UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

DÉVELOPPEMENT ET SYNTHÈSE D'UN PROCÉDÉ D'EXTRACTION DE COLLAGÈNE À METTRE EN OEUVRE SUR LA BASSE CÔTE NORD
(DEVELOPMENT AND SYNTHESIS OF COLLAGEN EXTRACTION PROCESS TO IMPLEMENT ON THE LOWER NORTH SHORE)

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Avant-propos

The following project focused on developing a collagen extraction process to transfer to the Lower North Shore. Working with a private enterprise from the area Shore Grow Inc., the project aims to develop a new industry to help diversify and support the rejuvenation of the economy of the Lower North Shore. The existing fishery has been erratic in terms of catch volume and price in recent years with different resources. The prospect of the information collected in this project can have an impact on future employment and capacity building of the local population. Given an established fishing industry, an approach was taken to synergistically develop higher-valued products with the pre-existing industry in the area. *Cucumaria frondosa*, a sea cucumber species present on the Lower North Shore, has the potential to be harvested commercially and collagen is a potential high-value biomolecule with a market demand that can overcome the area's logistical and implementation costs. Marine collagen has interesting bioactive and structural properties, interesting for cosmetic, nutraceutical, and biomedical industries. With the interest of various residents, and particularly Shore Grow, the project began first exploring different processes to see which worked best for the biomass and finally optimizing and scaling the process to the needs of the area. With the biomass valorization expertise of Université du Québec à Trois-Rivières and Centre de recherche sur les biotechnologies marines, a pre-commercial product was developed. The results in the following dissertation indicate that a collagen extraction process is technically feasible to implement in the isolated communities on the Lower North Shore. The processes developed had to minimize environmental impacts, keeping the land and waters pristine within the northern territory. The project has presented the unique opportunity of integrating engineering and scientific solutions to work on socio-economic impacts in a region with many opportunities.

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I would like to thank Kimberly Buffitt, the president of Shore Grow for making this project come to realization. Being a leader in bioresource development in the area, many of these projects begin from Kimberly's vision of developing the area in hopes of promoting jobs and rejuvenating the population and the economy. Kim has helped shape the way I see our resources along the Lower North Shore and provided the support needed to move the project forward.

I would also like to thank the Coasters Association for the support in helping to get the project mobilized and support bioeconomy projects such as this one. I would like to thank GIDC and Groupe Deric, my employer who has given me the adequate time and resources to execute the project while still working with the company. I would like to thank Pêcheries Shipek and Guy Vigneault for providing the sea cucumbers for the project and also Francois Otis and Normand Voyer of the Université Laval for executing our circular dichroism experiments and supporting the data analysis of the project. Finally, I would like to thank the Chaire de recherche municipale pour les villes durables, UQTR -Victoriaville and CRIBIQ (consortium de recherche et d'innovation en bioprocédés industriels au Québec) for making this master's research project possible with financial assistance.

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Résumé

Version française – substantiel (puisque ce mémoire est en anglais)

Les régions éloignées disposent d'un avantage unique par rapport aux zones métropolitaines, à savoir l'accès à des ressources vierges inexplorées et à des terres non occupées. Dans le cas de la Basse-Côte-Nord, le littoral regorge de nombreuses ressources terrestres et marines. La région cherche à développer de nouvelles industries pour contrer le déclin démographique et les taux de chômage élevés.

Une ressource marine intéressante est *Cucumaria frondosa* (*C. frondosa*), une espèce de concombre de mer d'eau froide présente dans les eaux marines du Québec et de l'Est du Canada. Cette espèce est actuellement exploitée pour les marchés alimentaires et traditionnels, mais elle contient également de nombreuses molécules bioactives qui peuvent être utilisées dans des secteurs tels que les cosmétiques, les biomédicaux et les industries pharmaceutiques. L'une de ces molécules est le collagène, une protéine structurelle de la matrice extracellulaire. Chez les concombres de mer, le collagène est principalement localisé dans la paroi corporelle. Selon sa structure, qu'elle soit native ou hydrolysée, le collagène possède de nombreuses propriétés bioactives, structurales et de signalisation.

Avant de présenter les résultats, une section couvrira une partie de la méthodologie utilisée pour mener les expériences. La première étape consistait à déterminer les propriétés de *C. frondosa*, telles que la teneur en humidité, en protéines et en collagène, pour évaluer les rendements et les performances tout au long du projet. Ensuite, différentes méthodes d'extraction ont été testées dans leur intégralité afin d'identifier celle qui fonctionnait le mieux, étant donné qu'il existait diverses approches disponibles dans la littérature pour extraire le collagène à partir de ressources marines. Une fois une méthode sélectionnée, son optimisation a été réalisée pour maximiser les rendements en tenant compte des contraintes chimiques, financières et en ressources auxquelles fait face la Basse-Côte-Nord.

Enfin, puisque l'objectif du projet était de développer une méthode applicable sur la Basse-Côte-Nord, un essai pilote à plus grande échelle (20 fois) a été réalisé. Cette étape était cruciale, car il est rare qu'un processus passe directement du laboratoire à une échelle

pilote sans ajustement. Cela permettrait à l'utilisateur final d'avoir une idée des besoins pour mettre en œuvre le processus à grande échelle dans la région. Par ailleurs, divers tests de caractérisation structurale, incluant la spectroscopie FTIR et le dichroïsme circulaire, des tests de pureté comme l'ICP-MS et UV-vis, ainsi que des tests d'identité tels que le SDS-PAGE et le Native PAGE, ont été réalisés pour analyser le produit final après chaque itération de la méthode.

Lorsqu'une étape spécifique du processus d'extraction était étudiée, divers paramètres tels que le pH, la température, le type de solvant et la durée étaient modifiés pour l'optimiser cette étape. Cependant, dans la plupart des cas, le processus complet était exécuté du début à la fin à chaque fois, afin de valider une ou deux hypothèses majeures lors d'un essai donné. Cette approche permettait de caractériser le produit final, car c'est ce que le consommateur utiliserait dans ses produits. Réduire le processus pour se concentrer uniquement sur une étape spécifique aurait entraîné une perte de produit final potentiel pouvant être envoyé à un client. De nombreuses optimisations ont été étudiées et mises en œuvre en fonction des préférences des clients pour les produits issus de chaque itération. De plus, nous disposions d'une quantité limitée de *C. frondosa*. Par conséquent, il a été décidé de produire autant de produit final que possible.

Enfin, il a été décidé de limiter le nombre de répétitions pour répondre à davantage de questions liées au processus global de production de collagène à mettre en œuvre sur la Basse-Côte-Nord. Les contraintes de temps étaient significatives, avec moins de six mois pour effectuer tous les travaux en laboratoire et passer d'un processus à l'échelle laboratoire à un essai pilote. De plus, le projet n'avait accès qu'à une quantité limitée de concombres de mer. Les ajustements et répétitions pourraient être réalisés sur place une fois que le processus serait livré à l'industriel qui planifie de l'exploiter sur la Basse-Côte-Nord. Ainsi, une approche systématique a été adoptée pour obtenir des réponses concrètes sur chaque étape – prétraitement, extraction et post-traitement – tout en gardant l'objectif principal en tête, parvenir à un processus simple pouvant être transféré aux communautés de la Basse-Côte-Nord.

Ce projet s'est concentré sur le développement d'un protocole d'extraction de collagène transférable à une industrialisation sur la Basse-Côte-Nord. Tout en maximisant le rendement, il était important que le protocole produise des concentrations significatives de collagène sous forme native, tout en maximisant la pureté et en minimisant le temps de traitement. Cette étude a montré que *C. frondosa* présentait des teneurs en protéines et en humidité similaires à celles d'autres espèces de concombre de mer, bien que la teneur en collagène soit légèrement inférieure à la moyenne. Les muscles des concombres analysés avait une teneur en humidité de 84 ± 3% une teneur en protéines de 590 ± 40 mg/g de muscle sec, et 1,6 ± 0.2 mg de collagène/g de muscle humide. Divers protocoles ont été testés et il a été déterminé que pour *C. frondosa*, le collagène solubilisé par pepsine était le plus efficace.

Lors des expérimentations d'optimisation, les résultats ont indiqué que les étapes de prétraitement de désagrégation et de déprotéinisation étaient essentielles pour rendre le muscle tégumentaire du concombre de mer dans un état permettant l'accès et l'extraction des fibres de collagène. Lorsqu'une tentative a été faite pour éliminer complètement la déprotéinisation, le rendement a diminué. Cela pourrait être dû à la présence potentielle d'autres protéines restantes dans l'extrait qui aurait pu affecter l'efficacité de la pepsine lors de l'étape d'extraction puisque la pepsine n'est pas spécifique aux protéines collagéniques mais agit sur des motifs de sites actifs de toutes les protéines.

Pour l'étape désagrégation, les facteurs examinés étaient les produits chimiques, le pH, le temps d'extraction et les ratios de solvants à 4 °C. Les conditions optimales consistaient à effectuer l'extraction pendant 48 heures, à un pH compris entre 5,5 et 8,0 avec un rapport solvant/substrat de 10 :1. En particulier, lorsque le rapport solvant/liquide lors de l'étape de désagrégation a été réduit à 4 :1, le rendement global du produit a diminué par un facteur de 5. Bien que tous aient eu un effet, l'utilisation de 2-mercaptoéthanol a permis d'augmenter le rendement par un facteur de trois par rapport à la même solution sans ce produit, probablement en raison de son impact sur les ponts disulfure de la matrice extracellulaire entre le collagène et d'autres molécules. Afin d'éviter l'utilisation de ce produit chimique toxique dans un cadre commercial dans une région éloignée, le processus d'extraction a ensuite été testé à température ambiante, avec des températures d'extraction

entre 20 °C et 40 °C, dans le but de perturber certains de ces liens entre le collagène et les molécules voisines. Bien que cela ait réduit le temps de traitement d'au moins trois fois, la formation de formes de collagène de poids moléculaire inférieur a commencé à apparaître. Bien qu'une dégradation ait été observée avec diverses combinaisons de température d'extraction et de concentration en pepsine, l'effet de dégradation était plus prononcé lorsque la température d'extraction était de 40 °C avec 0,1 g/L de pepsine, ou lorsque la température d'extraction était de 20 °C avec une concentration en pepsine de 0,7 g/L. Dans tous les cas, des portions de la structure native subsistaient encore. Sur la base de ces résultats, le point optimal a été déterminé pour une extraction de 90 minutes à 30 °C avec 0,1 g/L de pepsine. Le rendement en collagène produit était de 46 ± 3 mg de collagène par gramme de concombre de mer sec.

Ensuite, une première étape de mise à l'échelle du processus a été réalisée, passant de 200 g à 4000 g de biomasse transformée. Cependant, lors de l'extraction à l'échelle pilote, le concombre de mer entier a été utilisé dans le but de minimiser les pertes et le temps de traitement dans un cadre commercial. Le premier test à l'échelle pilote n'a pas permis d'obtenir les mêmes performances que le processus optimal à l'échelle de laboratoire. Entre les essais à l'échelle de laboratoire et ceux à l'échelle pilote, la teneur en protéines dans le produit final en poudre a diminué de 156 ± 2 mg de protéines par gramme de concombre de mer sec à 44 ± 1 mg de protéines par gramme de concombre de mer sec. Outre l'utilisation d'un équipement plus proche de celui utilisé en industrie, il est possible que les enzymes présentes dans les viscères aient dégradé les protéines et, en fin de compte, le collagène, réduisant ainsi le rendement. Des techniques de post-traitement ont été étudiées et optimisées, et la précipitation isoélectrique a été privilégiée au précipitation par salage, car la quantité de protéines récoltées était similaire, mais le temps de traitement était réduit. Pour la purification finale, l'ultrafiltration a permis d'éliminer plus de 97.66 ± 0.04 % de la concentration initiale de sodium dans 230 mL d'un échantillon brut de collagène en 9 heures, tandis que la dialyse a permis d'éliminer 98.56 ± 0.06 % du sodium d'un échantillon brut de collagène de 40 mL en 72 heures. Par conséquent, l'ultrafiltration a été choisie comme méthode de purification.

Les caractéristiques des produits à l'échelle de laboratoire et à l'échelle pilote étaient similaires et comparables à celles de la littérature, présentant des hélices α entre 120 - 130 kDa sur SDS-PAGE confirmant qu'il s'agit de collagène de type I, et un poids moléculaire natif d'environ 380 - 400 kDa confirmé par PAGE natif. La naturalité du produit à l'échelle de laboratoire a également été confirmée par dichroïsme circulaire, avec un maximum autour de 220 nm, indiquant des formes de collagène triple hélice intactes. L'analyse FTIR a montré les pics de collagène attendus, y compris les bandes amides A, B, I, II et III, tandis que les courbes UV-Vis avaient des pics maximums à 230 nm (représentant les groupes carbonyle et amide des liaisons peptidiques) avec des pics secondaires à 280 nm (représentant des signaux pour la tyrosine, la phénylalanine et le tryptophane), caractéristiques du collagène marin. Le pic des fonctions amide I observable sur le spectre FTIR se situait autour de 1645 cm^{-1}, ce qui est typique des hélices alpha intactes, et le rapport entre les pics des fonctions amide III et et le pic à la longueur d'onde 1450 cm^{-1} était d'environ 1 (entre 1,1 et 1,2), démontrant qu'une partie de l'extrait avait conservé sa structure secondaire. Chaque produit en poudre contenait également de faibles quantités de métaux lourds, répondant aux normes de divers organismes de réglementation internationaux. Le travail ci-dessus a permis de développer un produit et un processus d'extraction pouvant être mis en œuvre industriellement sur la Basse-Côte-Nord au Québec et techniquement faisable. Des travaux supplémentaires devraient être réalisés sur les paramètres technico-économiques afin de permettre une décision finale d'implantation dans ces communautés.

En résumé, un processus d'extraction de collagène a été développé pour une mise en œuvre industrielle sur la Basse-Côte-Nord. Grâce aux optimisations et aux apprentissages liés à chaque étape de l'extraction du collagène, un processus a été conçu et testé à l'échelle pilote, pouvant être transféré à la population de la Basse-Côte-Nord pour faire avancer le projet et poursuivre les étapes vers la commercialisation. Bien qu'il reste des itérations et des tests à effectuer, un processus et un produit viables ont été livrés dans le cadre de ce mémoire de projet.

English Version

Remote areas have a unique advantage over metropolis areas in the sense of having access to pristine untapped resources and unoccupied land. In the case of the Lower North Shore, the shoreline has many lands and sea resources. The area is looking for new industries to combat its population decline and unemployment rates.

An interesting marine-based resource is *Cucumaria frondosa* (*C. frondosa*), a cold-water sea cucumber species located in Quebec and Atlantic Canada. The species is currently being exploited for food and traditional markets but contains many bioactive molecules that can be used in markets such as cosmetics, biomedical, and pharmaceutical industries. One molecule is collagen, a structural extracellular matrix protein. In sea cucumbers, collagen is predominantly located in the body wall. Depending on its structure, *i.e*. native or hydrolyzed, it has a plethora of properties in terms of bioactivity, structure, and signaling.

