-essential amino acid - Isoelectric point= 5.41 - M_w= 132.1 g/mol - WS= 28.5 g/L
	Glutamine	- Non-essential amino acid - Isoelectric point= 5.65 - M_w= 146.1 g/mol - WS= 7.2 g/L at 37°C
Neutral heterocyclic	Hydroxyproline	- Non-essential amino acid - Isoelectric point= NR - M_w= 131.13 g/mol - WS= NR

M_w: molecular weight; WS: water solubility; NR: not reported.

2.1.1. Subcritical water hydrolysis mechanism

Exposure to high pressure at room temperature, or to high temperature under atmospheric pressure is limited to denaturation of protein (loss of quaternary, tertiary, and secondary structures), without breaking covalent bonds and changing the size of protein (Ziero et al., 2020). High pressure and high temperature together can hydrolyze protein into peptide fragments and free amino acids. Cleavage of covalent bonds (peptide bonds and disulfide bonds) of the proteins results in production of protein hydrolysates (oligopeptides and free amino acids). Moreover, at elevated temperature and prolonged treatment time, the amino acids may degrade to organic acids such as formic acid, acetic acid, and propionic acid (Quintain et al., 2001). The formation of hydronium (H_3O^+) and hydroxide (OH^-) ions in the aqueous medium, at high pressure and temperature within subcritical thermodynamic condition, promotes the reactivity of water as an acid, base, or bi-catalyst for hydrolysis reactions (Rivas-Vela et al., 2021). The increase in the concentration of ionic products modifies the pH of the medium that is correlated with the temperature. It was observed that the pH tends to decrease at temperatures lower than 180°C and increase above 200°C due to the degradation of amino acids and Maillard reaction products (Koh et al., 2019; Cho et al., 2020).

Fig. 2.2 shows the simplified stages of subcritical water mechanism in protein extraction and hydrolysis. During the early stages, high pressure and temperature disrupt weak interactions such as hydrogen bonds and result in loss of quaternary, tertiary, and secondary structures of protein (Rodiles-López et al., 2010; Tabilo-Munizaga et al., 2014). At the primary structure stage, the interaction between the positive charge of a hydronium ion (H^+) with the amino-terminal of one peptide bond results in atom excitation and cleavage of the peptide bond, then the hydroxide ion (OH^-) bonds to the new positively charged carboxy-terminal of the peptide bond. Due to the

bi-catalytic characteristic of subcritical water, the reaction could occur in the same way but firstly with the interaction of the hydroxide ion (OH^-) with carboxy-terminal and then the hydronium ion (H_3O^+) by the amino-terminal of peptide bond (Brunner, 2009). The selectivity of subcritical water in breaking peptide bonds is not clear and it is unknown which peptide bonds are more likely to hydrolyze (Espinoza et al., 2012; Melgosa et al., 2021; Lee et al., 2013). Powell et al. (2016) also reported that subcritical water probably has some limitations in cleaving of disulfide bonds; hence, they observed a significant increase in the peptide yield from 32.1% to 71.1% when bovine serum albumin (BSA) was pretreated with protein reducing agents (10 mM dithiothreitol and 55 mM iodoacetamide) prior to subcritical water treatment at 160°C/0.0 min.

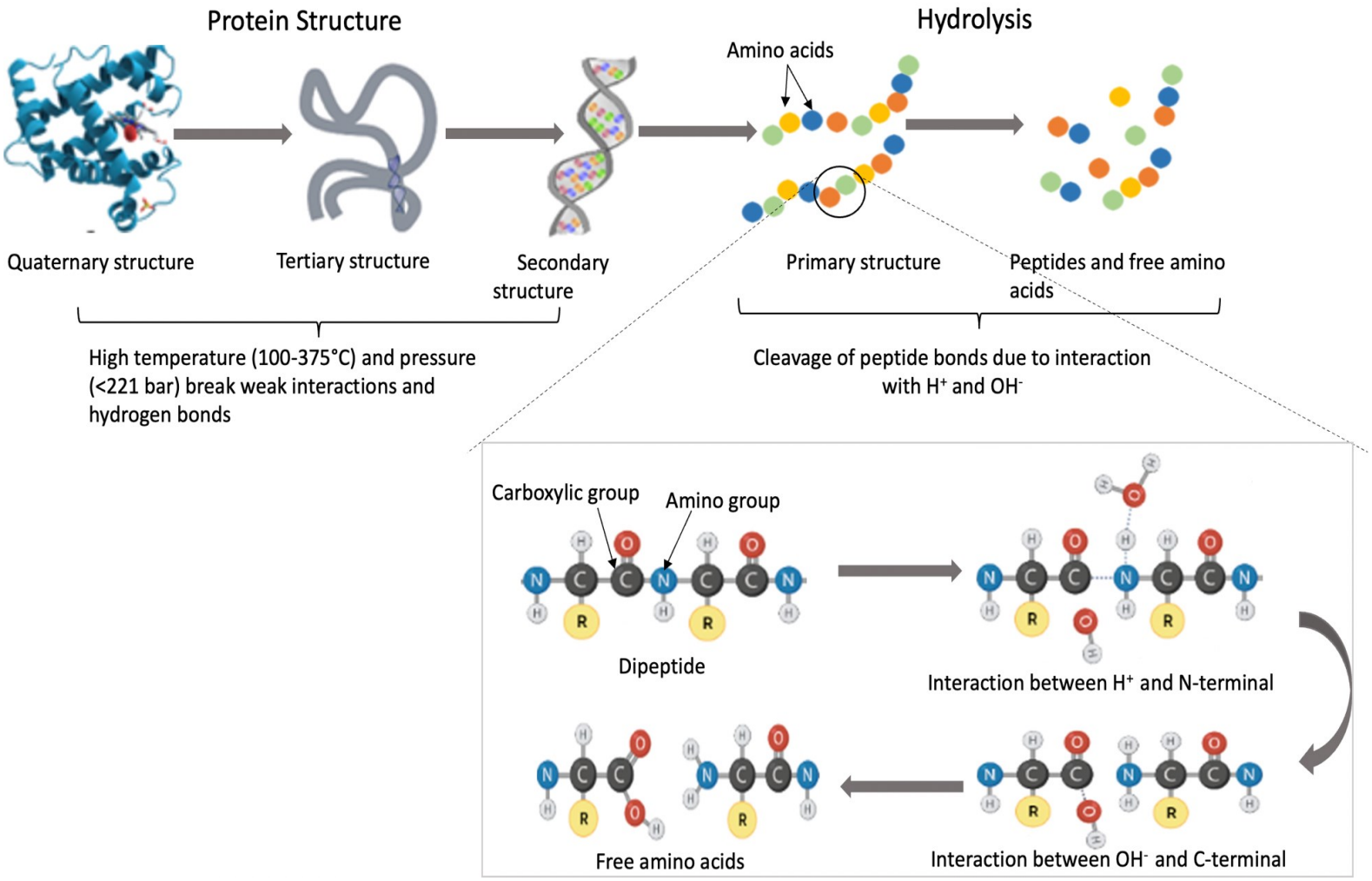

Figure 2.2. Simplified mechanism of subcritical water in protein extraction and hydrolysis (Adapted from Rivas-Vela et al., 2021; Ziero et al., 2020).

2.2. Chitosan

Chitosan, a natural polymer formed by β-(1 → 4)-2-acetamido-D-glucose and β-(1 → 4)-2-amino-D-glucose units, is a cationic amino polysaccharide produced by partially deacetylation (a structural modification) of chitin (Bakshi et al., 2020) (Fig. 2.3). Chitin is a long amino-polysaccharide polymer chain that contains N-acetylglucosamine units connected by β-1, 4-glycoside bonds (Irastorza et al., 2021). Chitin is the second most abundant natural polymer after cellulose. If the percentage of acetyl glucosamine group is more than 50%, it is named chitin, but if this percentage is less than that, the component is chitosan. Degree of deacetylation determines the number of free amino groups. Chitosan with degree of deacetylation in the range of 40-98% are in the market (Bakshi et al., 2020). Chitosan, in its pure state, is odorless, tasteless, and white, or yellowish color.

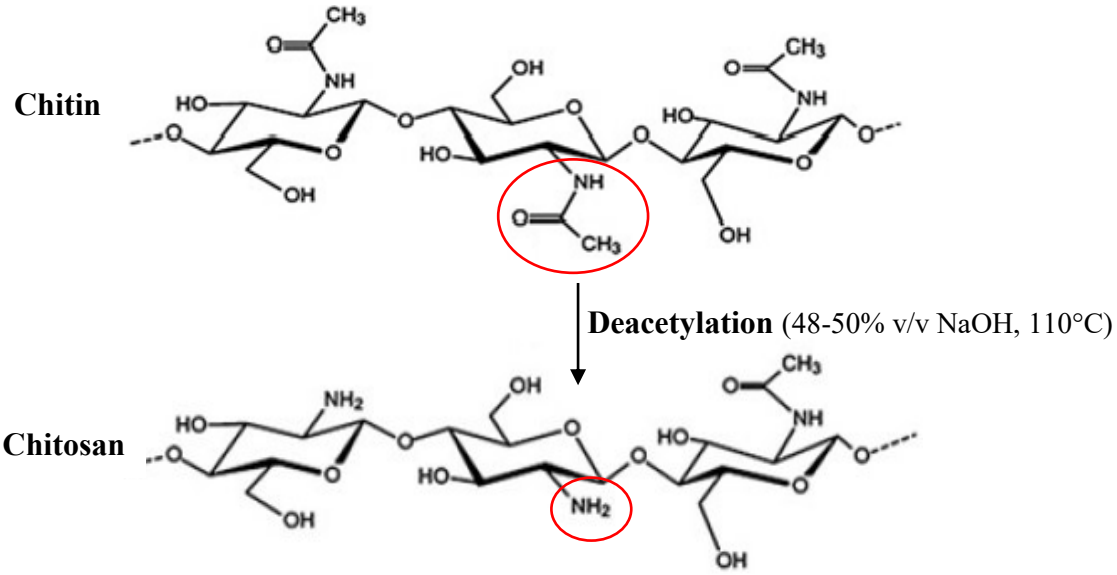

Figure 2.3. Deacetylation of chitin to chitosan.

The solubility of chitosan depends mainly on the free number of free amino groups (degree of deacetylation). The main difference between chitin and chitosan is their solubility. Chitosan is a water-soluble polysaccharide at pH below 7.0 and acts as a cationic polyelectrolyte due to the

presence of positively charged amino groups, while chitin's solubility is limited due to its semi-crystalline structure and excessive hydrogen bonding (Joseph et al., 2021; Bakshi et al., 2020). Solubility of chitosan plays an important role in its potential applications, as its solubilizing is challenging for industrial applications.

2.2.1. Source of chitosan

Chitosan can be obtained from natural resources such as crustacean shells (e.g., shrimp, crab, lobster, crayfish, and krill) and insects exoskeleton (e.g., scorpions, ants, cockroaches, spiders, beetles, brachiopods), invertebrate animals, or mollusks (e.g., octopus, cuttlefish, clams, oysters, geoducks, fossils, squids, fossils, snails), algae (e.g., diatoms, brown algae, green algae) and fungal cell walls (Arbia et al., 2013). Insects such as *Agabus bipustulatus*, and *Hydrophilus piceus* contain 10 and 20% chitin, respectively (Namboodiri & Pakshirajan, 2020). Shells from mollusks like cuttlefish contain 7.4% chitin. Crustacean shells such as tiger prawn, lobster, krill, and crab contain 16.7, 21.3, 20-30% and 15-30% chitin, respectively (Al Sagheer et al. 2009; Ngo & Kim, 2014; Osad et al. 2015). Shrimp shell contains 18-30% chitin (Al Hoqani et al. 2020; Joseph et al., 2021). Shrimp and crab shell are considered as the main source for chitosan production (Irastorza et al., 2021). In the study by Al Sagheer et al. (2009), different amounts of chitin were obtained for the shells from male (20.80%) and female (20.14%) blue swimming crab.

Around 5 million tons of shrimp were harvested worldwide in 2020, and this amount is anticipated to reach 7.28 million tons by 2025 (Nirmal et al., 2020). Shells account for 45-48% (depending on species) by weight of raw shrimp, which is discarded as inedible shrimp by-products during shrimp processing (Ambigaipalan & Shahidi, 2017). Shrimp shell comprises protein (30-40%), ash (30-50%) (Al Hoqani et al. 2020), fat (4.5-10%) (Bruno et al., 2019) and carotenoids (0.03%) (Prameela et al., 2017). Fig. 2.4 shows the chemical structure of shrimp shell. Proteins are

bonded to chitin nanofibrils with extensive covalent and hydrogen bonds. Chitin nanofibrils surrounded by protein form a bundle of fibers that are linked into a chitin-protein sheet embedded with minerals such as calcium carbonate and pigments such as astaxanthin. The chitin-protein planes are arranged into a multilayer structure in the shrimp shell cuticle. In Canada, shrimp shells were exported to China to be utilized for feed stock and glucosamine dietary supplement production (Ambigaipalan & Shahidi, 2017).

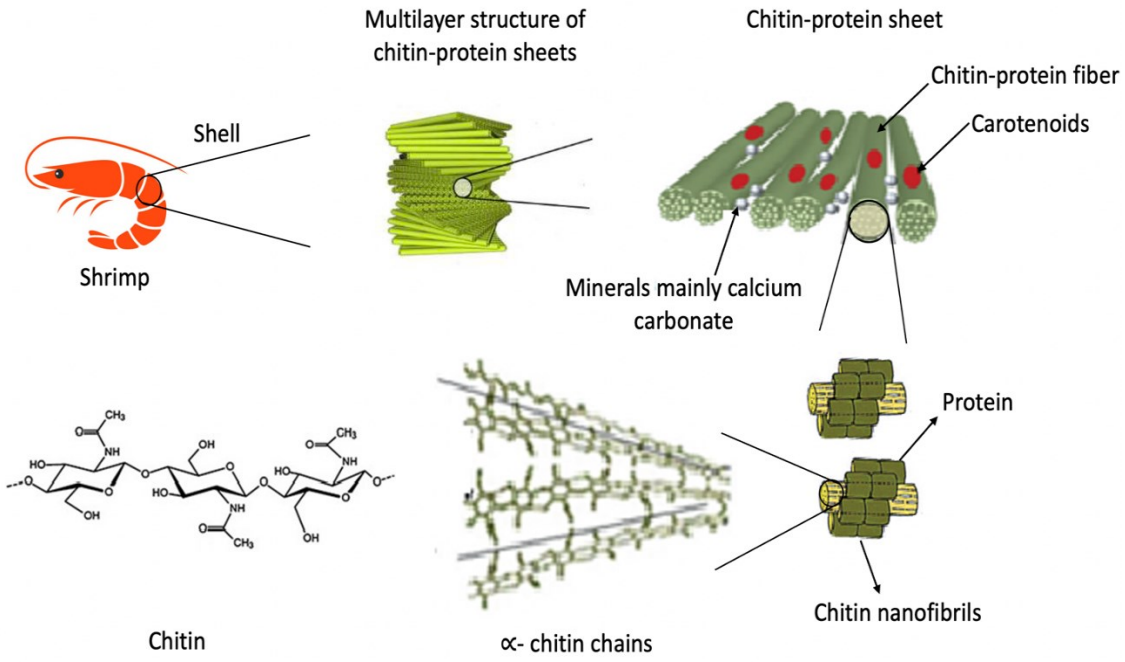

Figure 2.4. Shrimp shell structure (Adapted from Hülsey, 2018; Yang & Yan, 2018).

2.2.2. Applications of chitosan

Chitin and chitosan biopolymers both have excellent biodegradability and biocompatibility properties in the body (Bruno et al., 2019). Moreover, they have exhibited various biological properties such as antimicrobial, anticancer, anti-coagulant, antioxidant, anti-mutagenic, hypocholesterolemia, anti-proliferative, and immunity-enhancing property (Nouri et al., 2016). Chitin and chitosan are used in pharmaceutical industries like a drug delivery agent. Moreover, in the food industry, chitin and chitosan are used in encapsulation of flavoring agents, in food

formulations as binding, thickening, gelling, stabilizing, clarifying and antimicrobial agents (Bruno et al., 2019). A summary of various sources of chitosan and their characteristics and application is provided in Table 2.3.

Table 2.3. Properties and applications of chitosan obtained from different sources

Source	Property	Functionality	Application	Reference
Shrimp shell (*Penaeus semisulcatus*)	Polycationic nature	Antifungal and antimicrobial	Food and pharmaceutical industries	Al-Manhel et al. (2018)
Shrimp shell	Chelating activity	Removal of metal ions	Chemical and pharmaceutical industry	Dotto et al. (2015)
Crab shell (*Portunus trituberculatus*)	High reactivity	Antioxidant	Food and cosmetic industries	Huang et al. (2020)
Squid pen	Thermal, chemical, and mechanical stability	Nanofiber forming	Tissue engineering	Rolandi & Rolandi (2014)
Honeybee (*Apis mellifera*)	High binding capacity	Stabilizer, emulsifier	Food industry	Kaya et al. (2015)
Fungi (*Benjaminiell apoitrasii*)	Very low molecular weight (42.82 kDa compared to of commercial chitosan 464.83 kDa)	Antibiotic	Pharmaceutical industry	Mane et al. (2017)
House cricket (*B. portentosus)*	Biocompatibility	Nanofiber, hydrogel forming	Biomedical industry	Ibitoye et al. (2018)

Among the different chitosan properties, its antimicrobial activity is the most applicable in the food industry to enhance food safety and preservation. There is an increasing interest in the

application of this biopolymer in food packaging (Zhang et al., 2020; Yadav et al., 2021; Uranga et al., 2019; Zhao et al., 2018; Rambabu et al., 2019). Chitosan exhibits antimicrobial activity against a broad spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, filamentous fungi, and yeast (Kong et al., 2010). The antimicrobial mechanism of chitosan is described as a result of the interaction between positively charged amino group of chitosan and the negatively charged membrane of the microbial cell, which subsequently results in breakage of the cell membrane, eventually leakage of intracellular components, including proteins and nucleic acids (Gomes et al., 2017). Zhao et al. (2018) reported that chitosan-starch packaging film delayed the microbial spoilage of cooked ham pre-inoculated with a cocktail of ham spoilage microbiota containing *Brochothrix thermosphacta* FUA3558, *Carnobacterium maltaromaticum* FUA3559, *Leuconostoc gelidum* FUA3560 and FUA3561, and *Lactobacillus sakei* FUA3562 by two weeks.

2.2.3. Extraction of chitin and chitosan

Generally, extraction of chitin and its conversion to chitosan from biological sources involves four steps: (1) demineralization for removal of minerals, mainly calcium carbonate; (2) deproteination; (3) bleaching/decolorization; and (4) deacetylation (conversion of chitin to chitosan). However, the biological sources of chitin vary in composition and structural properties which result in some differences in the chitin/chitosan extraction protocols. For example, decolorization step is not necessary for squid sources, but it is required for pigmented sources like shrimp shell. Demineralization of fungi sources is not required due to the very low content of minerals (Namboodiri & Pakshirajan, 2020). Crustacean exoskeletons have strong networks to resist the agitated marine environment, while insects' exoskeletons have softer structure to assist their aerodynamic motions (Elieh-Ali-Komi & Hamblin, 2016; Vázquez et al., 2013).

2.2.3.1. Conventional methods

Chemical methods have been used for hydrolysis of chitin to chitosan from crustacean shells (Ifuku et al., 2009; Tolaimate et al., 2003; Al Sagheer et al., 2009; Ahing & Wid, 2016; Al Hoqani et al., 2020; Zaeni et al., 2017; Aneesh et al., 2020). However, chemical treatments require strong acid (e.g., HCl) solution for (Zaeni et al. 2017) demineralization and strong alkali solution (e.g., NaOH) for deproteination and deacetylation, respectively.

Al Hoqani et al. (2020) isolated chitosan from shrimp shell with chemical demineralization (3% hydrochloric acid, 1:10 w/v, 25°C, 1 h), deproteination (50% sodium hydroxide, 1:10 w/v, 110°C, 3 h), decolorization (10, 20, 30 % hydrogen peroxide, 1-5 h, 1:10 w/v) and deacetylation (50% sodium hydroxide, 1:10 w/v, 121°C, 15 min). The authors reported that the best condition for decolorization of chitin was using 30% hydrogen peroxide (1:10 w/v) for 3 h to obtain a yield of 4.7% of chitosan. The low yield of chitosan may be due use of harsh alkali treatment for deproteination that resulted in degradation of chitin. Ahing & Wid (2016) isolated chitin from shrimp shell using 1 M hydrochloric acid (1:16 w/v, room temperature, 24 h) for demineralization and 2 M sodium hydroxide (1:16 w/v, 48 h, room temperature) for deproteination. Deacetylation of isolated chitin was performed using 48% sodium hydroxide at room temperature for 48 h (ratio of solid: liquid not reported). Chitosan with a yield of 4.1% and a degree of deacetylation of 79.68% was obtained. In the study by Trung et al. (2020), shrimp shell was firstly pretreated with 0.35 M hydrochloric acid (1:2.5 w/v, room temperature, 24 h), then demineralized with 0.8 M hydrochloric acid (1:3.5 w/v, room temperature, 24 h) and deproteinized with 0.75 M sodium hydroxide (1:2.5 w/v, room temperature, 24 h) to obtain chitin. Afterward, chitin was deacetylated with 12 M sodium hydroxide (1:5 w/v, 65°C, 12 h). Chitosan with 84.76% degree of deacetylation was obtained and the excessive use of strong chemicals resulted in low crystallinity (30%). In other

study by Yen et al. (2009), chitosan with a yield of 32.2% and a degree of deacetylation of 93.3% was hydrolysed from crab shell using a chemical method as following: demineralization with 1 M hydrochloric acid (ratio not reported, 6 h, room temperature), deproteination with aqueous sodium hydroxide (concentration not reported, 1:10 w/v, 100°C, 3 h), and deacetylation with 40% sodium hydroxide (1:30 w/v) at 105°C/120 min.The mineral and protein hydrolysates obtained using strong acid and bases cannot be considered as a source for other applications due to the corrosive chemical contamination, and disposal of such valuable hydrolysates results in waste of resources and environmental concerns.

Hydrochloric acid has been primarily utilized for demineralization of crustacean shell. Yang et al. (2019) conducted a life cycle assessment of chemical fractionation of shrimp shell. The results of this study showed that the demineralization process using hydrochloric acid had the major contribution in the primary energy consumption and carbon footprint (Yang et al., 2019). Materials accounted for 57% of the primary energy, the energy required for hydrochloric acid production was 14-times the amount needed for sodium hydroxide. Materials (HCl and NaOH together) also had a contribution of 74% in the carbon footprint (Yang et al., 2019). In addition, the use of hydrochloric acid is costly because it is corrosive to the equipment and requires chitosan purification and wastewater treatment.

With these challenges, the use of organic acids such as acetic acid (Mahmoud et al., 2007), lactic acid (Ameh et al., 2014) and citric acid (Ameh et al., 2014; Devi & Dhamodharan, 2018) have been explored for demineralization of shrimp shell. Mahmoud et al. (2007) observed that use of 0.8 M lactic acid (1:50 w/v, 1 h, 100°C) effectively decreased the mineral content of shrimp shell from 30.65% to 3.7%, which was comparable with the mineral content of 3.1% of demineralized shrimp shell using 1 M hydrochloric acid (1:50 w/v, 1 h, 100°C). Ameh et al. (2014)

investigated different concentrations of citric acid (0.1 to 0.5 M) for demineralization of shrimp shell at a ratio of 1:13 w/v for 5 to 20 min. Citric acid with a concentration of 0.5 M and treatment for 20 min resulted in the highest mineral reduction of 83%. In addition, the use of weak organic acids results in less environmental issues, less chitosan impurity.

Moreover, the protein impurity of chitosan may cause a health risk on allergic people to protein (Espíndola-Cortés et al., 2017). Complete removal of protein has been a major challenge of chitosan isolation from crustacean shells due to the complexity of chitin-protein matrix network (Hao et al., 2021). Therefore, greener, and more efficient methods have been explored for fractionation of crustacean shell.

2.2.3.2. Green methods

Biotechnological methods (e.g., microbial fermentation and enzyme-assisted process) have been explored for removal of protein to fractionate crustacean shells (Pacheco et al., 2009; Aranday-García et al., 2017; Younes et al., 2014; Castro et al., 2018). *Lactobacillus* species such as L. *paracasei*, L. *plantarum*, and L. *helveticus*, *Serratia marcescens*, and *Brevibacillus parabrevis* are commonly used bacterial species. Chitin hydrolysis from crab shell by lactic fermentation using *Lactobacillus plantarum* sp. 47 (LPS47) was investigated (Castro et al., 2018). Fermentation was carried out at 32°C for 60 h and then the treated crab shell was bleached using methanol-chloroform-water solution (1:2:4 v/v, time and temperature not reported). The control sample was prepared by chemical demineralization (2 N hydrochloric acid, time, temperature, and ratio not reported), and deproteination (3.5% sodium hydroxide solution, 95°C, time and ratio not reported). Lactic fermentation resulted in comparable demineralization (99.55%) and deproteination (95.33%) degree as chemical treatment with 99.87% and 91.73%, respectively. Chitin hydrolysis from shrimp shell by co-fermentation using *Bacillus subtilis* and *Acetobacter*

pasteurianus was explored by Zhang et al. (2021). Shrimp shell (50 g) was deproteinized in 50 mL medium of 3% (v/v) B. *subtilis*, 50 g/L glucose, and 1 g/L yeast extract at 37°C and 250 rpm for 3 d. Demineralization was performed by adding 5 g/L monopotassium phosphate and 6% (v/v) ethanol into the fermentation liquid of B. *subtilis*, then 5 % (v/v) seed culture of A. *pasteurianus* was inoculated and kept at 30°C and 180 rpm for 2 d. After 5 d, chitin with a yield of 18% with deproteination degree of 94.5% and demineralization degree of 92% was obtained.

Bajaj et al. (2015) also hydrolyzed chitin from shrimp shell using bacterial enrichment cultures. In their study, an aerobic, chitinase-deficient, proteolytic enriched culture from ground meat and a mixed culture of lactic acid bacteria from bio-yoghurt was used for deproteination and demineralization, respectively. Protein removal of 89-91% was achieved within 40 h and 85-90% of calcium was removed in another 40 h. Chakravarty et al. (2018) also used a co-culture of *Serratia marcescens* db11 and *Lactobacillus plantarum* to fractionate lobster shell, resulting in 87.19% of total deproteination and 89.59% of demineralization with 82.56% yield of chitin. Protein hydrolysates obtained by deproteination by microbial fermentation can be considered as a safe protein source for other applications such as fertilizer and animal feed. The main advantage of this biological method is the minimum dependance on toxic chemicals. However, the main limitation in use of the biotechnological method is the long processing time of around two weeks, culture homogeneity, reproducibility, and the high cost of the process (Yang et al., 2019).

Microwave has been explored as an emerging green technology for chitin and chitosan hydrolysis (Apriyanti et al., 2018; El Knidri et al., 2016; Sebastian et al., 2019; Knidri et al. 2019; Zhang et al., 2016; Doan et al., 2021). Microwave irradiation can transfer heat very quickly to the biomass matrix due to the reversed heat transfer and homogeneous microwave field within the biomass matrix, consequently, increase the reaction and efficiency (Mohan et al., 2022). El Knidri

et al. (2016) investigated the effect of microwave irradiation on the hydrolysis time of chitosan (8 min) from shrimp shell. Microwave assisted hydrolysis was performed as following: demineralization with 3 M hydrochloric acid (1:10 w/v) at power 500 W for 8 min; deproteination with 10% sodium hydroxide (1:10 v/w) at power 160 W for 5 min and following with heating at 350 W for 3 min; and deacetylation with 50% sodium hydroxide (1:20 w/v) at power 350 W for 8 min. The control sample was prepared by conventional heating using a hot plate as following: demineralization with 3 M hydrochloric acid (1:10 w/v) at 75°C for 2 h; deproteination with 10% sodium hydroxide (1:10 v/w) at 80°C for 2 h; and deacetylation with 50% sodium hydroxide (1:20 w/v) at 100°C for 2 h 30 min. Chitosan with a degree of deacetylation of 82.73% was obtained in 24 min by microwave heating method using strong acid and alkali chemicals while chitosan with a similar degree of deacetylation (81.5%) was obtained in 6 h 30 min.

Nouri et al. (2016) also isolated chitosan from shrimp shell using microwave-assisted chemical method as following: deproteination with 2% sodium hydroxide (1:30 w/v, 80°C, 2 h), demineralization with 10% acetic acid (1:40 w/v, 50°C, 4 h), and deacetylation with 50% sodium hydroxide (ratio not reported) assisted by microwave with a power of 720 W for 20 s. Chitosan with a yield of 19.47% and degree of deacetylation 89.34% was obtained. Sebastian et al. (2019) also investigated the effect of microwave irradiation on chitosan hydrolysis from fungal biomass (R. *oryzae* NRRL 1526). Biomass was suspended in 1 N sodium hydroxide (1:50 w/v) and subjected to microwave at 300 W for 22 min, then treated with 2% acetic acid (1:50 w/v) at 95°C for 8 h. The control chitosan was hydrolyzed from fungal biomass with 1 N sodium hydroxide (1:50 w/v) in an autoclave at 121°C for 15 min and further treated with 2% acetic acid (1:50 w/v) at 95°C for 8 h. Microwave-assisted treatment resulted in chitosan with higher yield (13.43%) and degree of deacetylation of 94.6% than that of conventional heating (yield of 6.67% and degree of

deacetylation of 90.6%). The above studies showed that microwave technology reduced the extraction time from 48 h to 8 h and improved the yield from 6% to 19%, but strong chemicals were used.

Ultrasound is a non-thermal alternative to conventional methods for hydrolysis of biomolecules from biomass. This technology uses sound waves at frequency ranges of 20 kHz to 1000 kHz (Zou et al., 2019). The spread of ultrasound waves in a liquid medium causes cavitation (Kumari et al., 2018). Explosion of microbubbles, generated by the cavitation, on the cell wall of the biomaterials accelerate the penetration of the solvent into the biomass matrix, increase the mass transfer rate of biomolecules from matrix to solvent by increasing the porosity on the cell wall and breaking down the matrix (Sicaire et al., 2019; Pojić et al., 2018).

Ultrasound is considered a rapid, clean, and affordable technology in the food industry (Bruno et al., 2019). The incorporation of ultrasound as a pre-treatment prior to other extraction processes has benefited the extraction yield of valuable compounds from biomass (Iqdiam et al., 2019; Karki et al., 2010; Gulzar & Benjakul, 2018). Also, the use of ultrasound in extraction of valuable biomolecules from biomass has significant advantages including low temperature and consequently minimal thermal damage and oxidation (Ojha et al., 2020). The efficiency of ultrasound could be optimized by the control of frequency, nominal power, amplitude, type, and geometry of the probe (length and diameter of the probe) (Silva & Saldaña, 2020). In the study by Pezeshk et al. (2022), protein solubilization from shrimp shell increased from 82.3% to 95.31% with the assistance of ultrasonication. In their study, alkalin protein extraction from shrimp shell was performed by homogenizing shrimp shell (2 min, 8000 rpm) with cold distilled water (1:5 w/v), pH adjusted to 12.5 with 2 N NaOH then kept on ice for 20 min, followed by ultrasound treatment at 300 W and 20 kHz for 30 min. Singh et al. (2019) investigated the effects of amplitude

(20-82%), sonication time (8-50 min), and solid-solvent ratio (9-50 w/v) on ultrasound-assisted hydrolysis of chitin from squid pen. Demineralization was skipped because of low mineral content of squid pen. Deproteination was caried out with 1.0 M sodium hydroxide with power of 750 W and frequency of 20 kHz and the temperature was maintained at 30-40°C. Yield of chitin and remaining protein decreased with increasing the sonication time and amplitude, while solid-solvent ratio had no significant effect on the extraction yield and the remaining protein content. The optimum condition of 69% amplitude, 41.46 min sonication time, and 1:18 w/v solid-solvent ratio resulted in lower yield of chitin (34%) with lower remaining protein (3.5 mg/100 g sample) compared to a yield of 38% and remaining protein of 5.1 mg/100 g sample for chitin extracted by conventional method (1.0 M sodium hydroxide, 1:20 w/v, 50°C, 5 h). High amplitude and prolonged sonication caused break down and dissociation of chitin from squid pen matrix along with solubilization of protein and the small molecules could be removed during the washing process which result in lower yield of chitin (34%) (Singh et al., 2019). The effectiveness of ultrasound in solubilization of protein associated with chitin could be because of the cavitation phenomena which induces the depolymerization of macromolecules, breakage of hydrogen and inter/intramolecular covalent bonds in polymer chains and dispersion of aggregates (Singh et al., 2019; Kjartansson et al., 2006; Mohan et al., 2022). Thus, facilitating the extraction of proteins.

Similarly, ultrasonication for 1.0 h effectively solubilized proteins from shrimp shell from 44.01% to 10.59% with use of 0.25 M sodium hydroxide as a solvent; however, ultrasonication did not enhance the demineralization of shrimp shell (Kjartansson et al., 2006). In other study, the effects of ultrasonication time (10-150 min), temperature (40-80°C), and different concentrations of sodium hydroxide (30-50%) on deacetylation degree of chitosan was investigated (Huang et al., 2011). Deacetylation of squid pen chitin was carried out with assistance of ultrasound at 25 kHz

(power not reported) with a solid-solvent ratio of 1:10 w/v. The optimum condition of 120 min, 80°C, and 50% sodium hydroxide resulted in 92.92% degree of deacetylation. A decrease in degree of deacetylation was observed after 120 min of ultrasonication, which was attributed to the breakage of glycosidic bonds and degradation of the chitosan to soluble chitosan and/or D-glucosamine monomers (Huang et al., 2011). In the study by Vallejo-Domínguez et al. (2021), chitosan with a yield of 3.56% and degree of deacetylation of 90.97% was hydrolysed from shrimp shell by ultrasound assisted chemical treatment. First, shrimp shell was demineralized with 0.6 M hydrochloric acid (1:11 w/v, 30°C, 3h), deproteinized by sonication with deionized water (power and temperature not reported) for 20 min, and then deacetylated with 50% sodium hydroxide (1:4 w/v, initial heating at 70°C for 2 h, followed by heating at 115°C for 2 h). The positive effect of ultrasound on antimicrobial and dye absorption activities of chitin and chitosan was also observed (Kritchenkov et al., 2019; Zhu et al., 2019; Dotto et al., 2016) due to the reduction in molecular weight and viscosity and increase in the surface area of the biopolymers with ultrasonication.

Recently, subcritical water has also been investigated as a green approach for hydrolysis of crustacean shell. Hao et. (2021) deproteinized crab shell with subcritical water at 170°C for 1 h at a ratio of 1:15 w/v (pressure not reported), demineralized using 10% hydrochloric acid at a ratio of 1:10 (w/v) for 5 h at room temperature, then deacetylated using 30% sodium hydroxide at 120°C for 6 h in an autoclave. In their study, a yield of 11% of chitosan from crab shell was obtained. The authors did not observe significant changes in the functional groups, crystallinity, and morphological properties of chitosan. They also observed that the incorporation of citric acid (1%) in subcritical water hydrolysis of crab shell resulted in a significant increase in the degree of deacetylation of chitosan from 84.1% to 88.5%. Espíndola-Cortés et al. (2017) also investigated subcritical water hydrolysis of shrimp shell to obtain calcareous chitin. In their study, shrimp shell

was hydrolyzed at different sample-water ratio (0.05, 0.09, and 0.17 w/w), temperature (230, 260, and 280°C), and processing time (5, 15, and 30 min). The highest content of chitin (82.2%) with the highest deproteination of 96.06% was obtained at 260°C/53.6 bar for 30 min with a ratio of sample-water of 0.17 w/w.