This project focused on the development of a collagen extraction protocol that was transferable for industrialization in the Lower North Shore. While maximizing yield, it was important the protocol delivered significant concentrations of the native form of collagen while maximizing purity and minimizing process time. This study showed that *C. frondosa* had similar protein and moisture contents compared to other sea cucumber species, while collagen content was slightly below average compared to other species. Various protocols were tested, and it was determined that for *C. frondosa*, the pepsin-soluble collagen method was the most effective.

When the optimization experiments began, it was discovered that the initial steps of disaggregation and deproteination were crucial for exposing the collagen fibers in the sea cucumber's body wall, making it possible for extraction. Parameters such as chemicals, pH, extraction time, and solvent ratios were investigated for the disaggregation step at 4°C. While all had an effect, 2-mercaptoethanol increases the yield by 3-fold compared to the same solution without it, likely due to the impact on disulfide bonds in the extracellular matrix between collagen and other molecules. To pivot from using this toxic chemical in a commercial setting in a remote area, the extraction process was then tested at room temperature, and the actual extractions step between temperatures 20 ℃ to 40 ℃, with the

purpose of disrupting some of these bonds between collagen neighboring molecules. While this reduced the processing time by at least 3-fold, lower molecular weight forms of collagen began to develop. While degradation was observed in various extraction temperature-pepsin concentration combinations, the degradation effect was more pronounced when an extraction temperature of 40 °C and 0.1 g/L pepsin, or an extraction temperature of 20 °C and pepsin concentrations of 0.7 g/L were used. In all cases, portions of the native structure still existed. Based on the results obtained in this project, the optimal set point was deemed to do the extraction for 90 minutes at 30 ℃ using 0.1 g/L of pepsin. The collagen yield produced was 46 ± 3 mg collagen per gram of dry sea cucumber.

Next, the process was scaled up from 200 g to 4000 g. However, in the pilot scale extract, the complete sea cucumber was used to minimize wastage and process time in a commercial setting. The first scale-up test did not perform the same as the optimal lab scale run. In addition to the different equipment that were more like what would be seen in industry, the extraction performance may have been impacted by other components from the viscera such as other enzymes. Post-treatment techniques were studied and optimized, as isoelectric-like precipitation was favored over salting-out given the amount of protein harvested is similar but the processing time was less. For final purification, ultrafiltration removed more than 97.66 ± 0.04 % of the initial sodium concentration in 230 mL of a crude collagen sample within 9 hours while dialysis removed 98.56 ± 0.06% of sodium from 40 mL of a crude collagen sample in 72 hours. Therefore, ultrafiltration was selected as the purification method.

The characteristics of both lab scale and pilot scale were like each other and comparable to the literature, having α helices between 120-130 kDa on SDS PAGE confirm it to be type I collagen, and having a native molecular weight of around 380-400 kDa confirmed by Native PAGE. The nativity in the lab scale product was also confirmed by circular dichroism, having a maximum peak around 220 nm, indicating forms of intact triple helical collagen. FTIR analysis showed the given expected collagen peaks including amide A, B, I, II and III, while the UV-vis curves had maximum peaks at 230 nm (representing carbonyl and amide groups of peptide bonds) with smaller peaks at 280 nm (representing a signal for aromatic amino acids tyrosine, phenylalanine and tryptophan), common in

marine collagen. Each powdered product also had low amounts of heavy metals, complying with various regulatory bodies internationally. The work above developed a product and extraction process that can be industrially implemented on the Lower North Shore of Quebec and be technically feasible. Further work should be done on the technical-economical parameters to allow the final decision of implementation in these communities.

Mots Clés

Marine collagen, process development, sea cucumber, *Cucumaria frondosa*, pepsin soluble collagen, pilot scale-up, northern region development, bioextraction

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2ME	2-mercaptoethanol
AOAC	Association of Official Agricultural Chemist
ASC	Acid soluble collagen
ATR	Attenuated total reflectance
BCA	Bicinchoninic acid
CAGR	Compound annual growth rate
CD	Circular dichroism
CRBM	Centre de recherche sur les biotechnologies marines
CSA	Canadian Standards Association
DFO	Department of Fisheries and Oceans Canada
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
ESM	Electron scanning microscopy
FTIR	Fourier Transform Infrared
FDA	Food Drug Administration Agency
GAE	Gallic acid equivalents
ICP	Inductively coupled plasma
L-CPL	Left-handed circularly polarized light
LPL	Linearly polarized light
LNS	Lower North Shore
MCT	Mutable collagenous tissue
MELCCFP	Ministère de l'Environnement, de la Lutte contre les changements climatiques, de la Faune et des Parcs
MMP	Matrix metalloproteinase
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
NPL	Naturally polarized light
PAGE	Polyacrylamide gel electrophoresis
PEM	Photoelastic modulator
pI	Isoelectric point
PSC	Pepsin soluble collagen
R-CPL	Right-handed circularly polarized light
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SSC	Salt soluble collagen
Θ	Ellipticity
T_d	Thermal denaturation temperature
Tris	Tris(hydroxymethyl)aminomethane
UAE	Ultrasound assisted extraction
UQTR	Université du Québec à Trois-Rivières
USD	United States of America dollar
UV	Ultraviolet
UV-vis	Ultraviolet visible
XRD	X-ray diffraction

Chapitre 1 - Introduction

1.1 Creating sustainable economies in remote northern communities

Many northern communities remain untouched and are plentiful in natural resources, making them interesting candidates for bio-economic activities. A bioeconomy uses biotechnology to produce goods, services, or energy from natural or biological resources sustainably to create economic activity. Canada's bioeconomy focuses on creating government policy and regulation, biomass supply and stewardship, strong supply chains and company creation, and the creation of strong sustainable eco-systems (Bioindustrial Innovation Canada, 2019).

Even with the region's abundant resources and land, northern communities are faced with challenges getting into the bioeconomy. The isolation of the communities is an issue. There are limited, costly transportation options, which would affect the import of raw materials and the export of finished products. This puts local processing and transformation activities at a disadvantage compared to urban-situated ones. Also, being far away from the markets in which the products are used is a challenge, as it is harder to understand and communicate with consumers and other suppliers who are in the same industry. The current industries in such remote areas are predominantly traditional fishing, harvesting, and agriculture industries which perform minimum project transformations, and a lot of profit is being taken away from the regional economy because of this. The locals are more focused on maintaining these practices as opposed to trying new industries. Ultimately, local areas suffer when foreign firms establish temporary operations, such as factories or consultancy projects. Knowledge transfer to the community and agreed-upon regulations during these activities remain elusive, leading to potential long-term consequences (CSA Group, 2017).

For these northern communities to move forward with their natural resources and be a part of Canada's growing bioeconomy, they need to understand their natural resources, their characteristics, and how they can be transformed to benefit the economy. Sustainable industrial transformation needs to begin with harvesting and agriculture of the resource then to the manufacturing facility of the final product and final packaging to end consumer/ supply chain. An important part

of the bioeconomy is using renewable resources to have a secure resource for future production years (Diaz-Chavez et al., 2019). Finally, for these industries to thrive in such areas, the community needs to be a part of every aspect of the development of the resource which will require practices that may not be known to urban centers to be a success (Davidson-Hunt & Turner, 2012).

These steps need to be considered when trying to create a local bioeconomy on the Lower North Shore. An interesting species that has been identified is *Cucumaria frondosa*. The sea cucumber fishery is a good fishery from a commodity prospect given its economic value and increasing demand in international markets (Asriani & Tahang, 2020). Canada has an underfished market for sea cucumber which creates a great window of opportunity to create a viable industry (Purcell et al., 2013). Also, Canada predominantly produces dried sea cucumbers for the traditional market therefore this gives an interesting production opportunity to create high value transformed products in its remote regions. In combination with the best harvesting practices to protect the local environment, the Lower North Shore of Quebec has a chance to create an innovative bioeconomy with respect to the rest of the nation.

1.2 Sea cucumber industry and it's potential on the Lower North Shore

Sea cucumbers are a popular marine species in the fishery and aquaculture market and is growing each year, especially in Asia. Sea cucumbers have been harvested for at least 400 years (Purcell et al., 2013). As of 2015, the annual volume of sea cucumbers that undergo a transformation in a processing facility was up to 411 878 metric tonnes globally (Rahman et al., 2015). Around the year 2000, aquaculture surpassed wild capture of sea cucumber to supply the market demands. Currently the global sea cucumber market is valued at 4.31 billion dollars annually (Business Research Insights, 2024). In Canada, as of 2017, the sea cucumber landings were 9 922 metric tons with a total value of 18.3 million dollars (Hossain et al., 2020).

Sea cucumbers are utilized and consumed in a variety of markets such as pharmaceuticals, cosmetics, nutraceuticals, traditional medicine, and food. In Asian markets, sea cucumbers are a popular food source. In Canada, 90% of sea cucumber trade takes place in China where the product is

sold as a dried product for the traditional food industry (Hossain et al., 2020). Recently, sea cucumbers have made a splash in nutritional, personal care, and biomedical industries given their wide range of bioactive compounds (Rahman & Yusoff, 2017). These active compounds are being extracted to be used/studied in anti-cancer, anti-thrombotic, anti-microbial, anti-inflammatory, and wound healing products in the pharmaceutical world (Rahman & Yusoff, 2017). In the fields of nutrition and personal care, the active compounds can be used to create products like liquid extracts for cosmetics, powder capsules as nutraceuticals, collagen extracts for skincare, natural preservatives for the food industry, and photoprotective compounds can be used in personal care formulations to shield against UV radiation (Hossain et al., 2020; Siahaan et al., 2017). Liquid extracts are commonly collected and sold as active ingredients to be inputted into cosmetic products. These include phenolic acids, polysaccharides and phospholipids. Collagen is being used for products such as creams, lotions, lipsticks and gels. Currently, the market is trying to move away from the common bovine and porcine sources of collagen, given the potential of animal transmitted diseases and ethical issues. This is why marine sources have become prime candidates to potentially replace current sources of collagen.

While food and traditional markets are preferred commercialization pathways for sea cucumber products in Canada, they have lower economic profit margins compared cosmetic or pharmaceutical markets and require higher production/ harvesting volumes to create a sustainable business. The more transformations that an individual or a business can do in their respective industry or region, the more economic return they will receive on their product. To fully tap into the economic potential of sea cucumbers and their valuable bioactive components, Hossain et al. (2020) advocate for their utilization in the previously mentioned market sectors.

1.3 Collagen applications and market

The overall collagen market was valued at 9.75 billion in 2023 and is expected to grow at an annual rate of 9.6% (Grand View Research, 2024). Currently, 11% of this market is from marine sources. In 2023, the marine collagen market was valued at 1.08 billion USD and by 2032, is forecasted to grow to 2.32 billion USD, expecting compound annual growth rate (CAGR) increase of 8.94% (Fortune Business Insights, 2024). The majority of collagen is produced through acid hydrolysis

or pepsin acid hydrolysis processes. For the United States of America (USA), a particular increase is expected due to the demand for a healthier and a more environmentally sustainable resource, which marine sources can provide. In Asia, it is projected to see an expansion in its supply market share, with numerous manufacturers seeking to allocate resources towards the production of marine collagen in response to anticipated growth in demand (Fortune Business Insights, 2024). As a North American producer, it is crucial to bear in mind that production costs are generally higher here, partly due to more expensive labor. Strategies and marketing packages will need to be developed to remain competitive. European countries are an important target market for suppliers given they are expected to continue to be a large importer of collagen (Fortune Business Insights, 2024). South American, Middle Eastern, and African markets are becoming more popular and have evolved over recent years and should be monitored for potential importers in the coming years. Currently, large competitors include Ashland Inc (USA), COBIOSA (Spain), ETChem (China), and Vital Proteins LLC (US) (Fortune Business Insights, 2024). Another aspect of why collagen is an attractive product is because it can be used in a variety of applications including nutraceuticals, cosmeceuticals, pharmaceuticals and biomedical applications.

Within the collagen market, there are natured forms of collagen known as native collagen and denatured forms of collagen which are gelatin or hydrolyzed collagen. A demonstration of their structural difference is depicted in the figure below.

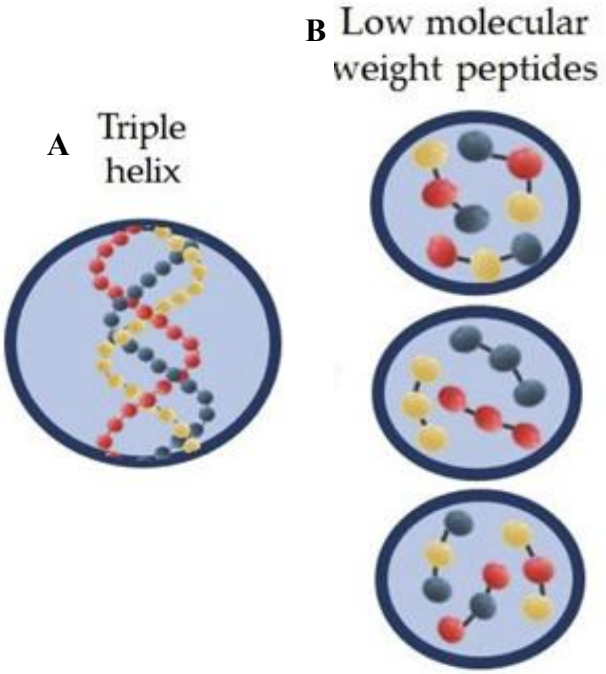

Figure 1.1. Structural comparison of (a) native collagen and (b) hydrolyzed collagen (Lopez et al., 2019).

Figure 1.1 illustrates that native collagen adopts a triple-helical arrangement of three alpha helices. Hydrolyzed collagen, meanwhile, results from collagen that has been subjected to denaturation and hydrolysis, breaking it down into smaller peptides. The processes of denaturation and hydrolysis will be explored further in Chapter 2. A summary of the properties of each form of collagen is summarized below:

Table 1.1. Comparison of characteristics of native collagen and hydrolyzed collagen (de Almagro, 2020; Lopez et al., 2019).

Property	**Native collagen**	**Hydrolyzed collagen**
Molecular weight	300 – 400 kDa	3 – 6 kDa
Structure	Triple helix intact	Denatured and degraded poly-peptide chains.
Solubility in water	Poor	Good
Absorption by human body	Poor	Good
Viscosity in solution	High	Low
Film forming properties	Good	Poor, needs support from other biomaterials

As described in table 1.1, native collagen remains in its triple-helical form, remaining viscous in solution. The native form has great biocompatibility and biodegradability, low immunogenicity, and excellent structural and scaffolding properties (de Almagro, 2020; Lopez et al., 2019). However, it is poorly soluble in water and has poor absorption properties. Typically, the extraction process of native collagen is complex and lengthy. Hydrolyzed collagen presents low viscosity solutions, excellent solubility in water, easily absorbed into the body and has much simpler extraction methods to generate this form (de Almagro, 2020; Lopez et al., 2019). Since the triple helical structure and intermolecular bonds are broken, it has poor structural properties. Due to the intricate nature of extracting native collagen, which necessitates stringent quality assurance measures, its industrial manufacturing and use fall behind those of hydrolyzed collagen. This has made the markets for native collagen more niche, therefore it has a higher economic value.