In other study by Osada et al. (2015), crab shell was hydrolyzed using subcritical water at different temperatures of 300, 350, and 400°C (pressure not reported) with a sample-water ratio of 1:15 w/w for 0.5-40 min. The authors observed that with subcritical water at 300°C for 30 min, all proteins of crab shell were hydrolysed to amino acids without degradation of chitin. The advantages of subcritical water technology are known as shorter reaction time and replacement of acid/basic solvents. The information on the use of subcritical water for hydrolysis of crustacean shell is limited. Further research is needed to investigate the effects of subcritical water on the functional properties of chitin and chitosan such as antimicrobial activity, fat and oil binding capacities.

Complete deproteination of shrimp shell (100%) has been a major challenge of chitosan production (Hao et al., 2021), as the remaining protein could be a health concern for protein allergic people (Espíndola-Cortés et al., 2017). Combination of the new technologies such as subcritical water with ultrasound technologies may be helpful to obtain 100% deproteination degree, optimize the process conditions of extraction/hydrolysis processes, and overcome some of the shortcomings.

2.3. Collagen

Collagen is a fibrous and structural protein of animal matrices, which contributes to physiological functions of tissues in cartilage, skin, bones, and tendons (Simpson et al., 2012). A single collagen molecule consists of three α-chains twisted together in a left-handed manner and

stabilized by covalent crosslinks, and intra/inter chain hydrogen bonds (Cao et al., 2020). Each α-chain has a molecular weight of 100 kDa and is composed of a specific repeating unit of glycine-X-Y, where X and Y are mostly proline and hydroxyproline, with around 1000 amino acids (Potti & Fahad, 2017; León-López et al., 2019). Triple helix polypeptides are linked into collagen fibril. Each collagen fibril is linked to its neighboring collagen fibrils into a collagen fiber (Fig. 2.5).

Collagen is a macromolecule with a molecular weight higher than 250 kDa (Irastorza et al., 2021), 280 nm length, and solubility in salts (sodium chloride, phosphates, or citrates) and acids (inorganic acids such as hydrochloric acid and organic acids such as citric acid, lactic acid, and acetic acid) (Potti & Fahad, 2017). Its isoelectric point is between pH 6.0 and 7.5 (Cassel & Kanagy, 1949). The shrinking temperature of mammalian collagen is between 62 and 65°C and the denaturation (helix to coil) temperature is less than the shrinking temperature by 25-30°C (Avila Rodríguez et al., 2018).

Currently, twenty-nine types of collagens have been identified based on the amino acid composition, sequence, structural, and functional properties (Pal & Suresh, 2016). They are categorized in eight different families based on the structure and supramolecular organization: fibril-forming collagens, basement membrane collagen, microfibrillar collagens, anchoring fibrils, hexagonal network-forming collagens, transmembrane collagens, multiplexin collagens, and fibril associated collagens (Hong et al., 2019; Ahmed et al., 2020; Gelse et al., 2003). Each type of collagen is present in different tissues for example type I in bones, tendons, and ligaments; type II in transparent tissue and cartilage; type III in lung, vessel, and liver; type IV in membranes; type V is adjacent to type I; type VI in placenta, cartilage, dermis, and lung; type VII in oral mucous, cervix, dermal and epiderma junctions, and skin (Ahmed et al., 2020). Animal matrices mostly contain collagen type I (90%) (Hong et al., 2019). Collagen could be converted into gelatin by

thermal treatment, resulting in partial cleavage of covalent and hydrogen bonds and eventually a disruption of crosslinking among α-chains and a helix to coil transition (Gómez-Guillén et al., 2011). Gelatin is a water-soluble protein with molecular weight of 15-250 kDa (Irastorza et al., 2021).

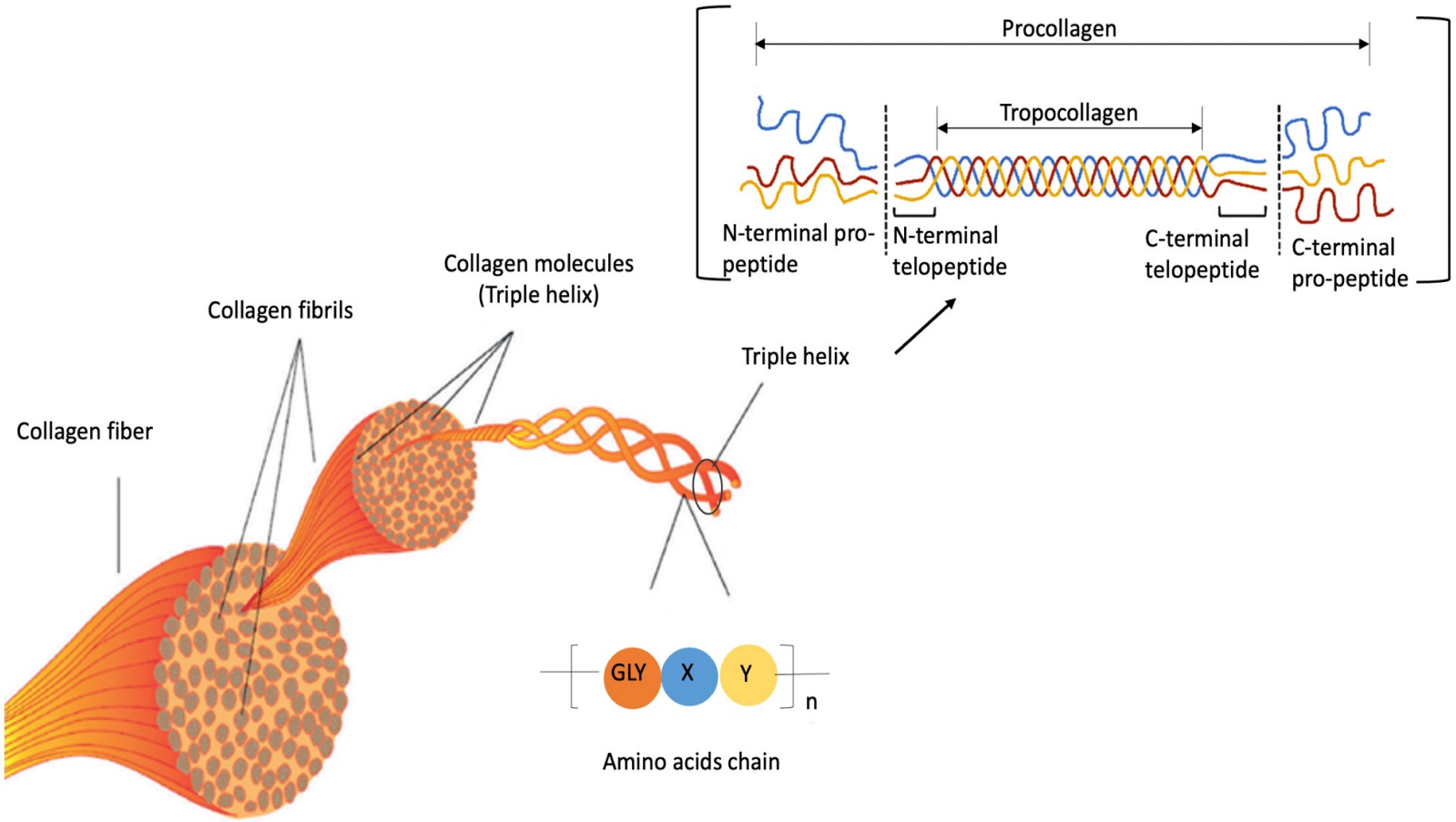

Figure 2.5. Diagram of collagen structure (Adapted from Song et al., 2022 & Reilly & Lozano, 2021). X and Y are mainly proline and hydroxyproline. GLY: glycine, n: repeating unit.

2.3.1. Molecular weight determination of collagen and collagen fragments

Various methods including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion (SEC)-gel permeation chromatography (GPC), matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry (MALDI-TOF MS), and a combination of HPLC and tandem mass spectrophotometry (HPLC-MS/MS) have been stablished to determine the molecular weight of protein and protein hydrolysates.

2.3.1.1. SDS-PAGE method

Laemmli–SDS-PAGE and Tricine–SDS–PAGE are the main SDS techniques to separate proteins and peptides. Laemmli–SDS-PAGE is mainly used to determine the molecular weight distribution of collagen in the hydrolysates. Tricine–SDS-PAGE is suitable for determination of molecular weight of proteins or peptides in a range of between 0.5 kDa to 30 kDa (Hong et al., 2019). In this method, the results are qualitative and incomplete separation of proteins and peptides by staking gel may results in weak bands.

2.3.1.2. SEC-GPC method

Presence of tryptophan and tyrosine in proteins or peptides results in a distinct absorption at a wavelength of 280 nm (Lakowicz, 1983). These two amino acids are mostly present in the telopeptide of collagen molecules (Hong et al., 2019). To determine the molecular weight of proteins and peptides, a standard curve (logarithm of standard's molecular weight against retention time) is required. When the protein hydrolysates are introduced to the size exclusion column, the larger protein elutes first (shorter retention time) then the smaller peptides elute at a longer retention time. However, in this method globular and linear proteins of the same molecular weight do not elute at the same time, globular protein elutes at longer retention time (Hong et al., 2019).

2.3.1.3. MALDI-TOF MS method

MALDI-TOF MS has been used for molecular weight determination of collagen peptides. This method is based on the time flight of ionized peptide. Briefly, to ionize peptides, the protein hydrolysates is mixed with a matrix compound like 2,5-dihydroxybenzoic acid (DHB). Then, the ionized peptides are vaporized with laser radiation (Lewis et al., 2000). This method may not be able to resolve peptides peak with a single matrix (Hong et al., 2019).

2.3.1.4. HPLC-MS/MS method

HPLC-MS/MS is an essential method to analyze the molecular weight and sequence of peptides based on the databases such as Swiss-prot and NCBI (Hong et al., 2019). However, there is a limitation in the identification of collagen peptides when glycan is attached to the peptide due to the limited information on collagen glycation PTM (Hong et al., 2019).

2.3.2. Collagen applications

Collagen has been widely used in food, pharmaceutical, biomedical, tissue engineering, and cosmetic industries due to its cell attachment ability, biodegradability, biocompatibility, low antigenicity, cross-linking, and film forming ability. In food, collagen has been used as dietary supplement and functional ingredient in food and beverages (Hashim et al., 2015). In cosmetics, collagen has been used as moisturizing and anti-aging agent (Avila Rodríguez ae al., 2018). In pharmaceutical, collagen has been used as drug delivery system. As collagen crosslinking property helps to entrap the therapeutics and maintain the biological activity and control the release from the system. In tissue engineering, collagen has been used in production of scaffolds for bone, skin cells, and nerve tissue regeneration, and injectable matrices due to its fibril forming property and biocompatibility (Lee & Arinzeh, 201; Reo et al., 2006; Oliveira et al., 2010). In biomedical,

collagen has been used in wound healing, contact lenses grafting, replacement of heart valve, and dental composites (Reimer et al., 2017; Chen et al., 2019; Buznyk et al., 2015).

Collagen is also used in its denatured form, gelatin, which has distinct characteristics, including water holding capacity, film forming, texture enhancer, colloid stabilization, crystallization, thickening, and emulsification (Pal & Suresh, 2016). Gelatin is not only used in food products but is extensively used in cosmetic, tissue engineering, pharmaceutical products, and bio-ink for 3D printing (Cao et al., 2020). Collagen and gelatin have been extensively used in developing biodegradable packaging materials due to their biodegradability and film forming ability (Irastorza et al., 2021; Jiang et al., 2020; Haghighi et al., 2109; Moreno et al., 2018). Interest in collagen applications have been increasing, with a global market of around US$ 4.2 billion in 2018, that is estimated to grow up to US$ 6.6 billion by 2025 (Ahmed et al., 2020).

2.3.3. Source of collagen

Collagen is mainly obtained from skin, bones, and tendons of bovine animals such as cows and cattle. But use of bovine collagen and gelatin are restricted due to the risk of bovine spongiform encephalopathy and allergenicity (Liu et al., 2001). Besides, there is a religious restriction in consumption of beef collagen and gelatin by Hindu. Bone and skin from porcine are considered as a safe source for collagen without health concerns. But use of porcine collagen is also prohibited in both Judaism and Islam (Mrázek et al., 2019). Marine sources especially fish by-products, including the skin, bone, scale, and viscera are considered as a reliable and alternative source of collagen. Marine sources have high content of collagen, less amount of biological toxins and containments, no risk of disease and no religious concerns (Ahmed et al., 2020). However, fish collagen has lower thermal stability and weaker rheological properties compared to the bovine and porcine collagen, which limits its applications in industrial products (Gómez-Guillén et al., 2011).

Furthermore, the strong odor and the risk of affecting allergic consumers limits the use of fish collagen in food and pharmaceutical industries (Chakka et al., 2017). For this reason, poultry by-products have gained considerable attention as a potential source of collagen in the past decade.

The poultry industry is a very fast-growing sector globally due to the increasing production and consumption of chicken meat and derived products. According to the statistics reported by Food and Agricultural Organization (FAO) in 2020, chicken meat production was 137 million tonnes worldwide. The United States had the highest share in chicken meat production among all countries, producing about 20.5 million tonnes of chicken meat, followed by China with 15 million tonnes and Brazil with 13.7 million tonnes of production. According to Statistics Canada, 1.3 million tonnes chicken were produced in Canada in 2020, and around 125 tonnes of chicken were produced in Alberta. Chicken feet are by-products during chicken processing which constitute 4% of chicken production. Chicken feet are rich in protein (17.42-21.58%), mainly collagen that could serve as a potential source of nutritional compounds and raw materials for value-added products (Liu et al., 2001; Potti & Fahad, 2017; Dhakal et al., 2018).

2.3.4. Extraction of collagen

There are two main steps in collagen extraction from animal by-products: (1) pre-treatment of raw material to remove non-collagenous proteins, lipids, and odor, break noncovalent inter/intra-molecular bonds, and enhancing the purity of extracted collagen (Gómez-Guillén et al., 2011), and (2) extraction of collagen by breaking down various inter/intramolecular covalent crosslinks, ester bonds and hydrogen bonds (Hong et al., 2019; Schmidt et al., 2016). Sodium hydroxide and calcium hydroxide are mainly used to pre-treat the raw material (Schmidt et al., 2016). Liu et al. (2015) reported 0.05-0.1 M sodium hydroxide as the best concentration to remove none-collagenous material with minimal loss of collagen and structural modifications.

Conventionally, there are different methods, including acid, salt, and enzymatic treatments for hydrolysis of collagen. The extracted collagens are referred as acid-solubilized collagen, salt-solubilized collagen, and enzyme-solubilized collagen. The process conditions of the methods which have been investigated for collagen hydrolysis from various by-products are summarized in Table 2.4. Overall, enzymatic treatment results in a higher yield (32-38.9%) than acid treatment (8-30%) while effectiveness of salt treatment is very limited (yield < 5%). Recently, incorporation of new technologies such as ultrasound as a pre-treatment step increased the hydrolysis of collagen by 5-124%.

Table 2.4. Yield of collagen hydrolysis by different methods.

By-product	Process Condition	Yield	Reference
Acid treatment			
Chicken feet (weight not reported, particle size of 0.5 x 0.5 cm^2)	- S: 0.5 M acetic acid - Solid-solvent ratio= 1:20 w/v - T= 25°C - t= 48 h	- Yield: 8.24% - Purity: not reported - M_w: 125-245 kDa	Potti & Fahad (2017)
Chicken feet (weight not reported, particle size of 10 mm and 0.4 mm)	- S: 5% lactic acid - Solid-solvent ratio= 1:8 w/v - T= 4-6°C - t= 36 h	- Yield: 28.4% - Purity: 7.72% - M_w: > 150 kDa	Liu et al. (2001)
Chicken feet skin (weight and particle size not reported)	- S: 0.5 M acetic acid - Solid-solvent ratio= 1:80 w/v - T= 4°C - t= 48 h	- Yield: 14.49% - Purity: not reported - M_w: 97, 116, >220 kDa	Zhou et al. (2016)
Duck feet (weight not reported, particle size of 10 mm)	- S: 5% lactic acid - Solid-solvent ratio= 1:8 w/v - T= 4-6°C - t= 12, 24, 36, and 48 h	- Yield: 28.75% - No difference with different soaking time - Purity: not reported - M_w: not reported	Theng et al. (2018)

Table 2.4. Continue.

By-product	Process Condition	Yield	Reference
Fish skin (weight and particle size not reported)	- S: 0.5 M acetic acid - Solid-solvent ratio= 1:100 w/v - T= 4°C - t= 24 h	- Yield: 37.42% pure collagen - M_w: >166 kDa and > 200 kDa	Wang et al. (2014)
Fish cartilage (weight and particle size not reported)	- S: 0.5 M acetic acid - Solid-solvent ratio= 1:100 w/v - T= 4°C - t= 24 h	- Yield: 27.04% pure collagen - M_w: >166 kDa and > 200 kDa	Liang et al. (2014)
Enzymatic treatment			
Chicken feet (weight and particle size not reported)	- E: Papain (1% w/v) - T=30 °C - t= 28 h	- Yield: 32.16% - Purity: not reported - M_w: 15 to <250 kDa	Dhakal et al. (2018)
Turkey tendon (weight and particle size not reported)	- E: Pepsin - S: 0.5 M acetic acid - Acid-enzyme ratio= 1:10 (unit not reported) - Solid-solvent ratio= NR - T=4°C - t= 48 h	- Recovery: 95.7% - Purity: 103.1% - M_w: 477.3 kDa	Grønlien et al. (2019)
Broiler chicken skin (50 g, particle size not reported)	- E: Pepsin (2500 U/mg) - S: 0.5 M acetic acid - Solid-solvent ratio= 1:10 w/v - T= 4°C - t= 48 h	- Yield: 38.9% with pepsin and 25.1% with ethylene diamine - Purity: 98.6% - M_w: not reported	Cliché et al. (2003)
Yellowfin tuna dorsal skin (weight and particle size not reported)	- E: Pepsin - S: hydrochloric acid solution (pH 2.0) - Solid-solvent ratio= 0.6 to 1.4 % w/v - T= 9°C - t= 12 to 36 h	- Yield: 27.1% with 0.98% w/v pepsin for 23.5 h extraction time. - Purity: not reported - M_w: not reported	Woo et al. (2008)
Cow's hide (weight not reported, particle size of 1 x 1 cm^2)	- E:1% (w/w) pepsin - S: 0.7 M acetic acid (pH 2.35) - Solid-solvent ratio= 1:20 w/v - T= 4°C - t= 66 h	- Yield: 12.5% - Purity: 75.13% - M_w: 95-340 kDa	Noorzai et al. (2020)

Table 2.4. Continue.

By-product	Process Condition	Yield	Reference
Catfish skin (100 g, particle size of 35 mm)	- E: pepsin (23.6 kU/g skin) - S: hydrochloric acid (pH 2.4) - Solid-solvent ratio= 1:5-1:50 w/v - Homogenized at 7000 rpm - T= 4°C - t= 5 min (5/5 s on and off)	- Recovery: highest of 59.03% at a ratio of 1:30 w/v of solid-solvent. - Purity: 100 % - M_w: 100-338.8 kDa	Tan & Chang (2018)
Salt treatment			
Fish skin (weight and particle size not reported)	- S: 0.45 M sodium chloride - Solid-solvent ratio= 1:100 w/v - T= 4°C - t= 24 h	- Yield: 4.55% pure collagen - M_w: >166 kDa and > 200 kDa	Wang et al. (2014)
Fish cartilage (weight and particle size not reported)	- S= 0.45 M sodium chloride - Solid-solvent= 1:100 w/v - T= 4°C - t= 24 h	- Yield: 2.18% pure collagen - M_w: >166 kDa and > 200 kDa	Liang et al. (2014)
Chicken feet skin (weight and particle size not reported)	- S: 0.45 M sodium chloride - Solid-solvent ratio= 1:80 w/v - T= 4°C - t= 48 h	- Yield: 1.13% - Purity: not reported - M_w: 97, 116, >220 kDa	Zhou et al. (2016)
Ultrasound-assisted treatment			
Chicken meat residue (weight and particle size not reported)	Ultrasound pre-treatment: - S: water - Solid-solvent ratio= 1:15 w/v - t= 30 min - Power= 400 W - Frequency= 24 kHz Collagen extraction: - S: 0.5 M acetic acid with pepsin - Enzyme-protein ratio= 1:2.5 w/w 48 h at 4°C. Collagen	Yield increased from 10.8% to 15.11% - Purity: 86.3-88.5% - M_w: 30-125 kDa	Schmidt et al. (2021)
Bovine tendon (weight and particle size not reported)	- E: pepsin (concentration not reported) - S: 0.5 M acetic acid - Solid-solvent ratio= NR - Power= 120 W - Frequency= 40 kHz - T= 20°C - t= 48 h (30 min acting time and 30 min resting time)	- Yield: increased by 124% compared to conventional enzymatic method - Purity: not reported - M_w: not reported	Li et al. (2009)

Table 2.4. Continue.

By-product	Process Condition	Yield	Reference
Calipash of soft-shelled turtle (weight and particle size not reported)	- S: 0.5 M acetic acid - Solid-solvent ratio= 1:20 w/v - Power= 200 W - Frequency= 24 kHz - T= 20°C - t= 5 min	- Yield: increased by 16.3% compared to conventional acid extraction - Purity: not reported - M_w: 116 kDa to >180 kDa	Zou et al. (2017)
Pulsed electric field			
Fish bone (weight not reported, ground sample passed through 100-mesh)	- E: pepsin 2100 U/mg - S: NR - Solid-solvent ratio= 1:11 w/v - Electric field strength= 22.79 kV/cm - Pulse number= 9	- Yield: 35% - Purity: not reported - M_w: not reported	He et al. (2017)
abalone viscera (weight not reported, passed through mesh-20)	- E: flavorenzyme and trypsin 4000 U/g - S: water - Solid-solvent ratio= 4:1 w/v - Electric field strength= 20 kV/cm - t= 600 μs	- Yield: 40% - Purity: not reported - M_w: not reported	Li et al. (2016)
Subcritical water			
Atlantic cod frame (60 g, particle size of 5 mm)	- S: pure water - Sample= 60 g - Flow rate= 10 mL/min - T= 90, 140, 190, and 250°C - P= 100 bar - t= 30 min	- Yield: collagen and collagen fragments yield increased from 13.2% to 53.9% by increasing temperature from 90 to 250°C. - M_w: decreased from 1500 kDa to <4 kDa by increasing temperature to 250°C. - Purity: not reported	Melgosa et al. (2021)

T: temperature; P: pressure; t: time, E: enzyme; S: solvent; NR: not reported. Yield (%) = (weight of collagen/weight of raw material) x 100. M_w: molecular weight.

2.3.4.1. Acid treatment

Traditionally, acid hydrolysis has been used for hydrolysis of collagen from chicken feet (Liu et al., 2001), chicken claws (Siswanto et al., 2020; Kimai et al., 2014), duck feet (Theng et al., 2018), and beef bone (Ferraro et al., 2017). Inorganic acids such as hydrochloric acid (0.5 M)

and organic acids such as citric acid (0.5 M), lactic acid (0.5 M), and acetic acid (0.5 M) are the main solvents utilized for acid hydrolysis. The acidic medium induces the repulsions between collagen molecules which improve the extraction of collagen (Ahmed et al., 2020). But low pH of <2.0 could cause denaturation and digestion of collagen (Theng et al., 2018). Wang et al. (2008) investigated the effect of different concentrations of acetic acid (0.3, 0.5, and 0.8 M), temperature (10, 20, and 30°C) and time (12, 24, 36 h) on collagen extraction from grass carp skin. The authors observed that a concentration of 0.54 M, temperature of 24.7°C and extraction time of 32.1 h were the optimum condition to obtain the highest yield of 19.7% (purity of the collagen not reported). The yield of acid-soluble collagen increased by increasing the acid concentration to 0.54 M and thereafter decreased. Due to denaturation of collagen at low pH value (pH 2.4). Also, the yield increased by extending the extraction time in the range of 12-24 h, beyond this range the increase of the yield was minimal. The positive effect of time could be explained by mass transfer rate, which is a time-dependant factor that plays key role in the efficiency of extraction. The optimal temperature varied when different acid concentrations and times were used.

Liu et al. (2001) explored different acids, including acetic acid, citric acid, lactic acid, and hydrochloric acid for extraction of collagen from chicken feet. Chicken feet were ground using a 10 mm and 0.4 mm plate in sequence. The process condition for all acid treatments were the same: 5% (unit not reported) acid solution, 1:8 w/v, 4°C, and 12, 24, 36, and 48 h. At the end of soaking time, chicken feet solutions were homogenized with a blender at 10000 rpm for 5 min (45 s on and 15 s off). The highest yields were achieved by acetic acid (30.86%) and lactic acid (28.4%) with times of 24 h and 36 h, respectively. The lowest yield was obtained by hydrochloric acid at various soaking times (7.88-13.82%) due to digestion of collagen into amino acids and peptides with concentrated hydrochloric acid. Glycine (23.7, 28.2, 32.6, and 36%) were found in collagen

hydrolysates obtained by hydrochloric acid, lactic acid, acetic acid, and citric acid, respectively. The amino acid composition of hydrolysed collagen also showed that collagen was significantly hydrolysed with hydrochloric acid. However, in the study by Potti & Fahad (2017), acid hydrolysis (0.5 M acetic acid, 1:20 w/v, at room temperature for 48 h) of chicken feet (cut into 0.5 x 0.5 cm) pretreated by 0.1 N sodium hydroxide (24 h, ratio not reported) resulted in lower collagen yield of 8.24%. The yield difference in the results of these two studies probably is due to the difference in the particle size (ground using10 mm and 0.4 mm plates vs cut into 0.5 x 0.5 cm) and the process temperature (4°C vs room temperature).

Theng et al. (2018) explored extraction of collagen from duck feet using lactic acid solution. In their study, duck feet were ground by a mincer using a 10 mm plate. First, grounded duck feet were pretreated with 0.5 M ethylenediaminetetraacetic acid (EDTA) solution (pH 7.5) at 4°C for 24 h then collagen was extracted using 5% lactic acid solution at a ratio of 1:8 w/v for 12, 24, 36, and 48 h at 4-6°C. The authors did not observe any significant difference in the collagen yield (28.32-28.49%) with different soaking times but swelling percentage of collagen increased from 233.10% to 241.31%, with increasing soaking time. Probably more water was trapped among the charged and uncharged polar groups of the collagen. In a study by Ferraro et al. (2017), the effect of age (4 and 7 years) and anatomy (femur and tibia) of cow milk bone on the yield of collagen extraction by acetic acid was investigated. Bone powder (particle size not reported) were soaked in 0.5 M acetic acid at a ratio of 1:5 w/v for 5 days at 4°C. Yield of collagen extracted from young bone, either tibia or femur, was significantly higher than from old bone, and the yield was higher for tibia bones with compared to femur bones (young tibia 16.9%, young femur 10.6%, old tibia 9.6%, and old femur 7.9%), probably due to the modification in the collagen crosslinking

with age and the particle size of the powder from bone with different anatomy even though all bones were crushed at same conditions.

2.3.4.2. Salt treatment

For salt hydrolysis of collagen, neutral saline media such as sodium chloride, Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride), phosphates or citrates have been investigated (Wang et al., 2014; Schmidt et al., 2016). Collagen was hydrolysed from the skin of Amur sturgeon (*Acipenser schrenckii*) fish with 0.45 M sodium chloride (1:100 w/v, 24 h, 4°C) and 0.5 M acetic acid (1:100 w/v, 24 h, 4°C). Salt treatment resulted in a lower yield (4.55%) than acid treatment (37.42%) (Wang et al., 2014). Similarly, collagen was hydrolyzed from cartilage of Amur sturgeon (*Acipenser schrenckii*) fish with 0.45 M sodium chloride (1:100 w/v, 24 h, 4°C) and 0.5 M acetic acid (1:100 w/v, 24 h, 4°C). Collagen with yields of 2.18% and 27.04% were obtained from salt and acid treatments, respectively (Liang et al., 2014). Zhou et al. (2016) investigated salt (0.45 M sodium chloride, 1:80 w/v, 48 h, 4°C) and acid (0.5 M acetic acid, 1:80 w/v, 48 h, 4°C) collagen extraction from chicken feet skin. Chicken feet skin collagen with yields of 1.13% and 14.49% were obtained by salt and acid extraction, respectively. The use of saline media in hydrolysis of collagen is limited due to its low solubility in salt and collagen molecules are mainly cross-linked.

2.3.4.3. Enzymatic treatment

Enzymatic hydrolysis of collagen from chicken feet (Dhakal et al., 2018), turkey tendon (Grønlien et al., 2019), broiler chicken skin (Cliché et al., 2003), bovine hide (Noorzai et al., 2020), and beef tendon (Ran & Wang, 2014) has been investigated. Pepsin (0.6-1.4% w/v) and papain (1% w/v) have been mainly utilized for collagen hydrolysis. Woo et al. (2008) studied enzymatic hydrolysis of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin. First, skin was

pretreated with 0.92 M sodium hydroxide (1:5 w/v) at 9°C for 24 h then collagen was hydrolysed using different concentrations of pepsin (0.6 to 1.4 % w/v) in hydrochloric acid solution (pH 2.0) at 9°C for duration of 12 to 36 h. Pepsin concentration (0.98% w/v) and extraction time (23.5 h) were the optimum conditions to obtain the highest yield of collagen (27.1%). Grønlien et al. (2019) also hydrolysed collagen from turkey tendons using pepsin enzyme. Small pieces of turkey tendons (size not reported) were soaked in 0.5 M acetic acid solution with pepsin (1:10 unit not reported) for 48 h at 4°C with constant stirring. The authors reported a 95.7% recovery of collagen from turkey tendons with a molecular weight of 477.3 kDa. In other study by Dhakal et al. (2018), enzymatic hydrolysis of collagen from chicken feet was investigated. Chicken feet were pretreated by 0.1 M sodium hydroxide (1:10 w/v) for 24 h then collagen was extracted using 1% w/v papain (30000 U/mg) at 30°C for 28 h, resulting in a yield of 32.16%.

In other study, Noorzai et al. (2020) investigated acid and acid-enzyme collagen hydrolysis from 4-year-old cow's hide. The hide samples were cut into 1 x 1 cm^2 pieces. For acid extraction, samples were pretreated by 0.1 M sodium hydroxide (1:10 w/v) for 6 h then soaked in 0.5 M acetic acid (pH 2.6) with a ratio of 1:30 w/v at 21°C for 24 h. For enzymatic treatment, samples were pretreated in 1:20 w/v solution of 0.1 M sodium hydroxide and 0.1 M sodium chloride for 6 h three times in each solution then were soaked in 1:20 w/v 0.7 M acetic (pH 2.35) acid and 1% (w/w) pepsin at 4°C for 48 h. Collagen with a higher yield of 12.5% was hydrolyzed by acid-enzymatic treatment (66 h) in comparison to the yield obtained by acid extraction (3.8% in 24 h). Collagen was hydrolysed from catfish *Ictalurus punctatus* skin with homogenization with and without enzymes. Catfish skins were mixed with hydrochloric acid (pH 2.4) at a ratio from 1:5 w/v to 1:50 w/v without and with pepsin (23.6 kU/g skin) (Tan & Chang, 2018). The mixture was homogenized at 7000 rpm for 5 min (5/5 s on and off). For treatment without enzyme, the mixture

was further stirred at 4°C for 1 h. Enzyme aided treatment resulted in the highest recovery (59.03%) at a ratio of 1:30 w/v and non-enzymatic treatment resulted in the highest recovery (60.38%) at a ratio of 1:50 w/v. The authors did not observe any significant difference in the highest recovery obtained by homogenization with and without enzymes, however the solvent volume (1:30 vs 1:50 w/v) and extraction time (5 min vs 65 min) were lower for the pepsin aided extraction.

Although enzymatic extractions have several advantages including high extraction yield due to hydrolysis of the telopeptides of the collagen molecules that mainly are stabilized with enzymatic crosslinks, which increases its solubility, enzymatic extractions are considered as a costly process (Ahmed et al., 2020).

Overall, the yield of collagen extraction depends on the age, species of animal, and process conditions such as temperature, time, raw material-solvent ratio, particle size, and the concentration of solvent.

2.3.4.4. Emerging technologies

Ultrasound is a rapid, safe, and simple technology which has been widely explored for protein extraction. Ultrasonication increases the porosity of the cell wall and consequently increases the mass transfer rate, decreases the extraction time, and improves the yield. Ultrasonication facilitates the extraction of collagen by opening the collagen fibril structure, without affecting the triple helix structure of collagen molecules (Ran & Wang, 2014; Schmidt et al., 2021). The extraction yield depends on the time of ultrasonication and amplitude of sound waves.

Schmidt et al. (2021) investigated the effect of ultrasound pre-treatment on the yield of enzymatic extraction of collagen from chicken meat residue. To remove non-collagenous proteins,

calcium, and fat chicken meat residue was treated with 0.1 M sodium hydroxide (1:20 w/v, 48 h, 4°C), 0.5 M EDTA-2Na (pH 7.5, 1:10 w/v, 5 days, 4°C), and 10% butyl alcohol (1:10 w/v, 48 h, 4°C), respectively. Sample was pretreated with ultrasonication in water at a solid-liquid ratio of 1:15 w/v for 30 min at 400 W and 24 kHz, then to extract collagen, pretreated sample was mixed with 0.5 M acetic acid as well as pepsin with a ratio of 1:2.5 w/w enzyme-protein and incubated in a shaker incubator at 150 rpm for 48 h at 4°C. Collagen yield increased from 10.8% to 15.11% with incorporation of ultrasound pre-treatment. Li et al. (2009) explored ultrasound-assisted enzymatic extraction of collagen from bovine tendon, where the tendon was rinsed with acetone and 0.15 M sodium chloride to remove none-collagenous protein. Tendon was mixed with pepsin (concentration and ratio not reported) in 0.5 M acetic acid in a flask and immersed in an ultrasonic bath at 120 W and 40 kHz for two days (30 min on and 30 min off) at 20°C. The authors observed a significant increase in the extraction yield (124%) in comparison to conventional enzymatic method without ultrasonication.

Zou et al. (2017) investigated the effects of ultrasonication on collagen extraction from calipash of soft-shelled turtle. Sample suspended in 0.5 M acetic acid at a ratio of 1:20 w/v then treated with ultrasound (200 W power, 24 kHz frequency, and temperature of 20°C) for 5 min. The authors observed that application of ultrasound increased the extraction yield by 16.3% compared to the conventional acid extraction (0.5 M acetic acid, 1:20 w/v, 24 h, temperature not reported). Kim et al. (2013) investigated the effect of ultrasonication on collagen extraction from skin of sea bass (*Lateolabrax japonicus*) and compared to conventional acid extraction (0.5 M acetic acid, 1:200 w/v, 24 h, 4°C). In their study, skin sample (1.0 x 1.0 cm) was soaked in 0.1 M acetic acid at a ratio of 1:200 w/v and introduced to ultrasonication at a power of 750 W, 20 kHz frequency and 20-80% amplitude at 4°C for 0-24 h (20/20 s on and off pulse). The authors observed

that ultrasonication at 80% amplitude for 3 h resulted in comparable recovery (approximately 20%) of intact collagen to the conventional without structural alteration. Furthermore, they observed a significant increase in the recovery (up to 90%) with increasing the time to 24 h and amplitude 80%, as well as presence of unknown components which are assumed to be products of collagen degradation (Kim et al., 2013).