Given the high value and favorable market trends, this makes native collagen an interesting molecule to explore for commercialization on the Lower North Shore, due to the high transport cost in the area, bioresource extracts need to have moderate to high sale values to offset this elevated cost. If the product is simply used for a lower grade market with low profit margins such as agricultural ingredients, the return on investment will not be enough to create a viable business. This is why

targeting industries like cosmetics, nutraceuticals, pharmaceuticals and biomedicine will produce the highest value. Also, there is no supply market established in the area; this presents a business opportunity to be studied.

There are several bioactive molecules including native type I collagen in the *C. frondosa* species that can be exploited for commercial applications including biomedical, cosmetic, nutraceutical, food and pharmaceutical domains (Senadheera et al., 2020). Some examples include the use in cosmetics for moisturizing, antioxidant and antiaging properties related to skin in lotions and creams (Senadheera et al., 2020). As for cosmetic injections, type I collagen has been used as botulinum type A and soft tissue filler (Naomi et al., 2019). Products such as Zyderm and Zyplast which contain porcine collagen are some of the most common dermal fillers in the USA to date (Naomi et al., 2019). In terms of nutraceutical and food industry potential, marine collagen is considered a healthy food supplement and antiaging agent. Collagen is also being used in spreads and confections, emulsifying agent, edible films that can be used as food casings, coatings and packaging to prevent meat oxidation. In biomedical fields, echinoderms have interesting tissue matrices know as mutable collagenous tissue (MCT) which is being studied for tissue regeneration applications. This is due to the fact the tissue can alter its flexibility and regenerate itself, depending on its environmental factors and stresses (Senadheera et al., 2020). This makes it very interesting for biomaterial and tissue scaffolding domains. Further details will be analyzed in chapter 2 in the literature review.

1.4 Quebec's sea cucumber

Sea Cucumbers are marine invertebrate animals which belong to the class of *Holothuroidea* and phylum of Echinodermata. These animals generally have elongated bodies with spike-like structures protruding perpendicularly from the body wall. *Cucumaria frondosa* is typically located in deep sea or benthic areas (Hossain et al., 2020). Sea cucumbers are known as deposit feeders and play an important role in their ecosystem as they remove sediments, organic matter, microalgae protozoa and detritus of macroalgae (Gao et al., 2011; Yang et al., 2006). Images of *C. frondosa* are shown in Figure 1.2 below.

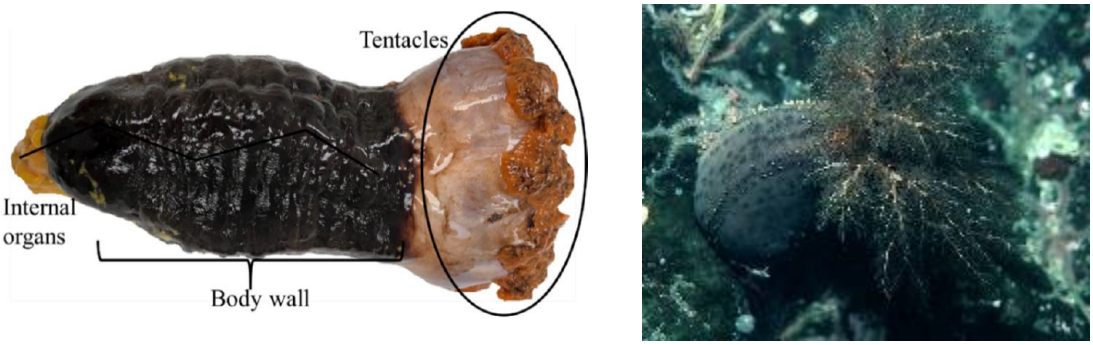

Figure 1.2. Visual representation of the sea cucumber Cucumaria frondosa and its anatomical structure (Hossain et al., 2020; UniProt, 2024).

As shown in Figure 1.2, these animals have elongated bodies with spike-like structures protruding perpendicularly from the dark brown body wall. There are approximately 1500 species of sea cucumber around the world with roughly 100 of them being used for food consumption (Hossain et al., 2020). In Atlantic Canada and Quebec, the most predominant and only commercially available sea cucumber species is the *Cucumaria frondosa*. This species generally grows roughly 40 to 50 cm long, 10 to 15 cm wide and can weigh up to 500 g (Hossain et al., 2020). These animals like to live in low and cold intertidal/tidal pools and are abundant in strong current areas that are rocky or contain stone, sand, gravel and shells. They are usually found in water depths ranging from 30 to 300 meters. (Nelson et al., 2012). Other species that have been identified in Atlantic Canada include Trachythyone nina (Mercier et al., 2010). In 2024, another species was identified: Pseudothyone labradoensis, found in the northern part of Labrador (Kremenetskaia et al., 2024). There are two harvesting techniques in Quebec which are by diving or by dragging using a net(DFO, 2017). However, diving fishing for sea cucumber is still relatively small and undeveloped compared to the dragging fishery in Atlantic Canada and Quebec (Seafood Watch, 2017). Currently, there are commercial quotas in place for Gaspé and the North Shore of Quebec, where processors handle the species (DFO, 2017),however they are still exploring the potential of zone of the Lower North Shore. Some exploratory trials have been performed by private enterprises in the region (Lavallee, 2020). Given the work has already begun and that the shoreline in this area

has many rocky and sandy bottoms, has a lot of current, and has good pristine water quality, establishing businesses have an opportunity to take advantage of the commercial sea cucumber products in the area.

1.5 Technology implementation on the Lower North Shore and constraints

For any successful industrial chemical production process, there is a balance between the engineering and physical science of the process, economics, and safety. This project will focus on scientific process synthesis and its potential scale-up. When analyzing cost, a major factor to the considered area is transport. Shipping costs are significant in the area and need to be minimized. All equipment to produce the product, chemicals and wastes need to be minimized. In terms of equipment, the simpler the better as equipment that require specialized labour to operate, and repair will need off the region support which is expensive. This also touches on an important safety aspect as with the lack of training, there will be a learning curve to ensure the equipment is operated efficiently and safely and if the equipment is not complex, this curve is minimized.

The area predominantly runs on hydroelectricity. However, all the inhabitants and businesses operate from a 20 MW hydroelectric dam (MELCCFP, 2024). In terms of the prospective plant's energy consumption, it needs to be minimized as there are not unlimited amounts of energy to be used. Unless new electric infrastructure is built by the government, the processing plant would have to operate within the regional capacity or implement its own power sources, this is an important design constraint.

There are very few waste management and wastewater treatment plants. All the waste generated would need to be stored and shipped off the region to be treated for now, which is extremely expensive. Other than two indigenous communities on the territory having lagoon systems, and one village having a bio disc treatment system, all other villages operate on septic or primary screening treatment stations removing large debris with no secondary or tertiary treatment is done (Moreira & Tremblay, 2014; Neegan Burnside, 2011). The chemicals that will be needed for this process will have to be environmentally friendly as the area and residents take pride in its clean

environment and would not like to see this change at any cost. The experiments in this thesis were carried out with the transfer of technology and the process to the Lower North Shore in mind.

1.6 Purpose, objectives and hypothesis of the project

English version

There is extensive research studying lab scale marine collagen processes and products including sea cucumbers and *C. frondosa.* Also, there are groups that look at native collagen, gelatin and hydrolyzed collagen products. Most of the research focuses more on the characterization of collagen produced from different species. There is limited research on the process synthesis and optimization of the critical steps (other than the extraction step) of native marine collagen production at lab scale and especially pilot scales. Also, many chemicals used by ingredient producers are becoming less accepted in modern days cosmetic and nutraceutical industries. The methodology and development of a protocol in this project considered the feasibility of implementing such a process on the Lower North Shore and the environmental and industrial limitations in that area. The main purpose and five sub-objectives are outlined in the figure below.

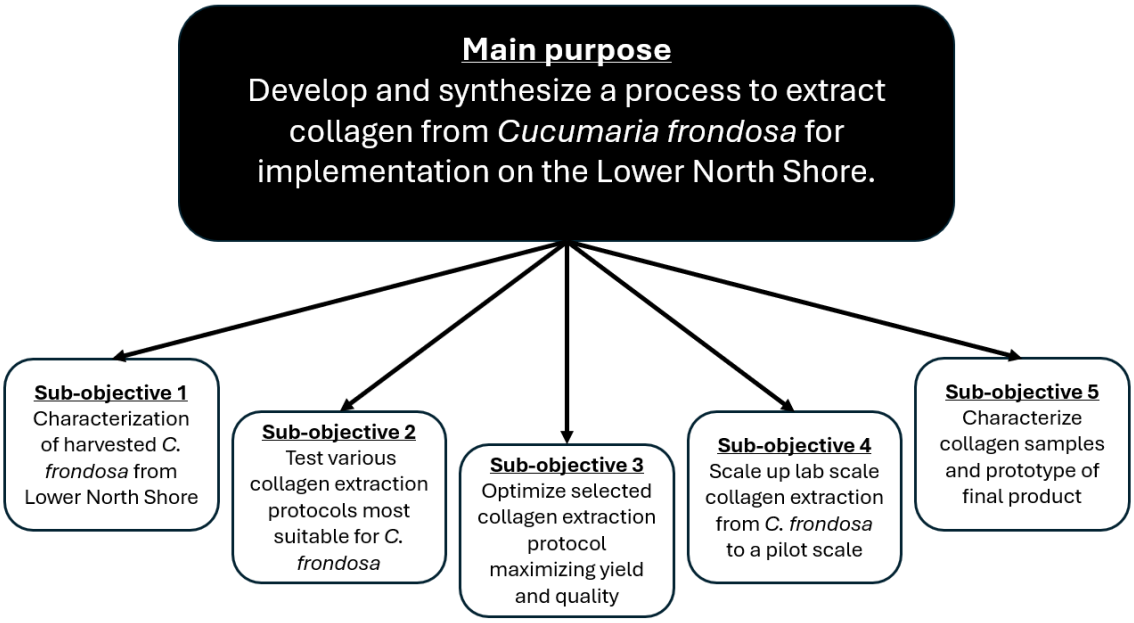

Figure 1.3. Outline of Masters project's main purpose and sub-objectives.

As described in Figure 1.3, the purpose of this project was to develop and synthesize a process to extract a collagen product from *C. frondosa* which can be implemented on the Lower North Shore of Quebec commercially. The objective of the project was to maximize the concentration, yield, and ratio of the native protein in collagen, which would increase the product's value and distinguish it in the market. This would also create the most opportunities in various industries. The following sub-objectives are identified to accomplish the purpose stated above and summarized in Figure 1.3.

The first sub-objective is to characterize the *C. frondosa* harvested from the Lower North Shore. Properties such as moisture content, total protein content, total collagen content, heavy metal and mineral contents were analyzed. It is hypothesized that the biomass would have properties like other commercially available products on the market.

The second sub-objective of the project is to test various collagen extraction protocols to determine the most suitable for *C. frondosa*. The objective is to focus on the production of solely native collagen but if poor yields are observed, other strategies will be used to obtain higher extraction rates and maximize the ratio of native collagen to denatured collagen. This includes an internal protocol available at CRBM developed for another resource, acid-soluble collagen process and pepsin soluble collagen process. These protocols were selected due to the fact they are conventional and do not require sophisticated material and chemicals. This would confirm which global extraction conditions are best suited for liberating the collagen from the resource. Yields will be used to track the impact of modifying the treatment protocol and extraction processes. Characterization methods will be used to determine if any change affects the nativity and structure of collagen. It is hypothesized that a native collagen will be able to be extracted with high purity and yield.

The third sub-objective is to take the extraction methodology selected from sub-objective 2 and investigate, synthesize and optimize process steps to maximize collagen yield and quality while reducing processing time. Various operating conditions will be tested such as temperature, solvent to solid ratio, pH, chemicals and time. These conditions will be studied in various steps of the extraction protocol. Like sub-objective 2, yields will be used to monitor the effects but also characterization techniques as described in sub-objective 5 will be used to monitor if a specific change

had an impact on the structure of collagen, with the goal to preserve as much native collagen as possible. It is believed that the processing parameters could be adapted in a way to further reduce processing time while maintaining the native structure of the collagen.

The fourth sub-objective is to execute a first pilot scale-up of the process developed throughout sub-objectives 2 and 3. This sub-objective will look at which conditions and equipment could be suitable though the product's industrialization pathway while maintaining the protein's structure, nativity and functionality. It is expected that the process will be scaled effectively to deliver a viable process that will preserve as much native formed collagen as possible for cosmetic and nutraceutical applications.

The fifth sub-objective is to characterize the extracted collagen product to compare to the literature and commercially available products. A panel of chemical and biomolecular analysis will be used to evaluate the structure, quality and purity. To ensure the purity, structure and identity of the extracted collagen, multiple techniques were used to monitor the same or similar sets of properties. This minimizes potential discrepancies. In this project, we aim to obtain a product that shares similar characteristics to other sea cucumber collagen products observed in the scientific literature and commercially. With all the objectives, an appropriate conclusion could be made on how scalable the product is, and the selected process.

It is important to note an adapted strategy was used throughout the project. The intricate native extraction process, which could take up to 14 days, presented a formidable challenge when compared to the project's mandate to extract, analyze, process and optimize collagen samples. The limited timeframe (less than 6 months of lab time) and resources available posed a significant constraint if each step of the process was to be evaluated. To ensure each step of the process was studied in the given the constraints mentioned, we chose not to fully duplicate the test trials. The lab scale could have been done on smaller proportions (on 20 g for example); more replicates could have been made. But considering the quantity needed for quality analysis, 20g doesn't give an adequate sample. It was decided to work on 100 g to 200 g batches, giving a volume of 2-4 kilograms for the 1:20 ratio in the process scale up. This gives enough product for the chemical and biomolecular analysis but limits the number of replications as the laboratory is not equipped to do

more replications at this scale, especially for the long tangential filtration steps (only 1 apparatus available for a 4-day step). Even if the equipment was available to do the appropriate replicates, the project only had a fixed amount of *C. frondosa* available to execute the most important iterations. Literature commonly uses 100 g batch sizes to execute its series of experiments. Since the research focuses on increasing the batch size to one kilogram, starting with a smaller quantity, such as 20 grams, will necessitate a second lab-scale operation, which will take more time. This is problematic, given that time constraints are a major factor in this project.

Version française

Il existe de nombreuses recherches sur les processus et produits de collagène marin à l'échelle de laboratoire, y compris les concombres de mer et *C. frondosa*. Parmi ces études, certaines se concentrent sur le collagène natif, la gélatine et les produits de collagène hydrolysé. La majorité des recherches s'intéressent davantage à la caractérisation du collagène produit à partir de différentes espèces. Cependant, il existe peu de recherches sur la synthèse des processus et l'optimisation des étapes critiques (autres que l'étape d'extraction) du collagène marin natif à l'échelle de laboratoire, et encore moins à l'échelle pilote. De plus, de nombreux produits chimiques utilisés par les producteurs d'ingrédients sont de moins en moins acceptés dans les industries modernes de la cosmétique et des nutraceutiques, et dans des communautés éloignées comme la Basse-Côte-Nord. La méthodologie et le développement d'un protocole dans ce projet ont pris en compte la faisabilité d'implémenter un tel processus sur la Basse-Côte-Nord, ainsi que les contraintes environnementales et industrielles de cette région. L'objectif principal et les cinq sous-objectifs sont présentés dans le graphique ci-dessous.