Ran and Wang (2014) investigated enzyme extraction of collagen from cattle tendons with and without ultrasonication. Minced tendons (less than 4 mm) were pretreated in 0.5 M acetic acid (1:15 w/v) for 12 h followed with ultrasonication at 20 kHz, 4°C for 3-24 h (20/20 s on and off pulse) and enzyme treatment with pepsin at 50 U/mg of tendon in 0.5 M acetic acid at 4°C for 24 h. In enzymatic treatment without ultrasonication, sample was pretreated under same conditions then collagen was hydrolysed with pepsin at 50 U/mg of tendon in 0.5 M acetic acid at 4°C for 48 h. The authors observed a higher yield for combination of ultrasonication for 24 h and enzymatic extraction (6.2%) than the enzymatic extraction without ultrasonication (2.4%). Their study showed that the use of ultrasonication with pepsin improved the yield of collagen extraction without affecting its helical structure and degradation of collagen (Ran & Wang, 2014).

Moreover, pulsed electric fields as a non-conventional method has been investigate for extraction of collagen. In a study by He et al. (2017), collagen with a yield of 35% was extracted from fish bone using high intensity pulsed electric fields: solid-liquid ratio of 1:11 w/v, electric field strength 22.79 kV/cm and pulse number 9. Also, Li et al. (2016) investigated the effect of pulsed electric fields on enzymatic protein extraction from abalone (*Haliotis discus hannai Ino*) viscera. The results showed that the extraction yield obtained by pulsed electric fields (40%) at intensity strength of 20 kV/cm, solid-solvent ratio of 4:1 w/v and treatment time of 600 μs was higher than the conventional enzymatic extraction (35.14% with, 4,000 U/g flavor enzyme and

trypsin, 50% water, 50°C, 3 h). However, the applicability of pulsed electric fields process depends on the electrical conductivity of the treated matrix. In addition, there is an initial high capital cost to develop the installations for pulsed electric fields treatment.

Lee et al. (2013) conducted subcritical water hydrolysis of porcine placenta at temperatures of 150, 170, and 200°C and a pressure of 375 bar for various holding time (0, 30, and 60 min). The authors reported that protein recovery increased from 58% to 71% when the temperature increased from 150°C to 170°C, thereafter protein recovery decreased to 55% at 200°C. At 170°C, protein recovery increased to 77% by increasing holding time to 30 min and decreased to 48% at prolonged holding time (60 min). The increase in the protein solubility was attributed to the ionization of water and decrease in the pH of water and conversion of collagen to gelatin at 150-170°C. The decrease in protein recovery was explained by decomposition of collagen to peptides and free amino acids at elevated temperature (200°C) and prolonged holding time (60 min) (Lee et al., 2013). Free amino acid content increased from 0.1 to 0.3 mM/g placenta by increasing temperature to 200°C and holding time to 60 min. Also, in their study, alanine, glycine, hydroxyproline, and proline contents increased approximately 5% by increasing holding time (0-60 min) and temperature (150-200°C). The authors also observed a shift in the molecular weight of the hydrolysates towards lower molecular weight from 20.2 kDa to 0.4 kDa and new peaks by increasing temperature and holding time that would account for decomposition of collagen. Higher pressure (1000 bar, 170°C, 30 min) was also investigated in their study, no difference was observed in protein recovery and amino acid content (data not reported). Similarly, high pressure treatment of 3000-4000 bar (2°C, 5 min) did not improve the solubility of intramuscular beef collagen (Suzuki et al., 1993). Collagen has been reported to be resistant against high pressure, as hydrogen bonds are pressure insensitive (Gekko & Koga, 1983).

Melgosa et al. (2021) hydrolysed 60 g grinded Atlantic cod (*Gadus morhua*) frames (5 mm particle size) using pressurized hot water with a flow rate of 10 mL/min at 90, 140, 190, 250°C and 100 bar for 30 min. The authors observed an increasing trend in the collagen and collagen fragments recovery from 13.2% to 53.9% by increasing the temperature from 90 to 250°C, and at 250°C, the protein recovery was 84.4%. Based on SDS-PAGE results, molecular weight bands corresponded to non-hydrolyzed β and γ-chains were observed at 90°C and less detected at 140°C and absent at 190 and 250°C. In their study, bands corresponded to α1 and α2-chains were not present even at 90°C, however few bands were observed at low molecular weights (less than 63 kDa), corresponding to products from α-chains decomposition (Melgosa et al., 2021). High and medium molecular weight proteins (20-1500 kDa), peptides (4-6 kDa), and small molecules with molecular weights less than 4 kDa were observed at 90, 140, and 190-250°C, respectively (Melgosa et al., 2021).

Subcritical water technology has the potential for hydrolysis of proteinaceous by-products to obtain collagen and collagen fragments. However, this technology requires high energy. Also, the effects of subcritical water treatment on the structural conformation of collagen and its triple helix structure should be investigated using FTIR and circular dichroism analysis (CD), respectively. A techno-economic assessment is required to optimize the operational cost.

Chapter 3: Production of chitosan from deproteinized shrimp shell using subcritical water hydrolysis assisted by ultrasound

3.1. Introduction

Around 5 million tons of shrimp were harvested worldwide in 2020, and it is predicted to reach 7.28 million tons by 2025 (Nirmal et al., 2020). Shells and heads comprise 45-48% of the shrimp weight that is discarded as inedible shrimp by-products (Ambigaipalan & Shahidi, 2017). The main components of shrimp shell are protein (15-50%), minerals mainly calcium carbonate (30-50%), and chitin (15-30%) (Liu et al., 2022; Kumari et al., 2017). Chitin is a polysaccharide ranked as the second most abundant biopolymer after cellulose (Nirmal, 2020). Chitosan, a natural polymer formed by β-(1 → 4)-2-acetamido-D-glucose and β-(1 → 4)-2-amino-D-glucose units, is produced by partially deacetylation of chitin (Bakshi et al., 2020). Owing to chitosan unique properties, including antimicrobial activity, biodegradability, and nontoxicity, chitosan is gaining an increasing attention in various applications in food, biomedical, textile, wastewater treatment and cosmetic industries (Trung et al., 2020).

Conventionally, chitosan production from crustacean shell involves three processes: (1) demineralization with strong acid like 1.0 M HCl treatment for removal of minerals, mainly calcium carbonate; (2) deproteination using strong alkali treatment like 2.0 N NaOH; (3) deacetylation using stromg alkali like 48-50% NaOH treatment (Ifuku et al., 2009; Tolaimate et al., 2003; Al Sagheer et al., 2009; Ahing & Wid, 2016; Al Hoqani et al., 2020; Zaeni et al., 2017; Aneesh et al., 2020). Traditional demineralization of shrimp shell has primarily been done using hydrochloric acid. Life cycle assessment of conventional fractionation of shrimp shell has shown that the demineralization process using hydrochloric acid has the major contribution in the primary energy consumption and carbon footprint (Yang et al., 2019). The use of this strong acid is also

not cost effective because it causes corrosion to the equipment and adds a purification treatment of chitosan and wastewater.

The use of organic acids such as acetic acid (Mahmoud et al., 2007), and citric acid (Devi & Dhamodharan, 2018) have been reported to be effective in demineralization of shrimp shell. Mahmoud et al. (2007) observed that mineral content of shrimp shell treated by 0.8 M lactic acid (1:50 w/v, 1 h, 100°C) decreased from 30.65% to 3.7%, which was comparable with the mineral reduction to 3.1% obtained by 1 M hydrochloric acid (1:50 w/v, 1 h, 100°C). In other study of Devi & Dhamodharan (2018), 70% of prawn shell minerals were recovered by citric acid. In their study, 8.5 g of shell ground with 0.044 mol citric acid for 10 min in a wet mill, then 300 mL of water added and soaked for 20 min (Devi & Dhamodharan, 2018). Furthermore, the use of weak organic acids results in less environmental issues, less chitosan impurity.

In addition, the protein extracts obtained using strong bases cannot be considered as a protein source for some applications, and disposal of such valuable extract results in waste of resources. Other approaches using enzymes or microorganisms have been explored for removal of protein, which have shown to be comparable to the chemical approach to fractionate crustacean shells (Pacheco et al., 2009; Aranday-García et al., 2017; Younes et al., 2014). Bajaj et al. (2015) extracted chitin from shrimp shell using bacterial enrichment cultures. In their study, an aerobic, chitinase-deficient, proteolytic enrichment culture from ground meat and a mixed culture of lactic acid bacteria from bio-yoghurt were used for deproteination and decalcification, respectively. Protein removal of 89-91% was achieved within 40 h and 85-90% of calcium was removed in additional 40 h. Chakravarty et al. (2018) also used a co-culture of Serratia marcescens db11 and *Lactobacillus plantarum* to fractionate lobster shell, resulting in 87.19% of total deproteination and 89.59% of demineralization with 82.56% yield of chitin. However, the main limitation in use

of the biotechnological method is the long processing time of around two weeks (Yang et al., 2019).

Alternatively, sCW hydrolysis of food by-products to recover valuable compounds such as protein (Lu et al., 2016; Pinkowska & Oliveros, 2014; Sereewatthanawut et al., 2008), amino acids (Kang et al., 2001; Esteban et al., 2010; Tavakoli & Yoshida., 2006), organic acids (Yoshida at el., 1999), calcareous chitin and hydroxyapatite (Espíndola-Cortés et al., 2017) has been reported. sCW refers to water at a temperature between 100°C and 374°C under sufficient pressure (0.2-221 bar) to maintain it in the liquid state. sCW hydrolysis is an environmentally friendly and non-chemical method to recovery biomolecules in short reaction time with low environmental impact. Quitain et al. (2001) investigated the hydrothermal treatment of shrimp shell at sub/supercritical conditions of 90-400°C and reaction times of 5-60 min. The highest yield of amino acids (70 mg/g dry shrimp shell) was obtained at 250°C in 60 min and all amino acids were decomposed to organic acids such as acetic acid and ammonia at 300°C and 30 min. In their study, at all investigated conditions, glucosamine was not detected probably due to its deamination to glucose and cellulose. The hydrolysis kinetics and mechanism of chitin in subcritical water were explored by Yang et al. (2018), where chitin began to be hydrolyzed at 283°C. The stability of chitin is due to the N-acetyl group, which could stabilize the crystallite structure of chitin by the additional hydrogen bonding between O-H group and N-acetyl group (Yang et al., 2018).

Calcium carbonate, the main mineral in crustacean shell, is insoluble in sub/supercritical water and stable up to 400°C and 40 bar (Osada et al., 2015). Hao et al. (2021) indicated that there was no significant difference in the structures of chitosan prepared by 4% sodium hydroxide with a ratio of 1:15 w/v at 90°C for 2 h and sCW at 170°C for 1 h and a ratio of 1:15 w/v (pressure not reported by the authors) treatments. However, the chitosan prepared by sCW had higher

compactness and thermal stability and lower molecular weight (445.2 kDa) than the one prepared using sodium hydroxide (598.7 kDa) treatment.

Also, ultrasonication has been used for protein recovery from food by-products in various studies (Dong et al., 2011; Karki et al., 2010; Ochoa-Rivas et al., 2017; Xu et al., 2017; Zhu & Fu, 2012). Ultrasound is considered a green and affordable extraction method (Silva & Saldaña, 2020). Ultrasound uses sound waves at frequency range from 20 kHz to 1000 kHz. The spread of ultrasound waves in a liquid medium causes cavitation phenomenon. Explosion of microbubbles, generated by cavitation, on the cell wall of the particles accelerate the penetration of solvent into the matrix by increasing the porosity of the cell walls (Silva & Saldaña, 2020; Ekaette & Saldaña, 2021).

The effect of ultrasound on chitin extraction from shrimp shell by the co-fermentation of *Bacillus subtilis* and *Acetobacter pasteurianus* was investigated by Zhang et al. (2022), where protease activity in the fermentation solution increased significantly from 81.8 U/mL (without ultrasound pre-treatment) to 96.9 U/mL after pre-treatment of shrimp shell with high-intensity ultrasound at 800 W for 30 min (5s on and 5s off). Protein extraction from shrimp shell pretreated with ultrasound was about 50% higher than the control sample. Moreover, chitin purified from shrimp shell pre-treated with ultrasound exhibited lower molecular weight (11.2 kDa), higher purity (89.8%), and higher degree of deacetylation (21.1%) compared to chitin extracted without ultrasound pre-treatment (13.5 kDa, 86.6%, 18.5%, respectively). The same effects on the molecular weight and deacetylation degree of chitosan were observed when high-frequency ultrasonic bath was employed for deproteination of shrimp shell with deionized water (Vallejo-Domínguez et al., 2021). The authors observed that ultrasonication for 15, 20 and 25 min with deionized water (power and frequency not reported) led to chitosan with molecular weight of

73.61, 86.82, and 55.66 kDa, and deacetylation degree of 80.60, 92.86 and 94.03%, respectively. Pezeshk et al. (2022) explored the effect of ultrasonication (300 W; 0, 10, 20, and 30 min) combined with alkaline pH-shifting processing (sample homogenized with cold distilled water at a ratio of 1:5 w/v, 8000 rpm for 2 min, pH adjusted to 12.5 with 2 N sodium hydroxide, 20 min) on deproteination of shrimp shell. Ultrasound-assisted alkaline processing resulted in a significantly higher protein recovery (25.12%) than the alkaline processing without ultrasonication (14.41%). To the best of our knowledge, there is no study on deproteination of shrimp shell using sCW hydrolysis assisted by ultrasound. Therefore, the main objective of this study was to investigate the process conditions of sCW assisted by ultrasound for protein hydrolysis of shrimp shell. Then, the deproteinized shrimp shell was demineralized, bleached, and deacetylated to obtain chitosan-rich residue. The hydrolysates were characterized for total nitrogen content and free amino acid content. Also, physico-chemical properties of the chitosan-rich residue were analyzed and compared with properties of that obtained by alkali deproteination.

3.2. Materials and methods

3.2.1. Raw material and chemicals

Shrimp shells were provided by Marisquería Diego restaurant (Mexico City, Mexico). Shrimp shells were washed under tap water and sun dried for three days. To obtain a uniform particle size of 1.0 mm, the dried shells were ground in a centrifugal mill (Retsch, Haan, Germany), packed in plastic bags and stored at 4°C.

The chemicals and solvents used in this study, including hexane (HPLC grade), ethylene diamine tetra acetic acid (EDTA), ethanol (100%), acetone (>99.9), anhydrous sodium sulfate (>99.0%), sodium chloride (ACS, analytical reagent), sodium hydroxide (reagent grade), hydrochloric acid (ACS, analytical reagent), hydrogen peroxide (30%), sodium carbonate (ACS,

analytical reagent), copper (II) sulphate (>99%), sodium potassium tartrate (ACS, analytical reagent), Folin reagent (2.0 N), bovine serum albumin (>96%), sodium tetraborate (999%), sodium dodecyl sulfate (SDS) (>98.5%), o-phthaldialdehyde (OPA) (HPLC grade), methanol (HPLC grade), ß-mercaptoethanol (>99%), chitosan (75–85% deacetylated with medium molecular weight of 190–310 kDa), and lysine (>99%) were acquired from Sigma Aldrich (Oakville, ON, Canada). Nitrogen gas (99.9% purity) was purchased from Praxair (Edmonton, AB, Canada). Milli-Q water was obtained from Milli-Q system (18.2 MΩ.cm, Millipore, Billerica, MA, USA).

3.2.2. Proximate composition analysis

3.2.2.1. Moisture content

Moisture content of shrimp shell was determined using a gravimetric method. Briefly, 2.0 g of sample was weight in a pre-weighed aluminum dish and then kept in a convection oven set at 105°C for 3 h. The dish with dried sample was weighed after cooling in a desiccator. The change in total weight of sample was determined gravimetrically.

3.2.2.2. Ash content

Crude ash content of shrimp shell was determined gravimetrically. The sample (1.0 g) was weighed in a pre-weighed porcelain crucible, then kept in a muffle furnace (Model F-A1730, Thermolyne Corporation, Dubuque, IA, USA) at 550°C overnight. After cooling the crucible in a desiccator, ash content was calculated.

3.2.2.3. Protein content

Crude protein content of shrimp shell was analyzed by the Dumas combustion method using a LECO TruSpec nitrogen analyser (LECO Instruments Ltd., Mississauga, ON, Canada). The system was calibrated using orchard leaves and ethylene diamine tetra acetic acid (EDTA) as standards. Nitrogen released from combustion of samples was quantified and used to calculate

crude protein with a conversion factor of 6.25, which represents an average nitrogen content of proteins being equal to 16% w/w.

3.2.2.4. Fat content

Crude fat content of shrimp shell was analyzed in test tubes following the method earlier described by Liu et al. (2023). Briefly, 0.5 g of shrimp shell was weighed in a pre-weighed test tube, then 10 mL of hexane was added to the test tube and caped with a polytetrafluoroethylene (PTFE) lined phenolic cap. The sample was allowed to sit for 24 h at room temperature to ensure fat extraction. After 24 h, the sample was centrifuged at 470 *xg* for 10 min. Hexane containing fat was transferred into a new pre-weighed test tube. Then, hexane was flushed out using a nitrogen stream. Total extracted crude fat was quantified using eqs. (3.1-3.5):

$$(\text{Test tube} + \text{Sample} + \text{Hexane weight}) - \text{Test tube weight} - \text{Sample weight} = \text{Initial hexane weight} \quad (3.1)$$

$$(\text{Test tube} + \text{Hexane} + \text{Crude fat weight}) - (\text{Test tube} + \text{Crude fat weight}) = \text{Hexane transferred weight} \quad (3.2)$$

$$(\text{Hexane transferred weight}/\text{Initial hexane weight}) \times \text{Sample weight} = \text{Corrected sample weight} \quad (3.3)$$

$$(\text{Test tube} + \text{Crude fat weight} - \text{Test tube weight}) = \text{Crude fat weight} \quad (3.4)$$

$$(\text{Crude fat weight}/\text{Corrected sample weight}) \times 100 = \%\ \text{Crude fat in the original sample} \quad (3.5)$$

3.2.3. Deproteination from shrimp shell

3.2.3.1. Subcritical water hydrolysis assisted by ultrasound

Shrimp shell was pretreated with ultrasound, as described by Huerta et al. (2020), using a 10 mm probe at 20 kHz (Model FS-1200N, Shanghai Sonxi Ultrasonic Instrument Co., Shanghai,

ZJ, China) and nominal powers of 600 and 1200 W for 5 min, using water as solvent with a ratio of 1:20 w/v. Then, hydrolysis of shrimp shell was carried out using a semi-continuous subcritical water apparatus, earlier described by Ciftci & Saldaña (2015). The sCW system mainly consisted of a HPLC pump (Gilson 307, Villiers-le-Bel, France), a pre-heater, a stainless-steel high-pressure reactor (2.54 cm ID × 10 cm length, with inlet and outlet filters of 20 µm), a digital pressure gauge, a cooling system (Swagelok, Edmonton, AB, Canada), an oven (Binder, Bohemia, NY, USA) and a back pressure regulator (Tescom, Elk River, MN, USA) (Fig. 3.1).

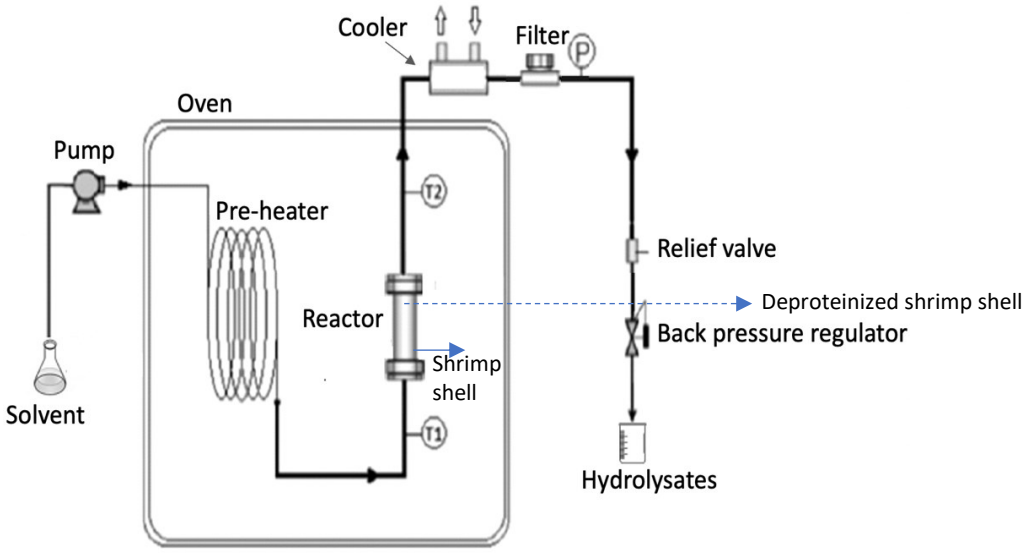

Figure 3.1. Schematic of the subcritical fluid system: P= pressure gauge, T1 and T2= thermocouples (Adapted from Aghashahi, 2020).

Pretreated shrimp shell (2 g) was mixed with 18 g of glass beads and then loaded into the stainless-steel reactor. Degassed Milli-Q water was delivered to the system at a flow rate of 5 mL/min by the HPLC pump. This flow rate was the same used by Ciftci & Saldana (2015), who evaluated different flow rates. An oven was used for heating and temperature was monitored using a thermometer. When the desired temperature and pressure were reached, the hydrolysates were collected at 10, 20, 40 and 60 min and stored at -18°C for further analysis. Experiments were

carried out at a pressure of 50 bar and temperatures of 140, 180, 220, and 260°C (Table 3.1). The pressure of 50 bar was the same as previous studies in our laboratory (Liu et al., 2023). All experiments were performed in duplicates. The solid residues (deproteinized shrimp shell) left in the reactor after each experiment were collected and dried in an oven at 40°C overnight for further treatments.

Table 3.1. Experimental design for sCW deproteination of shrimp shell.

Temperature (°C)	Time (min)	Pressure (bar)	Flow rate (mL/min)
140	0-10	50	5
	10-20		
	20-40		
	40-60		
180	0-10	50	5
	10-20		
	20-40		
	40-60		
220	0-10	50	5
	10-20		
	20-40		
	40-60		
260	0-10	50	5
	10-20		
	20-40		
	40-60		

3.2.3.2. Alkali treatment

For comparison, deproteination of shrimp shell was performed using conventional alkali treatment following the method described by Ahing & Wid (2016). First, shrimp shell was mixed with 2.0 M sodium hydroxide at a ratio of 1:16 w/v and left for 48 h at room temperature (~25°C).

Then, the sample was vacuum filtered. The supernatant was stored at -18°C for further analysis. Solid residues were washed with distilled water until neutral pH of 6.5-8.0 and dried in an oven at 40°C overnight.

3.2.4. Further treatments of deproteinized shrimp shell to obtain chitosan

Deproteinized shrimp shell obtained by sCW hydrolysis assisted by ultrasound and alkali treatment in Section 3.2.2 were further treated to remove minerals, pigment, and deacetylate chitin to obtain chitosan-rich residue (Fig. 3.2). The treatments are described in the following sections.

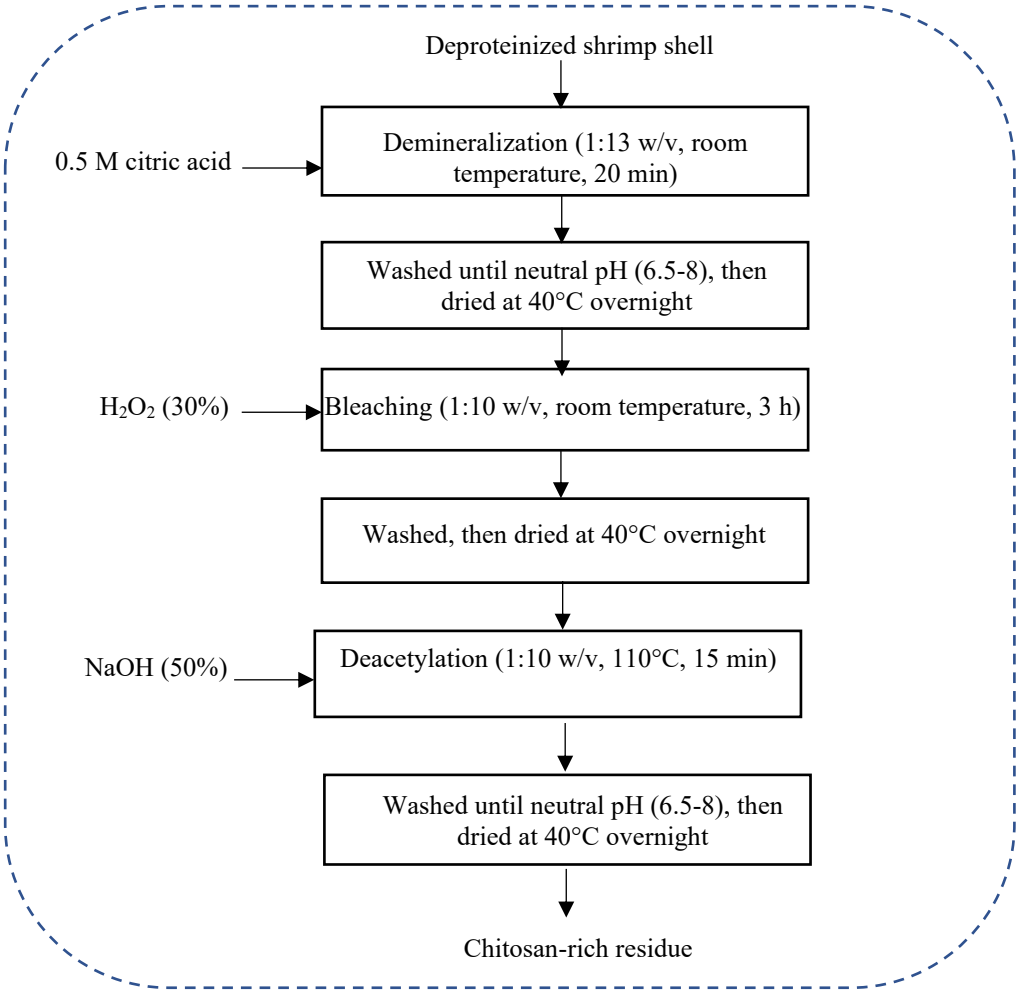

Figure 3.2. Flow chart of processes to obtain chitosan-rich residue from deproteinized shrimp shell.

3.2.4.1. Demineralization of deproteinized shrimp shell

Demineralization of deproteinized shrimp shell was done according to the method described by Ameh et al. (2014). Deproteinized shrimp shell (0.7-1.0 g) was mixed with 0.5 M citric acid at a ratio of 1:13 w/v and left for 20 min at room temperature (~25°C). Then, the sample was vacuum filtered. Solid residues were washed with distilled water (~ 500 mL) until neutral pH (6.5-8.0) and dried in an oven at 40°C overnight.

3.2.4.2. Bleaching of demineralized shrimp shell

Bleaching was done according to the method described by Al Hoqani et al. (2020). Deproteinized and demineralized shrimp shell (0.4-0.5 g) was mixed with H_2O_2 (30%) at a ratio of 1:10 w/v and left for 3 h at room temperature (~25°C). The H_2O_2 (30%) was the best concentration in the range of concentrations studied (Al Hoqani et al., 2020). Then, the sample was vacuum filtered, and chitin (solid residue) was washed with distilled water (~ 500 mL) and dried in an oven at 40°C overnight.

3.2.4.3. Deacetylation of chitin

Deacetylation of chitin was carried out according to the method described by Al Hoqani et al. (2020) with slight modifications. Chitin (~ 0.5 g) was mixed with 50% NaOH solution at a ratio of 1:10 w/v in a test tube and caped with a PTFE lined phenolic cap. The test tube was immersed in an oil bath at 121°C for 15 min. Then, the sample was washed with distilled water until neutral pH (6.5-8.0) and vacuum filtered. Obtained chitosan was dried in an oven at 40°C overnight, weighed for calculating the yield and stored at 4°C for further analysis. Yield and degree of deproteination of chitosan were calculated using eqs. (3.6-3.7):

$$\text{Yield (\%)} = \frac{\text{Weight of chitosan}}{\text{Initial weight of shrimp shell}} \times 100 \tag{3.6}$$

$$\text{Degree of deproteination (\%)} = [1\text{- } ((m_c \times p_c)/(m_s \times p_s))] \times 100 \tag{3.7}$$

where, m_c and p_c are mass and protein content of chitosan and m_s and p_s are mass and protein content of shrimp shell.

3.2.5. Characterization of hydrolysates

3.2.5.1. Total nitrogen content

The total nitrogen content of hydrolysates was determined following the Lowry method (Waterborg & Matthews, 1994). Complex-forming solution was prepared immediately before use by mixing 2% (w/v) sodium carbonate in distilled water, 1% (w/v) copper sulphate in distilled water, and 2% (w/v) sodium potassium tartrate in distilled water at a ratio of 100:1:1 (v/v/v). An aliquot of each sample (0.1 mL) was mixed with 0.1 mL of 2 N sodium hydroxide and kept at 100°C for 10 min in a boiling water bath. The sample was cooled to room temperature, mixed with 1 mL of freshly prepared complex-forming solution, and kept at room temperature for 10 min. Then, 0.1 mL of 1 N Folin reagent was added to the sample and mixed using a vortex mixer. The sample was kept at room temperature for 40 min. Absorbance was read at a wavelength of 550 nm in a spectrophotometer (JENWAY 6230D, Stone, Staffordshire, UK) as protein concentrations were high. For low concentration protein samples, some literature used 750 nm (Waterborg & Matthews, 1994). Blank was prepared with the same protocol using distilled water instead of the sample. Bovine serum albumin (BSA) with concentrations of 100, 250, 500, 1000, and 1500 µg/mL were used for standard curve (See Fig. A1).

3.2.5.2. Free amino acid content

Free amino acid content of hydrolysates was measured by the OPA spectrophotometric assay. The OPA reagent was prepared daily following the method described by Church et al. (1983). Briefly, the OPA solution was prepared by mixing 50 mL of 0.1 M sodium tetraborate, 5 mL of 20% (w/w) SDS, 80 mg of OPA (dissolved in 2 mL of methanol), and 200 µL of ß-

mercaptoethanol and diluting to a final volume of 100 mL with distilled water. Then, 2 mL of OPA reagent was added to 100 µL of sample, mixed for 2 min at ambient temperature, and the absorbance was read at a wavelength of 340 nm in a spectrophotometer (JENWAY 6230D, Stone, Staffordshire, UK). Free amino acid content was calculated from a standard curve prepared using lysine standard solution at concentrations of 0.0, 0.2, 0.3, 0.4, and 0.6 mg/mL (See Fig. A2).

3.2.6. Characterization of chitosan

3.2.6.1. Color

The color parameters (L, a, and b) of the obtained chitosan powder from shrimp shell were measured using a Hunter Lab colorimeter (CR-400/CR-410, Konica Minolta, Ramsey, NJ, USA) that uses a D65 illuminant with an opening of 14 mm and a 10° standard observer according to the ASTM D2244 method (ASTM, 2011). Same amount (300 mg) of each chitosan powder sample was place onto the surface of a transparent plastic cup of 1 cm height x 3.5 cm diameter, which was then placed on the surface of a white standard plate. The whiteness index (WI) was calculated using eq. (3.8):

$$\mathrm{WI} = 100 - [(100 - \mathrm{L})^2 + \mathrm{a}^2 + \mathrm{b}^2]^{0.5} \tag{3.8}$$

where, “L” represents the luminosity, “a” represents red and green colors, and “b” represents the yellow and blue colors of the sample.

3.2.6.2. Degree of deacetylation

Degree of deacetylation (DDA) of chitosan obtained from shrimp shell was calculated according to eq. (3.9) (Zhang et al., 2022):

$$\mathrm{DDA}\ (\%) = 100 - \left[\frac{(\mathrm{C}/\mathrm{N}) - 5.14}{1.72} \times 100\right] \tag{3.9}$$

where, C and N are carbon and nitrogen contents as determined by the Dumas combustion method using a LECO TruSpec nitrogen analyser (LECO Instruments Ltd., Mississauga, ON, Canada).

3.2.6.3. Fourier transform infrared (FTIR) spectroscopy

Transmittance spectra characterization of chitosan was done using a Fourier transform infrared spectrometer (FTIR) Nicolet iS50 (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with an Omnic software, following the methodology of Zhao & Saldaña (2019). The analyses were conducted in the frequency range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans per sample.

3.2.6.4. X-Ray diffraction

The crystallinity of the prepared chitosan was analyzed at scanning range of 5 to 55° (2θ) with a scanning speed of 2°/min in a D8 Discover X-ray diffraction system (Bruker, Billerica, MA, USA) equipped with Cu-source and high throughput LynxEYE 1-dimensional detector with high intensity and speed. The detector was operated with Cu radiation at 40 kV and 38 mA. The relative crystallinity (RC) was calculated as area under the crystalline peak relative to the total area under the curve and expressed as percentage (%) in eq. (3.10) (Trung et al., 2020).

$$RC\ (\%) = \frac{\text{Area under the crystalline peak}}{\text{Total area under the curve}} \times 100 \tag{3.10}$$

3.2.6.5. Surface morphology

The surface morphology of chitosan was measured using EVO 10 Scanning Electron Microscope (Carl Zeiss Canada Ltd., Toronto, Canada) operating at 10 kV following the methodology of Huerta et al. (2020). The sample was mounted onto a stub using a conductive carbon adhesive tab, and sputter-coated with Au/Pd before imaging using Hummer 6.2 Sputter Coater (Anatech Ltd., Richmond, Canada). Then, the sample was photographed, and the images were collected using SmartSEM imaging software.

3.2.7. Statistical analysis

Data are shown as mean ± standard deviation according to the results obtained from at least duplicate experiments and analysis. One-way analysis of variance (ANOVA) and the multiple comparisons of the means with Tukey's test at $p< 0.05$ were performed using Minitab version 18.0 (Minitab Inc., State College, PA, USA) at 95% confidence interval.

3.3. Results and discussion

3.3.1. Proximate composition of shrimp shell

Crude protein, fat, ash, moisture, and carbohydrates contents of shrimp shell are reported in Table 3.2. Protein (48.65%) and ash (22.54%) were the main constituents present in shrimp shell. Similarly, protein (30%) and ash (40%) were the main components in crab shell (Osada et a., 2015). The protein content was in agreement with the value of 45.93% (Aneesh et al., 2020) for shrimp shell, but was higher than the values of 23.07% (Younes et al., 2016) and 28.84% (Al Sagheer et al., 2009) obtained for shrimp shell. The ash content obtained in this study was similar to the amount (21.50%) reported by Tolaimate et al. (2003). Chitin content, the main source of total carbohydrates in shrimp shell, was consistent with the amount (19.13%) reported by Al Sagheer et al. (2009). Fat content was lower than 3.93% (Klomklao et al., 2009) and higher than 0.69% (Younes et al., 2016). The slight differences in the results of this study and other studies probably was due to the difference in shell composition of different species and different gender of shrimp. Earlier, Al Sagheer et al. (2009) found a slight difference in the composition of shell from male and female blue swimming crab.

Table 3.2. Proximate composition of shrimp shell.

Constituent	This study %(w/w)	Literature %(w/w)
Crude protein	48.65 ± 1.21	45.93[a]
Fat	1.65 ± 0.14	0.69[b]
Ash	22.54 ± 0.27	21.5[c]
Moisture	7.23 ± 0.03	-
Total carbohydrates	18.25 ± 0.41	18.1[d]

Data are expressed as mean ± standard deviation of two replicates. [a]Aneesh et al. (2020); [b]Younes et al. (2016); [c]Tolaimate et al. (2003); [d]Hu et al. (2020).

3.3.2. Characterization of hydrolysates

Shrimp shell hydrolysates obtained from alkali (2.0 M NaOH, 1:16 w/v, room temperature, 2880 min), sCW (140-260ºC, 50 bar, 10-60 min, 5 mL/min), and sCW (140-260ºC, 50 bar, 10-60 min, 5 mL/min) assisted by ultrasound (1:20 w/v shrimp shell/water, 600 and 1200 W, 20 kHz, 5 min) were analyzed for total nitrogen content and free amino acids content.

Fig. 3.3 shows the effect of temperature and time on total nitrogen content of hydrolysates obtained by sCW (A), sCW assisted by ultrasound (600 W) (B), and sCW assisted by ultrasound (1200 W) (C). The sCW assisted by ultrasound resulted in better hydrolysis of shrimp shell protein than sCW without ultrasound pre-treatment. Also, sCW assisted by ultrasound resulted in the higher yields of total nitrogen of 74.06 mg/g shrimp shell (7.4%) with 600 W and 99.01 mg/g shrimp shell (9.9%) with 1200 W at a lower temperature (180ºC) in comparison to the yield of sCW at 260ºC (34.2 mg/g shrimp shell (3.4%)) without ultrasound pre-treatment. Ultrasound pre-treatment favored the hydrolysis of protein. The better yield is because ultrasonic waves produced acoustic cavitation that increases the porosity on the matrix, and eventually promoted the penetration of solvent into the shrimp shell matrix (Pezeshk et al., 2022). In the study by Zhang et al. (2022), incorporation of ultrasound pre-treatment also increased deproteination degree of

shrimp shell by co-fermentation from 4.5% (without pre-treatment) to 9.1% (with pre-treatment). In their study, first, shell was pretreated with ultrasound at a power of 800 W for 30 min with water (20 kHz, 2.5:20 w/v), then deproteinized with fermentation media containing 1g/L yeast extracts, 50 g/L of glucose at a ratio of 1:1 w/v for 5 d at 37°C. The increase in the dissolution of shrimp shell protein was attributed to the effect of ultrasonication on protein unfolding and disruption of hydrophobic interactions and loss of tertiary and secondary structure based on the fluorescence and Far-UV CD spectra of protein (Zhang et al., 2022).

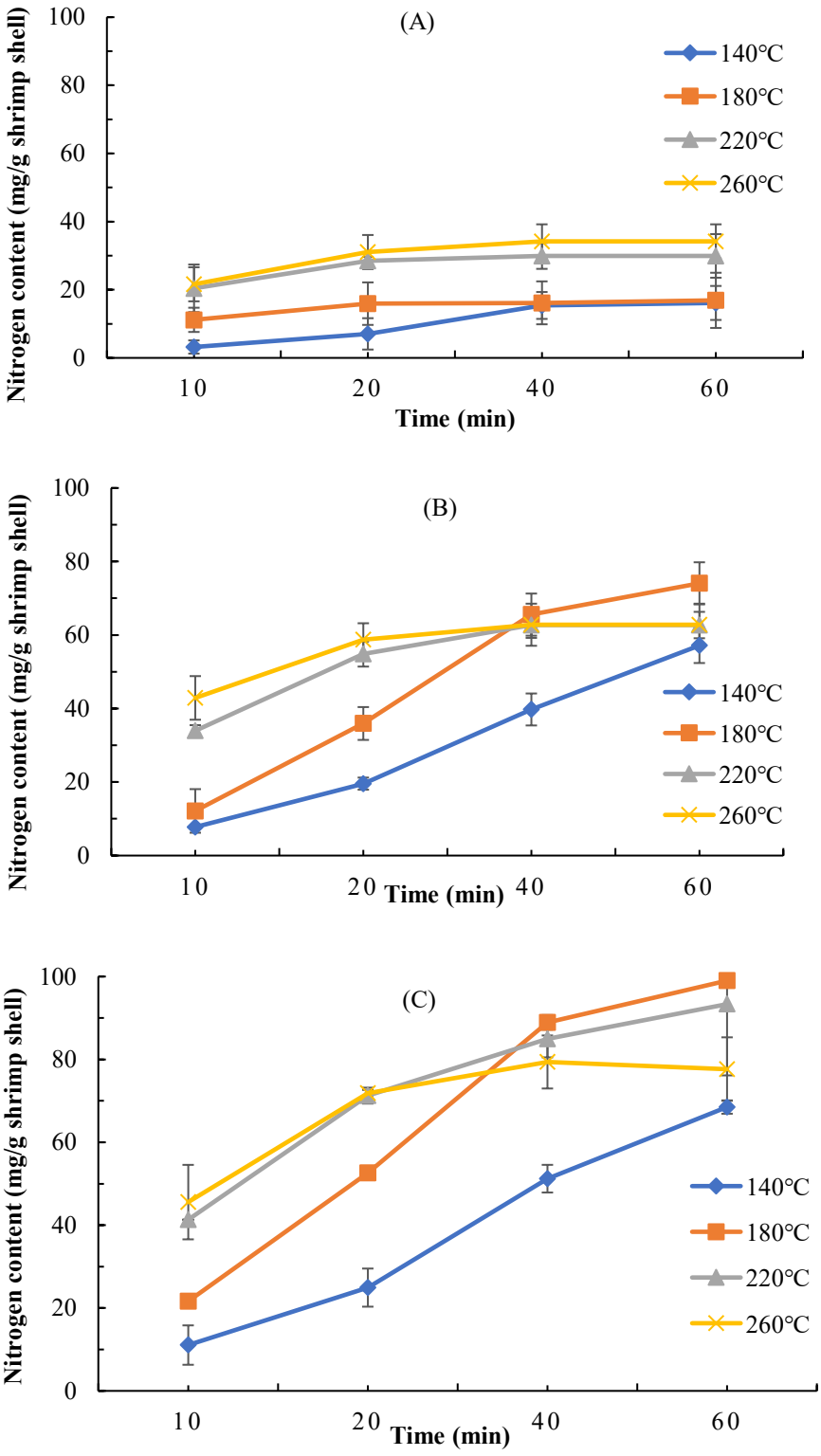

Figure 3.3. Effect of temperature and time on total nitrogen content of shrimp shell hydrolysates obtained by: (A) only SCW, (B) ultrasound (600 W, 5 min) + SCW, and (C) ultrasound (1200 W, 5 min) + SCW treatments at 50 bar.

The increase in the hydrolysis of protein with sCW by increasing the temperature was because of the increase in the concentration of hydronium and hydroxide ions, as the dissociation constant of water increased at high temperatures (K_w from 10^{-14} mol^2L^{-2} at ambient condition to 10^{-11} mol^2L^{-2} at 300°C). In the study by Chun et al. (2022), comb pen shell was hydrolyzed with sCW at 120-220°C and 30 bar with a ratio of 1:30 w/v for 30 min. A higher protein content of 613.7 mg/g sample obtained at 180°C. This could be due to the difference in the protein content of the raw material. In other study, sCW hydrolysis of shrimp shell resulted in higher yield of protein (140.73 mg/g shrimp shell, batch mode) (Espíndola-Cortés et al., 2017) compared to our study. Shrimp shell was hydrolysed at a solid-liquid ratio of 9:100 (w/w) at 260°C and 53.6 bar for 5 min (Espíndola-Cortés et al., 2017). However, in their study, the hydrolysis was conducted in a batch reactor, so the mode of process might have an influence on the yield of hydrolysis, also different species, gender of shrimp may have shell with different composition.

Above 40 min of sCW hydrolysis, there was not an increase in the total nitrogen content (Fig. 3.3A). Moreover, there was a reduction in the total nitrogen content of hydrolysates obtained with sCW assisted by ultrasound at temperatures higher than 180°C (Fig. 3.3B & C). This reduction was attributed to the cleavage of protein into peptides and free amino acids, which could also further break down into organic acids with low molecular weight such as formic acid, acetic acid, propionic acid, etc. (Quitain et al., 2002).

Fig. 3.4 shows free amino acids content of hydrolysates obtained by sCW (A), sCW assisted by ultrasound (600 W) (B), and sCW assisted by ultrasound (1200 W) (C). At 140°C, sCW resulted in comparable amino acid yield with of sCW assisted by ultrasound. While at 260°C, sCW assisted by ultrasound (1200 W, 5 min) resulted in higher amino acid yield (41.93-70.92 mg/g shrimp shell) than sCW (20.99-49.06 mg/g shrimp shell) within 10 to 60 min (Fig. 3.4A &

C). The highest amino acid content of 70.92 mg/g shrimp shell was achieved by sCW assisted by ultrasound at a power of a 1200 W for 5 min, and then hydrolysing with water at subcritical condition of 260ºC and 50 bar and a solid-liquid ratio of 1:150 (w/v) for 60 min (Fig. 3.4C). However, alkali treatment resulted in a lower free amino acids content of 21.31 mg/g shrimp shell. This result was consistent with the result of Quitain et al. (2001), who reported a yield of 70 mg amino acid/g shrimp shell with sCW hydrolysis of shrimp shell at 250°C, 40 bar and a solid-liquid ratio of 1:125 (w/v) for 60 min.

Osada et al. (2015) indicated that all proteins of crab shell were hydrolysed, and α-chitin was stable at 300°C for 30 min (pressure not reported) during sCW hydrolysis. Also, amino acids of crab shell were found to be stable during sCW hydrolysis at 170°C (Hao et al., 2021). In a study by Aneesh et al. (2020), amino acid profile of shrimp shell showed that lysine and glutamic acid were the major amino acids, followed by alanine, aspartic acid, glycine, and leucine. Similarly, Liu et al. (2022) reported lysine, glycine, and alanine as the main amino acids in the shrimp shell hydrolysates obtained after sCW hydrolysis at 140-220°C and 50 bar for up to 60 min. However, tyrosine, followed by lysine, arginine, and histidine were the main amino acids in the hydrolysates obtained at a higher temperature of 260°C. Quitain et al. (2001) also observed that with the increase of temperature from 90 to 250°C during hydrothermal treatment of shrimp shell, there was an increase in the amount of glycine from 0 to 28 mg/g shrimp shell and alanine from 4 to 17 mg/g shrimp shell. The increase in the amount of these amino acids was attributed to the decomposition of complex amino acids such as arginine and aspartic acid to form simple amino acids like glycine and alanine, and the stability of glycine and alanine during hydrothermal treatment. Also, they observed the decomposition of all amino acids at 300°C to organic acids (acetic acid) and ammonia (NH_3) by decarboxylation and deamination, respectively.

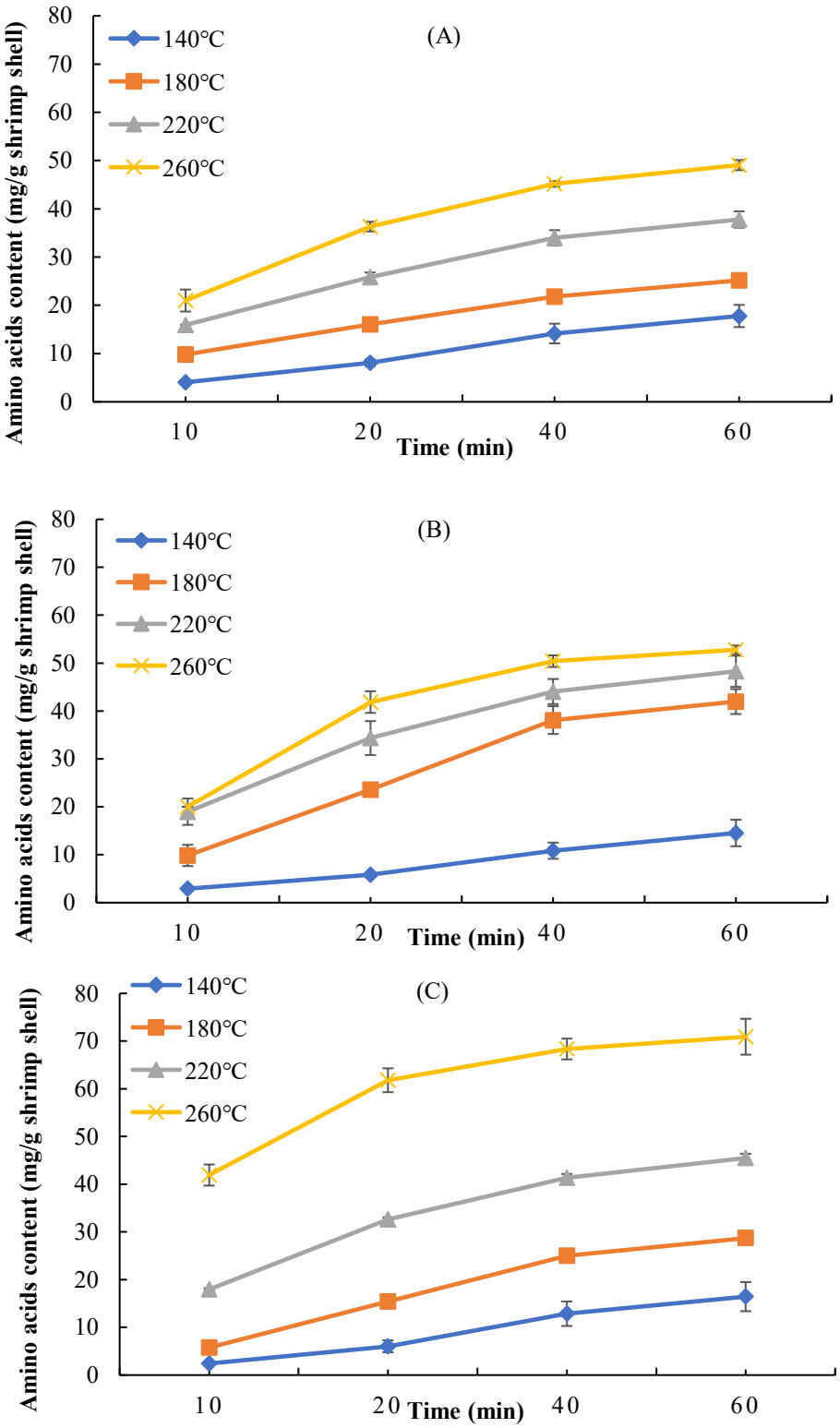

Figure 3.4. Effect of temperature and time on amino acids content of shrimp shell hydrolysates obtained by: (A) only SCW, (B) ultrasound (600 W, 5 min) + SCW, and (C) ultrasound (1200 W, 5 min) + SCW treatments at 50 bar.

Cuticular proteins present in the shell of shrimp, crab, and insects are mainly lipophilic protein (Suzuki et al. 2001). Nitrogen content of hydrolysates obtained after deproteination of shrimp shell and the degree of deproteination using alkali treatment with 2.0 M sodium hydroxide at a ratio of 1:16 w/v at room temperature for 48 h, sCW at 260ºC/50 bar for 40 and 60 min, and sCW hydrolysis assisted by ultrasound (1200 W for 5 min) at 180ºC/50 bar and 260ºC/50 bar for 60 min are summarized in Table 3.3. There was not a significant difference in the nitrogen content and degree of deproteination obtained by alkali and sCW at 260ºC and 50 bar for 40 and 60 min treatments. Alkali treatment resulted in hydrolysates with nitrogen content of 46.09 mg/g shrimp shell and degree of deproteination of 58.84%. Hydrolysates with the highest nitrogen contents (99.01 mg/g shrimp shell) was obtained by sCW hydrolysis assisted by ultrasound (1200 W for 5 min) at 180ºC and 50 bar for 60 min followed by sCW hydrolysis assisted by ultrasound (1200 W for 5 min) at 260ºC and 50 bar for 60 min (77.67 mg/g shrimp shell). The highest degree of deproteination of 80.93% was obtained by sCW hydrolysis assisted by ultrasound (1200 W for 5 min) at 260ºC followed by 72.57% with sCW hydrolysis assisted by ultrasound (1200 W for 5 min) at 180ºC. The higher degree of deproteination at 260ºC was consistent with higher free amino acids content at this condition, that was discussed earlier (Fig. 3.4C). This indicated that the rate of hydrolysis of protein to free amino acids was higher than the rate of protein extraction at temperatures higher than 180ºC.

Table 3.3. Nitrogen content of hydrolysates and degree of deproteination of residue obtained after alkali, sCW, and ultrasound + sCW treatments.

Deproteination method	Nitrogen content (mg/g shrimp shell)	Degree of deproteination (%)
Alkali (2.0 M NaOH, 1:16 w/v, room temperature, 2880 min)	46.09 ± 1.85[a]	58.84 ± 0.95[a]
sCW (260ºC, 50 bar, 40 min)	34.02 ± 2.90[a]	58.05 ± 0.30[a]
sCW (260ºC, 50 bar, 60 min)	34.02 ± 2.90[a]	60.61 ± 0.89[a]
Ultrasound (1200 W, 5 min) + sCW (180ºC, 50 bar, 60 min)	99.01 ± 0.75[b]	72.57 ± 0.65[b]
Ultrasound (1200 W, 5 min) + sCW (260ºC, 50 bar, 60 min)	77.67 ± 7.66[c]	80.93 ± 0.55[c]

Data are expressed as mean ± standard deviation, n = 2. [a-c]Values with different letters in same column are significantly different at $p<0.05$. Degree of deproteination (%) $= [1- ((m_c \times p_c)/(m_s \times p_s))] \times 100$, where, m_c and p_c are mass and protein content of residue and m_s and p_s are mass and protein content of initial shrimp shell.

3.3.3. Characterization of chitosan-rich residue

Deproteinized shrimp shells obtained with different deproteination methods in the previous section, sCW (260°C, 50 bar, 40 min), sCW (180°C, 50 bar, 60 min) assisted by ultrasound (1200W, 5 min), and sCW (260°C, 50 bar, 60 min) assisted by ultrasound (1200W, 5 min), along with the deproteinized shrimp shell with alkali solution (2.0 M NaOH, 1:16 w/v, room temperature, 2880 min) were demineralized, bleached, and deacetylated to obtain chitosan-rich residue. Yield, degree of deacetylation, color, chemical structure, crystallinity, and surface morphology of residue were analyzed.

3.3.3.1. Physicochemical parameters

Table 3.4 shows the yield, degree of deacetylation, and whiteness index of chitosan-rich residues obtained from deproteinized shrimp shells after different deproteination methods. Yields of 17.76, 19.11, 13.04, and 10.56% were obtained with alkali, sCW, and sCW assisted by ultrasound at 180°C and 260°C, respectively. The yields obtained by alkali and sCW were similar

to the yield of 19.47% chitosan (Nouri et al., 2016) isolated from shrimp shell using deproteination with 2% sodium hydroxide (1:30 w/v, 80°C, 2 h), demineralization with 10% acetic acid (1:40 w/v, 50°C, 4 h), and deacetylation with 50% sodium hydroxide (ratio not reported) assisted by microwave with a power of 720 W for 20 s. The lower yields of chitosan-rich residue obtained with sCW assisted by ultrasound at 180°C and 260°C could be due to the lower protein content in the residue after deproteination of shrimp shell and maybe degradation to glucosamine or glucose. As, it can be seen from Table 3.3, the degree of deproteination of shrimp shell after alkali, sCW, and sCW assisted by ultrasound at 180°C and 260°C treatments were 58.84, 58.05, 72.57, 80.93%, respectively.

However, in other study by Zhang et al. (2022), a lower deproteination degree of shrimp shell of 9.1% (with pre-treatment) was obtained, where the shell was pretreated with ultrasound at a power of 800 W for 30 min with water (20 kHz, 2.5:20 w/v) and deproteinized with fermentation media containing 1g/L yeast extracts, 50 g/L of glucose at a ratio of 1:1 w/v for 5 d at 37°C. Al Hoqani et al. (2020) reported a yield of 4.7% chitosan obtained from shrimp shell. The low yield of their study could be due to hydrolysis of chitin to glucosamine and/or glucose at the more severe acidic and basic conditions applied for demineralization (3% hydrochloric acid, 1:10 w/v, 25°C, 1 h), deproteination (50% sodium hydroxide, 1:10 w/v, 110°C, 3 h), and deacetylation (50% sodium hydroxide, 1:10 w/v, 121°C, 15 min) treatments. The yields obtained with sCW assisted by ultrasound deproteination at 180°C (13.04%) and 260°C (10.56%) were comparable with the value reported by Hao et. (2021), where a yield of 11% of chitosan from crab shell was obtained. In their study, the shell was deproteinized with sCW at 170°C for 1 h at a ratio of 1:15 w/v (pressure not reported), demineralized using 10% hydrochloric acid at a ratio of 1:10 (w/v) for 5 h at room temperature, then deacetylated using 30% sodium hydroxide (ratio not reported) at

120°C for 6 h in an autoclave. In other study by Yen et al. (2009), chitosan with a yield of 32.2% was isolated from crab shell by demineralization with 1 N hydrochloric acid (ratio not reported, 6 h, room temperature), deproteination with aqueous sodium hydroxide (concentration not reported, 1:10 w/v, 100°C, 3 h), and deacetylation with 40% sodium hydroxide (1:30 w/v) at 105°C for 120 min. The lower yield of crab shell chitosan (11%) obtained by Hao et al. (2021) compared to the value (32.3%) reported by Yen et al. (2009), probably is due to the higher degree of deproteination of crab shell in their study. Hence, they used sCW at 170°C for 1 h for deproteination and used a higher temperature (120°C) and prolonged time (6 h) for deacetylation compared to the Yen et al. (2009). Moreover, the species, gender, and season of harvest may affect the crustacean shell composition in terms of chitin content and eventually the yield of chitosan.

Table 3.4. Physicochemical properties of chitosan-rich residue obtained from shrimp shell with (A) alkali (2 M NaOH, 1:16 w/v, 48 h, room temperature), (B) sCW (260°C, 50 bar, 40 min), (C) ultrasound (1200W, 5 min) + sCW (180°C, 50 bar, 60 min), (D) ultrasound (1200 W, 5 min) + sCW (260°C, 50 bar, 60 min).

	Yield (%)	Degree of deacetylation (%)	Whiteness index
This study			
Chitosan-rich A	17.76 ± 0.50[a]	65.09 ± 0.15[a]	67.80 ± 0.01[a]
Chitosan-rich B	19.11 ± 0.20[a]	66.29 ± 0.84[a]	63.97 ± 0.02[b]
Chitosan-rich C	13.04± 0.58[b]	60.70 ± 0.48[b]	63.27± 0.01[b]
Chitosan-rich D	10.56 ± 1.24[b]	64.27 ± 0.21[a]	60.42 ± 0.48[c]
Commercial chitosan	NR	≥75	68.21 ± 0.02[a]
Literature			
Shrimp shell chitosan (Nouri et al., 2016)	19.50	89.30	NR
Shrimp shell chitosan (Al Hoqani et al., 2020)	4.70	NR	NR
Shrimp shell chitosan (Zaeni et al., 2017)	NR	61.80	NR
Shrimp shell chitosan (Ahing & Wid, 2016)	4.09	79.68	NR
Crab shell chitosan (Hao et al., 2021)	11.00	84.10-88.50	NR
Crab shell chitosan (Yen et al., 2009)	32.20	83.30-93.30	55.80-56.60

Data are expressed as mean + standard deviation, n = 2. [a-c]Values with different letters in same column are significantly different at $p<0.05$. NR: not reported.

The degree of deacetylation determines the free amino group content of polysaccharides that generally has been used to distinguish between chitin and chitosan. Commercial chitosan has a degree of deacetylation between 40 to 98% (Bakshi et al., 2020). For example, chitosan purchased from Simga Aldrich is labeled with a degree of deacetylation of ≥75%. A range of 60.7-66.29% of degree of deacetylation was observed for chitosan-rich residue obtained with alkali, sCW, and sCW assisted by ultrasound in Table 3.4, so the chitosan-rich residues obtained in our study were comparable with the commercial chitosan regardless of the deproteination

method. The lower degree of deacetylation of 60.70% obtained by subcritical water assisted by ultrasound at 180°C could be due to the lower pH of the subcritical water media at this temperature. Hence, a basic pH required to promote deacetylation of chitin. While at a higher temperature than 200°C the pH of the media tends to increase. These results were consistent with 61.78% degree of deacetylation for chitosan obtained from shrimp shell by Zaeni et al. (2017). In their study, shrimp shell was demineralized with 1.0 N hydrochloric acid (1:10 w/v, 1 h, 75°C), deproteinized with 3.5% sodium hydroxide (1:10 w/v, 2 h, 65°C), and deacetylated by 50% sodium hydroxide (ratio not reported, heated with microwave for 11 min, power not reported). However, Ahing & Wid (2016) reported a higher degree of deacetylation of 79.68% for chitosan from shrimp shell probably because deacetylation of isolated chitin was performed using 48% sodium hydroxide at room temperature for an extended time of 48 h (ratio of solid:liquid not reported).

Yen et al. (2009) also reported a high degree of deacetylation of 83.3-93.3% for chitosan isolated from crab shell, where purified crab chitin was deacetylated with 40% sodium hydroxide (1:30 w/v) at 105°C for 60, 90, and 120 min, an increasing trend observed with increasing the time of treatment. Hao et al. (2021) observed that the incorporation of citric acid (1%) in sCW hydrolysis of crab shell resulted in a significant increase from 84.1% to 88.5% in the degree of deacetylation of chitosan. Also, Nouri et al. (2016) observed that microwave irradiation with a power of 720 W for 20 s (50% NaOH, ratio not reported) increased the degree of deacetylation of shrimp shell chitosan from 81.69% to 89.34% in comparison to the conventional deacetylation (50% NaOH, ratio not reported, 100°C, 60 min). The lower degree of deacetylation of our study in comparison to the literature may be due the short time (15 min at 121°C) of deacetylation treatment.

A range of 60.42-67.8 of whiteness index (WI) of chitosan-rich residue obtained with alkali, sCW, and sCW assisted by ultrasound at 180°C and 260°C were observed in Table 3.4. The results were consistent with the yellow to whitish color of shrimp shell chitosan reported by Al Hoqani (2020). They reported that the best condition for decolorization of chitin was using 30% hydrogen peroxide (1:10 w/v) for 3 h. In other study by Vallejo-Domínguez et al. (2021), a maximum WI of 43.31 was observed for chitosan from shrimp shell where shrimp shell was demineralized with 0.6 M hydrochloric acid (1:11 w/v, 30°C, 3h), deproteinized by sonication with deionized water (power not reported) for 20 min, and deacetylated with 50% sodium hydroxide (1:4 w/v, initial heating at 70° for 2 h, followed by heating at 115°C for 2 h). However, in their study decolorization of chitin was skipped. Also, a more yellowish color (WI= 55.8-56.6) was observed for crab chitosan (Yen et al., 2009). The WI of chitosan-rich residues obtained with sCW assisted by ultrasound at 260°C was significantly lower than of the other treatments. Previous studies reported that the more yellowish color of chitosan from sCW hydrolysis might be due to Maillard reaction between the amino group and carbonyl group present in chitosan (Zhao & Saldaña, 2019). Also, oxidation of astaxanthin induces the yellowish colour of chitin and consequently of chitosan (Vallejo-Domínguez et al., 2021).

3.3.3.2. Fourier transform infrared (FTIR) spectroscopy

The FTIR analysis was done to investigate the effects of different deproteination methods on the chemical structure of chitosan-rich residue. Fig. 3.5 shows the FTIR curves of chitosan-rich residue obtained by different treatments (A) and commercial chitosan (B), which were comparable in terms of chitosan characteristic functional groups but had different intensities. Absorbance peaks at 3416, 1670, 1580, 1390, 2890, and 1030-1170 cm^{-1} corresponding to O–H, amide I, amide II, C–H, C–O–C, and C–O, respectively, which attributed to chitosan characteristics were observed

regardless of deproteination method with lower intensities compared to commercial chitosan. Besides, peak at 1320 cm^{-1} corresponding to amide III was observed only in the chitosan-rich residues obtained by sCW assisted by ultrasound at 260°C and alkali treatments but with lower intensity compared to commercial chitosan (Fig. 3.5A). The higher intensity of the peaks in the spectra of commercial chitosan reflects its higher degree of deacetylation (Fig. 3.5B).

The absence of new absorbance peaks in the spectra indicated that none of the deproteination methods altered the chemical structure of chitosan-rich residue. The results of the present study were consistent with the results reported by Trung et al. (2020) where O–H, amide II, amide III, C–H, C–O–C, and C–O were split at similar bands for chitosan from shrimp shell. The results of the present study were also consistent with the results reported by Hao et al. (2021) where O–H, amide I, and amide II were split at similar bands for chitosan from crab shell. In the spectra of chitosan-rich residue regardless of deproteination method, there were no peaks splitting at 1430 and 870 cm^{-1} characteristics of carbonate groups (Osada et al., 2015), indicating the absence of calcium carbonate, the main mineral of shrimp shell. However, a peak at 1430 cm^{-1} corresponding to calcium carbonate was present in the commercial chitosan with low intensity (Fig. 3.5B). Splitting of amide I indicated the deacetylation of chitin in chitosan-rich residue (Al Sagheer et al., 2009). Similar results were observed in chitin isolated from prawn shell pre-treated by hot glycerol (18.9:200 w/w, 200°C, 4 min), then demineralized and deproteinized with citric acid (8.5 g shell, 0.044 mole citric acid, 300 mL water, 30 min) (Devi & Dhamodharan, 2018). Similarly, Zaeni et al. (2017) observed amide I in the FTIR spectra of chitosan obtained from shrimp shell. Where deacetylation was performed using 50% sodium hydrochloride and heated by microwave (power not reported) for 7-15 min.

The absorbance peak corresponding to N−H stretching of chitin splitting at 3261 cm^{-1} was not observed in none of the samples that reflects the deacetylation of chitin, while Yang et al. (2019) observed a very intense peak for shrimp chitin. The weaker intensities of C–H and O−H stretching for the chitosan-rich residue in comparison to the commercial chitosan, indicating a remarkable disruption of the inter/intramolecular hydrogen bonding network.

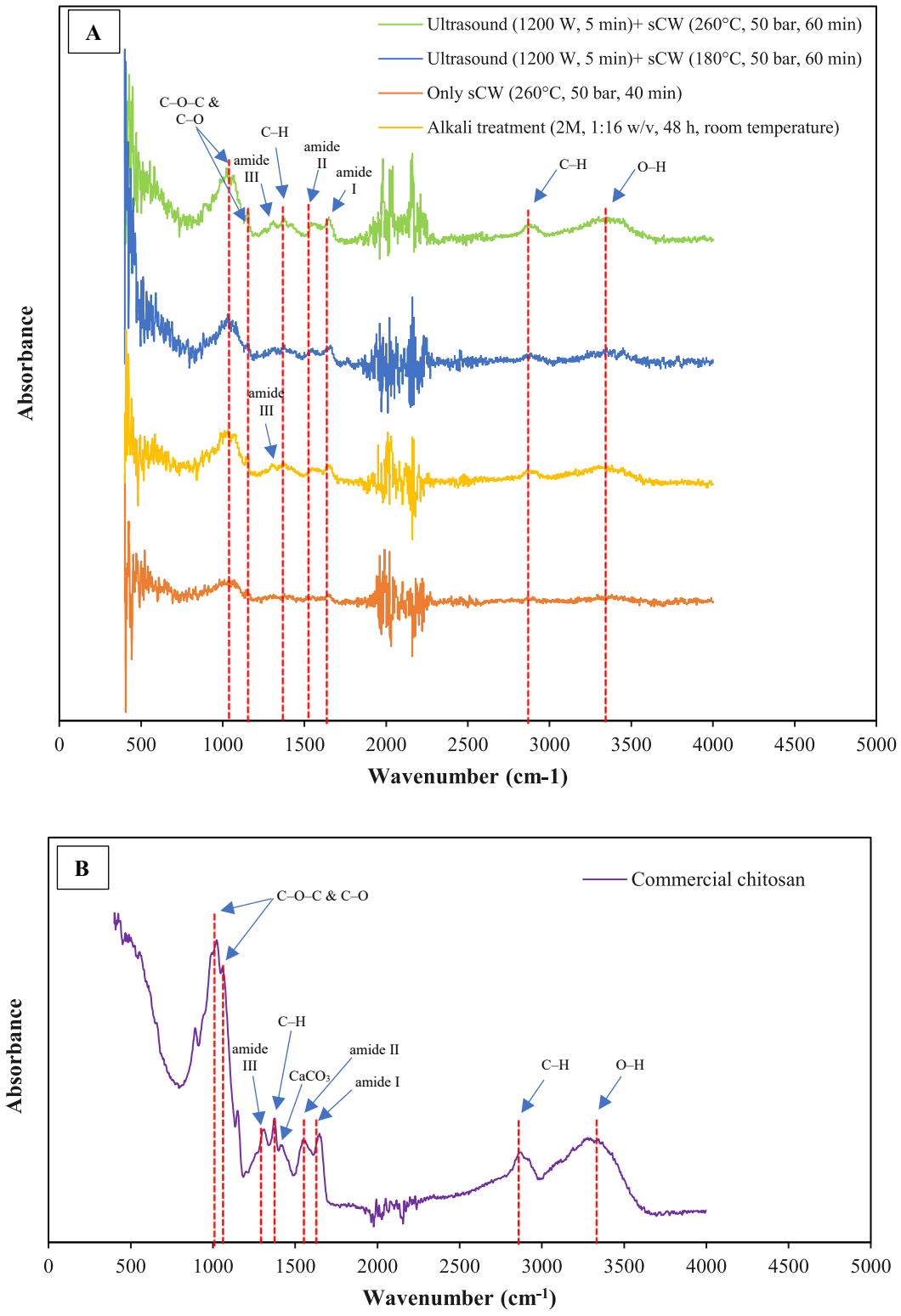

Figure 3.5. FTIR spectra of chitosan-rich sample obtained using different methods for deproteination of shrimp shell (A) and commercial chitosan (B).

3.3.3.3. X-ray diffraction

Fig. 3.6 shows crystalline structure of the chitosan-rich residue obtained using different methods for deproteination of shrimp shell (A) and commercial chitosan (B) analyzed by X-ray powder diffraction within two-theta range of 5-55°. The X-ray of chitosan-rich residue obtained by different deproteination methods had similar patterns but different intensities. Only two crystalline reflections corresponding to two-theta of around 9.3° and 20° were observed regardless of the deproteination method (Fig. 3.6A). These results were comparable with the X-ray of commercial chitosan where only two reflections at 11.5° and 23.6° were observed (Fig. 3.6B). The crystalline reflections of calcium carbonate at two-theta of 30° (Osada et al., 2015) was not observed in the chitosan-rich residue obtained by any deproteination method used, which indicated that the residue was free of minerals.