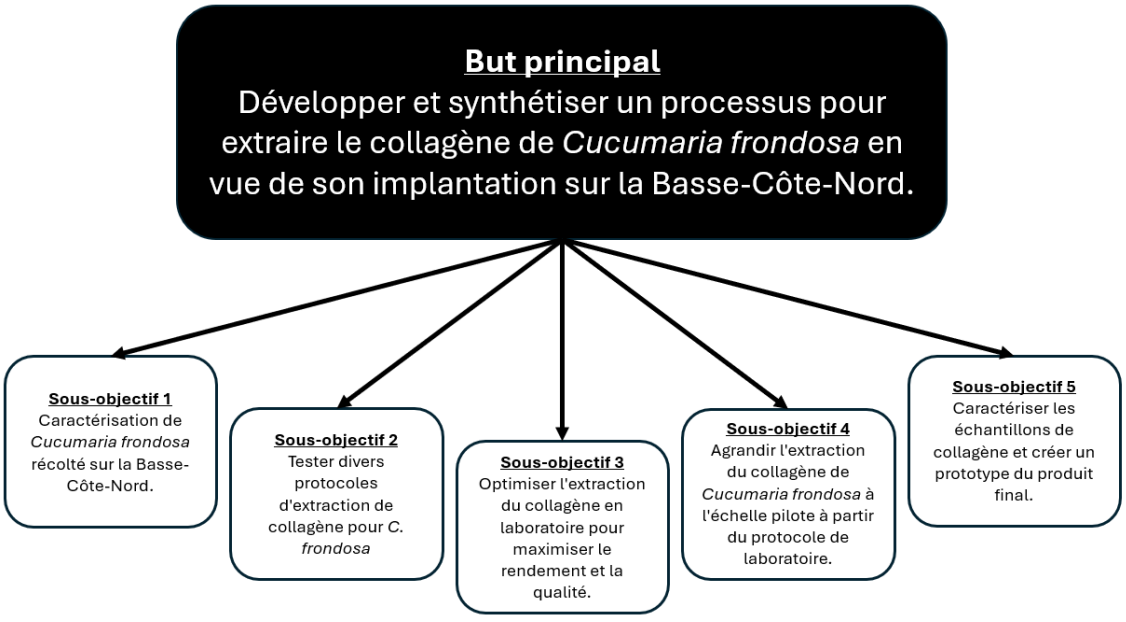

Figure 1.4. Aperçu de l'objectif principal et des sous-objectifs du projet de maîtrise.

Comme mentionné dans la Figure 1.4., le but de ce projet est de développer et de synthétiser un processus permettant d'extraire un produit de collagène à partir de *C. frondosa* pouvant être mis en œuvre sur la Basse-Côte-Nord du Québec. La qualité recherchée du collagène dans ce projet consiste à maximiser la concentration, le rendement et le ratio de la protéine sous sa forme native, afin d'ajouter de la valeur et de différencier le produit sur le marché tout en ouvrant le plus d'opportunités dans divers secteurs industriels. Les objectifs suivants ont été identifiés pour atteindre ce but. Les sous-objectifs suivants ont été identifiés pour accomplir l'objectif énoncé ci-dessus et résumé dans la Figure 1.4.

Le premier sous-objectif est de caractériser le *C. frondosa* récolté sur la Basse-Côte-Nord. Des propriétés telles que la teneur en humidité, la teneur totale en protéines, la teneur totale en collagène, les métaux lourds et les contenus minéraux ont seront analysées. L'hypothèse est que la biomasse contiendrait des propriétés similaires à d'autres produits commercialement disponibles sur le marché.

Le deuxième sous-objectif était de tester divers protocoles d'extraction de collagène pour déterminer le plus adapté à *C. frondosa*. Le sous-objectif est de se concentrer sur la production uniquement de collagène natif. Cependant, si des rendements faibles sont observés, d'autres stratégies seront utilisées pour obtenir des taux d'extraction plus élevés tout en maximisant le ratio de collagène natif par rapport au collagène dénaturé. Cela inclut un protocole interne disponible au CRBM développé pour une autre ressource, ainsi que des processus de collagène soluble dans l'acide et la pepsine. Ces protocoles ont été sélectionnés, car ils sont conventionnels et ne nécessitent pas de matériels ou de produits chimiques sophistiqués. L'hypothèse est qu'un collagène natif pourra être extrait avec une grande pureté et un bon rendement.

Le troisième sous-objectif est d'optimiser les étapes du processus pour maximiser le rendement et la qualité du collagène tout en réduisant le temps de traitement. Divers paramètres d'exploitation seront testés, tels que la température, le ratio solvant/solide, le pH, les produits chimiques et le temps pour les étapes de désagrégation, de déprotéinisation, d'extraction, de récupération et de purification. Comme pour le sous-objectif 2, les rendements seront utilisés pour évaluer les effets, ainsi que des techniques de caractérisation pour observer si des changements spécifiques ont un

impact sur la structure et la nativité du collagène. Le sous-objectif est de conserver autant de collagène natif que possible.

Le quatrième sous-objectif est d'effectuer une première mise à l'échelle pilote du processus développé dans les sous-objectifs 2 et 3. Cet objectif consiste à déterminer quelles conditions et quels équipements seraient adaptés à une voie d'industrialisation du produit tout en maintenant la structure, la nativité et la fonctionnalité de la protéine. Il est attendu que le processus soit mis à l'échelle de manière efficace pour fournir un processus viable qui conserve le plus de collagène formé natif possible pour les applications cosmétiques et nutraceutiques.

Le cinquième sous-objectif est de caractériser le produit de collagène extrait afin de le comparer avec les produits de la littérature et ceux disponibles sur le marché. Différentes analyses chimiques et biomoléculaires seront utilisées pour évaluer la structure, la qualité et la pureté. L'utilisation de multiples méthodes pour confirmer les données sur la pureté, la structure et l'identité vise à réduire les incertitudes concernant la nature du collagène extrait et sa qualité.

Il est important de noter qu'une stratégie adaptée a été utilisée tout au long du projet. Compte tenu de la complexité du processus complet d'extraction, qui peut prendre jusqu'à 14 jours, et de l'obligation d'extraire et d'analyser les itérations du processus et du produit, le délai (moins de 6 mois de laboratoire) et la quantité de ressources représentaient une contrainte importante si l'on voulait évaluer chaque étape du processus. C'est pourquoi il a été décidé de ne pas procéder à des essais de reproduction et à diverses caractérisations. Les expériences à l'échelle laboratoire auraient pu être réalisées sur de plus petites proportions (sur 20 g par exemple) ; plus de répétitions auraient pu être faites. Mais compte tenu de la quantité nécessaire pour l'analyse de la qualité, 20 g ne donnent pas beaucoup de produit. Il a été décidé de travailler sur des lots de 100 à 200 g, ce qui donne un volume de 2 à 4 kilogrammes pour un ratio de 1:20 dans la mise à l'échelle. Cela donne assez de produit pour l'analyse chimique et biomoléculaire mais limite le nombre de réplications car le laboratoire n'est pas équipé pour faire plus de réplications à cette échelle, en particulier pour les longues étapes de filtration tangentielle (seulement 1 installation disponible pour une étape de 4 jours). Même si l'équipement était disponible pour effectuer les réplications appropriées, le projet ne disposait que d'une quantité fixe de *C. frondosa* pour exécuter les itérations les plus importantes.

La littérature utilise généralement des lots de 100 g pour exécuter ses séries d'expériences. Étant donné que la thèse visait à augmenter la taille des lots jusqu'à un kilogramme, le fait de commencer avec un lot plus petit (par exemple 20 g) oblige à d'intégrer une deuxième mise à l'échelle du laboratoire et, comme mentionné ci-dessus, le temps est un facteur limitant dans ce projet.

Chapitre 2 - Literature review

2.1 Characteristics of sea cucumbers

2.1.1 Characteristic overview

Sea cucumber have many characteristics, nutrients and biomolecules that have been explored in the food, nutraceutical, cosmetic and pharmaceutical industry. The body wall and viscera contain different active biomolecules. In this study, the body wall was investigated in more detail given that structural protein collagen is more concentrated in this area. Like other marine species, the concentration of various nutrients can vary by season, environment or external stresses such as feed regimes. As discovered by David and MacDonald (2020), the lipid content and glycogen content tend to increase in the summer months while the protein content tends to be higher in the fall. A compositional breakdown of these molecules and characteristics are described in Figure 2.1 below:

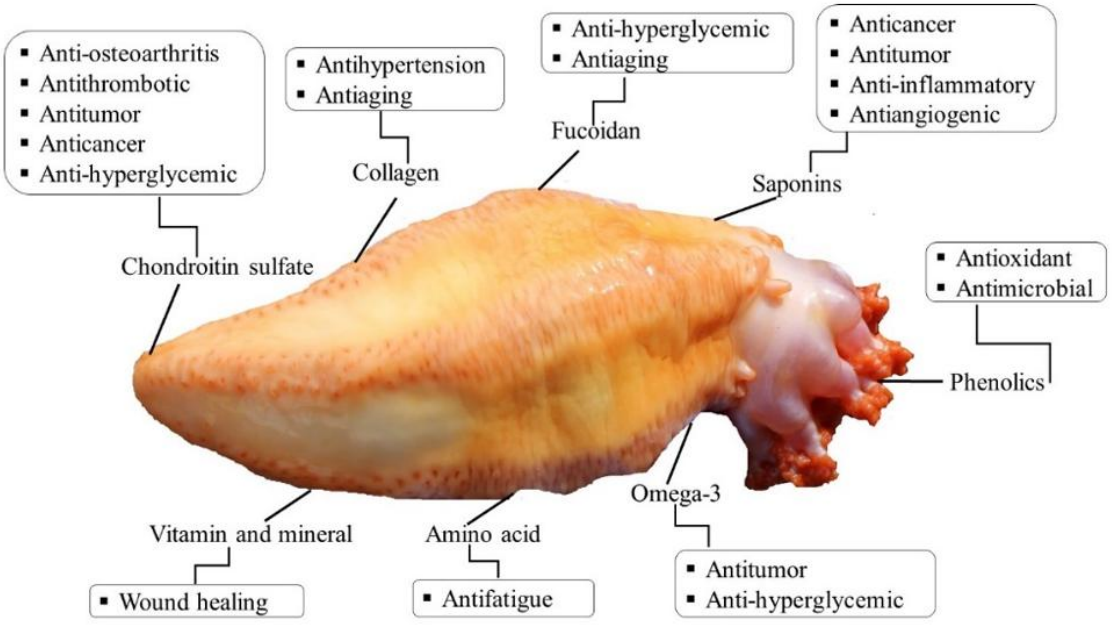

Figure 2.1. Characteristics and biomolecule breakdown of sea cucumber and its potential benefits (Hossain et al., 2020).

Molecules such as chondroitin, saponins, triterpene glycosides and mycosporine have anti-cancer/tumor properties while fucoidan, and phenolic acids can be used for antioxidant supplements. Collagen is in abundance in sea cucumbers, making up roughly 70% of the total proteins within body wall tissue, and is useful in antiaging and wound healing applications (Kim & Pangestuti, 2011; Saito et al., 2002). In addition to the molecules presented above, there are also many nutrients, vitamins, peptides and fatty acids. These molecules are often concentrated in one organ and/or section of the sea cucumber. For example, amino acids can differentiate in concertation depending on if one is studying the body wall versus the viscera (Mamelona & Saint-louis, 2010; Y. Zhong et al., 2007).

2.1.2 Collagen

Collagen is found in the extracellular matrix and dermis of animals. It provides flexibility and strength to connective tissue. A depiction of the primary amino acid backbone is shown in Figure 2.2 below.

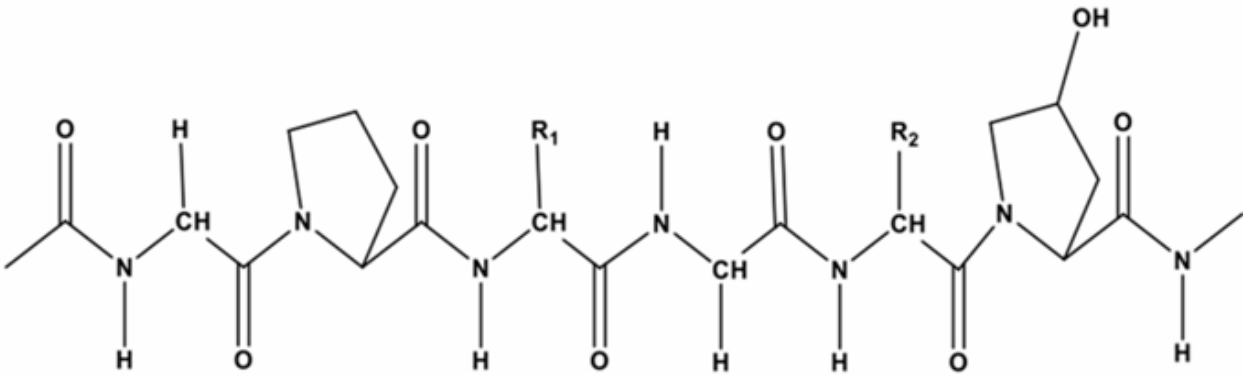

Figure 2.2. Chemical structure of type I collagen (Senadheera et al., 2020).

As shown in Figure 2.2, the collagen backbone is made up of carboxyl , amino and other functional groups. The carboxyl and amino groups of neighboring amino acids undergo a condensation reaction to form a peptide bond. Each functional group specific to an amino acid is represented by R. The sequence of these groups gives rise to the structure and function of a protein, such as collagen. This molecule has a wide range of beneficial biological and mechanical properties for use in food,

pharmaceuticals, biomedicine and cosmetics. This thesis will explore these properties in more detail in section 2.2.

2.1.3 Phenolic acids

Phenolic acids are antioxidants which are not only in marine invertebrates but also seaweed and other plants. Structures of various phenolic acids that are found in sea cucumbers are summarized in the figure below.

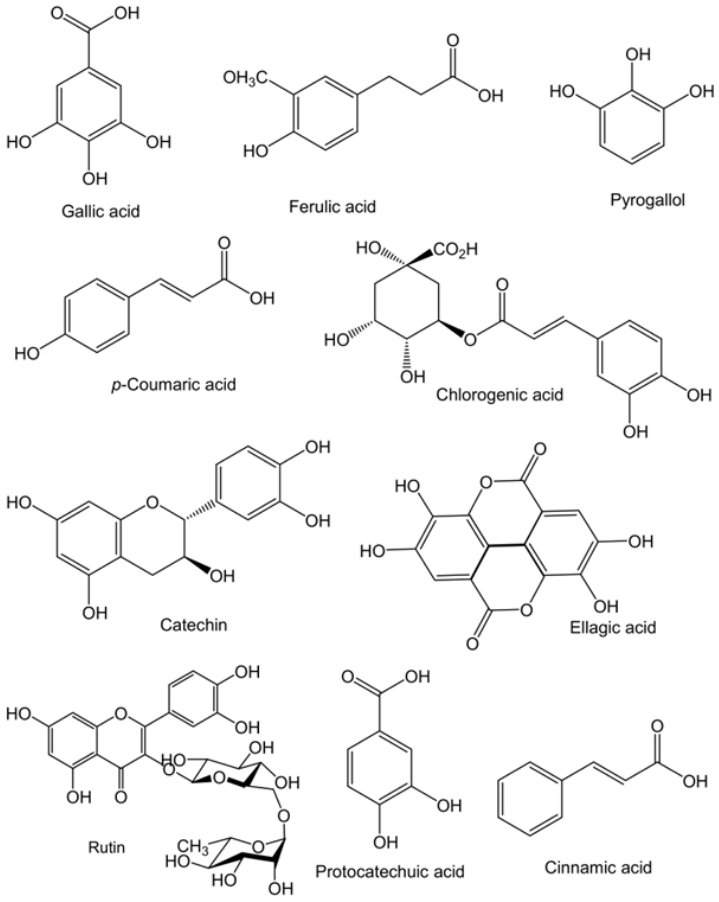

Figure 2.3. Chemical structure of various phenolic acids found in sea cucumbers (Hossain et al., 2020).