Similar crystalline reflection angle positions were observed for chitosan obtained from crab shell deproteinized with sCW at 170°C for 1 h and a ratio of 1:15 w/v (pressure not reported), demineralized using 10% hydrochloric acid at a ratio of 1:10 w/v for 5 h at room temperature, then deacetylated using 30% sodium hydroxide (ratio not reported) at 120°C for 6 h in an autoclave (Hao et al., 2021). Earlier, for shrimp shell chitosan, two crystalline reflections were also observed at around 11.7° and 22.3° (Nouri et al., 2016). The results were also comparable with crystalline reflections observed for chitosan at 10.2° and 19.8-20.1° (Vallejo-Domínguez et al., 2021). In their study, chitosan was isolated from shrimp shell using 0.6 M hydrochloric acid (1:11 w/v, 2 h, 30°C) for demineralization, high frequency ultrasonication (10-40 min, power and frequency not reported) for deproteination, and 50% sodium hydroxide (1: w/v, initially heated at 70°C for 2 h, followed by heating at 115°C for 2 h) (Vallejo-Domínguez et al., 2021).

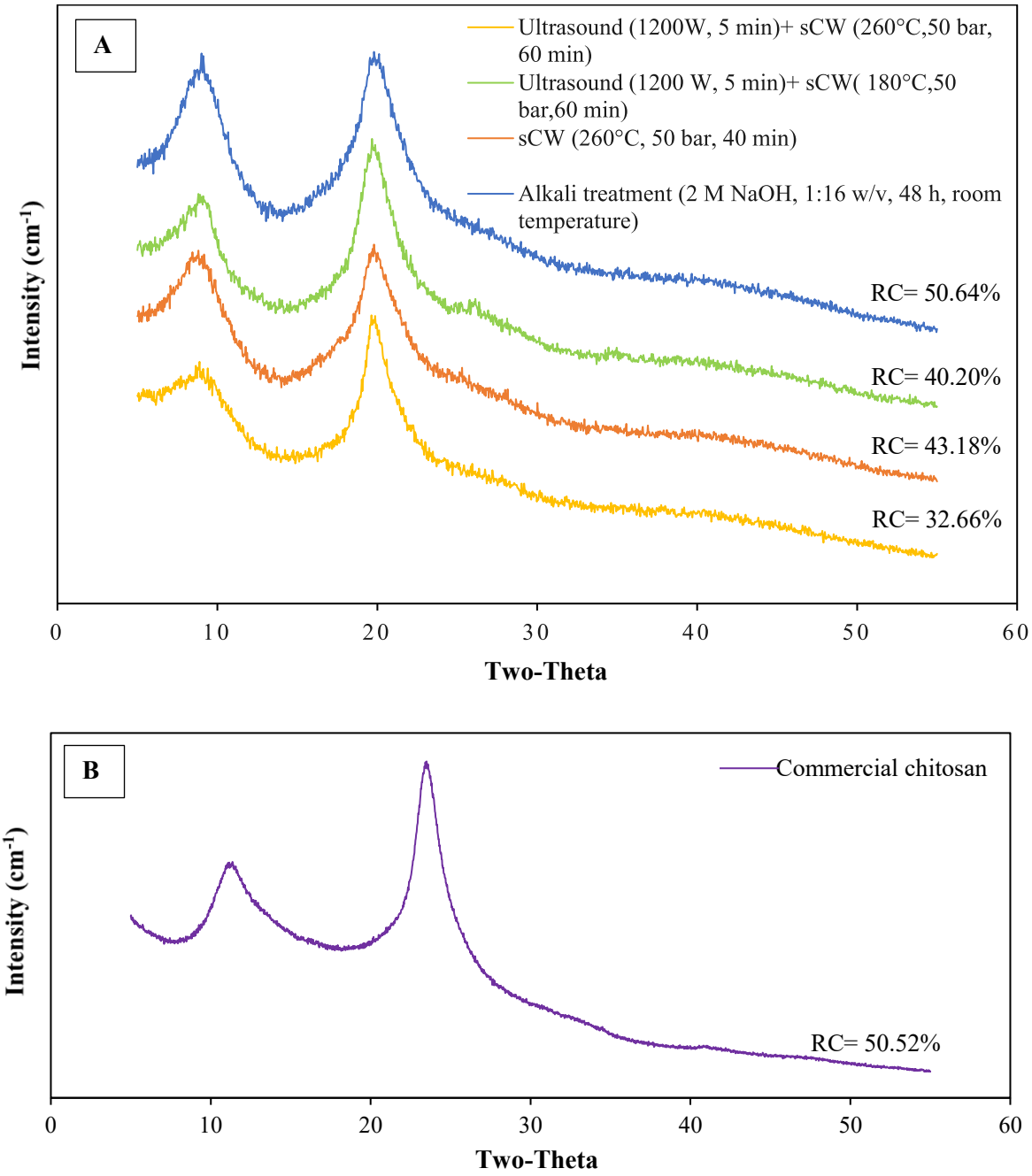

Figure 3.6. X-ray pattern of chitosan-rich residue obtained using different methods for deproteination of shrimp shell (A) and commercial chitosan (B). RC: relative crystallinity.

The crystalline reflections of chitosan-rich residue obtained by alkali deproteination were more intense than chitosan-rich residue obtained with sCW and sCW assisted by ultrasound. Crystalline reflections with lower intensity indicate the partial breakage of hydrogen bonds within

the chitosan-rich residue structure during sCW and sCW assisted by ultrasound. Generally, hydrogen bonds decrease the rotation degree of sugar residues by obstructing the spinning of them along the glycoside bonds, eventually result in the formation of crystalline structure (Hao et al., 2021). However, there was not a significant difference in the intensity of the reflections when chitosan hydrolyzed from shrimp shell by deproteination with 2% sodium hydroxide (1:30 w/v, 80°C, 2 h), demineralization with 10% acetic acid (1:40 w/v, 50°C, 4 h), and deacetylation with 50% sodium hydroxide (ratio not reported) assisted by microwave with a power of 720 W for 20 s (Nouri et al., 2016).

The relative crystallinity of chitosan-rich residue obtained from deproteinized shrimp shell using alkali, sCW, sCW assisted by ultrasound at 180°C, and sCW assisted by ultrasound at 260°C were 50.64%, 43.18%, 40.2%, and 32.66% respectively (Fig. 3.6A). The relative crystallinity of commercial chitosan was 50.52% (Fig. 3.6B), that was similar to the value of residue obtained by alkali deproteination and higher than the ones deproteinized with sCW with/without ultrasound treatments. The results indicated that sCW assisted by ultrasound at 260°C was more effective in destruction of the crystalline region than the other methods and the chitosan-rich residue had more amorphous region. This also indicated that sCW benefited from the ultrasound pre-treatment in breaking the intramolecular hydrogen bonds (Noori et al., 2016). Hongkulsup et al. (2016) reported that chitin with lower crystallinity had higher solubility.

In other study, a relative crystallinity of 80.9% was observed for prawn shell chitin isolated from prawn shell pre-treated by hot glycerol (18.9:200 w/w, 200°C, 4 min), then demineralized and deproteinized with citric acid (8.5 g shell, 0.044 mole citric acid, 300 mL water, 30 min) (Devi & Dhamodharan, 2018). In the study by Trung et al. (2020), chitosan isolated from shrimp shell had similar X-ray pattern as observed in our study, and with comparable relative crystallinity of

30%. Shrimp shell was firstly pretreated with 0.35 M hydrochloric acid (1:2.5 w/v, room temperature, 24 h), then demineralized with 0.8 M hydrochloric acid (1:3.5 w/v, room temperature, 24 h) and deproteinized with 0.75 M sodium hydroxide (1:2.5 w/v, room temperature, 24 h) to obtain chitin. Afterward, chitin was deacetylated with 12 M sodium hydroxide (1:5 w/v, 65°C, 12 h).

3.3.3.4. Surface morphology

Fig. 3.7 shows SEM images with 400x magnifications of chitosan-rich residues obtained using different methods for deproteination of shrimp shell (A, B, C, and D) and commercial chitosan (E). The surface morphology reflects the extent of the surface destruction on the chitosan-rich residue during deproteination, demineralization, decolorization, and deacetylation of shrimp shell. A flake shape characteristic was observed for the chitosan-rich residue, regardless of the deproteination method. The same structure was observed for chitin where alkali treatment was used for deproteination of prawn shell (Devi & Dhamodharan, 2018). More compact particles with some microfibrillars were observed for chitosan-rich residue obtained with alkali deproteination (Fig. 3.7A), which is associated to the higher crystallinity observed by X-ray analysis. Similarly, a compact structure with aggregates on the surface was observed for commercial chitosan (Fig. 3.7E) that had a similar crystallinity as the obtained with alkali treatment. Flakes with more microfibrillar structure were observed for chitosan-rich residue obtained with sCW with or without ultrasound. However, a smoother surface with more uniformity was observed for chitosan-rich residue obtained with sCW (Fig. 3.7B) in comparison to the one obtained with sCW assisted by ultrasound (Fig. 3.7C & D).

Some crumbling flakes were observed on the chitosan-rich residues obtained with sCW assisted by ultrasound at 180°C and 260°C. However, the commercial chitosan had higher number

of crumbling flakes. The presence of crumbling flakes on the chitosan-rich residue obtained with sCW assisted by ultrasound could be attributed to the destruction of chitosan microfibrils caused by acoustic phenomena and turbulence occurred during ultrasonication.

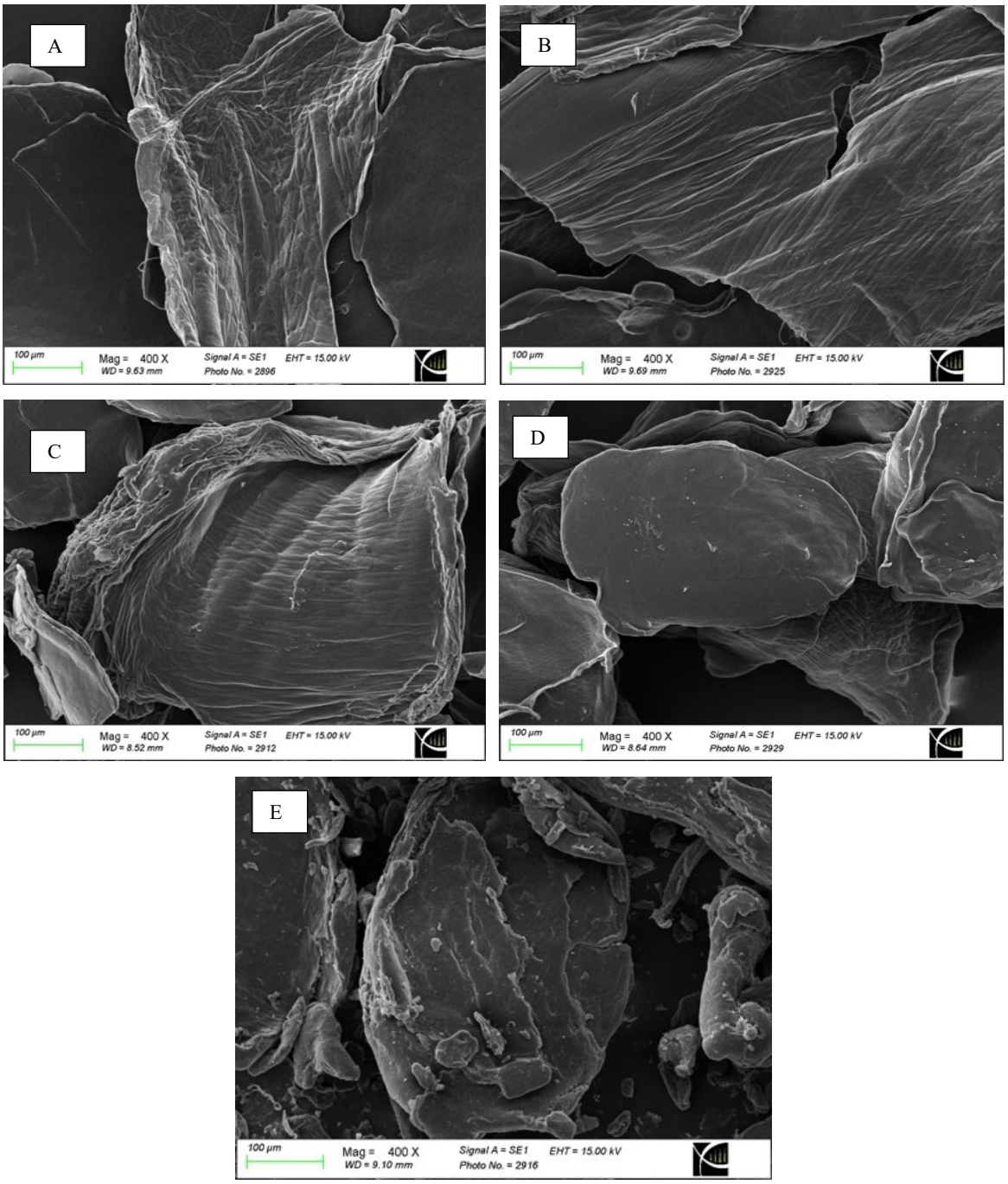

Figure 3.7. SEM images of chitosan-rich residue obtained by: (A) alkali, (B) sCW (260ºC, 50 bar, 40min), (C) ultrasound (1200W, 5 min) + sCW (180ºC, 50 bar, 60 min), (D) ultrasound (1200W, 5 min) + sCW (260ºC, 50 bar, 60 min), and commercial chitosan (E).

Similar results were observed when chitosan was isolated from shrimp shell using 0.6 M hydrochloric acid (1:11 w/v, 2 h, 30°C) for demineralization, high frequency ultrasonication (10-40 min, power and frequency not reported) for deproteination, and 50% sodium hydroxide (1: w/v, initially heated at 70°C for 2 h, followed by heating at 115°C for 2 h) (Vallejo-Domínguez et al., 2021). Yen et al. (2009) reported that chitin purified from crab shell exhibited microfibrillar structure, but chitosan showed layers with crumbling flakes. Chitin was isolated from crab shell by demineralization with 1 N hydrochloric acid (ratio not reported, 6 h, room temperature) and deproteination with aqueous sodium hydroxide (1:10 w/v, 100°C, 3 h). Deacetylation of chitin was performed with 40% sodium hydroxide (1:30 w/v, 105°C, 2 h). The presence of crumbling flakes could be due to the long time of deacetylation process which resulted in dehydration and destruction of chitosan microfibrils (Yang et al., 2014; Zhao et al., 2007).

3.4. Conclusions

This study investigated the effects of temperature and time on deproteination of pretreated shrimp shell with ultrasound using sCW at 50 bar with a flow rate of 5 mL/min. The hydrolysate with the highest yield of nitrogen content (99.01 mg/g shrimp shell) with degree of deproteination of 72.57% was obtained from shrimp shell pretreated with ultrasound at 1200 W for 5 min and then hydrolysed with subcritical water at 180^{o}C and 50 bar for 60 min. However, at 260°C/50 bar/60 min, sCW assisted by ultrasound (1200 W, 5min) resulted in the highest free amino acid yield (70.92 mg/g shrimp shell with the highest degree of deproteination of shrimp shell (80.93%). These values were significantly higher than those of the alkali treatment where nitrogen content of 46.09 mg/g shrimp shell, free amino acids content of 21.31 mg/g shrimp shell, and 58.84% degree of deproteination were obtained. The process benefited from the use of ultrasound pre-treatment since it enhanced the protein hydrolysis compared to sCW without incorporation of ultrasound

pre-treatment (34.2 mg/g shrimp shell total nitrogen content, 49.06 mg/g shrimp shell free amino acids content, and 60.61% degree of deproteination at 260°C, 50 bar, 60 min).

Deproteinized shrimp shell were further processed to remove minerals and pigments, ultimately deacetylated to produce chitosan. The yield (10.56%) and whiteness index (60.42) of chitosan-rich residue obtained from deproteinized shrimp shell using sCW assisted by ultrasound at 260°C were lower than the alkali treated sample (17.76% and 67.8, respectively). But chitosan-rich residue obtained from deproteinized shrimp shell using sCW assisted by ultrasound at 260°C had comparable degree of deacetylation (64.27%) and similar functional groups to the alkali treated sample (65.05% degree of deacetylation) and commercial chitosan (>75% degree of deacetylation) that indicates this process did not alter the functional group of the residue. However, it had lower relative crystallinity (32.66%) than the alkali treated sample (50.64%) and the commercial chitosan (50.52%), which indicated its better solubility (the lower relative crystallinity the better solubility). Therefore, the results of this study suggested that sCW assisted by ultrasound was a promising treatment for deproteination of shrimp shell in short time (60 min) in a greener way. Also, it is possible to improve the yield and physico-chemical properties of chitosan by manipulating the power and time of ultrasound pre-treatment.

Chapter 4: Hydrolysis of chicken feet to produce collagen/collagen fragments using pressurized hot water technology

4.1. Introduction

Collagen, a macromolecule with a helical structure, consists of three α-chains. Each α-chain is composed of around 1000 amino acids with a specific repeating unit of glycine-proline-hydroxyproline (Potti & Fahad, 2017). The structural and rheological properties of collagen make it an excellent biopolymer for food, pharmaceutical, biomedical, leather, and cosmetic industries (Pati et al., 2010; Ferraro et al., 2016). Collagen is commonly used in its denatured form, gelatin, which has distinct functional properties such as water holding capacity, film forming, texturizing, thickening, and emulsification (Pal & Suresh, 2016; Ahmed et al., 2020). Collagen can be converted into gelatin by thermal treatment at 70-80°C for 7-8 h (Ames, 1952), resulting in partial cleavage of covalent and hydrogen bonds and eventually a helix to coil transition (Gómez-Guillén et al., 2011). Gelatin has been used in food, cosmetic, tissue engineering, pharmaceutical, and bio-ink for 3D printing products (Cao et al., 2020).

Collagen, a fibrous protein of the extracellular matrix of animals, can be extracted from the animal skin, bones, and tendons (Hong et al., 2019). Beef and pork by-products are the main source for collagen. However, there are some religious restrictions and health concerns in the consumption of porcine and bovine collagen. Seafood by-products such as skin, bone, and scale are considered as alternative sources of collagen. But fish collagen has low thermal stability and weak rheological properties compared to the bovine and porcine collagen (Gómez-Guillén et al., 2011), which limits its applications in industrial products. For this reason, poultry by-products have gained considerable attention as a potential source of collagen in the past decade.

There are two main steps for collagen extraction from animal by-products: (1) pre-treatment of raw material to remove non-collagenous protein, lipid, odor, and pigment, also break noncovalent inter/intramolecular bonds and disrupt the triple-helix structure of collagen (Gómez-Guillén et al., 2011), and (2) hydrolysis of collagen based on its solubility in neutral saline, acidic media with or without enzymes to break inter/intramolecular covalent crosslinks, ester bonds and other bonds with saccharides (Hong et al., 2019). Sodium hydroxide and calcium hydroxide are mainly used for pre-treatment of the raw material (Schmidt et al., 2016). Liu et al. (2015) reported 0.05-0.1 M sodium hydroxide as the best concentrations to remove non-collagenous material without losing acid soluble collagen and disturbing triple helix structure of collagen (analyzed by FTIR).

Traditionally, acid treatment has been used for extraction of collagen from chicken feet (Liu et al., 2001), chicken claws (Siswanto et al., 2020), duck feet (Theng et al., 2018), and beef bone (Ferraro et al., 2017). Inorganic acids such as hydrochloric acid and organic acids such as citric acid, lactic acid, and acetic acid are the main solvents utilized for acid hydrolysis. Wang et al. (2008) investigated the effect of acetic acid concentration (0.3, 0.5, and 0.8 M), temperature (10, 20, and 30°C) and time (12, 24, and 36 h) on collagen extraction (ratio of solid-solvent not reported) from grass carp skin. They reported an acetic concentration of 0.54 M, temperature of 24.7°C and extraction time of 32.1 h as optimum conditions to obtain the highest yield of collagen (19.3%). Liu et al. (2001) explored different acid solutions (5% unit not reported), including acetic acid, citric acid, lactic acid, and hydrochloric acid at 4°C with a solid-solvent ratio of 1:8 w/v for 12, 24, 36, and 48 h for collagen extraction from chicken feet. The highest yields were achieved by acetic acid (30.86% within 24 h) and lactic acid (30.88% within 36 h), where the amino acid composition showed that with hydrochloric acid collagen was significantly hydrolysed. For

extraction of salt soluble collagen, neutral saline media such as sodium chloride, Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride), phosphates or citrates have been investigated (Wang et al., 2014; Schmidt et al., 2016). The use of saline media in extraction of collagen is limited because the collagen molecules are mainly cross-linked.

Also, collagen extraction by enzymatic hydrolysis of chicken feet (Dhakal et al., 2018), turkey tendon (Grønlien et al., 2019), broiler chicken skin (Cliché et al., 2003), bovine hide (Noorzai et al., 2020), and beef tendon (Ran & Wang, 2014) has been investigated. Pepsin and papain were the main enzymes utilized for collagen extraction. Woo et al. (2008) studied collagen extraction from yellowfin tuna skin employing pepsin (0.6 to 1.4% w/v) in hydrochloric solution (pH 2.0) at 90°C for 12 to 36 h. Pepsin concentration of 0.98% w/v and extraction time of 23.5 h were the optimum extraction conditions to obtain collagen with a yield of 27.1% (molecular weight not reported). In other study, collagen was extracted from chicken feet using papain (30000 U/mg, 1% w/v) at different temperatures of 4, 30, and 56°C for various extraction times of 20, 24, and 28 h (Dhakal et al., 2018). The highest yield of 32.16% was obtained at 30°C for 28 h with a molecular weight distribution of 25-150 kDa. Water holding capacity of chicken feet collagen (1.9 g/g) was higher than the values reported for yak bone (0.29 g/g) and pig skin (0.21 g/g). The oil holding capacity (5.3 g/g) was also higher than yak bone collagen (1.67 g/g) (Dhakal et al., 2018). However, enzymatic treatment is a costly process.

Non-conventional methods such as pulsed electric field (He et al., 2017) and ultrasound (Kim et al., 2012; Ran & Wang 2014; Kim et al., 2013) have been investigated for extraction of collagen from animal by-products. Li et al. (2009) explored assisted enzymatic treatment of collagen from bovine tendon where the flask was immersed in the ultrasonic bath (120 W, 40 kHz) during the enzymatic treatment of 2 d (30 min on and 30 min off) (temperature not reported). They

observed a significant increase in the extraction yield (by 124%) in comparison to enzymatic extraction without ultrasound. However, the effect of ultrasonication on molecular weight of collagen was not analyzed.

Alternatively, pressurized hot water (PHW) has shown to be a promising and eco-friendly technology to recover valuable compounds from food by-products. Pressurized hot water refers to hot water at temperature above its boiling point and below its critical point (subcritical region) (Saldaña et al., 2021) under sufficient pressure to maintain the water in the condensed phase. At this condition, dissociation constant of water increases, and results in a change in the pH of water and the relative permittivity of water reaches values like of organic solvents such as ethanol or methanol (Huerta & Saldaña, 2019). Other terms such as near critical water, subcritical water (sCW), high temperature extraction and extraction using hot compressed water have also been used. The PHW has been used in the extraction of total sugar and protein from defatted rice bran (Hata et al., 2008), lignan, protein, and carbohydrate from flaxseed meal (Ho et al., 2007), phenolic compounds from potato peel (Singh & Saldaña, 2011), anthocyanins and total phenolics from cranberry pomace (Saldaña et al., 2021).

Recently, Melgosa et al. (2021) obtained collagen and collagen fragments (peptides and amino acids) with a recovery of 57.7% and 100% from Atlantic cod (*Gadus morhua*) frames using pressurized hot water at 250°C and 100 bar for 30 min. In other study, a collagen/gelatin mixture with a yield of 32-36% was obtained from different marine sponges using pressurized hot water at 37°C/50 bar for 16 h (Noor et al., 2021). Squid muscle was also hydrolyzed by subcritical water at 160-258°C and 6-66 bar for 3 min, the highest yield of free amino acids (421.53 mg/100g) and structural amino acids (380.58 mg/100g) were obtained at 250°C and 220°C (60 bar for 3 min), respectively (Asaduzzaman & Chun, 2015). Similarly, Ahmed & Chun (2018) reported the highest

degree of hydrolysis (14.47%) of de-oiled tuna skin at 250°C and 50 bar for 5 min. Lee et al. (2013) conducted subcritical water hydrolysis of porcine placenta at 20-200°C and 375 bar for 30-60 min. They reported that more than 60% of collagen was solubilized, alanine, glycine, hydroxyproline, and proline contents increased approximately 5% by increasing holding time and temperature, where time had no effect on the molecular weight. Molecular weight decreased by increasing temperature and holding time from >20 kDa to 1.4-4.2 kDa. Also, they reported that increasing the pressure from 375 bar (170°C, 30 min) to 1000 bar (170°C, 30 min) did not make any difference in protein recovery and free amino acid content. To the best of our knowledge, there is no study on collagen/collagen fragments hydrolysis from chicken feet using PHW. Therefore, the main objective of this study was to investigate the process conditions of temperature and time of PHW for collagen/collagen fragments hydrolysis from chicken feet. Then, the hydrolysates were characterized for degree of hydrolysis, free hydroxyproline content, collagen/collagen fragments content, amino acid profile, and molecular weight distribution.

4.2. Materials and methods

4.2.1. Raw material and chemicals

Chicken feet were kindly provided by Maple Leaf Poultry (Edmonton, AB, Canada). Chicken feet were rinsed with tap water. To obtain a uniform particle size, the chicken feet were ground in a grinder with a particle size of less than 2.0 mm. Then, the ground chicken feet were freeze dried and packed in opaque plastic container and stored in a cool room at a temperature of 4°C.

The chemicals and solvents used in this study, including sodium hydroxide, hydrochloric acid (ACS, analytical reagent), hydrogen peroxide (30%), sodium tetraborate (99%), sodium dodecyl sulfate (SDS) (>98.5%), o-phthaldialdehyde (OPA) (HPLC grade), methanol (HPLC grade), ß-mercaptoethanol (>99%), protein standard mixture 69385, phosphate buffer, sodium azide, B-

amino-n-butyric acid, ethanolamine, sodium acetate, tetrahydrofuran, copper (II) sulfate (>99%), sulfuric acid, p-dimethylaminobenzaldehyde (ACS reagent, 99%), trans-4-Hydroxy-L-proline (>99%) and glycine (>99%) were acquired from Sigma Aldrich (Oakville, ON, Canada). Nitrogen gas (99.9% purity) was purchased from Praxair (Edmonton, AB, Canada). Milli-Q water was obtained from Milli-Q system (18.2 MΩ.cm, Millipore, Billerica, MA, USA).

4.2.2. Proximate composition

Crude protein, ash, moisture, and fat contents of chicken feet were analyzed according to the methods described earlier in Chapter 3, Section 3.2.2.

4.2.3. Pressurized hot water hydrolysis

First, ground chicken feet were pretreated with 0.1 N sodium hydroxide at a ratio of 1:10 (w/v) at room temperature for 24 h (Fig. 4.1). Then, the sample was washed with distilled water until neutral pH (7-7.5). The PHW hydrolysis of pretreated sample was carried out using a Parr 4590 system (Parr Instrument Company, Moline, IL, USA), described earlier by Valdivieso Ramirez et al. (2021). The Parr 4590 system is equipped with a 100 mL batch reactor, nitrogen cylinder, temperature controller, thermocouple, heater, pressure gauge, and stirring system. Pretreated chicken feet and milli-Q water at a ratio of 1:50 w/v were loaded into the 100 mL batch reactor. Before pressurizing, the reactor containing the slurry was flushed with nitrogen gas at 10 bar for 12 min if the medium is water (the time vary depending on the viscosity of the medium) to remove air. Afterwards, the reactor was pressurized with nitrogen gas. In the beginning, the pressure was set below the desire pressure because when heating starts, the pressure increases to reach the desired pressure by the increase of the temperature. In the case of hydrolysis at 50 bar, the pressure was set in the range of 35-48 bar, depending on the target temperature. A heater was used, and temperature was adjusted using the controller, and the temperature inside the reactor was

maintained with the thermocouple. When the desired temperature was reached after 10-15 min depending on the target temperature, timing for the experiment started. Experiments were carried out at a pressure of 50 bar, and temperatures of 40, 80, 120, 140, 160 and 180°C for 10, 20, 40, and 60 min. When the experiment ended, to safely depressurized the system, the heater was turned off and the reactor cooled down by circulating distilled water. Then, the stirrer was turned off and started to depressurize by gently opening the pressure release valve. When there was no pressure in the reactor, the hydrolysates and solid residues were unloaded and separated using cheese cloth and stored at -18°C for further analysis.

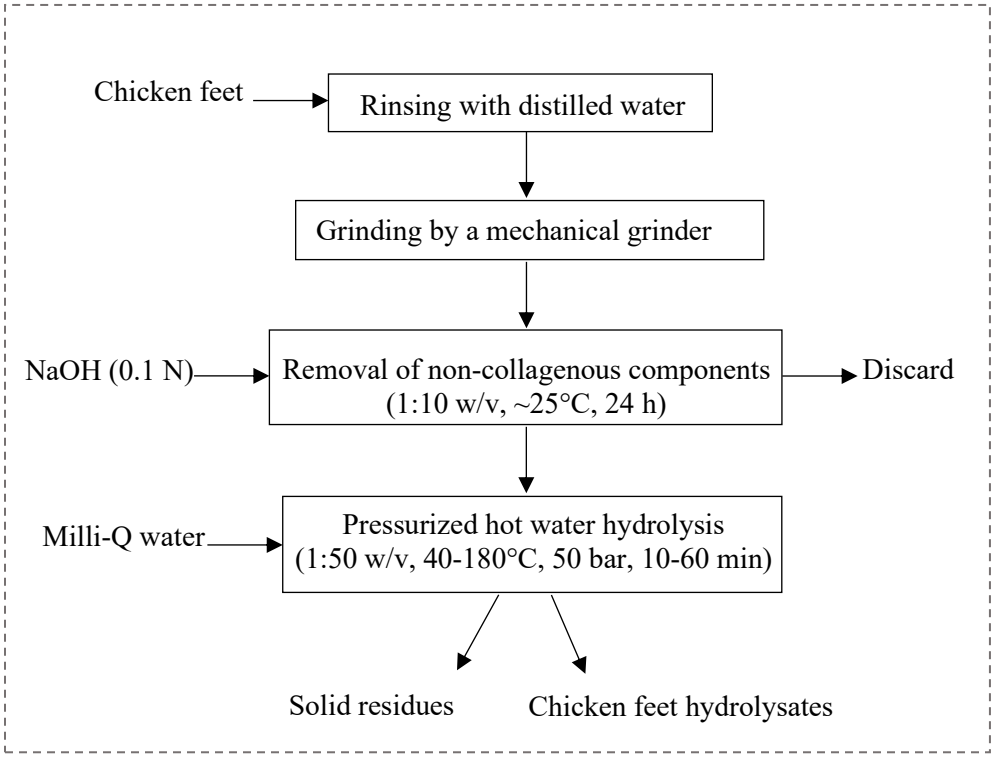

Figure 4.1. Experimental design for extraction/hydrolysis of chicken feet using pressurized hot water.

4.2.4. Conventional acid treatment

To compare, acid treatment of chicken feet was conducted according to the method described by Potti & Fahad (2017). Briefly, chicken feet were pretreated with 0.1 N sodium

hydroxide at a ratio of 1:10 (w/v) at room temperature for 24 h. Then, the pretreated sample was soaked with 0.5 M acetic acid with a ration of 1:20 w/v at a temperature of 4°C for 48 h. Collagen was precipitated by adjusting the pH to 7.0 using 0.1 N sodium hydroxide, followed by centrifugation with 8000 *xg* at 4°C for 15 min. The precipitate was freeze dried to obtain dry collagen.

4.2.5. Characterization of hydrolysates

4.2.5.1. Hydroxyproline quantification

Free and total hydroxyproline contents of hydrolysates were analyzed according to the method of Neuman and Logan (1950). For total hydroxyproline content, the hydrolysates obtained from pressurized hot water were further hydrolyzed with hydrochloric acid following the method described by Bruce et al. (2022). An aliquot of hydrolysate (1.0 mL) was mixed with 1.0 mL of 12 M hydrochloric acid and 4 mL of 6 M hydrochloric acid, then vortexed gently and flushed with nitrogen. Samples were placed in an oven at 110°C for 24 h, followed by cooling in an ice water bath for 15 min. Then, samples were filtered using Whatman paper #4 (Fisher Scientific, Mississauga, Ontario), and the acid solution evaporated using a rotary evaporator (Heidolph Collegiate, Brinkmann, Mississauga, ON) which was equipped with an Ultra auto-purge vacuum system (DistiVac, Brinkmann, Mississauga, ON) at 42°C. The dried samples were dissolved in 2 mL of distilled water and the pH was adjusted to neutral. For the second time, the solvent was evaporated, and the dried sample redissolved in 3 mL of distilled water.

For analyzing the content of free hydroxyproline content, the hydrolysates were analyzed as is, where acid hydrolysis was omitted. Briefly, in a test tube, an aliquot of each sample (1.0 mL) was mixed with 1.0 mL of 0.01 M copper (II) sulfate solution, 1.0 mL of 2.5 M sodium hydroxide solution, and 1.0 mL of 6% hydrogen peroxide solution. The test tubes were kept in a water bath

at 80°C for 5 min with constant mixing and then cooled in an ice water for 10 min. Then, 4 mL of 3 N sulfuric acid and 2 mL of 5% p-dimethylaminobenzaldehyde (dissolved in isopropanol) were added to each test tube for development of color and vortexed for one minute. Test tubes were then kept in the water bath at 70°C for 16 min, and then cooled in cold water for 5 min. Absorbance of each sample was measured at 550 nm in a spectrophotometer against the blank. The blank was prepared with distilled water instead of the sample. The hydroxyproline content of the hydrolysates were calculated from a standard curve prepared using hydroxyproline standard solution with concentrations of 0.0, 2.5, 5, 10, 20, and 40 µg/mL (See Fig. A3).

A conversion factor of 7.7 was used for calculating the collagen content based on the hydroxyproline content (Zhou et al., 2016).

4.2.5.2. Degree of hydrolysis

Free amino acids content of hydrolysates and total amino acid content of raw material were measured by the OPA spectrophotometric assay following the method described by Church et al. (1983). Raw material was treated with hydrochloric acid before determination of total amino acid content; by mixing 40 mg of sample with 3 mL of 6 M HCl at 110ºC for 24 h. The OPA reagent was prepared daily. Briefly, the OPA solution was prepared by mixing 50 mL of 0.1 M sodium tetraborate, 5 mL of 20% (w/w) SDS, 80 mg of OPA dissolved in 2 mL of methanol, and 200 µL of ß-mercaptoethanol and diluting to a final volume of 100 mL with distilled water. Then, 2 mL of the OPA reagent was added to 100 µL of sample, mixed for 2 min at ambient temperature, and the absorbance was measured at a wavelength of 340 nm in a spectrophotometer. Free amino acid content was calculated from a standard curve prepared using glycine (0.0, 0.05, 0.1. 0.125 mg/mL) (See Fig. A4). The degree of hydrolysis (DH) was calculated using eq. (4.1):

$$DH\ (\%) = \frac{Amount\ of\ free\ amino\ acid\ in\ the\ hydrolysate}{Amount\ of\ total\ amino\ acids\ in\ the\ raw\ material} \times 100 \qquad (4.1)$$

4.2.5.3. Amino acid profile

Amino acid profile of the hydrolysates analyzed following the method described earlier by Liu et al. (2023). Briefly, the hydrolysate mixed with 0.2 mL of internal standard (B-amino-n-butyric acid and ethanolamine at 25 µmol/mL) and centrifugated at 2500 rpm for 15 min. The vials for HPLC injection contain 50 µL of the supernatant, 50 µL of 4.29 M NaOH, and 400 µL of milli-Q water. Maintaining pH of the solution at 10 is crucial for derivatization. The mobile phase included two eluents: (A) 1600 mL of 0.1 M sodium acetate buffer pH 7.2, 180 mL methanol, 10 mL tetrahydrofuran, and 210 mL of milli-Q water; (B) 100% methanol. The elution gradient was: 0-1 min, isocratic 100% A; 5-25 min, isocratic 85% A and 15% B; 38-39 min, linear from 55% to 35% A and 45% to 65% B; 40-42.5 min, isocratic 100% B, and 43-48 min, isocratic 100% A. A Supelcosil LC-18 column (150 mm x 4.6 mm, 3 µm, Sigma Aldrich, Oakville, ON, Canada), with a flow rate of 1.1 mL/min used for separation of amino acids. The fluorescence intensity measured at wavelength of 340 nm (excitation) and 450 nm (emission).

4.2.5.4. Molecular weight distribution by Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of the hydrolysates obtained by PHW hydrolysis of chicken feet performed by the method of Laemmli, (1970). Briefly, 1.8 mL of 2x Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromphenol blue, 0.125M Tris-Cl, pH 6.8) was mixed with 0.2 mL of 2-mercaptoethanol. Then, 3:1 v/v of hydrolysates and the buffer were mixed and incubated at 95-99°C for 6 h. Later, the samples and the protein marker (FroggaBio, BLUeye Prestained Protein ladder, PM008-0500) were loaded onto the separating gel and stacking gel. Electrophoresis was conducted with 10x running buffer (30.3 g TRIS base, 144.4 g glycine, 10 g SDS, 1.0 L distilled water) at 100 V for 5 min then increased to 150 V and run for 50 min until the dye reached the

bottom. The gels stained by 1 g/L Coomassie brilliant blue R-250 solution (320 mL of 0.1 M aluminum nitrate, 125 mL of isopropanol, 50 mL of glacial acetic acid, 5 mL of Triton X-100, 500 mg of Coomassie brilliant blue R-250) for 1-2 h and distained with 10% acetic acid overnight by changing the solution after 1, 2, 4, and 8 h.

4.2.5.5. Molecular weight distribution by size exclusion-gel permeation chromatography

Molecular weight distribution of the protein hydrolysates obtained by PHW hydrolysis of chicken feet were determined by size exclusion-gel permeation chromatography according to the method described by Melgosa et al. (2021). Briefly, the hydrolysates were filtrated through syringe filters (0.22 μm pore diameter). An aliquot (20 μL) of each sample was introduced to a Phenomenex Yarra SEC-2000 column (300 x 7.8 mm i.d., 3μm, 145 Å) at a temperature of 25°C, and eluted at a flow rate of 1.0 mL/min. The elution gradient was 0.1 M phosphate buffer (pH 6.8), including 0.025% sodium azide. The UV/Vis intensity of the samples was measured at 280 nm using a UV/Vis detector. Protein standard mix 69385 from Sigma-Aldrich (Oakville, ON, Canada) was used as molecular weight standards. The standard curve was plotted using the retention times of the standards (log Molecular weight versus retention time) (See Fig. A5). The molecular weight (M_w) distribution was analyzed based on the retention times of the unknown peaks present in the chromatograms of the hydrolysates.

4.2.6. Statistical analysis

Data are shown as mean ± standard deviation according to the results obtained from at least duplicate experiments and analysis. One-way analysis of variance (ANOVA) and the multiple comparisons of the means with Tukey's test at $p < 0.05$ were performed using Minitab version 18.0 (Minitab Inc., State College, PA, USA) at 95% confidence interval.

4.3. Results and discussion

4.3.1. Proximate composition of chicken feet

Crude protein, fat, ash, moisture, and total carbohydrates contents of chicken feet are reported in Table 4.1. Protein (20.85%) and moisture (65.86%) contents were the major constituents present in chicken feet, and the minor constituents were fat (5.79%), ash (5.20%), and total carbohydrates contents (2.30%). The protein content was slightly lower than the value of 21.85% reported for protein content of chicken feet by Dhakal et al. (2018). However, the protein content obtained in our study was higher than the values of 18.10% (Potti & Fahad, 2017) and 17.42% (Liu et al., 2001) reported for chicken feet. Chicken feet's fat content in our study (5.79%) was higher than the fat content of 4.44% (Dhakal et al., 2018) and 3.9% (Potti & Fahad, 2017), and considerably lower than 12.04% reported by Liu et al. (2001). Ash content (5.20%) was similar to the amount (5.98%) reported by Liu et al. (2001). However, the amount of ash in chicken feet of our study was considerably lower than the contents of 14.28% and 7.94% reported by Dhakal et al. (2018) and Potti & Fahad (2017), respectively. The difference in the chicken feet proximate composition may be due to the difference in the breed (American, Mediterranean, English, and Asiatic) and the category (laying, broiler, and dual-purpose) of the chicken. Ash and fat constituents of chicken feet could be due to the presence of bone and skin, respectively. The moisture content of chicken feet in our study was similar to previously reported for porcine skin (59.22%) (Jo et al., 2015) and bovine hide (64%) (Noorzai et al., 2020).

Table 4.1. Proximate composition of chicken feet.

Constituent	% (w/w)
Crude protein	20.85 ± 0.58
Fat	5.79 ± 0.67
Ash	5.20 ± 0.06
Moisture	65.86 ± 0.55
Total carbohydrate	2.30 ± 0.34

Data are expressed as mean ± standard deviation of two replicates.

4.3.2. Collagen/collagen fragment extraction yield

Chicken feet was pretreated with 0.1 N sodium hydroxide to remove none-collagenous components such as fat, pigment, odor and facilitate the penetration of solvent into the sample by swelling the chicken feet matrix. The total collagen content of the chicken feet was 208.62 mg/g chicken feet. Pretreated sample was hydrolyzed using pressurized hot water at different temperatures of 40, 80, 120, 140, 160, and 180°C with different hydrolysis times of 10, 20, 40, 60 min at 50 bar (according to literature that pressure does not have significant influence on the hydrolysis) and a sample-water ratio of 1:50 w/v (to avoid the increase in viscosity due to gelatinization and/or flooding in the system). Fig. 4.2 shows the effects of temperature and hydrolysis time on collagen/collagen fragment content in chicken feet hydrolysates obtained after pressurized hot water treatment. In general, there was not a significant difference in the collagen and collagen fragment content of hydrolysates obtained at different times of hydrolysis (10-60 min), indicating hydrolysis for 10 min as the best time. However, the content of collagen/collagen fragment in the hydrolysates increased from 6.50 mg/g chicken feet to 152.04 mg/g chicken feet

(15.2% yield) when increasing the temperature from 40°C/10 min to 140°C/10 min. There was also a significant increase in the collagen/collagen fragment content when the temperature increased from 80°C to 120°C due to the changes in the thermodynamic properties of water at subcritical water condition such as decrease in dielectric constant and increase in diffusivity of water. At subcritical condition, water dissociate into hydronium (H_3O^+) and hydroxide (OH^-) ions. The formation of ion products modifies the pH of the medium. The pH tends to decrease at temperatures lower than 180°C (Koh et al., 2019; Cho et al., 2020), thus promotes the reactivity of water as an acid or bi-catalyst for hydrolysis reactions (Rivas-Vela et al., 2021). Moreover, the fluctuations in density of water (994.36 kg/m^3 at 40°C/50 bar to 889.65 kg/m^3 at 180°C/50 bar, NIST Chemistry WebBook) within subcritical condition cause agitation in the media that facilitates the heat and mass transfer during the extraction/hydrolysis process (Ziero et al., 2020). When the temperature increased from 120°C/10 min to 140°C/10 min, collagen/collagen fragment content further increased and reached a value of 152.04 mg/g chicken feet. However, further increasing the temperature up to 180°C/10 min did not make a significant difference in the collagen/collagen fragment content in comparison to the value obtained at 140°C/10 min. However, the collagen/collagen fragment content at 180°C/40 min (200.92 mg/g chicken feet) was higher than at 140°C/10 min (152.04 mg/g chicken feet) due to the higher concentrations of hydronium (H_3O^+) and hydroxide (OH^-) ions in the aqueous medium at high temperatures that induce the hydrolysis of proteins to peptides and free amino acids.

Overall, treatment at 140°C/10 min was the best condition to obtain collagen/collagen fragment yield of 15.2% that was almost two times higher than the conventional acid treatment that resulted in a collagen yield of 8.16%. However, in the study by Lee et al. (2013), porcine placenta was hydrolyzed using subcritical water at 150, 170, and 200°C and 375 bar with zero

(0.0) holding time (solid-solvent ratio not reported). The authors observed an increase in protein content of hydrolysates from 58% to 71% by increasing temperature from 150°C to 170°C, that decreased to 55% with further increasing the temperature to 200°C. They also investigated the effect of time (0, 30, and 60 min) on protein content of porcine placenta hydrolysates at 170°C. Protein content of hydrolysates reached 77% by increasing holding time to 30 min and decreased to 48% at 60 min. Similarly, Jo et al. (2015) reported a lower nitrogen content of 3.82% for porcine skin hydrolysates obtained by subcritical water with a solid-solvent ratio of 1:2 w/w at 300°C and 80 bar after 60 min of hydrolysis. Melgosa et al. (2021) observed an increasing trend in the collagen and collagen fragments extraction yield (13.2%) to 53.9% from Atlantic cod (*Gadus morhua*) frames by increasing the temperature from 90°C up to 250°C. In their study, 60 g Atlantic cod frames was hydrolyzed using pressurized hot water at 90, 140, 190, and 250°C and 100 bar with a flow rate of 10 mL/min for 30 min in a semi-continuous reactor. The difference in the trend of the extraction yield of our study (batch operated) and their study by the increasing the temperature probably is due to the highest amount of collagen in the codfish frames (306 mg/g codfish frame) in comparison to chicken feet (208.62 mg/g chicken feet), and operation mode of the process (batch versus semi-continuous). In the batch process, the extracted proteins and peptide remained exposed to the hydronium (H_3O^+) and hydroxide (OH^-) ions and more likely to break down into free amino acids during the reaction time (10-60 min).

In this study, 10 min was the shorter time of hydrolysis. At a higher temperature of 160°C, chicken feet collagen may already be degraded within hydrolysis for 10 min. Hydrolysis for 2.5 or 5 min at this temperature maybe is sufficient to avoid degradation of collagen and improve the yield. The use of a higher pressure (e.g., 100 bar) also may help to avoid the degradation of chicken

feet collagen. Also, it may be possible to improve the yield of collagen by employing some pre-treatments such as ultrasound or addition of organic acids such as citric acid or malic acid.

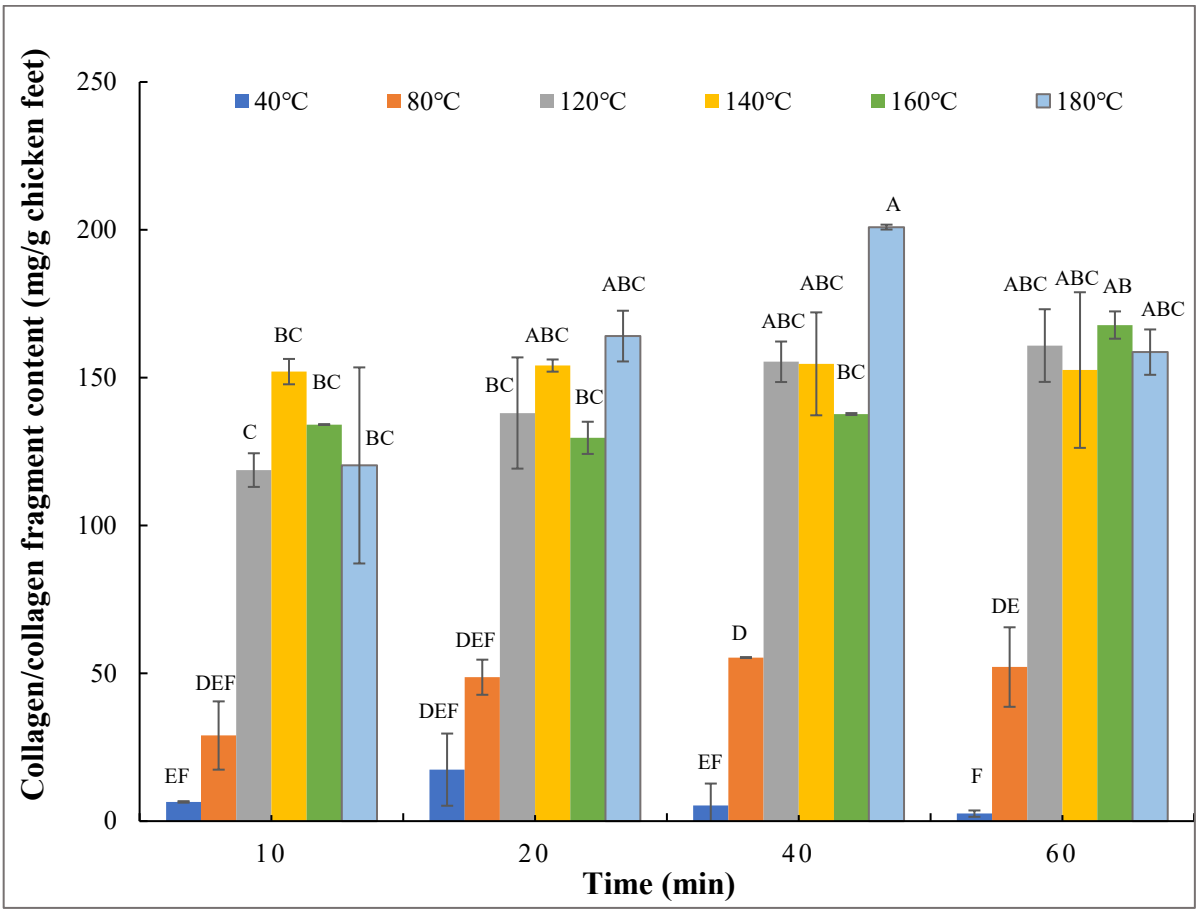

Figure 4.2. Effect of temperature and time on collagen and collagen fragment content in chicken feet hydrolysates obtained by pressurized hot water hydrolysis at 50 bar and a sample-water ratio of 1:50 w/v. [A-F]Bars that do not share a letter are significantly different.

Hydroxyproline is the amino acid that is almost exclusively present in collagen. The presence of free hydroxyproline in the hydrolysates indicates the hydrolysis of collagen during the extraction/hydrolysis process. Fig. 4.3 shows free hydroxyproline content in the chicken feet hydrolysates obtained at different temperatures (40-180°C) and hydrolysis times (10-60 min) by pressurized hot water treatment. At temperatures of 40 to 140°C, the free hydroxyproline content

increased significantly by increasing the reaction time from 10 to 40 min, and further increasing the time up to 60 min did not make a significant change at 120°C and 140°C.

However, at a higher temperature of 160°C, there was a significant decrease in the free hydroxyproline content by increasing the reaction time from 10 min (8.26 mg/g chicken) to 60 min (3.6 mg/g chicken feet), also at 180°C, from 10 min (4.04 mg/g chicken feet) to 20 min (3.15 mg/g chicken feet). In terms of the effect of temperature, there was not a significant difference in the free hydroxyproline content at low temperatures of 40°C and 80°C during 10-60 min (0.09-1.15 mg/g chicken feet). However, by increasing the temperature from 80°C to 160°C, free hydroxyproline content increased significantly and reached the maximum content (8.26 mg/g chicken feet) at 160°C and 10 min, but further increase up to 180°C for 60 min resulted in a significant decrease to 3.02 mg/g chicken feet. Decrease in the free hydroxyproline content was attributed to the breakdown of amino acids at high temperature and extended experiment time into organic acids with low molecular weight such as formic acid, acetic acid, etc. (Quitain et al., 2002). However, in the study by Melgosa et al. (2021), free hydroxyproline was increased from 1.59 mg/g extract to 12.07 mg/g extract by increasing the hydrolysis temperature from 90°C to 250°C at 100 bar with a flow rate 10 mL/min for 30 min in a semi-continuous process. The higher free hydroxyproline content indicates the higher degree of hydrolysis of collagen in their study. The higher degree of hydrolysis could be due to the lower thermal stability of fish collagen (Gómez-Guillén et al., 2011). The denaturation temperature of collagen from fish scale is 35°C (Liu et al., 2018), where of collagen from chicken skin is 41°C (Cliché et al., 2003).

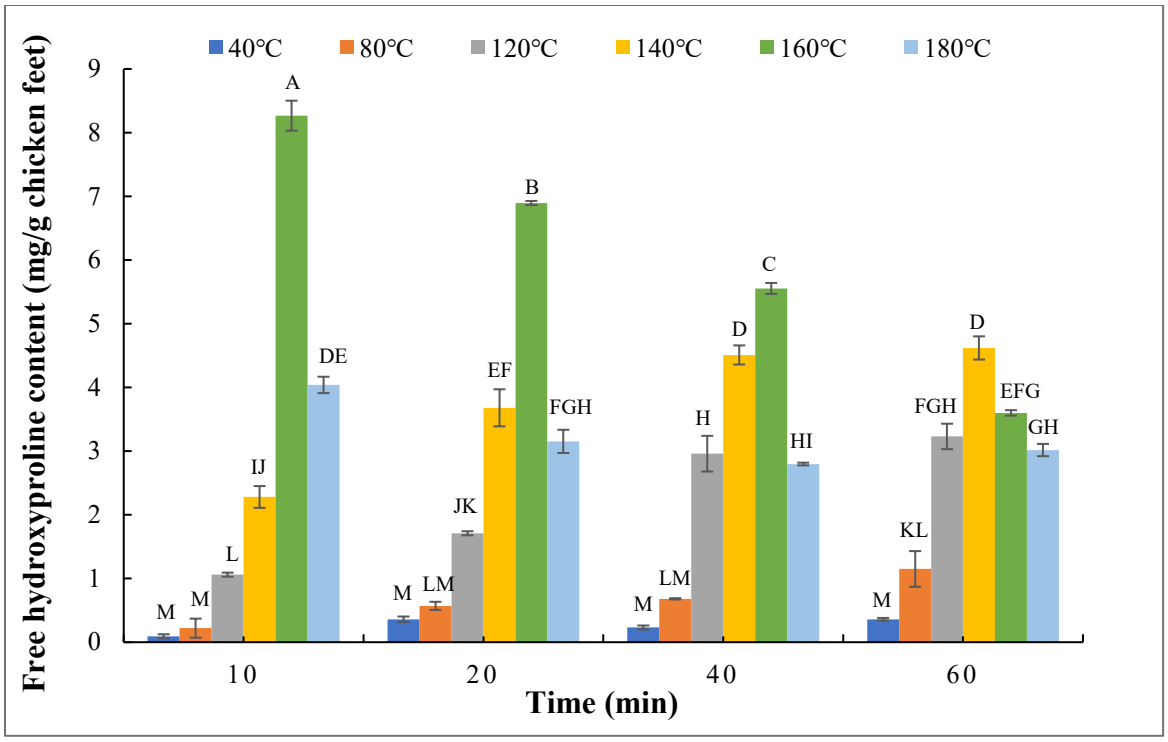

Figure 4.3. Effect of temperature and time on free hydroxyproline content in chicken feet hydrolysates obtained by pressurized hot water hydrolysis at 50 bar and a sample-water ratio of 1:50 w/v. [A-M]Bars that do not share a letter are significantly different.

4.3.3. Amino acid profile of chicken feet hydrolysates

Amino acid profile of chicken feet hydrolysates obtained by pressurized hot water hydrolysis at 40-180°C and 10-60 min are summarized in Table 4.2. The OPA assay used for amino acid composition analysis of hydrolysates does not react with hydroxyproline and proline amino acids. For this reason, the hydroxyproline content of hydrolysates was analyzed according to the method of Neuman and Logan (1950) as discussed earlier in Section 4.3.2. Briefly, maximum free hydroxyproline content of 8.26 mg/g chicken feet was obtained at 160°C and 10 min, which decreased by further increasing the temperature to 180°C and time up to 60 min.

From Table 4.2, methionine, glutamine, threonine, and citrulline amino acids were not present or were in low quantities (0.0-0.6 mg/g chicken feet) in all hydrolysates obtained at 40-180°C and 10-60 min. These amino acids were earlier reported in minor quantities (0.0-3.27%) in

chicken feet collagen (Liu et al., 2001). Also, aspartic acid, glutamic acid, asparagine, arginine, alanine, tyrosine, and phenylalanine were not present or were in low quantities (0.0-0.1 mg/g chicken feet) in hydrolysates obtained at 40-160°C and 10-60 min. However, these amino acids increased by increasing the temperature to 180°C and increasing the time of hydrolysis from 10 to 60 min. Serine, isoleucine, leucine, and lysine contents also increased by increasing temperature from 40°C to 180°C with no trend with increasing time. This observation agreed with the considerable increase in the DH at 180°C/10-60 min (Fig. 4.4B). The quantity of glycine increased by increasing the temperature and time of hydrolysis and reach the maximum value of 2.3 mg/g chicken feet at 180°C/60 min.

Overall, hydroxyproline (Fig. 4.3), lysine and glycine were the main amino acid constituents in the hydrolysates obtained at all conditions, followed by serine, leucine, and isoleucine in the hydrolysates obtained at temperatures of 120-180°C. Where lysine, leucine, and isoleucine are essential amino acids. As reported earlier, glycine (33%), proline and hydroxyproline (22%) were the main amino acids of collagen (León-López et al., 2019). Lysine is a hydrophilic amino acid therefore higher quantity of this amino acid in chicken feet hydrolysates were obtained even at a low temperature of 40°C that increased with increasing temperature. Hydronium (H_3O^+) and hydroxide (OH^-) ions generated in the aqueous medium at subcritical water conditions and their concentrations increased at higher temperatures that enhanced the dissolution of non-polar molecules (Ziero et al., 2020), this explained the presence of uncharged amino acid (serine) and hydrophobic amino acids that mainly have non-polar side chains (leucine, isoleucine, alanine, and phenylalanine) in the hydrolysates obtained at temperatures of 120-180°C.

Similarly, glycine (30%), proline (10%), and hydroxyproline (10%) were the main amino acids of porcine placenta obtained at 150-200°C and 375 bar for 0-60 min (Lee et al., 2013). The

authors did not observe a significant difference in the content of the amino acids by the increase in temperature and time. However, the contents of glycine, proline, hydroxyproline, and alanine in codfish frame hydrolysates increased significantly (5 to 10 times) by increasing the temperature from 90°C to 250°C at 100 bar for 30 min (Melgosa et al., 2021). In other study, glycine (28%) and proline (22%) were the major amino acids while serine, lysine, leucine, and isoleucine had low quantities (0-2.5%) in porcine skin hydrolysates obtained at 300°C and 80 bar for 1 h (1:2 w/w) (Jo et al., 2015). The faster decomposition of amino acids with high molecular weight than the ones with low molecular weights like glycine explains the low concentrations of serin, lysine, leucine, and isoleucine at 300°C (Jo et al., 2015). The difference in the amino acids profile of collagen hydrolysates could be due to the difference in the source of by-products (animals versus marine), the age of animal, and the process conditions.

Table 4.2. Amino acid (mg/g chicken feet) profile of chicken feet hydrolysates obtained by pressurized hot water hydrolysis at 40-180°C, 50 bar, 10-60 min, and a sample-water ratio of 1:50 w/v.

Time (min)	Temperature (°C)	Acidic			Basic			Uncharged polar	
		Aspartic acid	Glutamic acid	Total acidic	Arginine	Lysine	Total basic	Glycine	Threonine
10	40	0.00±0.00	0.01±0.00	0.01	0.00±0.00	0.25±0.02	0.25	0.00±0.00	0.01±0.01
	80	0.00±0.00	0.00±0.00	0.01	0.00±0.00	0.22±0.04	0.22	0.00±0.00	0.01±0.01
	120	0.00±0.00	0.00±0.00	0.01	0.00±0.00	0.27±0.08	0.27	0.02±0.03	0.02±0.02
	140	0.01±0.00	0.01±0.00	0.01	0.00±0.00	0.45±0.35	0.46	0.06±0.09	0.09±0.11
	160	0.02±0.00	0.01±0.00	0.04	0.02±0.00	0.69±0.01	0.71	0.16±0.02	0.00±0.00
	180	0.21±0.03	0.05±0.00	0.25	0.04±0.01	0.98±0.17	1.02	0.47±0.09	0.00±0.00
20	40	0.00±0.00	0.00±0.00	0.01	0.00±0.00	0.27±0.07	0.27	0.00±0.00	0.01±0.01
	80	0.00±0.00	0.00±0.00	0.01	0.00±0.00	0.26±0.09	0.26	0.00±0.00	0.01±0.01
	120	0.01±0.00	0.01±0.00	0.01	0.00±0.00	0.42±0.34	0.42	0.06±0.08	0.11±0.14
	140	0.01±0.00	0.01±0.00	0.02	0.01±0.00	0.56±0.59	0.57	0.12±0.17	0.24±0.32
	160	0.04±0.00	0.02±0.00	0.06	0.02±0.00	0.74±0.04	0.75	0.11±0.01	0.00±0.00
	180	0.46±0.04	0.09±0.00	0.54	0.08±0.01	1.64±0.04	1.72	0.82±0.04	0.00±0.00
40	40	0.00±0.00	0.01±0.00	0.01	0.00±0.00	0.26±0.09	0.27	0.01±0.00	0.00±0.00
	80	0.00±0.00	0.00±0.00	0.01	0.00±0.00	0.26±0.12	0.26	0.02±0.00	0.00±0.00
	120	0.01±0.00	0.01±0.00	0.02	0.01±0.00	0.53±0.54	0.54	0.27±0.05	0.01±0.01
	140	0.02±0.00	0.03±0.00	0.05	0.02±0.00	0.78±0.80	0.80	0.47±0.02	0.01±0.02
	160	0.12±0.01	0.03±0.00	0.15	0.02±0.02	0.74±0.07	0.76	0.26±0.02	0.00±0.00
	180	0.79±0.03	0.19±0.00	0.97	0.16±0.01	1.36±0.79	1.52	1.61±0.04	0.00±0.00
60	40	0.00±0.00	0.00±0.00	0.00	0.00±0.00	0.20±0.06	0.21	0.01±0.00	0.00±0.00
	80	0.00±0.00	0.00±0.00	0.01	0.00±0.00	0.24±0.15	0.25	0.06±0.04	0.00±0.00
	120	0.02±0.00	0.01±0.00	0.03	0.02±0.00	0.67±0.72	0.69	0.43±0.03	0.01±0.02
	140	0.03±0.00	0.03±0.00	0.06	0.03±0.00	0.58±0.60	0.61	0.65±0.04	0.02±0.02
	160	0.20±0.00	0.05±0.00	0.25	0.04±0.00	1.01±0.09	1.05	0.46±0.01	0.00±0.00
	180	0.88±0.03	0.46±0.27	0.90	0.28±0.08	2.13±0.37	2.41	2.30±0.09	0.00±0.00

Table 4.2. Continue.

Time (min)	Temperature (°C)	Uncharged polar					Hydrophobic		
		Tyrosine	Serine	Asparagine	Glutamine	Total uncharged polar	Alanine	Phenylalanine	Isoleucine
10	40	0.02±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.04	0.00±0.00	0.00±0.00	0.00±0.00
	80	0.02±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.03	0.00±0.00	0.00±0.00	0.00±0.00
	120	0.02±0.00	0.02±0.01	0.00±0.00	0.00±0.00	0.08	0.01±0.00	0.00±0.00	0.00±0.00
	140	0.02±0.00	0.05±0.02	0.02±0.01	0.00±0.00	0.24	0.02±0.01	0.02±0.02	0.12±0.17
	160	0.02±0.00	0.07±0.01	0.02±0.00	0.01±0.00	0.29	0.02±0.00	0.01±0.00	0.24±0.01
	180	0.02±0.00	0.16±0.03	0.06±0.01	0.02±0.00	0.74	0.06±0.01	0.02±0.01	0.21±0.06
20	40	0.02±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.04	0.00±0.00	0.00±0.00	0.00±0.00
	80	0.02±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.05	0.00±0.00	0.00±0.00	0.00±0.01
	120	0.02±0.00	0.05±0.01	0.01±0.00	0.00±0.00	0.25	0.02±0.00	0.02±0.02	0.08±0.11
	140	0.02±0.00	0.10±0.01	0.02±0.01	0.00±0.00	0.51	0.02±0.02	0.03±0.03	0.13±0.18
	160	0.02±0.00	0.03±0.01	0.01±0.02	0.00±0.00	0.17	0.02±0.00	0.01±0.00	0.19±0.01
	180	0.02±0.00	0.23±0.01	0.09±0.00	0.03±0.00	1.19	0.11±0.01	0.04±0.00	0.18±0.02
40	40	0.02±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.03	0.00±0.00	0.00±0.00	0.00±0.00
	80	0.02±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.05	0.00±0.00	0.00±0.00	0.00±0.00
	120	0.02±0.00	0.13±0.03	0.01±0.01	0.00±0.00	0.44	0.03±0.02	0.01±0.01	0.08±0.12
	140	0.02±0.00	0.20±0.01	0.03±0.01	0.00±0.00	0.74	0.05±0.03	0.02±0.01	0.13±0.18
	160	0.02±0.00	0.08±0.01	0.05±0.01	0.01±0.00	0.42	0.04±0.00	0.02±0.00	0.18±0.04
	180	0.07±-0.02	0.39±0.01	0.16±0.01	0.06±0.00	2.29	0.23±0.01	0.09±0.01	0.12±0.10
60	40	0.02±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.03	0.00±0.00	0.00±0.00	0.00±0.01
	80	0.02±0.00	0.03±0.02	0.00±0.00	0.00±0.00	0.11	0.01±0.01	0.00±0.00	0.01±0.01
	120	0.02±0.00	0.19±0.01	0.03±0.02	0.00±0.00	0.68	0.05±0.03	0.01±0.00	0.16±0.23
	140	0.02±0.00	0.27±0.01	0.06±0.04	0.00±0.00	1.01	0.06±0.02	0.01±0.00	0.14±0.19
	160	0.02±0.00	0.14±0.00	0.07±0.01	0.02±0.00	0.72	0.07±0.00	0.03±0.00	0.21±0.02
	180	0.15±-0.01	0.48±0.03	0.19±0.02	0.03±0.05	2.89	0.37±0.03	0.13±0.01	0.20±0.07

Table 4.2. Continue.

Time (min)	Temperature (°C)	Hydrophobic				Citrulline	Total amino acid (mg/g chicken feet)
		Tryptophan	Methionine	Leucine	Total hydrophobic		
10	40	0.00±0.00	0.00±0.00	0.02±0.02	0.04	0.00±0.00	0.36±0.04
	80	0.00±0.00	0.00±0.00	0.02±0.01	0.02	0.00±0.00	0.29±0.06
	120	0.01±0.00	0.00±0.00	0.02±0.02	0.04	0.01±0.00	0.41±0.09
	140	0.00±0.01	0.00±0.00	0.17±0.03	0.26	0.02±0.01	1.06±0.58
	160	0.02±0.00	0.00±0.00	0.24±0.00	0.53	0.04±0.00	1.61±0.04
	180	0.04±0.01	0.01±0.00	0.26±0.13	0.60	0.08±0.02	2.70±0.24
20	40	0.00±0.00	0.00±0.00	0.02±0.01	0.03	0.00±0.00	0.34±0.09
	80	0.00±0.00	0.00±0.00	0.02±0.01	0.03	0.00±0.00	0.36±0.12
	120	0.00±0.00	0.00±0.00	0.06±0.08	0.18	0.01±0.00	0.88±0.60
	140	0.00±0.00	0.00±0.00	0.21±0.05	0.31	0.03±0.01	1.54±0.97
	160	0.02±0.01	0.00±0.00	0.22±0.03	0.46	0.03±0.00	1.48±0.05
	180	0.05±0.01	0.02±0.00	0.15±0.03	0.55	0.09±0.01	4.09±0.22
40	40	0.00±0.00	0.00±0.00	0.02±0.01	0.03	0.00±0.00	0.34±0.11
	80	0.00±0.00	0.00±0.00	0.01±0.01	0.02	0.00±0.00	0.35±0.12
	120	0.00±0.00	0.00±0.00	0.07±0.10	0.20	0.02±0.01	1.23±0.63
	140	0.00±0.00	0.01±0.00	0.14±0.20	0.35	0.04±0.02	2.00±1.14
	160	0.06±0.01	0.01±0.00	0.23±0.07	0.53	0.05±0.01	1.90±0.26
	180	0.07±0.01	0.04±0.01	0.16±0.09	0.73	0.13±0.01	5.67±0.65
60	40	0.00±0.00	0.00±0.00	0.01±0.01	0.02	0.00±0.00	0.27±0.07
	80	0.00±0.00	0.00±0.00	0.02±0.02	0.04	0.00±0.00	0.42±0.09
	120	0.00±0.00	0.00±0.00	0.21±0.02	0.34	0.03±0.01	1.87±0.91
	140	0.00±0.00	0.01±0.00	0.30±0.03	0.38	0.04±0.02	2.25±0.91
	160	0.08±0.00	0.01±0.00	0.26±0.04	0.65	0.06±0.00	2.72±0.03
	180	0.10±0.01	0.06±0.01	0.10±0.03	0.95	0.15±0.02	8.04±1.01

4.3.4. Degree of hydrolysis (DH) of chicken feet protein/collagen

Fig. 4.4 shows DH of the hydrolysates obtained at different temperatures (40-180°C) and hydrolysis times (10-60 min). At the lower temperatures of 40°C and 80°C, there was not a significant DH during 10 to 60 min of pressurized hot water treatment. But the DH increased considerably by increasing the temperature up to 160°C where it reached 7.7% using eq. 4.1 in 10 min. At 120°C and 140°C, an increasing trend was observed with the increase in time of hydrolysis from 10 min (5.18%) to 60 min (8.46%). But the DH remained almost constant by increasing the time at 160°C. However, when temperature was further increased to 180°C, the DH increased by increasing the time and reached the highest value of 11.64% in 60 min. The significant increase in DH at 180°C was attributed to the increase in the concentration of water ion products that is correlated with the temperature. Where basic amino acids such as lysine, arginine, and asparagine were significantly increased when the temperature increased to 180°C (Table 4.2). Also, these amino acids increased considerably by increasing the time from 10 to 60 min at 180°C (Table 4.2).