Figure 2.3 shows that these compounds have one or more rings that are aromatic, with various functional groups (such as hydroxyl or carboxyl) or other molecular chains connected to them (Hossain et al., 2022). These compounds can be involved in acid reactions, hydrogen bonding,

esterification reactions, etherification reactions, and oxidation due to their chemical structure (Mamari, 2021). These chemical functionalities give this molecule great antioxidant, antimicrobial and anticancer properties (Hossain et al., 2022). *C. frondosa* contain significant amounts of phenolic acids (22.5 to 236.0 mg gallic acid equivalents (GAE)/ 100 g dry weight of sample) which can be found in its digestive tract and tentacles (Hossain et al., 2020). Common phenolic acids found in sea cucumbers include gallic acid, ferulic acid, p-coumaric acid, catechin, rutin, ellagic acid, protocatechuic acid, cinnamic acid and pyrogallol (Hossain et al., 2022). It gains a lot of these phenolic compounds through eating phytoplankton. Ethanol and other solvent extraction techniques can be used to remove phenolic acids.

2.1.4 Polysaccharides

Polysaccharides from marine creatures have garnered much attention and research over the past few years due to their potential health benefits. Sea cucumbers are very rich in polysaccharides and in particular, sulphated polysaccharides which have gained interest from pharmaceutical industries (Siahaan et al., 2017). The following image illustrates the structure of a polysaccharide commonly found in sea cucumbers.

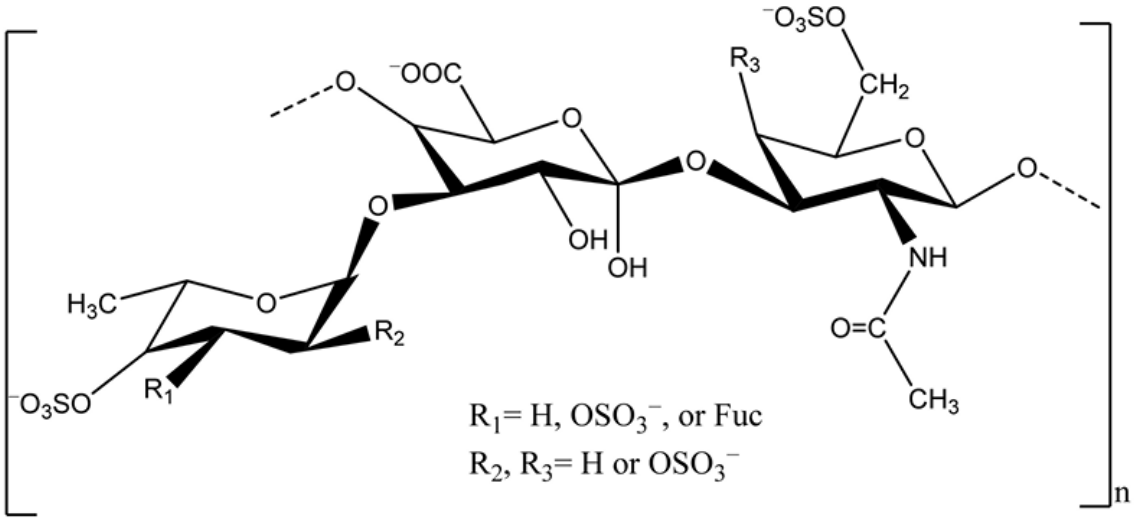

Figure 2.4. Example of a polysaccharide structure found in sea cucumbers. In this case, it is fucosylated chondroitin sulphate (Hossain et al., 2022).

Figure 2.4. shows that the polysaccharide chain consists of many sugar monomers and functional group attachments. The conformation of the main carbohydrate chain has an impact on its antioxidant activities (Hossain et al., 2022). In the body wall of sea cucumbers, fucosylated chondroitin sulphate have gained interest. This carbohydrate possesses intriguing biological properties for medical use. However, extracting and purifying this molecule is a complex and time-consuming process that requires advanced techniques such as size exclusion or ion exchange chromatography. Another noteworthy polysaccharide is fucoidan, which is already employed in the functional food industry. Additionally, it boasts promising anti-clotting, anti-coagulant, and anti-diabetic properties, making it an alluring prospect for biomedical research (Hossain et al., 2020).

2.1.5 Saponins

Saponins are bioactive compounds that play an important role in chemical defense and pharmacological activities (Siahaan et al., 2017). Saponins in sea cucumbers are typically triterpene glycosides. Their chemical structure is shown in the following figure.

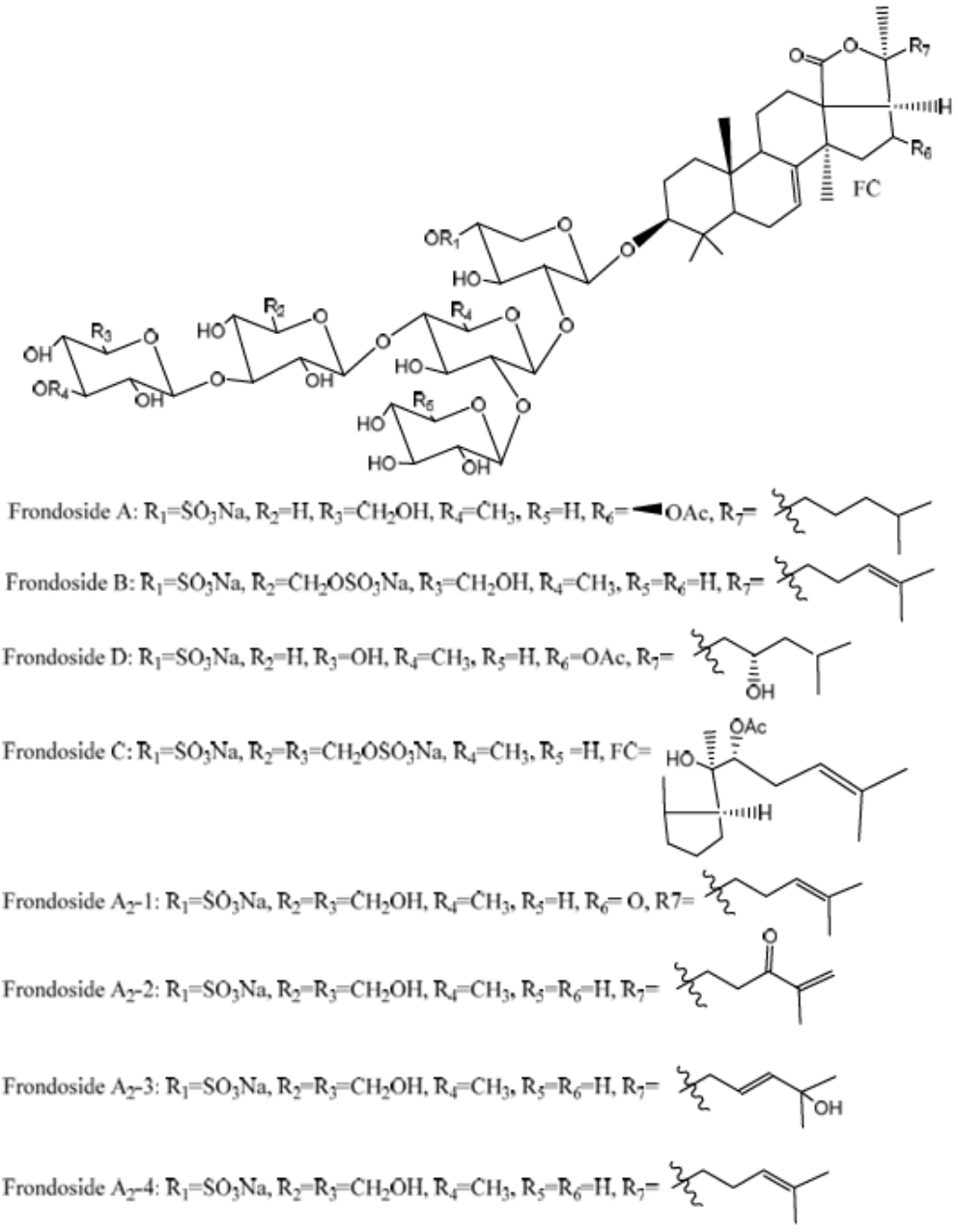

Figure 2.5. Example of saponins found in sea cucumbers (Hossain et al., 2020).

As observed in Figure 2.5, saponins are made up of a chain of up to 6 monosaccharides (Hossain et al., 2020). The sugar moiety is polar, while the non-polar aglycone is connected to it by a glycosidic bond (Fagbohun et al., 2023). This molecule has a polar and a non-polar end, which allows it to interact with various biological structures and events related to the cell membrane and their

signaling pathways (Fagbohun et al., 2023). The predominant saponin in *C. frondosa* is frondoside A but also have significant amounts of frondoside B and C. These compounds have interesting antiviral, antifungal and anti-cancer properties and are typically separated through liquid-liquid extractions and purified by high pressure liquid chromatography HPLC (Hossain et al., 2020).

2.1.6 Gelatin and collagen hydrolysates

Upon further hydrolysis of collagen, one can produce gelatin which has average molecular weight between 15 kDa and 200 kDa or collagen hydrolysates which have a molecule weight between 3 kDa and 6 kDa (Farooq et al., 2024; Lopez et al., 2019). Figure 2.6 demonstrates type I collagen and the formation of gelatin and hydrolyzed peptides.

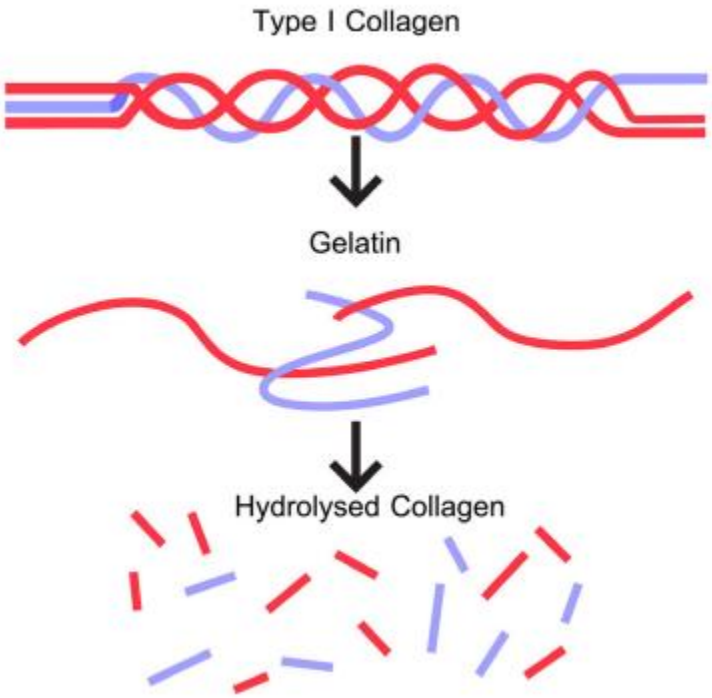

Figure 2.6. Schematic demonstrating the degradation of type I collagen into gelatin and collagen hydrolysates (Koutsoumanis et al., 2020).

As seen in Figure 2.6, gelatin is formed when the collagen triple helix is denatured which can be done by heat or chemical processes (Farooq et al., 2024). There are two types of gelatin, type A

and B which can be obtained by acid or alkaline pretreatment (Farooq et al., 2024). The size of the molecules is determined by the degree of hydrolysis and is typically a variety range of sizes. The distribution of molecular weights is impacted by the source in which gelatin is being extracted from, the enzymes performing the extraction and its operating conditions (D. Liu, Nikoo, et al., 2015). The molecular weight of the peptides in a gelatin sample is not well correlated or consistently distributed (Haug & Draget, 2009). However, the molecular weight distribution is important information that manufacturers must provide to their customers. The distribution can affect sample properties, for example, if there are too many high-molecular-weight gelatin chains (γ-chains of 300 kDa or more) in the sample, the gelatin will harden quickly in applications such as capsules and gels. (Haug & Draget, 2009). Therefore, it is important that manufacturers study and monitor this property of their products. This will ensure that customers know what to expect from the products they receive. Gelatin has interesting antioxidants and antithrombotic properties (D. Liu, Nikoo, et al., 2015). It is also commonly used in the food industry as an additive, filler and gelling agent.

If the molecules are further hydrolyzed as seen in Figure 2.6, collagen hydrolysates are formed. Given the amino acid composition is still like that of collagen, they still share similar antioxidant and other biologically active properties. As shown by Abedin et al. (2015), they were able to optimize a procedure to extract collagen hydrolysates from *S. vastus* that could be used as an ACE inhibitor and had radical scavenging capabilities.

2.1.7 Vitamins, minerals and other

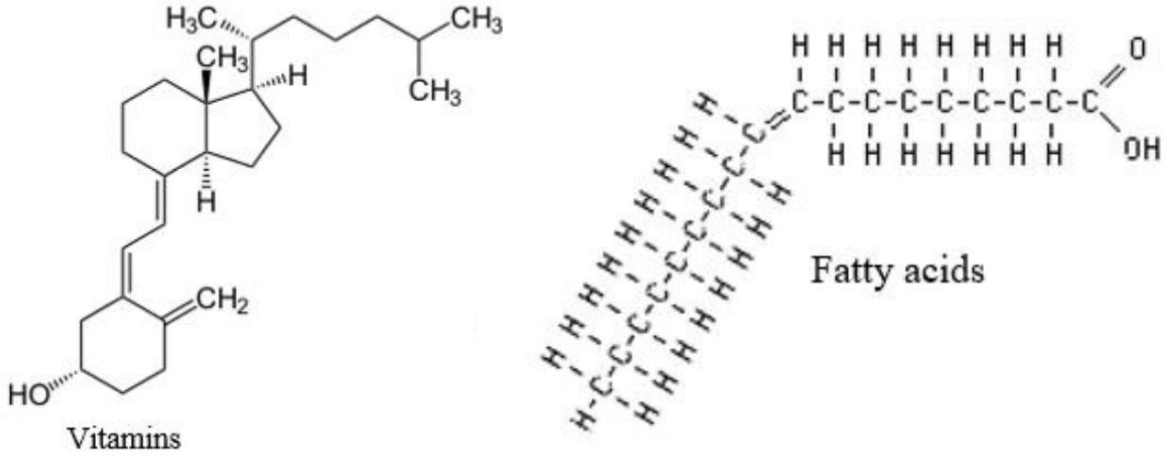

Figure 2.7. Example of the chemical structures of (a) vitmains and (b) fatty acids that are found in sea cucumbers (Fagbohun et al., 2023).