However, DH of 3.9% and 10.5% were obtained for tuna skin (1:50 w/v) with subcritical water hydrolysis in a batch reactor at 120°C/50 bar and 180°C/50 bar with a very shorter time (5 min) in comparison to our study (60 min) (Ahmed & Chun, 2018). This could be due to the lower thermal stability of tuna skin collagen/protein. In other study, free amino acids content in codfish frame hydrolysates increased by increasing the temperature from 90°C to 250°C and reached its maximum of 755.40 mg/g extract at 250°C (Melgosa et al., 2021). However, the highest free amino acids content (818.5 mg/g extract) in de-oiled sardine waste hydrolysates was obtained at 190°C, 100 bar, 10 mL/min flow rate, 30 min, that decreased (738.9 mg/g extract) by increasing the temperature of hydrolysis to 250°C (100 bar, 10 mL/min flow rate, 30 min) (Melgosa et al., 2020). According to the literature, the nature of raw material, temperature and time of pressurized

hot water hydrolysis, and the mode of the operation (batch, continue or semi-continue) influence the production or decomposition of free amino acids during hydrolysis.

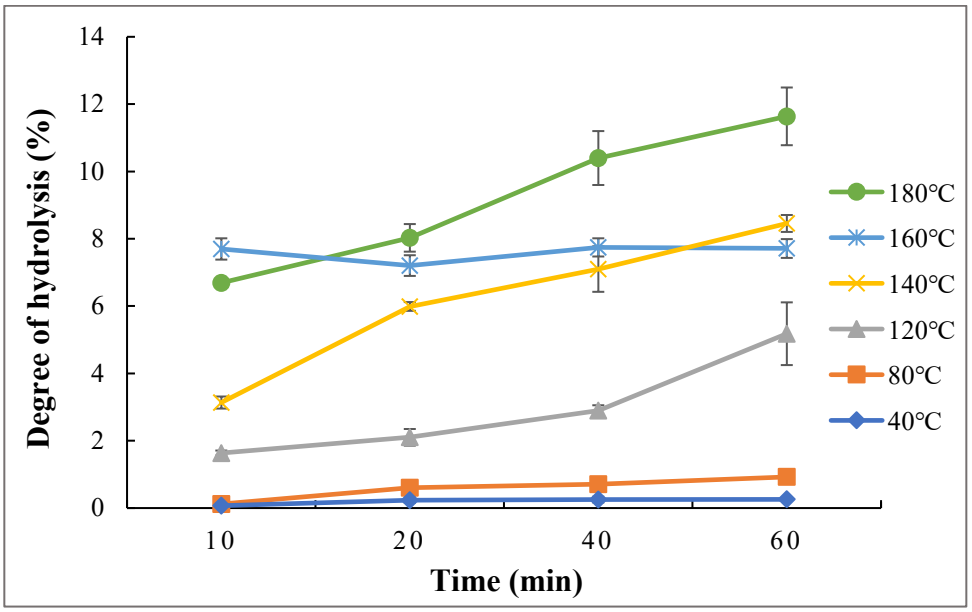

Figure 4.4. Effect of temperature and time on the degree of hydrolysis of chicken feet.

4.3.5. Molecular weight distribution of chicken feet hydrolysates

The molecular weight distribution of chicken feet hydrolysates obtained after pressurized hot water treatment at different temperatures of 40, 80, 120, 140, 160, and 180°C and times of 10, 20, 40, and 60 min was investigated using SDS-PAGE and SEC-GPC methods.

Fig. 4.5 shows electrophoretic profiles of chicken feet hydrolysates obtained after pressurized hot water treatment at different temperatures of 40, 80, 120, 140, 160, and 180°C and times of 10, 20, 40, and 60 min. At 40°C and 10 min of hydrolysis, no band was present. However, by increasing the time up to 60 min, some bands were observed in the range of 63 to higher than 245 kDa. At 80°C/10 min, weak bands were present at molecular weights of 180 kDa and when the time increased to 20 min, the intensities of these bands increased and new bands at higher than

245 kDa appeared, however by further increasing the time up to 60 min, the intensities of the bands decreased, and no new distinct bands appeared. Intense band at molecular weight higher than 245 kDa was observed in the profiles of hydrolysates obtained at 120°C that correspond to non-hydrolyzed collagen. By increasing the time up to 60 min, this band was more defined, and no new band was observed. Similarly, at 140°C, an intense band at molecular weight higher than 245 kDa and a weak band at molecular weight of 5 kDa were observed, and there was not a considerable change with increasing the time from 10 to 60 min. However, this band was less visible at 160°C for 10-20 min and not present at 180°C.

At 160°C, the bands in the range of 63-5 kDa were diffused that could be due to the higher viscosity of the samples at this temperature, and with the increase in time of the hydrolysis, the band at 5 kDa became more defined. At 180°C and 10 min, there was a distinct band at 5 kDa, but its intensity decreased when the time increased up to 60 min. These results indicated the hydrolysis of collagen to gelatin (15-250 kDa) and collagen peptides (0.3-8 kDa) at high temperatures (>140°C). Similar observation was reported by Melgosa et al. (2021) for codfish frame hydrolysates obtained at 90-250°C and 100 bar for 30 min. In their study, bands at molecular weights higher than 245 kDa were observed at 90°C. These bands disappeared by increasing the temperature up to 250^{o}C and new bands at molecular weights less than 17 kDa were observed (Melgosa et al., 2021).

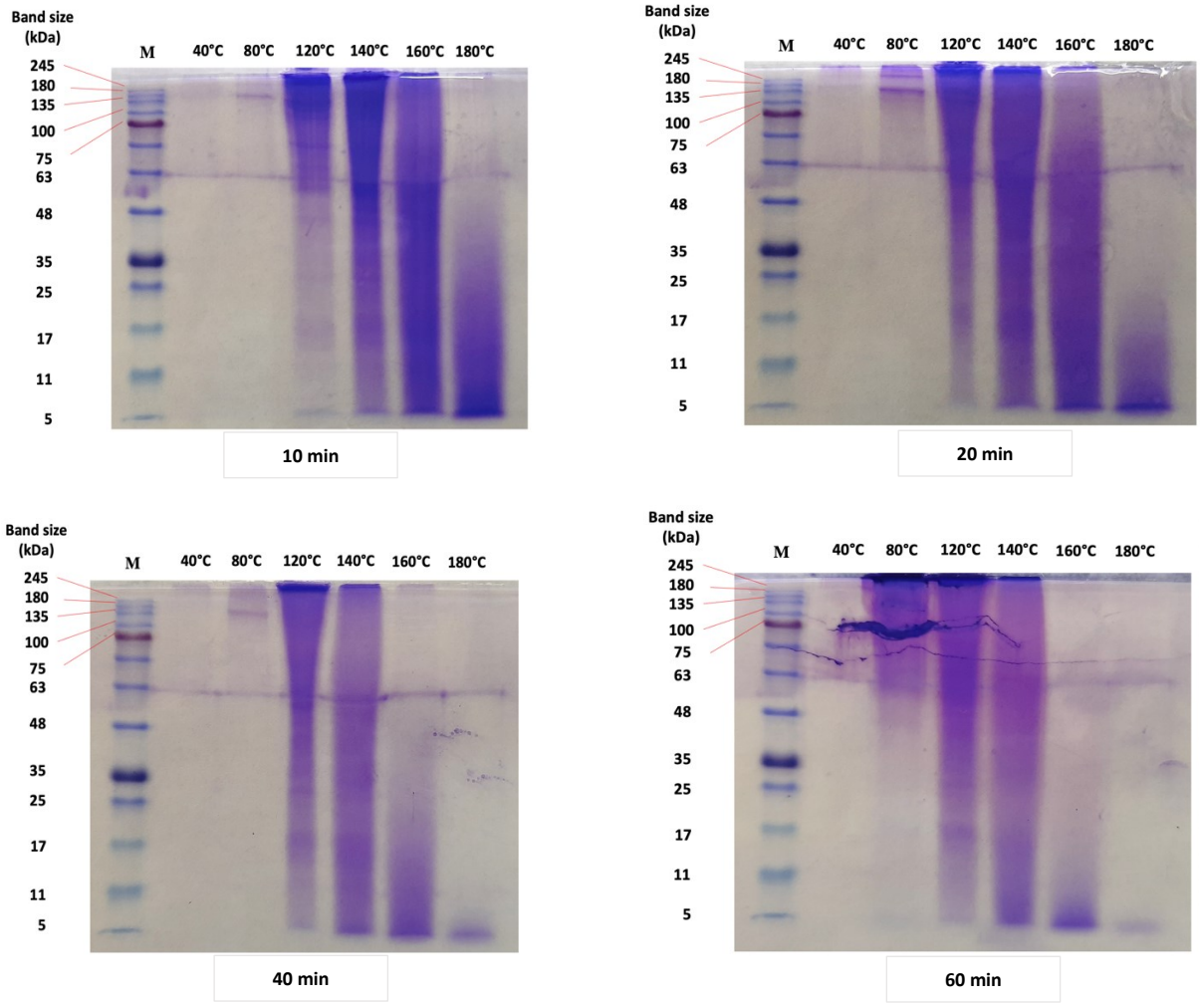

Figure 4.5. Electrophoretic profiles of chicken feet hydrolysates obtained after pressurized hot water treatment at different temperatures and times. M: Marker.

Molecular weight distribution of the chicken feet hydrolysates was also investigated by SEC-GPC following the method previously used by Melgosa et al. (2021) to analyze the collagen hydrolysates obtained after subcritical water hydrolysis of codfish frame. A protein mixture of thyroglobulin (660 kDa), γ-globulin (150 kDa), albumin (67 kDa), and ribonuclease-A (13.7 kDa) was used as standards, where the retention times were 5.39, 6.61, 8.1, and 9.3 min, respectively. Fig. 4.6 shows the chromatograms of hydrolysates obtained at different temperatures (40-180°C) and same time (10 min) (Fig. 4.6a), and different temperatures (120, 140, 160, and 180°C) and

times (10-60 min) (Fig. 4.6b-e). Collagen, gelatin, and collagen peptides have molecular weights of >250 kDa, 15-250 kDa, and 0.3-8 kDa, respectively (Irastorza et al., 2021). Overall, the highest peak with the highest percentage of the area under the curve observed in the chromatograms of the hydrolysates at 40, 80, 120, 140, 160, and 180°C were at retention times of 5.1 min (843.33 kDa), 5.17 min (788.61 kDa), 4.81 min (1113.52 kDa), 4.83 min (1092.38), 8.6 min (29.46 kDa), and 9.55 min (11.85 kDa) (Fig. 4.6a), respectively.

These results indicated that the highest peak was shifted to a longer retention time (lower molecular weight) by the increase in temperature. Similar trend was observed when the time of hydrolysis increased from 10 to 60 min (Fig. 4.6b-e). However, there is a possibility of overestimation of the molecular weights of the hydrolysates since elongated proteins would elute in shorter retention times than spherical proteins of equal molecular weights (Hong et al., 2019). But these results were consistent with the results observed by SDS-PAGE analysis reported earlier (Fig. 4.5).

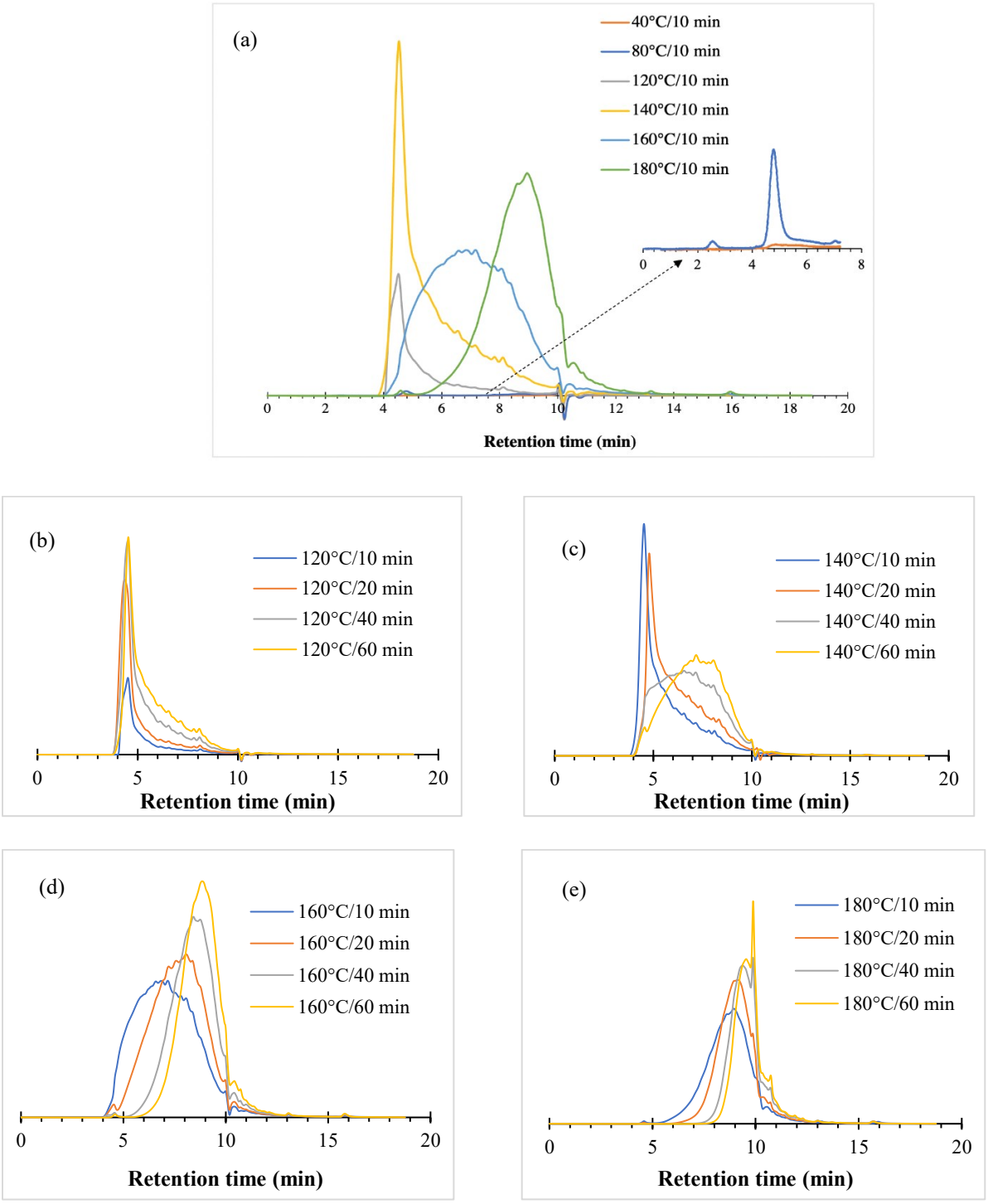

Figure 4.6. Effect of temperature and time on molecular weight distribution of chicken feet hydrolysates obtained after pressurized hot water treatment.

At low temperatures of 40-140°C, collagen was the dominant protein in the hydrolysates; and, if the area under the peak represents the quantity, the collagen content increased by increasing the temperature from 40 to 140°C, which agreed with the results earlier presented in Fig 4.2. However, at 140°C, when the time of hydrolysis increased to 40 and 60 min, the peaks shifted to retention times of 7-8.5 min without a good definition, indicating the hydrolysis of collagen to gelatin. By further increasing the temperature to 160°C and 180°C, collagen was hydrolyzed to gelatin and collagen hydrolysates, respectively.

The broad peaks of chromatograms at 160°C reflect the gelatinization of collagen where the proteins were not separated well due to the higher viscosity of the hydrolysates. This indicated the hydrolysis of larger proteins to smaller proteins and protein hydrolysates at temperatures higher than 140°C that was also observed by Koomyart et al. (2014), who hydrolysed krill using subcritical water at 100-240°C (pressure not reported) for 10 min and reported a shift in the peaks towards higher retention times by the increase in the temperature. At temperatures of 100-140°C, they observed groups of peaks at retention times of 6-13 min, corresponding to molecular weights around 1000 kDa and several peaks appeared at higher temperatures than 160°C and 15-20 min, corresponding to 10 kDa. Similarly, Melgosa et al. (2021) observed several peaks at retention times of 4.05, 5.92, and 6.65 min in chromatograms of codfish hydrolysates obtained at 90°C/100 bar/30 min. However, they observed the highest peaks at 12.92 min (1.1 kDa), 11.25 min (5.7 kDa), 11.25 min (5.7 kDa), and 11.28 min (3.2 kDa) at temperatures of 90, 140, 190, and 250°C/100 bar/30 min, respectively. This could be attributed to lower thermal stability of fish collagen. Jo et al. (2015) also observed that with increasing the temperature during subcritical water hydrolysis of porcine skin from 200°C/40 bar to 350°C/80 bar, peaks for high molecular weights disappeared while peaks for low molecular weights appeared. They reported molecular

weights of 6.95, 2.8, 0.5, and 0.2 kDa for hydrolysates at 200°C/40 bar, 250°C/40 bar, 300°C/80 bar, and 350°C/80 bar during 60 min of hydrolysis.

Lee et al. (2013) also observed that peaks were shifted from high molecular to low molecular weight region for porcine placenta hydrolysates obtained at 150, 170, and 200°C and 375 bar (zero holding time). At 150°C, they observed four peaks in the molecular weight region of > 20.1 kDa that were attributed to gelatin and high molecular weight peptides and two peaks in the region of < 0.1 kDa that were attributed to low molecular weight peptides. At 200°C, they did not observe any peak in the high molecular weight region, only 4.2, 1.4, and 0.4 kDa. Considering that in our study a protein mix with a molecular weight range of 13.7-660 kDa was utilized as a standard, there was a limitation to investigate the presence of peptides with lower molecular weights of 0.3-8 kDa.

The results demonstrate that pressurized hot water at low temperatures up to 140°C hydrolyzed/extracted collagen from animal by-products, and at higher temperatures promoted hydrolysis of larger proteins to lower molecular weight fragments (gelatin, peptides, and free amino acids). The collagen global market was around US$ 4.2 billion in 2018 and is estimated to grow up to US$ 6.6 billion by 2025 (Ahmed et al., 2020), and the global collagen peptides market was US$ 631 million in 2021 and is estimated to grow up to US$ 828 million by 2026 (Chaturvedi, 2022). Collagen is the most used biopolymer in tissue engineering (O'brien, 2011), not only meets the criteria to be used in biomedicine, also contributes to active regeneration of tissues (Irastorza et al., 2021). High biocompatibility, biodegradability, and malleability are some characteristics of collagen (Irastorza et al., 2021). Gua et al. (2020) reported that electrospun collagen-chitosan membranes resulted in *in-vivo* restoration of calvarial bone defect by generation of massive bone tissue. Also, Whu et al. (2013) observed that collagen-chitosan scaffolds induced generation of

new cartilage in rabbit with articular cartilage defects in one month, and after 6.5 months the properties of new cartilage were similar to the normal cartilage.

Collagen has also wide applications in the food industry. Collagen-based films and coatings have been developed as sustainable packaging material to preserve food quality and extend its shelf life. Collagen and gelatin have been widely used in industrial production of sausage casings (Irastorza et al., 2021). Encapsulation based on gelatin has also been widely studied for formulation of functional food (Kuai et al., 2020). Antioxidant and antihypertensive activities have been reported for collagen peptides (Ishak & Sarbon, 2018). Collagen peptides have been mainly applied in cosmetic applications. The effectiveness of supplementation of collagen peptides on skin elasticity, hydration, and wrinkling has been reported (Ito et al., 2018; Kim et al., 2018). Fig. 4.7 shows a simplified schematic of subcritical water hydrolysis of chicken feet to produce collagen/collagen fragments (peptides and amino acids).

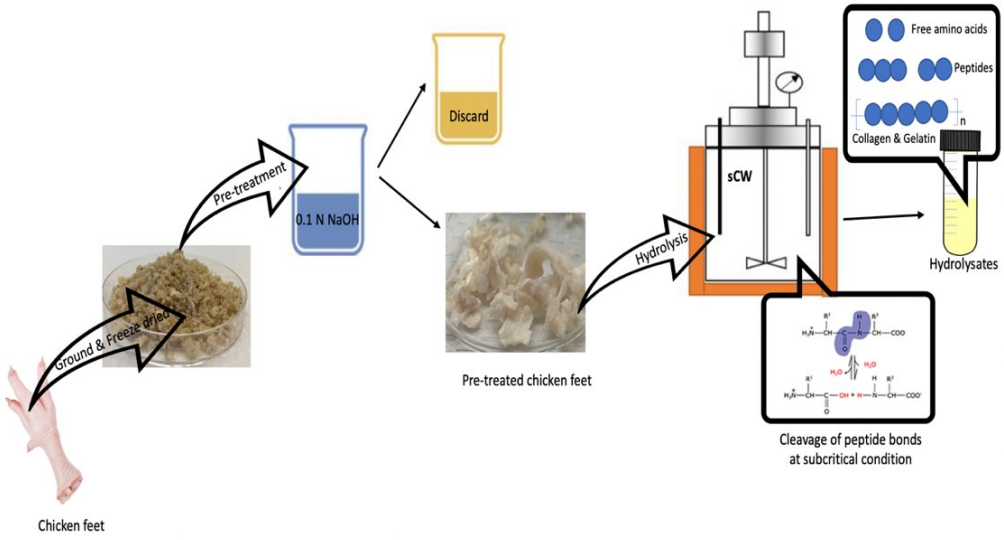

Figure 4.7. A simplified schematic of subcritical water hydrolysis of chicken feet.

4.4. Conclusions

This study investigated the effects of temperature and time on pressurized hot water hydrolysis of chicken feet to obtain collagen. Collagen/collagen fragments content of the hydrolysates increased significantly by increasing temperature from 40°C to 140°C, and a further increase up to 180°C did not make a significant change. Increasing the time of hydrolysis (10-60 min) did not significantly affect the content of collagen/collagen fragments in the hydrolysates. However, the content of collagen/collagen fragments was further increased at 180°C when treatment was prolonged to 40 min. Overall, hydrolysis at 140°C/50 bar/10 min was the best condition to obtain hydrolysates with collagen/collagen fragments (152.04 mg/g chicken feet) with a yield of 15.2% that was almost two times higher than the conventional acid treatment (8.16%).

Free hydroxyproline content increased with increasing temperature and time and reach the highest value of 8.27 mg/g chicken feet at 160°C/50 bar/10 min. However, further increase in time up to 60 min and temperature to 180°C resulted in its degradation. Overall, hydroxyproline, lysine and glycine were the main free amino acids of chicken feet hydrolysates. The degree of hydrolysis also increased by increasing temperature and time with the highest of 11.64% at 180°C/50 bar/60 min using pressurized hot water. At low temperatures (40-140°C), collagen was the main protein in the hydrolysates. Collagen was hydrolyzed to gelatin (15-250 kDa) and collagen hydrolysates (<15 kDa) when temperature increased to 160°C and 180°C, respectively. Therefore, the results of this study suggest that pressurized hot water at low temperatures up to 140°C was a promising technology to hydrolyze/extract collagen, while with the use of temperatures higher than 140°C could hydrolyze/extract gelatin and/or collagen hydrolysates from animal by-products.

Chapter 5: Conclusions and recommendations

5.1. Conclusions

The animal by-products such as shrimp shell and chicken feet are promising sources for chitosan and collagen production, respectively. These biopolymers have wide applications specially in food (e.g., dietary food additive) and biomedical industries (e.g., tissue engineering). In 2020, globally, 5 million tons of shrimp were produced, with a predicted increase up to 7.3 million tons by 2025 (Nirmal et al., 2020). Currently, shrimp shell is discarded as inedible by-products with approximately 45-48% (depending on species) by weight of raw shrimp (Ambigaipalan & Shahidi, 2017). But shrimp shell can be considered as the main source of chitosan production that contains 18-30% of chitin (Al Hoqani et al., 2020; Joseph et al., 2021). On the other hand, in 2018, the market size of collagen was around US$ 4.2 billion worldwide that is estimated to reach US$ 6.6 billion by 2025 (Ahmed et al., 2020). Chicken feet with collagen content of 20% (w/w) is a promising source for collagen production.

In the first study of this thesis research, shrimp shell was investigated to obtain chitosan. Removal of protein, minerals, and pigment from shrimp shell led to enrichment of chitin for further hydrolysis to obtain chitosan. The use of alkali hydrolysis with sodium hydroxide is a conventional treatment for deproteination of shrimp shell but this treatment not only is time consuming, also results in protein hydrolysates that cannot be considered as a safe source of protein because of the remaining sodium hydroxide. Therefore, subcritical water assisted by ultrasound was investigated as a green approach for deproteination of shrimp shell. First, shrimp shell was pretreated with ultrasound using water at 600 and 1200 W for 5 min followed by subcritical water hydrolysis at 140, 180, 220, and 260°C and 50 bar for 10, 20, 40, and 60 min with a flow rate of 5 mL/min. To compare, alkali treatment using 2.0 M sodium hydroxide with a ratio of 1:16 w/v at room

temperature for 48 h was performed to deproteinize shrimp shell. Both temperature and time had significant effect on subcritical water deproteination of shrimp shell. The use of ultrasound pre-treatment benefited the subcritical water deproteination of shrimp shell by increasing the yields of nitrogen and free amino acids of the hydrolysates as well as improving the degree of deproteination of the shell. Nitrogen content of 34.2 mg/g shrimp shell and free amino acids content of 49.06 mg/g shrimp shell with 60.61% degree of deproteination was obtained using subcritical water only treatment at 260°C/50 bar and 60 min. The highest yield of nitrogen content (99.01 mg/g shrimp shell) with 72.57% of deproteination was obtained using ultrasound pre-treatment at 1200 W/5 min followed by subcritical water at 180ºC/50 bar for 60 min. The highest free amino acid content of 70.92 mg/g shrimp shell and the highest degree of deproteination of 80.93% were obtained using ultrasound pre-treatment at 1200 W/5 min followed by subcritical water at 260ºC for 60 min. These values were significantly higher than the alkali treatment where free amino acid content of 21.31 mg/g shrimp shell was obtained with only 58.84% deproteination degree.

Deproteinized shrimp shell were demineralized using 0.5 M citric acid with a ratio of 1:13 w/v at room temperature for 20 min, bleached with 30% hydrogen peroxide with a ratio of 1:10 w/v at room temperature for 3 h, and deacetylated with 50% sodium hydroxide at a ratio of 1:10 w/v at 121°C for 15 min. Chitosan-rich residue with a yield of 10.56% and whiteness index of 60.42 was obtained from deproteinized shrimp shell using subcritical water assisted by ultrasound at 260°C and 50 bar. These values were lower than the alkali treated sample (17.76% and 67.8, respectively). However, the degree of deacetylation (64.27%) and functional groups of chitosan-rich sample obtained from deproteination using subcritical water assisted by ultrasound at 260°C and 50 bar were comparable to the alkali treated sample (65.05% degree of deacetylation) and lower than the commercial chitosan (>75% degree of deacetylation). Also, its lower relative

crystallinity of 32.66% compared to the alkali treated sample (50.64%) and commercial chitosan (50.52%) indicated its better solubility property, the less crystalline area, the better solubility, which is important for its industrial applications. The findings of this study suggested that subcritical water assisted by ultrasound is a promising treatment to obtain chitosan from shrimp shell in a greener approach with a shorter time of processing. Also, the resultant protein hydrolysates could be utilized in some applications in the food industry like dietary food additives.

Pre-treatment of chicken feet led to removal of lipids, minerals, and odor to produce collagen with less impurities. Conventionally, acid treatment with hydrochloric acid or enzymatic treatments with papain have been used to extract/hydrolyse collagen. The use of acids is corrosive to the equipment and enzymatic treatment is a costly process. However, pressurized hot water is considered as an eco-friendly technology for hydrolysis of collagen from chicken feet.

In the second study of this thesis research, chicken feet were investigated to obtain collagen using pressurized hot water technology. Chicken feet were pretreated using 0.1 N sodium hydroxide with a ratio of 1:10 w/v at a temperature of ~25°C for 24 h to remove lipids, minerals, and odor. Then, the pressurized hot water hydrolysis of pretreated chicken feet was carried out at 40, 80, 120, 140, 160, and 180°C and 50 bar for 10, 20, 40, and 60 min with a ratio of 1:50 w/v in a 100 mL batch reactor. To compare, the conventional acid treatment of pretreated chicken feet was conducted using 0.5 M acetic acid with a ratio of 1:20 w/v at 4°C for 48 h. The increase in temperature from 40°C to 140°C significantly increased the collagen/collagen fragments content of the chicken feet hydrolysates. However, further increase up to 180°C did not make a significant change in the collagen/collagen fragments content. Increasing the time of hydrolysis from 10 to 60 min also did not make a significant change in the content of collagen/collagen fragments maybe because the rate of degradation to free amino acid was higher than the rate of hydrolysis. Which

agreed with the increase in the free hydroxyproline content by increasing the temperature and time where the highest value of 8.27 mg/g chicken feet was obtained at 160°C/10min. This amino acid is exclusively present in collagen. But, at 180°C, the collagen/collagen fragments content had an increase from 10 to 40 min. However, it decreased by further increasing the time up to 60 min and temperature to 180°C, that may be due to its further hydrolysis to organic acids. Treatment at 140°C for 10 min was the best condition to obtain collagen/collagen fragments of 152.04 mg/g chicken feet (yield of 15.2%) from chicken feet that resulted in almost the double yield compared to the conventional acid treatment (yield of 8.16%).

By increasing the temperature and time, the degree of hydrolysis also increased with a maximum of 11.64% at 180°C and 60 min. According to the amino acids profile of chicken feet hydrolysates obtained at different temperatures and times, hydroxyproline, lysine and glycine were the main free amino acids. Hydrolysis at 140°C/50 bar/10 min was the best condition to obtain collagen from chicken feet.

The findings of this study suggested that pressurized hot water treatment at temperatures below 140°C was promising to obtain collagen from chicken feet, while at temperatures above 140°C could hydrolyze collagen to gelatin and/or collagen hydrolysates (e.g., peptides). The chicken feet collagen could find a wide application in tissue engineering due to its biocompatibility and biodegradability. Also, it can be utilized in food and pharmaceutical industries with no limitations in production of halal and kosher products.

Overall, the objectives of this research were achieved, and the findings were promising for the application of pressurized hot water technology for utilization of animal by-products to recover valuable biopolymers, such as chitosan and collagen. Also, the use of ultrasound technology

together with pressurized hot water technology was promising to enhance the deproteination process of shrimp shell.

5.2. Recommendations and future work

Some recommendations to advance this research for Chapter 3 are:

- Investigate the kinetics and the order of the reactions to have a better understating of the mechanisms in the hydrolysis of shrimp shell.
- Ultrasound pre-treatment was conducted for 5 min. It is recommended to investigate a longer time of pre-treatment (e.g., 15 and 30 min) to optimize the deproteination of shrimp shell.
- Deacetylation of chitin was performed within 15 min. Optimization of the process condition (e.g., time and temperature) is suggested to obtain a higher degree of deacetylation. However, deacetylation was performed using the conventional alkali treatment (50% sodium hydroxide) that is not an eco-friendly method. Therefore, investigating a greener method like use of the ultrasound technology as a greener method could help to reduce the environmental burden and improve the degree of deacetylation of chitosan.
- Quantification of glucosamine and glucose in the shrimp shell hydrolysates using HPLC is recommended to better understand the hydrolysate composition.
- The final chitosan should be characterized in terms of its physico-chemical properties such as its average molecular weight using a Zetasizer and solubility. In addition, functional properties of chitosan such as antimicrobial activity and water and oil binding capacity should be investigated for its future applications.

Some recommendations to advance this research for Chapter 4 are:

- To produce collagen in a greener way, investigation of a green technology like ultrasonication with a diluted acid (e.g., malic acid) instead of using alkali solution for pre-treatment of chicken feet is recommended.
- Pressurized hot water treatment of chicken feet was conducted with a solid-solvent ratio of 1:50 v/w. It is recommended to investigate a lower ratio as with a higher volume of water, a higher concentration of water ions product (H_3O^+ and OH^-) may be present in the reaction media.
- Investigate the effect of pressurized hot water treatment on the structural conformation (FTIR) and triple helix structure (circular dichroism) of collagen.
- Investigate the functional and mechanical properties of collagen (e.g., oil and water binding capacities) and collagen peptides (e.g., antioxidant activity) obtained by pressurized hot water treatment of chicken feet for its future applications. In addition, ultrafiltration using a membrane with a molecular weight cut-off at 250 kDa or higher is recommended to purify the final collagen product.

5.3. Limitations

Throughout this research, there were some limitations that could affect the results:

For the process:

- Grinding chicken feet to obtain a good representative sample with uniform particle size was challenging due to the presence of hard tissue like bones and cartilage. Therefore, the samples were first grinded using an industrial grinder, freeze dried, and grinded for second time using a coffee grinder to obtain an uniform size of sample.
- According to the method protocol, there was a need of an extensive washing step after each chemical treatment for the samples (e.g., demineralization, deacetylation, etc.).

After these treatments, distilled water was used to neutralize the samples, which was time consuming and caused loss of sample with small particle size.

- The need of filtration after each washing step of the residues also resulted in loss of sample as some of them remained in the filtering paper.
- The need of cleaning the filter located after the cooling system after each experiment to minimize plugging that might results in the increase of pressure.

For the analysis:

- The use of the spectrophotometer can affect the results due to the possibility of interference of other compounds besides the compound of interest.
- The need of further acid hydrolysis of the hydrolysates for quantification of collagen content based on hydroxyproline amino acid was a time-consuming process as the evaporation of the acid solvent (HCl) and neutralization of samples were required.

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APPENDIX

Appendix A. Calibration curves

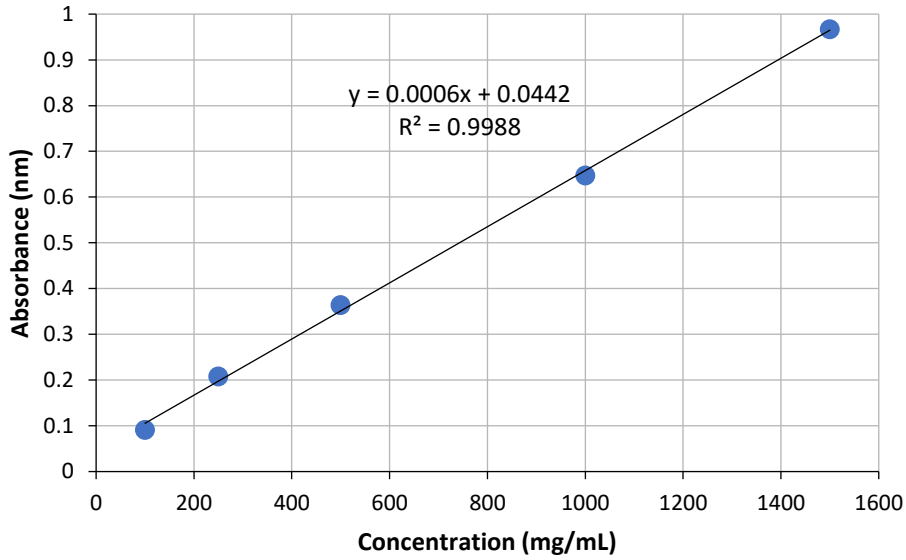

Figure A1. Bovine serum albumin standard curve to determine nitrogen content using spectrophotometer.