Sea cucumbers have nutrients such as Vitamin A, Vitamin B1, B2, B3 and minerals including calcium, magnesium, iron, zinc and other elements that provide health benefits. The chemical structure of vitamins is summarized in Figure 2.7.a. These molecules also provide benefits to extracts used in cosmetic formulations (Siahaan et al., 2017). Also, sea cucumbers contain omega-3 fatty acids in its body wall, tentacles and viscera that have anti-hyperglycemic properties and is commonly used in folk medicine in Eastern Asian countries (Hossain et al., 2020). An example of the chemical structures of vitamins and fatty acids are found in Figures 2.7.a and 2.7.b respectively.

Other molecules include functional amino acids that are biochemical backbones of many biomedical, cosmetic and nutraceutical applications.

2.2 Collagen

2.2.1 Collagen properties, structure and sources

Collagen is the most abundant protein in human extracellular matrices, making up almost 30% of the total protein found in connective tissues. The following diagram depicts a typical example of an invertebrate's extracellular matrix.

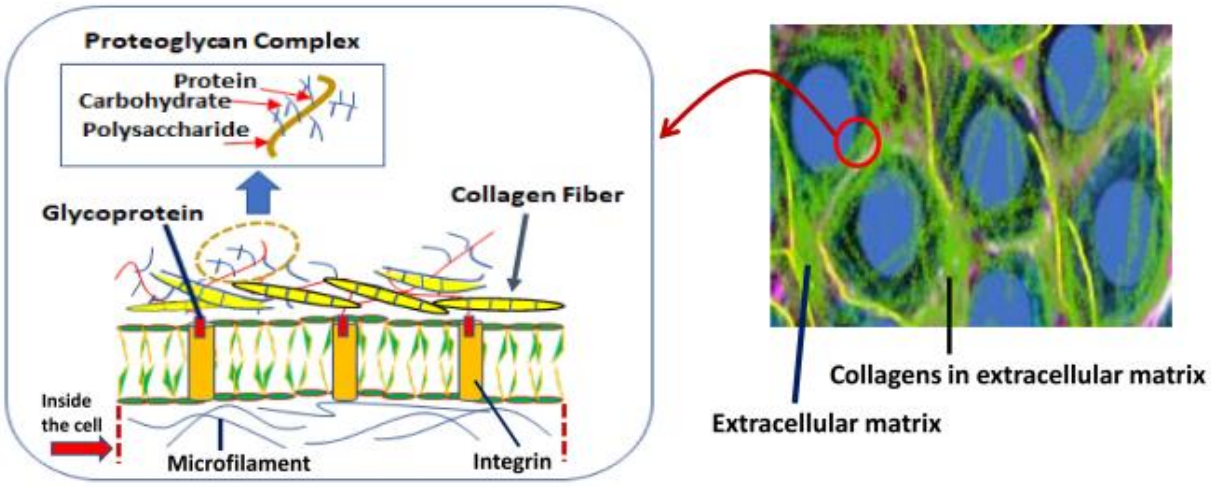

Figure 2.8. Visual representation of the extracellular matrix in invertebrates, as well as the arrangement of its components. The left image illustrates the structural components and their interactions, while the right image depicts a cellular model and the distribution of collagen throughout the network (Azizur Rahman, 2019).

As shown in Figure 2.8, collagen, a key component of the extracellular matrix (right image of Figure 2.8.), interacts with a variety of molecules, including proteins, glycosaminoglycans, glycoproteins, polysaccharides, phospholipids, and other substances (left image Figure 2.8). Collectively, these elements create a sturdy yet flexible framework that enables many organisms and animals to function effectively. As shown by Trotter et al. (1996) in *Cucumaria frondosa*, stiparin is one of these molecules that crosslinks different collagen fibrils together, strengthening the matrix. Many of these interactions of the molecules in Figure 2.8 occur by surface interactions through ionic, hydrogen, Van der Waals interactions that can be disrupted through various extraction techniques (Trotter et al., 1996).

Collagen is made up of 3 alpha helices, which normally form together a molecular weight between 300 kDa to slightly greater than 400 kDa and 300 nm in length (Kwon et al., 2014; Rajabimashhadi et al., 2023; Senadheera et al., 2020). Chains can be homomeric, where all three alpha helices are identical, or heteromeric, where at least one alpha chain differs from the other two. When two of the alpha helical chains come together it is called a beta-chain, when all three alpha helices come together, it is known as a gamma chain. Each individual alpha helix lefthanded symmetry but when

brought together to form its tertiary structure, a right handed helix is created as shown in Figure 2.9 below (Senadheera et al., 2020).

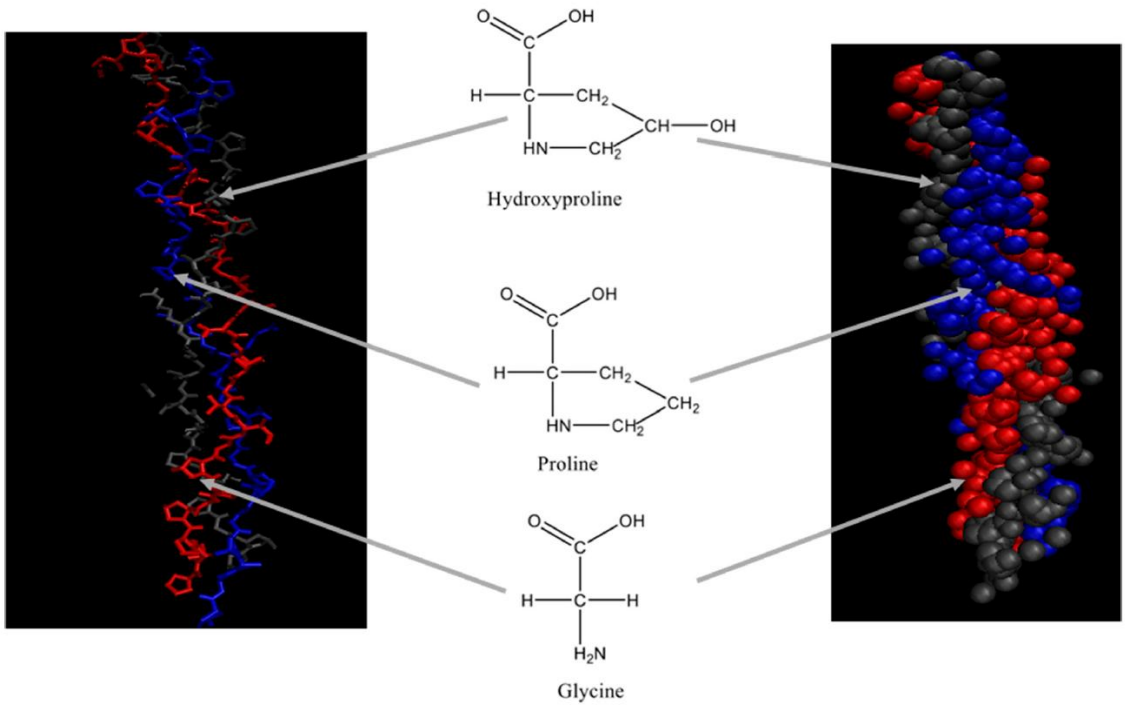

Figure 2.9. 3-D representation and key amino acids of collagen (Senadheera et al., 2020).

As depicted in Figure 2.9, each alpha chain has a repeating sequence which is glycine-X-Y where X and Y are typically proline and hydroxyproline (Senadheera et al., 2020). Glycine amino acids arrange themselves in a tight, triple helix, while proline and hydroxyproline residues line the outside surface of the protein. The tight packing of the glycine molecules is due to interchain N-H and carboxyl group bonds on neighboring strands, hydrogen bonds and electrostatic interactions. Glycine in this sequence is important because it does not sterically hinder neighboring amino acids, allowing it to pack tightly in the center of the molecule (Jafari et al., 2020). Different helical lengths from one type of collagen to the next can be due to the presence of non-helical regions, helical interruptions and different terminations of the helical domains (Senadheera et al., 2020).

The hierarchical structure and overview the assembly process of collagen molecules to form fibers as seen in extracellular matrices are shown in Figure 2.10.

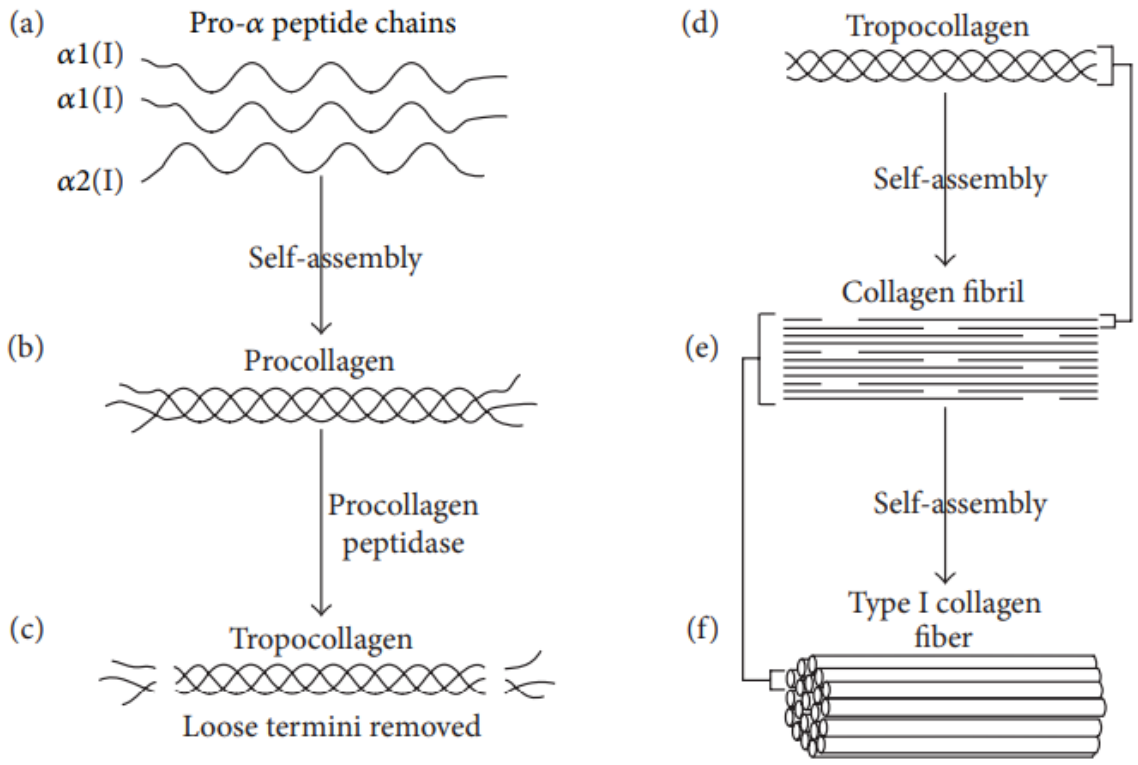

Figure 2.10. Overview of hierarchical structure and assembly pathway of type 1 collagen (Kruger et al., 2013).

As shown in Figure 2.10, three alpha helices self-assemble to form procollagen. Peptidic enzymes then cleave the molecule at both termini, creating tropocollagen (Kruger et al., 2013). Tropocollagen refers to the fundamental unit of collagen, which assembles into collagen fibrils, and eventually collagen fibers that help compose highly ordered extracellular matrices for various organisms and animals. When these fibers are in a network with one another, there are many cross links that help strengthen the network but over crosslinking can cause the networks to become brittle (Rajabimashhadi et al., 2023). A more detailed explanation of the biosynthesis and assembly is presented in section 2.2.2 of this thesis.

Tropocollagen has three domains which consist of two non-helical regions, the C-terminal (-COOH) and N-terminal ($-NH_2$). The two terminal ends of the molecule are known as the telopeptide regions and are very important in cross linking other collagen molecules, developing the highly ordered fibril assembly through oxidative deamination reactions at neighboring lysine and

hydroxylysine residues by lysyl oxidase (Holmes et al., 2017; D. Liu, Nikoo, et al., 2015). The C-terminal is typically 25 amino acids long, while the N-terminal is 16 amino acids long but may vary from one species to the next. These regions are also responsible as antigen determinants for collagen and when removed can improve the immunogenicity of the molecules (Holmes et al., 2017). Lysine and hydroxylysine are also crosslinked intermolecular through glycosylation of these amino acids.

Hydroxyproline plays a few important roles in collagen. One aspect is the more hydroxyproline in the amino acid sequence of collagen, the more stable the molecule is at higher temperatures (Oslan et al., 2022). This is due the ability of the pyrrolidine ring be able to form more intramolecular hydrogens bonds, stabilizing the structure (Rajabimashhadi et al., 2023). This is typically one reason why warm water marine collagen is more stable than collagen from cold water species, as typically hydroxyproline concentrations in warm water species are higher (Oslan et al., 2022). For example fivespot flounder (*Pseudorhombus pentopthalmus*, warm water fish) has 72 hydroxyproline residues per 1000 amino acids where pacific grenadier (*Coryphaenoides perctoralis*, cold water fish) has 49 hydroxyproline residues per 1000 amino acids (Akita et al., 2020). The flounder collagen is more stable thermally as compared to the grenadier species. The same argument can be made when comparing bovine or porcine collagen to marine sourced collagen. These collagens are typically more stable than marine counterparts as they have higher concentrations of hydroxyproline.

The distinctive feature of alpha helices can help identify one type of collagen from another. Type I collagen is the predominant collagen being reviewed in this study, however there are types I up to XXIX (Farooq et al., 2024). Table 2.1 demonstrates the different types of collagen and their diversity.

Table 2.1. Summary of characteristics and distribution of different types of collagen (Senadheera et al., 2020).

Collagen Type	Chains	Sub Family	Distribution
I	α1(I) α2(I)	Fibrillar collagen	Skin, tendon, bone, dermis, intestine, uterus
II	α1(II)	Fibrillar collagen	Hyaline cartilage, vitreous, nucleus pulposus
III	α1(III)	Fibrillar collagen	Dermis, intestine, large vessels, heart valve
IV	α1(IV) α2(IV) α3(IV) α4(IV) α5(IV) α6(IV)	Basement membrane and associated collagen	Basement membranes
V	α1(V) α2(V) α3(V)	Fibrillar collagen	Cornea, placental membranes, bone, large vessels
VI	α1(VI) α2(VI) α3(VI)	Beaded filament forming collagen	Descement's membrane, skin, heart muscles
VII	α1(VII)	Basement membrane and associated collagen	Skin, placenta, lung, cartilage, cornea
VIII	α1(VIII) α2(VIII)	Short chain collagen	Produced by endothelial cells, descemet's membrane
IX	α1(IX) α2(IX) α3(IX)	Fibril associated and related collagen	Cartilage
X	α1(X)	Short chain collagen	Hypertrophic and mineralizing cartilage
XI	α1(XI) α2(XI) α3(XI)	Fibrillar collagen	Cartilage, intervertebral disc, vitreous humor
XII	α1(XII)	Fibril associated and related collagen	Chicken embryo tendon, bovine periodontal ligament
XIII	α1(XIII)	Trans membrane collagens and collagen like proteins	Cetal skin, bone, intestinal mucosa

Table 2.1 summarizes the collagen type, its alpha chains, its subfamily, and the tissues in which it is distributed. For example, type I collagen contains α 1 and α 2 helices. It belongs to the fibrillar collagen subfamily, and it can be found in the skin, tendon, bone, dermis, intestine and uterus. As for the nomenclature used for identifying different types of collagen, roman numerals are used to indicate the type and Arabic numerals are used to number the type of α chains that are present in the molecule. An interesting fact discovered through studies was that type III collagen contained type I collagen and together, they form mixed fibrils in different tissues (Senadheera et al., 2020). Table 2.1 reveals that collagen is a highly diverse and intricate group of proteins that is widespread throughout various tissues.