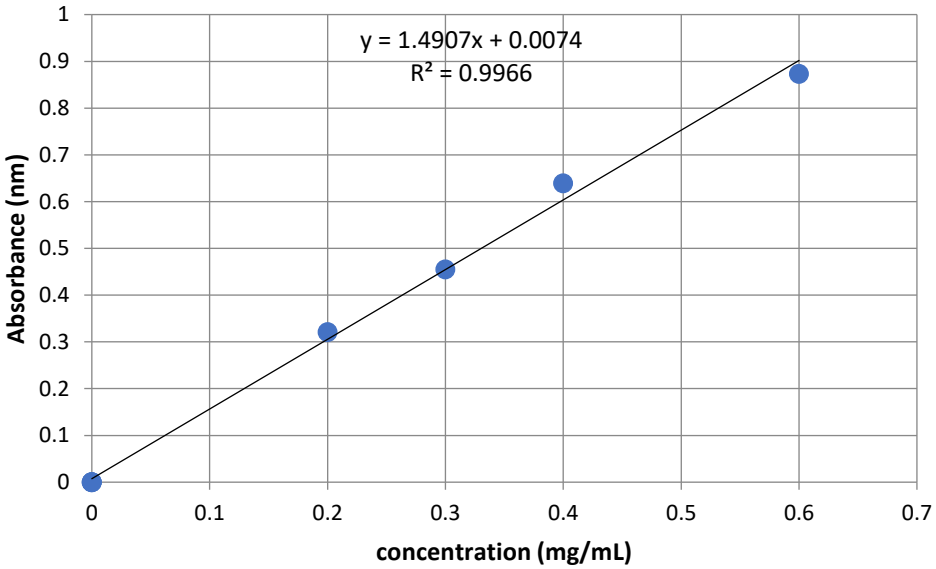

Figure A2. Lysine standard curve to determine free amino acid content using spectrophotometer.

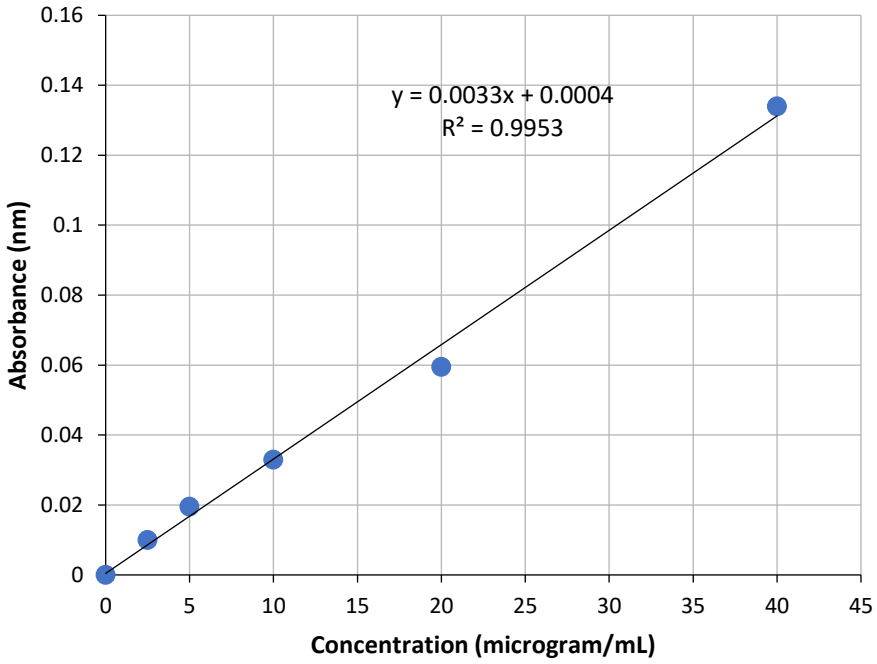

Figure A3. Hydroxyproline standard curve to determine collagen/collagen fragments content using spectrophotometer.

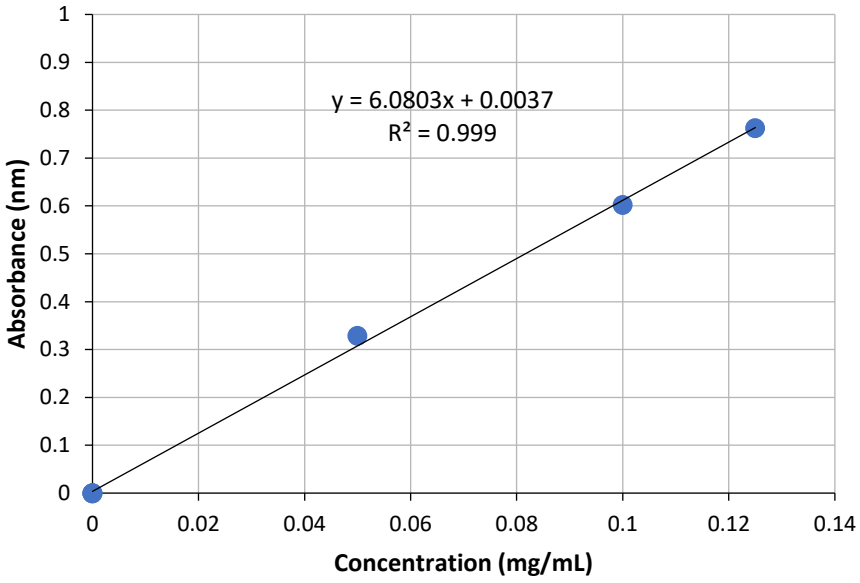

Figure A4. Glycine standard curve to determine the degree of hydrolysis using spectrophotometer.

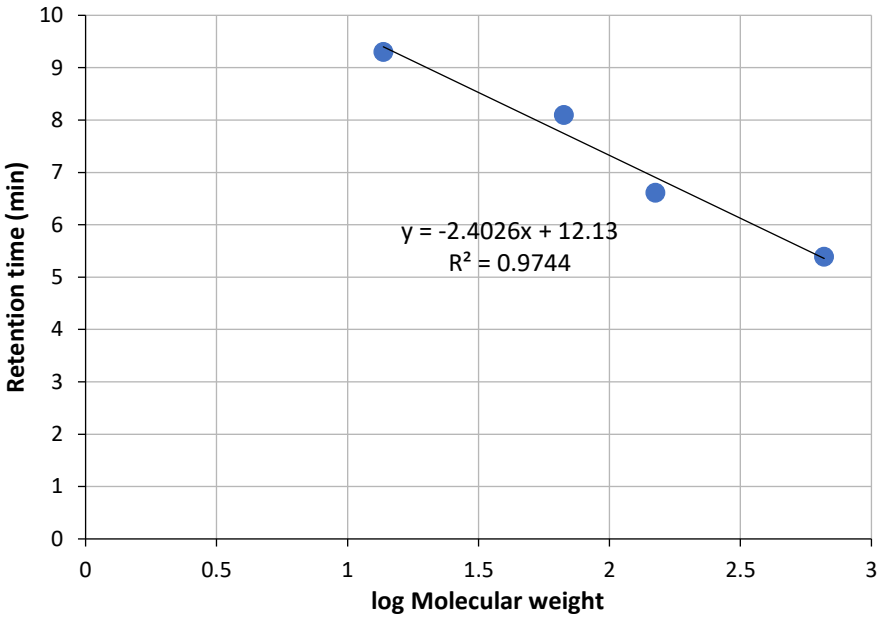

Figure A5. Protein mix (thyroglobulin (660 kDa), γ-globulin (150 kDa), albumin (67 kDa), and ribonuclease-A (13.7 kDa)) standard curve to determine molecular weight using size exclusion chromatography.

Appendix B. Subcritical water assisted by ultrasound deproteination of shrimp shell

Table B1. Nitrogen content of shrimp shell hydrolysates obtained using sCW at 50 bar and 5 mL/min.

Temperature (°C)	Time (min)	Nitrogen content (mg/mL)	Nitrogen content (mg/g shrimp shell)	Average nitrogen content (mg/g shrimp shell)
140	10	0.18	4.57	3.19 ± 1.90^{f}
	10	0.07	1.82	
	20	0.41	10.30	7.05 ± 4.61^{ef}
	20	0.15	3.80	
	40	0.73	18.18	15.39 ± 3.93^{de}
	40	0.50	12.60	
	60	0.79	19.65	16.13 ± 4.97^{de}
	60	0.50	12.60	
180	10	0.59	14.82	$11.17 \pm 5.18d^{ef}$
	10	0.30	7.53	
	20	0.72	18.05	15.93 ± 3.03^{de}
	20	0.55	13.8	
	40	0.72	18.05	16.16 ± 2.64^{de}
	40	0.57	14.27	
	60	0.72	18.05	16.89 ± 1.57^{cde}
	60	0.63	15.73	
220	10	0.91	22.69	20.42 ± 3.12^{bcd}
	10	0.73	18.15	
	20	1.17	29.26	28.47 ± 1.09^{abc}
	20	1.11	27.68	
	40	1.21	30.31	29.94 ± 0.53^{ab}
	40	1.18	29.56	
	60	1.21	30.31	29.94 ± 0.53^{ab}
	60	1.18	29.56	
260	10	0.85	21.19	21.61 ± 0.58^{bcd}
	10	0.88	22.03	
	20	1.29	32.34	31.07 ± 1.78^{ab}
	20	1.19	29.8	
	40	1.45	36.22	34.2 ± 2.84^{a}
	40	1.29	32.18	
	60	1.45	36.23	34.2 ± 2.85^{a}
	60	1.29	32.18	

[a-f]Numbers in the same column that do not share a letter are significantly different ($p < 0.05$).

Table B2. Nitrogen content of shrimp shell hydrolysates obtained using ultrasound (600 W, 5 min) + sCW at 50 bar and 5 mL/min.

Temperature (°C)	Time (min)	Nitrogen content (mg/mL)	Nitrogen content (mg/g shrimp shell)	Average nitrogen content (mg/g shrimp shell)
140	10	0.22	7.37	7.76 ± 0.55^{g}
	10	0.24	8.14	
	20	0.62	20.73	19.57 ± 1.65^{efg}
	20	0.55	18.40	
	40	0.70	46.36	39.75 ± 9.35^{bcde}
	40	0.50	33.13	
	60	0.98	65.20	57.14 ± 11.39^{abcd}
	60	0.74	49.09	
180	10	0.33	10.92	12.14 ± 1.72^{fg}
	10	0.40	13.37	
	20	1.17	38.96	35.93 ± 4.28^{cdef}
	20	0.99	32.90	
	40	1.09	73.02	65.55 ± 10.57^{ab}
	40	0.87	58.08	
	60	1.27	84.42	74.06 ± 14.65^{a}
	60	0.96	63.70	
220	10	0.99	32.87	33.95 ± 1.53^{defg}
	10	1.05	35.03	
	20	1.57	52.40	54.82 ± 3.42^{abcd}
	20	1.72	57.23	
	40	0.90	60.24	62.72 ± 3.50^{abc}
	40	0.98	65.19	
	60	0.90	60.24	62.72 ± 3.50^{abc}
	60	0.98	65.19	
260	10	1.41	47.09	42.89 ± 5.93^{bcde}
	10	1.16	38.70	
	10	1.86	61.90	58.73 ± 4.48^{abcd}
	20	1.67	55.57	
	40	1.00	66.86	62.8 ± 5.73^{abc}
	40	0.88	58.75	
	60	1.00	66.86	62.8 ± 5.73^{abc}
	60	0.88	58.75	

[a-g]Numbers in the same column that do not share a letter are significantly different ($p < 0.05$).

Table B3. Nitrogen content of shrimp shell hydrolysates obtained using ultrasound (1200 W, 5 min) + sCW at 50 bar and 5 mL/min.

Temperature (°C)	Time (min)	Nitrogen content (mg/mL)	Nitrogen content (mg/g shrimp shell)	Average nitrogen content (mg/g shrimp shell)
140	10	0.23	7.7	11.06 ± 4.75^{g}
	10	0.43	14.42	
	20	0.65	21.68	24.93 ± 4.60^{fg}
	20	0.85	28.18	
	40	0.73	48.86	51.22 ± 3.34^{e}
	40	0.80	53.58	
	60	1.01	67.37	68.51 ± 1.61^{cd}
	60	1.04	69.65	
180	10	0.62	20.70	21.62 ± 1.30^{g}
	10	0.68	22.53	
	20	1.57	52.29	52.6 ± 0.43^{de}
	20	1.59	52.90	
	40	1.32	87.69	88.94 ± 1.77^{ab}
	40	1.35	90.19	
	60	1.49	99.53	99.01 ± 0.75^{a}
	60	1.48	98.48	
220	10	1.24	41.37	41.31 ± 0.08^{ef}
	10	1.24	41.26	
	20	2.10	69.90	71.26 ± 1.92^{c}
	20	2.18	72.62	
	40	1.23	81.74	84.99 ± 4.60^{abc}
	40	1.32	88.24	
	60	1.22	81.14	83.34 ± 3.10^{abc}
	60	1.28	85.53	
260	10	1.56	51.92	45.56 ± 8.99^{e}
	10	1.18	39.20	
	10	2.14	71.23	71.82 ± 0.82^{c}
	20	2.17	72.40	
	40	1.12	74.86	79.38 ± 6.40^{bc}
	40	1.26	83.91	
	60	1.08	72.26	77.67 ± 7.66^{bc}
	60	1.25	83.09	

[a-g]Numbers in the same column that do not share a letter are significantly different ($p < 0.05$).

Table B4. Free amino acids content of shrimp shell hydrolysates obtained using sCW at 50 bar and 5 mL/min.

Temperature (°C)	Time (min)	Free amino acids content (mg/mL)	Free amino acids content (mg/g shrimp shell)	Average free amino acids content (mg/g shrimp shell)
140	10	0.15	3.78	4.04 ± 0.37^{i}
	10	0.17	4.30	
	20	0.30	7.60	8.09 ± 0.69^{hi}
	20	0.34	8.57	
	40	0.25	12.72	14.16 ± 2.04^{fg}
	40	0.31	15.60	
	60	0.32	16.16	17.79 ± 2.30^{def}
	60	0.39	19.42	
180	10	0.44	10.88	9.8 ± 1.51^{gh}
	10	0.35	8.73	
	20	0.67	16.69	16.03 ± 0.94^{ef}
	20	0.61	15.37	
	40	0.44	21.94	21.77 ± 0.25^{cd}
	40	0.43	21.59	
	60	0.50	24.85	25.18 ± 0.46^{c}
	60	0.51	25.50	
220	10	0.64	15.96	15.93 ± 0.04^{ef}
	10	0.64	15.91	
	20	1.00	25.21	25.87 ± 0.94^{c}
	20	1.06	26.54	
	40	0.66	32.85	33.98 ± 1.60^{b}
	40	0.70	35.11	
	60	0.73	36.56	37.79 ± 1.74^{b}
	60	0.78	39.02	
260	10	0.78	19.38	20.99 ± 2.28^{cde}
	10	0.90	22.60	
	20	1.42	35.61	36.32 ± 1.01^{b}
	20	1.48	37.04	
	40	0.90	44.75	45.16 ± 0.58^{a}
	40	0.91	45.58	
	60	0.97	48.33	49.08 ± 1.06^{a}
	60	0.10	49.82	

[a-i]Numbers in the same column that do not share a letter are significantly different ($p < 0.05$).

Table B5. Free amino acids content of shrimp shell hydrolysates obtained using ultrasound (600 W, 5 min) + sCW at 50 bar and 5 mL/min.

Temperature (°C)	Time (min)	Free amino acids content (mg/mL)	Free amino acids content (mg/g shrimp shell)	Average free amino acids content (mg/g shrimp shell)
140	10	0.08	2.56	2.91 ± 0.46^{i}
	10	0.10	3.23	
	20	0.18	5.84	5.83 ± 0.02^{hi}
	20	0.17	5.82	
	40	0.18	12.04	10.84 ± 1.69^{fghi}
	40	0.14	9.65	
	60	0.25	16.49	14.54 ± 2.77^{efgh}
	60	0.19	12.58	
180	10	0.34	11.42	9.85 ± 2.21^{ghi}
	10	0.25	8.29	
	20	0.68	22.64	23.56 ± 1.31^{e}
	20	0.73	24.49	
	40	0.54	36.03	38.10 ± 2.93^{cd}
	40	0.60	40.17	
	60	0.60	40.13	41.98 ± 2.61^{bcd}
	60	0.66	43.82	
220	10	0.63	20.92	18.98 ± 2.75^{efg}
	10	0.51	17.03	
	20	1.11	36.86	34.35 ± 3.54^{d}
	20	0.96	31.85	
	40	0.69	45.92	44.06 ± 2.62^{abc}
	40	0.63	42.21	
	60	0.76	50.60	48.29 ± 3.26^{ab}
	60	0.69	45.99	
260	10	0.66	21.95	20.03 ± 0.00^{ef}
	10	0.54	18.10	
	20	1.30	43.45	41.86 ± 2.26^{bcd}
	20	1.21	40.26	
	40	0.77	51.26	50.4 ± 1.22^{ab}
	40	0.74	49.54	
	60	0.80	53.39	52.76 ± 0.90^{a}
	60	0.78	52.12	

[a-i]Numbers in the same column that do not share a letter are significantly different ($p < 0.05$).

Table B6. Free amino acids content of shrimp shell hydrolysates obtained using ultrasound (1200 W, 5 min) + sCW at 50 bar and 5 mL/min.

Temperature (°C)	Time (min)	Free amino acids content (mg/mL)	Free amino acids content (mg/g shrimp shell)	Average free amino acids content (mg/g shrimp shell)
140	10	0.18	5.92	2.42 ± 0.39^{h}
	10	0.17	5.60	
	20	0.46	15.48	6.03 ± 1.25^{f}
	20	0.46	15.30	
	40	0.38	25.21	12.86 ± 2.57^{e}
	40	0.37	24.85	
	60	0.43	28.82	16.44 ± 3.05^{de}
	60	0.43	28.59	
180	10	0.54	18.13	5.76 ± 0.22^{f}
	10	0.53	17.77	
	20	0.99	32.92	15.39 ± 0.13^{d}
	20	0.97	32.32	
	40	0.63	41.94	25.03 ± 0.25^{c}
	40	0.61	40.84	
	60	0.69	46.12	28.7 ± 0.16^{c}
	60	0.67	44.85	
220	10	1.21	40.37	17.95 ± 0.25^{c}
	10	1.30	43.50	
	20	1.80	60.03	32.62 ± 0.43^{b}
	20	1.91	63.56	
	40	1.00	66.81	41.39 ± 0.78^{ab}
	40	1.05	69.90	
	60	1.02	68.27	45.49 ± 0.90^{a}
	60	1.10	73.59	
260	10	0.08	2.70	41.93 ± 2.21^{h}
	10	0.06	2.14	
	20	0.21	6.91	61.8 ± 2.50^{gh}
	20	0.15	5.15	
	40	0.22	14.68	68.35 ± 2.18^{fg}
	40	0.17	11.04	
	60	0.29	18.60	70.93 ± 3.76^{f}
	60	0.21	14.28	

[a-h]Numbers in the same column that do not share a letter are significantly different ($p < 0.05$).

Appendix C. Pressurized hot water hydrolysis of chicken feet to obtain collagen/collagen fragments

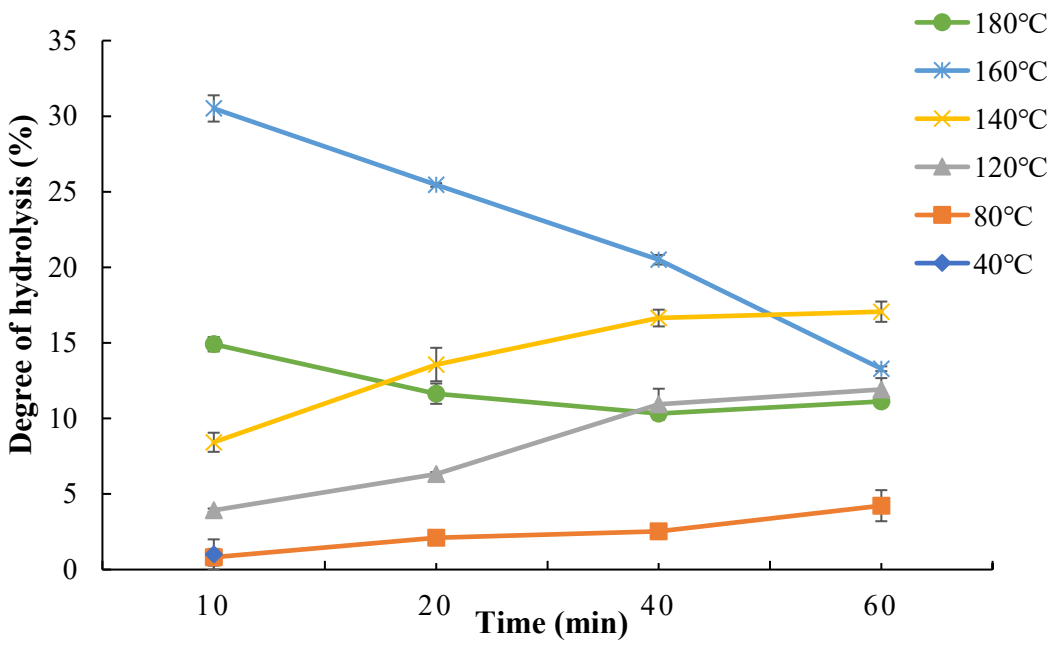

Figure C1. Effect of temperature and time on the degree of hydrolysis of chicken feet based on free hydroxyproline amino acid content.

Table C1. Collagen/collagen fragments content of chicken feet hydrolysates obtained after pressurized hot water at 50 bar and a ratio of 1:50 w/v.

Temperature (°C)	Time (min)	THC (mg/g chicken feet)	FHC (mg/g chicken feet)	C/CFC (mg/g chicken feet)	Average C/CFC (mg/g chicken feet)
40	10	3.21	0.07	6.67	6.50 ± 0.25^{ef}
	10	3.07	0.12	6.32	
	20	2.53	0.39	8.77	17.41 ± 12.21^{edf}
	20	1.48	0.33	26.04	
	40	0.35	0.25	10.52	5.26 ± 7.44^{ef}
	40	0.00	0.21	0.00	
	60	0.18	0.34	1.77	2.53 ± 1.07^{f}
	60	1.59	0.37	3.29	
80	10	0.94	0.12	37.12	28.96 ± 11.55^{def}
	10	0.94	0.33	20.79	
	20	1.53	0.52	44.47	48.67 ± 5.94^{def}
	20	3.71	0.61	52.87	
	40	1.62	0.68	55.21	55.32 ± 0.16^{d}
	40	0.00	0.69	55.44	
	60	0.57	1.34	61.62	52.12 ± 0.16^{de}
	60	0.80	0.95	42.61	
120	10	4.94	1.08	122.76	118.73 ± 5.69^{c}
	10	3.03	1.03	114.71	
	20	6.30	1.74	151.34	138.04 ± 18.81^{bc}
	20	7.48	1.69	124.74	
	40	7.85	2.77	160.21	155.37 ± 6.85^{abc}
	40	7.89	3.16	150.52	
	60	9.35	3.08	152.16	160.85 ± 12.29^{abc}
	60	6.48	3.37	169.54	
140	10	21.75	2.40	149.01	152.04 ± 4.29^{bc}
	10	22.30	2.16	155.07	
	20	23.71	3.89	152.62	154.08 ± 2.06^{abc}
	20	23.66	3.46	155.54	
	40	22.89	4.40	142.36	154.67 ± 17.41^{abc}
	40	26.30	4.62	166.97	
	60	21.89	4.49	133.96	152.57 ± 26.32^{abc}
	60	26.98	4.75	171.17	
160	10	25.85	8.43	134.07	134.13 ± 0.08^{bc}
	10	25.53	8.10	134.19	
	20	23.21	6.87	125.79	129.64 ± 5.44^{bc}
	20	24.25	6.92	133.49	
	40	23.35	5.50	137.46	137.69 ± 0.33^{bc}
	40	23.53	5.62	137.92	
	60	25.85	3.63	171.06	167.79 ± 4.62ab
	60	24.94	3.57	164.52	

Table C1. Continue.

180	10	22.62	3.95	143.76	120.31 ± 33.16^{bc}
	10	16.71	4.13	96.86	
	20	25.12	3.02	170.12	164.06 ± 8.58^{abc}
	20	23.80	3.28	157.99	
	40	28.80	2.78	200.34	200.92 ± 0.82^{a}
	40	28.98	2.81	201.51	
	60	24.25	2.94	164.06	158.63 ± 7.67^{abc}
	60	22.98	3.08	153.21	

THC: Total hydroxyproline content; FHC: Free hydroxyproline content; C/CFC: Collagen/collagen fragments content. Numbers in the same column that do not share a $^{a\text{-}f}$letter are significantly different ($p < 0.05$).

Table C2. Amino acids content of chicken feet hydrolysates and degree of hydrolysis obtained after pressurized hot water at 50 bar and a ratio of 1:50 w/v.

Temperature (°C)	Time (min)	AAs content (mg/mL)	AAs content (mg/g chicken feet)	DH (%)	Average DH (%)
40	10	0.01	0.48	0.19	0.07 ± 0.01^{g}
	10	0.01	0.58	0.23	
	20	0.00	0.13	0.054	0.24 ± 0.08^{g}
	20	0.00	0.12	0.05	
	40	0.01	0.31	0.12	0.25 ± 0.09^{g}
	40	0.01	0.48	0.19	
	60	0.00	0.10	0.04	0.26 ± 0.04^{g}
	60	0.01	0.45	0.18	
80	10	0.00	0.15	0.06	0.12 ± 0.04^{g}
	10	0.00	0.20	0.08	
	20	0.01	0.45	0.18	0.61 ± 0.03^{fg}
	20	0.01	0.74	0.30	
	40	0.01	0.48	0.19	0.71 ± 0.03^{fg}
	40	0.02	0.78	0.31	
	60	0.01	0.58	0.23	0.92 ± 0.01^{fg}
	60	0.01	0.71	0.28	
120	10	0.001	0.38	0.15	1.63 ± 0.07^{g}
	10	0.00	0.22	0.09	
	20	0.03	1.56	0.63	2.10 ± 0.25^{fg}
	20	0.03	1.46	0.58	
	40	0.03	1.71	0.69	2.90 ± 0.16^{efg}
	40	0.04	1.84	0.74	
	60	0.05	2.32	0.93	5.18 ± 0.93^{efg}
	60	0.05	2.30	0.92	
140	10	0.08	3.95	1.58	3.13 ± 0.18^{def}
	10	0.08	4.21	1.69	
	20	0.11	5.68	2.28	5.99 ± 0.13^{de}
	20	0.10	4.81	1.93	
	40	0.15	7.51	3.01	7.10 ± 0.68^{d}
	40	0.14	6.96	2.79	
	60	0.14	14.58	5.84	8.46 ± 0.25^{c}
	60	0.11	11.29	4.52	
160	10	0.10	18.66	7.47	7.70 ± 0.32^{b}
	10	0.10	19.79	7.92	
	20	0.09	17.44	6.99	7.20 ± 0.31^{b}
	20	0.09	18.53	7.42	
	40	0.09	18.86	7.55	7.74 ± 0.27b
	40	0.10	19.81	7.94	
	60	0.09	18.76	7.51	7.71 ± 0.28^{b}
	60	0.10	19.75	7.91	
180	10	0.08	16.65	6.67	6.69 ± 0.03^{b}
	10	0.08	16.75	6.71	
	20	0.10	19.32	7.74	8.03 ± 0.41^{b}

Table C2. Continue.

	20	0.10	20.77	8.32	
	40	0.12	24.55	9.83	10.40 ± 0.80^{a}
	40	0.14	27.38	10.97	
	60	0.14	27.54	11.03	11.64 ± 0.86^{a}
	60	0.15	30.57	12.24	

AAs: Amino acids; DH: Degree of hydrolysis. [a-g]Numbers in the same column that do not share a letter are significantly different ($p < 0.05$).

Table C.3. Amino acids profile of chicken feet hydrolysates obtained by pressurized hot water hydrolysis at 40-180°C, 50 bar, 10-60 min, and a sample-water ratio of 1:50 w/v.

Time (min)	Temperature (°C)	Amino acid content (µg/mL)								
		Aspartic acid	Glutamic acid	Asparagine	Serine	Glutamine	Glycine	Threonine	Citrulline	Arginine
10	**40**	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	80	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	120	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	140	0.4 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.1	1.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
	160	4.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	1.4 ± 0.0	0.2 ± 0.0	3.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0. 4 ± 0.0
	180	0.0 ± 0.0	1.0 ± 0.0	1.2 ± 0.0	3.2 ± 0.1	0.4 ± 0.0	9.4 ± 0.2	0.0 ± 0.0	2.0 ± 0.0	0.8 ± 0.0
20	**40**	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	80	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	120	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.1	2.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
	140	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	2.4 ± 0.1	4.8±0.3	0.0 ± 0.0	0.2 ± 0.0
	160	0.8 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	0.0 ± 0.0	2.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0
	180	9.2 ± 0.1	1.8 ± 0.0	1.8 ± 0.0	4.6 ± 0.0	0.6 ± 0.0	16.4 ± 0.1	0.0 ± 0.0	2.0 ± 0.0	1.6 ± 0.0
40	**40**	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	80	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	120	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	2.6 ± 0.1	0.0 ± 0.0	5.4 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
	140	0.4 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	4.0 ± 0.0	0.0 ± 0.0	9.4 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.4 ± 0.0
	160	2.4 ± 0.0	0.6 ± 0.0	1.0 ± 0.0	1.6 ± 0.0	0.2 ± 0.0	5.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0
	180	15.8 ± 0.1	3.8 ± 0.0	3.2 ± 0.0	7.8 ± 0.0	1.2 ± 0.0	32.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.0	3.2 ± 0.0
60	**40**	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	80	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.1	0.0 ± 0.0	1.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	120	0.4 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	3.8 ± 0.0	0.0 ± 0.0	8.6 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.4 ± 0.0
	140	0.6 ± 0.0	0.6 ± 0.0	1.2 ± 0.1	5.4 ± 0.0	0.0 ± 0.0	13.0 ± 0.1	0.4 ± 0.1	0.0 ± 0.0	0.6 ± 0.0
	160	4.0 ± 0.0	1.0 ± 0.0	1.4 ± 0.0	2.8 ± 0.0	0.4 ± 0.0	9.2 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	0.8 ± 0.0
	180	17.6 ± 0.1	9.2 ± 0.5	3.8 ± 0.1	9.6 ± 0.2	0.6 ± 0.1	46.0 ± 0.2	0.0 ± 0.0	2.0 ± 0.0	5.6 ± 0.1

Table C.3. Continue.

Time (min)	Temperature (°C)	Amino acid content (µg/mL)								
		Alanine	Tyrosine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Total
10	**40**	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	5.0 ± 0.0	6.4 ± 0.0
	80	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	4.4 ± 0.0	5.4 ± 0.0
	120	0.2 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	5.4 ± 0.1	8.0 ± 0.0
	140	0.4 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	2.4 ± 0.2	3.4 ± 0.0	9.0 ± 0.3	21.0 ± 0.1
	160	0.4 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	4.8 ± 0.0	4.8 ± 0.0	13.8 ± 0.0	34.8 ± 0.0
	180	1.2 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.8 ± 0.0	0.4 ± 0.0	4.2 ± 0.1	5.2 ± 0.1	19.6 ± 0.2	50.0 ± 0.3
20	**40**	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	5.4 ± 0.1	6.6 ± 0.0
	80	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	5.2 ± 0.1	6.4 ± 0.0
	120	0.4 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	1.6 ± 0.1	1.2 ± 0.1	8.4 ± 0.3	17.4 ± 0.3
	140	0.4 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.0	2.6 ± 0.2	4.2 ± 0.0	11.2 ± 0.6	29.6 ± 0.3
	160	0.4 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	3.8 ± 0.0	4.4 ± 0.0	14.8 ± 0.0	29.0 ± 0.0
	180	2.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	3.6 ± 0.0	3.0 ± 0.0	32.8 ± 0.0	82.2 ± 0.0
40	**40**	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	5.2 ± 0.1	6.4 ± 0.0
	80	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	5.2 ± 0.1	6.4 ± 0.0
	120	0.6 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	1.6 ± 0.1	1.4 ± 0.1	10.6 ± 0.5	23.8 ± 0.3
	140	1.0 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	2.6 ± 0.2	2.8 ± 0.2	15.6 ± 0.8	38.6 ± 0.7
	160	0.8 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	1.2 ± 0.0	0.4 ± 0.0	3.6 ± 0.0	4.6 ± 0.1	14.8 ± 0.1	37.4 ± 0.3
	180	4.6 ± 0.0	1.4 ± 0.0	0.8 ± 0.0	1.4 ± 0.0	1.8 ± 0.0	2.4 ± 0.1	3.2 ± 0.1	27.2 ± 0.8	112.0 ± 0.5
60	**40**	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	4.0 ± 0.1	4.8 ± 0.3
	80	0.2 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2± 0.0	0.4 ± 0.0	4.8 ± 0.1	7.8 ± 0.2
	120	1.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	3.2 ±0.2	4.2 ± 0.0	13.4 ± 0.7	36.6 ± 1.1
	140	1.2 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	2.8 ± 0.2	6.0 ± 0.0	11.6 ± 0.6	44.2 ± 0.9
	160	1.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	1.6 ± 0.0	0.6 ± 0.0	4.2 ± 0.0	5.2 ± 0.0	20.2 ± 0.1	55.4 ± 0.3
	180	7.4 ± 0.0	3.0 ± 0.0	1.2 ± 0.0	2.0 ± 0.0	2.6 ± 0.0	4.0 ± 0.1	2.0 ± 0.0	42.6 ± 0.4	159.2 ± 0.5

Table C.4. Molecular weight distribution of chicken feet hydrolysates obtained by pressurized hot water at different temperatures (50 bar, 5 mL/min, 10 min) analyzed by SEC-GPC.

Temperature (°C)	Peak No.	Retention time (min)	Molecular weight (kDa)	Height	Area (%)
40	1	5.10	843.33	18600	75.87
	2	7.52	82.94	418	0.58
	3	7.91	57.07	202	0.35
	4	8.35	37.44	1493	2.54
	5	8.71	26.51	1487	3.45
	6	9.23	16.11	3475	10.93
	7	9.41	13.56	2743	6.28
80	1	5.17	788.61	494	25.14
	2	5.57	537.50	186	5.69
	3	7.94	55.46	271	7.70
	4	8.79	24.56	749	31.09
	5	9.08	18.60	525	14.81
	6	9.33	14.64	510	15.58
120	1	4.81	1113.52	500389	97.66
	2	6.55	210.13	4646	0.26
	3	6.99	137.83	4857	0.26
	4	7.59	77.56	1737	0.09
	5	7.87	59.30	0	0.03
	6	8.34	37.60	4998	0.26
	7	8.66	27.81	15102	1.31
	8	9.41	13.56	1768	0.12
140	1	4.83	1092.38	1011810	95.25
	2	7.00	136.52	18793	0.64
	3	7.63	74.64	16175	0.75
	4	8.34	37.80	17914	0.70
	5	8.65	28.08	36975	2.66
160	1	7.02	133.93	117051	10.84
	2	7.31	101.43	149176	12.24
	3	7.65	73.22	184871	27.78
	4	8.33	38.16	187001	10.11
	5	8.60	29.46	203906	39.04
180	1	4.90	1021.50	19134	1.15
	2	9.55	11.85	254837	98.85