Collagen can be sourced from invertebrates and vertebrates but is predominantly sourced from bovine and porcine. There is a growing trend towards considering alternative options, taking into account societal factors such as religion and culture, low antigenicity, extensive land needs, and the spread of disease (Farooq et al., 2024). Other animal sources include human placenta, rat, chicken, and kangaroo (Senadheera et al., 2020). Studies have also looked at producing human recombinant collagen using prokaryotic and eukaryotic cells to mass produce the protein (T. Wang et al., 2017). Marine vertebrates and invertebrates offer an intriguing source of collagen, as they are free from religious and ethical constraints, pose a lower risk of disease transmission, and maintain excellent biocompatibility, adsorption and biodegradability (Senadheera et al., 2020). There are some drawbacks to marine collagen compared to land-based animal collagen. They have lower thermal stability, poor mechanical properties and higher degradation rate (Subhan et al., 2021). The lower thermal stability is due to the lower concentration of hydroxyproline in the protein's amino acid backbone (Subhan et al., 2021). To combat these issues, researchers have tried incorporating different synthetic and natural polymers to alter or link to the functional groups of collagen in hopes of stabilizing it. Other treatments include UV irradiation, chemical treatments an dehydrothermal treatment but these methods can generate cytotoxicity and poor biocompatibility (Subhan et al., 2021). Figure 2.11 summarizes various marine sources of collagen. Collagen from marine species derived products have been met with industrial production scale up issues related to their low extraction yields and low temperature processing conditions which lead to increase cost (Rajabimashhadi et al., 2023).

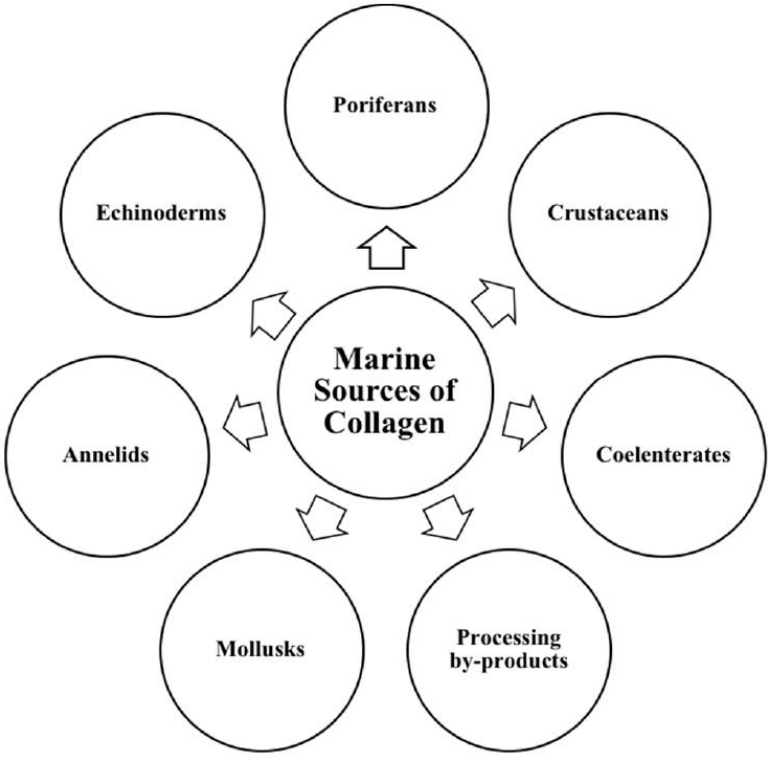

Figure 2.11. Sources of marine collagen (Senadheera et al., 2020).

As shown in Figure 2.11, marine sources of collagen include poriferans, crustaceans, coelenterates, echinoderms, annelids, mollusks and processing by-products. Fish and its processing byproducts make up a lot of the supply of marine collagen. The bones, scales, skin and cartilages are great sources of collagen but are often discarded, roughly 7.3 million tons/year (Rajabimashhadi et al., 2023). The collagen structure and stability vary from one fish source to the next, depending on the amino acid content which has shown to be linked to their natural habitats and environment, but mainly being type I collagen (Farooq et al., 2024; Prajaputra et al., 2024).

Echinoderms are a marine resource that contains a lot of collagen. Sea cucumbers, an echinoderm, will be discussed in more detail in the present thesis. Marine sponges have gelatinous internal tissues which contain collagen. The collagen helps form the exoskeleton, forming a fibrous network to provide flexural rigidity to the network (Farooq et al., 2024). Despite having over 15000

types of sponges, these species remain an untapped resource to supply collagen. Jellyfish are also interesting in terms of collagen, as 75% of the species total protein content is collagen. From one species to the next, the collagen content is vastly different (Farooq et al., 2024). Crustaceans have very small amounts of collagen, and therefore not appealing for commercial extraction purposes for this biomaterial (Farooq et al., 2024). Finally, Mollusca (squids, scallops, etc.) are great sources of collagen. They have higher amino acid contents compared to other marine species and have significant crosslinking in the telopeptide regions, making them more like terrestrial collagen sources. Their collagen is also more thermally stable (Farooq et al., 2024).

2.2.2 Collagen synthesis

Regardless of the source and type, collagen is biosynthesized in a similar fashion and summarized in the figure below.

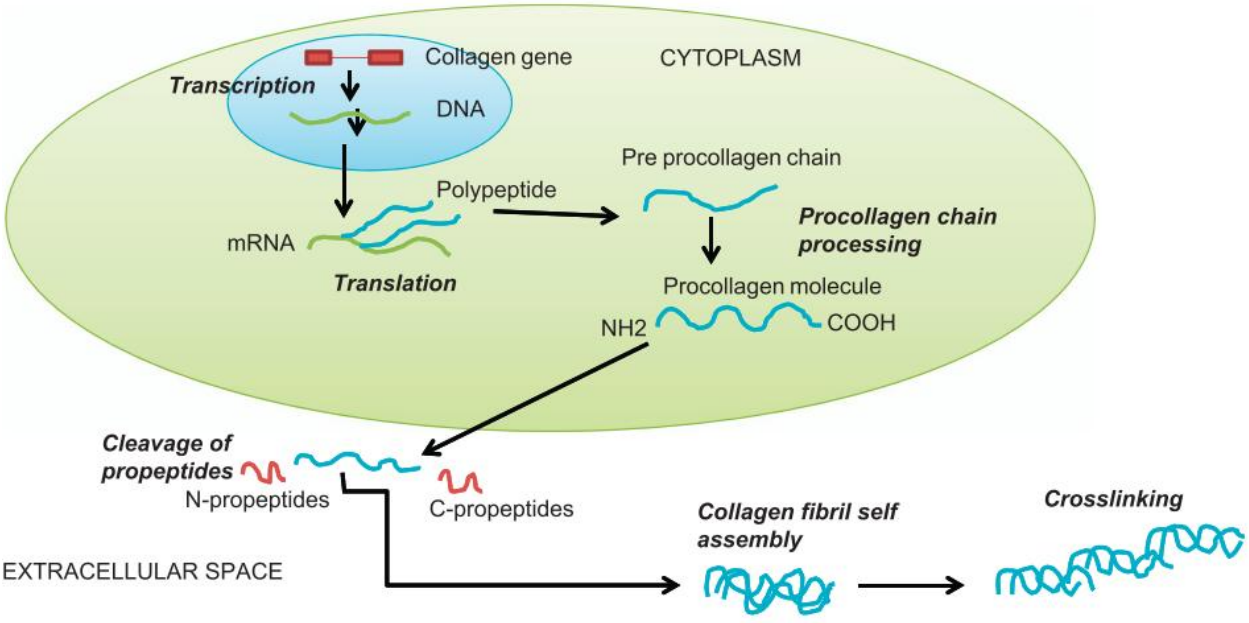

Figure 2.12. Biosynthesis pathway of collagen (Hookana et al., 2014).

As depicted in Figure 2.12, within the nucleus of cells, such as mammalian fibroblasts, genetic material is converted into RNA (Naomi et al., 2019). These genes are encoded for the pro alpha 1 and pro alpha 2 chains for type 1 collagen. Upon RNA maturation through various intron and exon

splicing, the mRNA is transported to the cytoplasm before being translated into their pro-alpha 1 or pro-alpha 2 chains. The processes of gene transcription and RNA splicing can lead to protein diversity. Through post-translation modifications mediated by intracellular enzymes, the three alpha helices are brought together to become a triple helical structure (Naomi et al., 2019). In fibril forming collagen, nearly 50% of the prolines are hydroxylated (Naomi et al., 2019). One of the important post translation modifications is the hydroxylation of proline at the 4 position and also lysine (Naomi et al., 2019). All intramolecular bonds will take place which include disulfide bonds between cysteine and the interchain hydrogen bonds that keep the helix tightly packed forming what is known as procollagen. The protein is then packed into a vesicle in the Golgi apparatus and secreted in the extracellular matrix where procollagen peptidases cleave regions of procollagen (non-helical N and C terminal regions), transforming them into tropocollagen. Through lysine, hydroxylysine cross-linking between tropocollagen molecules make collagen fibrils (Noorzai & Verbeek, 2020). These bundles then self-assemble through hydrophobic and charged amino acids, creating a staggered yet densely packed structure, forming striations every 680 angstroms (Noorzai & Verbeek, 2020). As these collagen fibers mature, these bundles become more cross linked through oxidases converting amino groups on lysine and sidechains of hydroxylysine to aldehydes, followed by a condensation reaction between these groups creating the link. With age, more and more crosslinks are formed, creating a more rigid fiber.

2.3 Collagen extraction techniques

Collagen extraction consists of many pre-treatment and post-treatment steps along with the extraction step. The overall process is dependent on the species and composition of the biomass. For sea cucumbers in general, it consists of steps outlined in the block diagram in Figure 2.13.

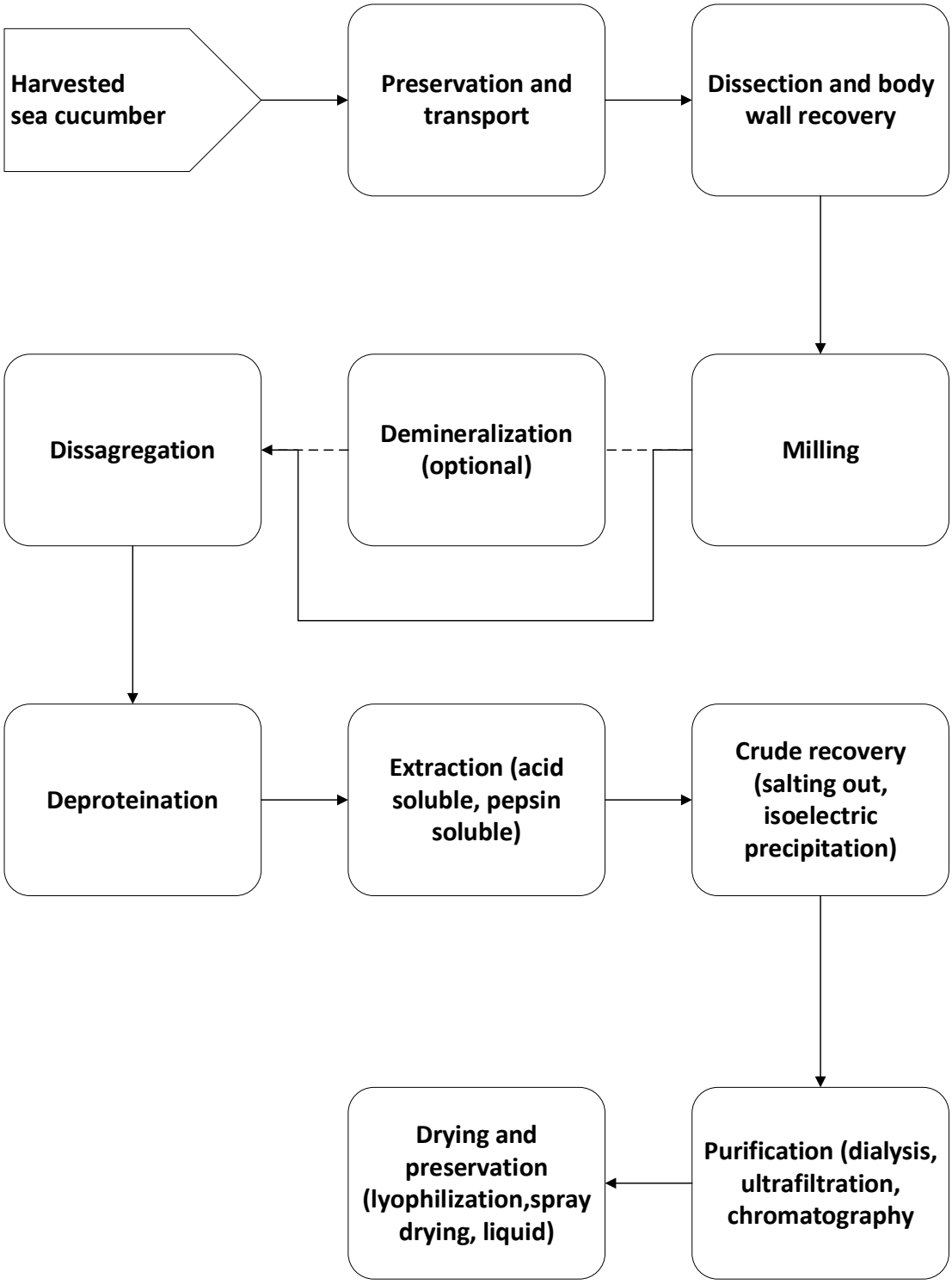

Figure 2.13. Block flow diagram of sea cucumber collagen extraction process.

2.3.1 Pre-extraction processing

To extract collagen from sea cucumbers, the resource needs to be processed and conditioned for the extraction chemicals to be able to appropriately penetrate the material and liberate collagen. Collagen interacts with itself forming fibrils but also other proteoglycans and glycosaminoglycans which form a highly organized and complex matrix (Farooq et al., 2024). There are non-covalent, hydrogen and ionic interactions with these molecules but there are also covalent intramolecular cross-links that need to be disrupted to ensure the collagen can be more readily extracted. The collagen fibrils in sea cucumber are very insoluble because of these interactions (Matsumura et al., 1973). Marine biomasses can be exposed to a variety of pre-treatments including size reduction, dilapidation, deproteination, discoloration, demineralization and heavy metal removal. As shown in Figure 2.13., the pre-treatment steps involved in collagen production from sea cucumbers in undergo size reduction, deproteination and sometimes heavy metal removal. The use of a disaggregation solution is a unique step for echinoderms. It breaks down different intermolecular and intramolecular bonds, which increases the overall collagen extraction yield (Matsumura et al., 1973; Trotter et al., 1996).

2.3.1.1 Sample Preservation

Upon capture and death, sea cucumbers secrete endogenous proteases that begin to break down their bodies (Fan et al., 2024). Autolysis can be brought upon by UV damage, cutting, mechanical damage and environmental conditions. Cathepsin B, D, E and rMMMP-2 enzymes are thought to be directly linked to the cleavage of collagen (Fan et al., 2024). In the food industry, they are blanched to inactivate these proteases. (Fan et al., 2024). In the collagen market, this is not an effective method as it can break down the collagen in the body wall by thermal degradation. The sea cucumber can also be dried or dehydrated before shipment. If using conventional drying techniques such tray drying, similar issues with thermal degradation can damage collagen in the body wall. The product can also be lyophilized but this method is costly and since collagen must be extracted with acids, water and other materials, removing this moisture and rehydrating it later can be seen as counterproductive. Freezing is a common way to preserve the material, however it has been observed that collagen degradation and tissue softening can occur (S. Li et al., 2022). S. Li

et al. (2022) showed that frozen sea cucumber and their myofibril proteins degraded over a 7-day period compared to live storage methods. The low temperatures had an impact on the proteins. The optimal approach would be to extract the protein as soon as possible after catching sea cucumbers. However, industrial processing capacity and logistical limitations make this option impractical. Additionally, there is a greater chance of microbial contamination due to the materials' high-water content and the favorable temperatures for bacterial growth in the surrounding environment. Freezing can reduce the risk of bacterial contamination. The frozen method also does not allow the protease enzymes to degrade sea cucumber, given these enzymes do not function at these temperatures.

The number of times a resource is frozen and thawed before its body wall is extracted is another crucial factor when using the freezing method. According to research, properties like water retention, color, texture, and protein content gradually deteriorate with each cycle (Tan et al., 2018). Other observers noted that the freeze-thaw cycle could help loosen the collagen in fish skin tissue, increasing the yield (Feng et al., 2025). Regarding the properties, they are generally affected. For instance, the thermal denaturation temperature of cow smooth muscle collagen decreases by about 10 ℃, and the shear force decreases by approximately 30% after 5 freeze-thaw cycles (Cao et al., 2022). The phenomenon arises from the transformation and recrystallization of ice inside the substance, leading to cell bursting and harming adjacent tissues. It additionally promotes protein denaturation through enhanced oxidation. However, after a single freeze-thaw cycle, the effects were much less: the thermal denaturation temperature only decreased by 4°C, and the shear force of the material remained unchanged (Cao et al., 2022). Freezing was used for this research project as the product needed to be stored for long periods of time (greater than 12 months) and the amount of freeze-thaw cycles of the starting biomass was limited to no more than twice but the majority of sea cucumber specimens underwent only one freeze thaw cycle. As will be discussed in section 3.2.1, the impact of freeze-thaw cycles on the biomass before extraction and the resulting yield and product characteristics were not examined.

2.3.1.2 Size reduction

The first pretreatment step is to rinse the sea cucumber and reduce the size of the body wall. The main goal of this is to reduce the size to increase the surface area favoring the penetration of solvent and enzymes and thus promoting extraction. Diffusion predominantly controls the mass transfer process. Reducing particle size will reduce the path that the chemicals need to travel in order to reach the solvent and perform their actions, which increases yield (Laasri et al., 2023).

2.3.1.3 Heavy metal removal/demineralization

Once the sea cucumber body wall has been reduced in size, the biomass can undergo a treatment to remove heavy metals. This is not done in all cases and is largely dependent on the environment of the species and the presence of industrial and commercial activities that emit waste containing contaminants such as heavy metals (S. Lin et al., 2017). To remove these metals, S. Lin et al. (2017) have developed a method to use chelating agent ethylenediaminetetraacetic acid (EDTA), which forms complexes with heavy metals and removes them for the body wall. The four carboxylic and two amino groups present in EDTA form coordination links with the positively charged metal counterparts (Spigolon et al., 2022). The body wall is left to soak in the EDTA mixture for 48-72 hours. The EDTA will also remove other common minerals present in the body, reducing the overall hardness of the body wall and increasing extraction efficiency (Kittiphattanabawon et al., 2010).

2.3.1.4 Disaggregation solution

As mentioned earlier, the body wall of sea cucumbers is highly ordered and complex. Figure 2.8, which can be found in Section 2.2.1, illustrates how collagen interact with other molecules within the extracellular matrix. These bonds, including covalent, electrostatic, and hydrogen bonds, come together to create a robust yet flexible network. To disrupt these interactions, two approaches for the disaggregation of these links. The Matsumura lab developed a solution using EDTA, NaCl, 2-mercaptoethanol (2ME) and Tris-HCl to break these bonds. To promote degradation of the bonds, the solution and biomass would be left for 24-72 hours (Matsumura et al., 1973). 2-mercaptoethanol is a reducing agent and is toxic. In an industrial setting, it may not be the ultimate option. In

the mid 1990's, the Trotter lab developed a protocol which used only EDTA and Tris-HCl to interfere with these interactions. The biomass was placed in this solution for 24-48 hours then followed by a treatment in water for another 24 to 48 hours to disaggregate the material.

The key difference between the two methods is the use of 2-mercaptoethanol in the Matsumura et al. (1973) studies and the Trotter et al. (1996) findings. 2-mercaptoethanol was used to break down disulfide bonds between various proteoglycans and collagen fibrils as it was shown that the denaturation of these fibrils is correlated with the reduction of disulfide bonds (Trotter et al., 1996). There are no interchain disulfide bonds in type I collagen. There are disulfide cysteine-cysteine bonds in the telopeptides regions of the protein when in its procollagen form, but they are cleaved before becoming the fully mature protein (Benjakul et al., 2012; Matsumura et al., 1973; Thurmond & Trotter, 1996). The Matsumura group thought that this was an essential chemical to disaggregate the sea cucumber body wall tissue. The Trotter group was able to disaggregate the *C. frondosa* body wall using only Tris and EDTA. This solution disrupts the ionic and electrostatic interactions between the collagen fibrils and other tissue components (Trotter et al., 1996). The Tris solution is also at a pH 8.0 which causes collagen to become negatively charged. Associated glycosaminoglycans are negative at this pH as well and the two molecules repel each other. In the Matsumura procedure, 0.5M NaCl has also been used to increase the ionic strength of the solution, which causes the collagen fibrils to interact with themselves and not the surrounding solvents, precipitating out of solution (Trotter et al., 1996). In certain cases, the use NaCl has been include in variations of the Trotter protocol as well. In the Trotter protocol, water is added after the Tris-EDTA solution. The collagen fibrils, which have complexed with each other, will then precipitate out of the water solution. A direct comparison to which disaggregation method is better in terms of yield has yet to be shown, but the Trotter method is more sustainable at an industrial scale due to its lack of toxic chemicals.

Matsumura et al. (1973) also say the EDTA does not have a major effect on the disaggregation. However as mentioned, it can help demineralize solutions and can inhibit metalloprotease activities, which reduce collagen yields and overall protein quantities (Laronha & Caldeira, 2020).

2.3.1.5 Deproteination

Before extracting collagen from sea cucumbers, a common procedure is removing any non-collagenous proteins. The least common method consists of using mildly acidic treatments. These are suitable for materials with a low number of crosslinks. In the case of sea cucumbers, the method is unsuitable given the number of cross links in the network (Oslan et al., 2022).

The alternative approach is alkaline pretreatment, such as sodium hydroxide. This method depolymerizes intermolecular and intramolecular links which allows the removal of non-collagenous proteins (Oslan et al., 2022). This pretreatment method is based on the understanding that most proteins are insoluble in alkaline solutions (Spigolon et al., 2022). Compared to other alkaline chemicals such as calcium hydroxide, sodium hydroxide also has great swelling capabilities which increases the extraction efficiency further down the processing scheme (Nurhayati et al., 2021). Sodium hydroxide to an extent also removes lipids through saponification reactions (Nurhayati et al., 2021).

Typical concentrations of sodium hydroxide used at this stage range from 0.05 to 0.1 M (Oslan et al., 2022). Concentrations above 0.5 M can permanently denature the protein (D. Liu, Wei, et al., 2015). Concentrations between 0.2M and 0.5 M have been shown to decrease the production of soluble collagen at the end of the process (D. Liu, Wei, et al., 2015). Another parameter that had an impact on the deproteination step was temperature. At 20 ℃, higher deproteination rates are observed compared to doing this step at 4 ℃. In the case of marine collagen, the final product structure can be impacted given the low thermal stability properties of marine collagen. In industrial production, this step has been the subject of scrutiny and attempts have been made to replace it by increasing the enzyme concentration (1200–1300 U/g) and the acid concentration (0.6 M acetic acid). While this approach has been effective, it can also affect the collagen structure due to the harsher extraction conditions (Rajabimashhadi et al., 2023).

2.3.1.6 Other pre-treatments

Another pre-treatment step seen in collagen processing is delipidation. This step is not commonly seen in collagen production from sea cucumbers given the low amounts of lipids in this animal.

Solvents such as butyl alcohol and ethanol are used for this purpose (Farooq et al., 2024). This uses the properties of miscibility to allow fat substances to separate in these solvents. As for depigmentation, hydrogen peroxide is a common chemical used (Senadheera et al., 2020). Hydrogen peroxide forms radicals which perform oxidoreduction reactions on the pigments which causes them to lose their color. It has also been shown that the addition of hydrogen peroxide does not impact the yield and in fact, can slightly increase it (Lee et al., 2022).

2.3.2 Extraction processing

The following section will present different techniques of the extraction step in order to liberate collagen from the source material.

2.3.2.1 Acid soluble collagen (ASC) extraction

Chemical/acid hydrolysis is one of the most common extraction methods used to extract native collagen from the tissues of animals. Once the chosen biomass has undergone the various pre-treatments described earlier and depicted in Figure 2.13., it is subjected to an acid treatment. The mixture is constantly stirred for 24 to 72 hours at a temperature below the thermal denaturation point of collagen. This temperature depends on the raw material in which the collagen was derived from (A. Liu & Zhang, 2018; L. Wang et al., 2014). Acid is used as a solvent as opposed to water given collagen is much more soluble in acid. This is because of the cross-links in the triple helix structure of collagen and the hydrophobic side chains in its native fibrous form. Acetic acid can disrupt various intermolecular cross-links within a collagen molecule and intramolecular bonds between molecules in a fibril. (Ahmad et al., 2010; Benjakul et al., 2012; Jafari et al., 2020; Nurilmala et al., 2019). The following diagram, Figure 2.14 depicts a bond disruption of two collagen molecules caused by acetic acid.

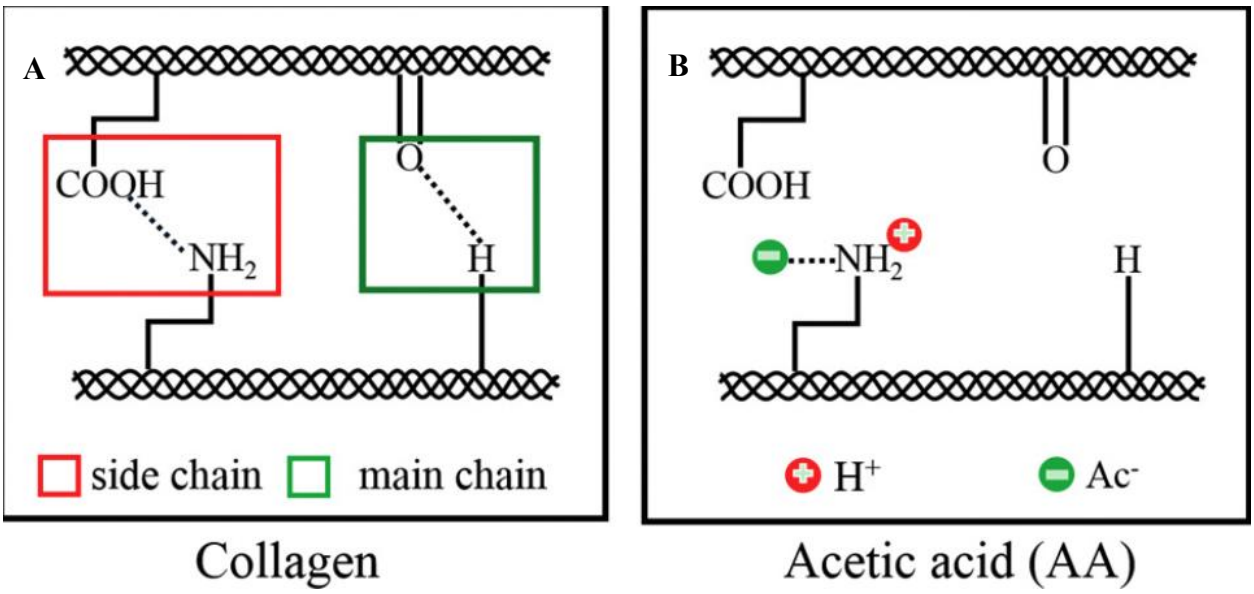

Figure 2.14. Schematic of (a) the interactions between native collagen molecules forming fibrils and (b) acetic acid disruption of intermolecular cross-links within collagen fibrils. Ac^- refers to the acetate anion and H^+ refers to the hydronium ion upon dissociation of acetic acid in water. Image is apart of a graphical abstract (Yang et al., 2018).

Figure 2.14 shows an intramolecular hydrogen bond between two neighboring molecules (Ahmad et al., 2010; Nurilmala et al., 2019) . Other than the hydrogen bonds, electrostatic bonds are disrupted. Each tropocollagen molecule becomes more positively charged, causing electrostatic repulsion that separates two molecules (Benjakul et al., 2012; Senadheera et al., 2020). Similarly, non-covalent intermolecular cross-links are disrupted to further increase solubility (Davison et al., 1972; Jafari et al., 2020; Oslan et al., 2022). This allows individual molecules to dissolve freely in the acidic solution.

Other important parameters which are tuned to optimize the extraction are the mixing rate, temperature, solvent type, and solvent: mass ratio. Temperature is an operating parameter which has important trade off with marine collagen. Raising the temperature of the extraction solution can boost mass transfer by lowering its viscosity. Also, collagen extraction has been shown to be an endothermic process and increases in temperature will increase product yield (Ahmad et al., 2010). However, it can also damage marine collagens, which, depending on the species, can start to deteriorate at temperatures above 20 °C (Jafari et al., 2020). All of these are maximized to increase the

mass transfer of collagen from the solid phase to the liquid phase. To determine the optimal solvent for the extraction process, various organic acids, such as acetic acid, citric acid, and lactic acid, as well as inorganic acids like hydrochloric acid, were subjected to testing (Sadowska, 2007). For each acid, the collagen was not completely soluble due to fact some intermolecular cross-links in the collagen were still present. Organic acids provide better solubilization as opposed to inorganic acids since the very low pH's produced by inorganic acids cause the positively charged amine groups of collagen to bind free anions in solution. This reduces the electrostatic repulsive forces causing the collagen to tighten its fibers, limiting the access and solubility in water (Sadowska, 2007).

While this process has shown to be easy and affordable, it is very time consuming, uses harsh solvents and has relatively low yields (Felician et al., 2018; Schmidy et al., 2016). To increase the yields, enzymes were used to further help solubilize the collagen in the aqueous phase and degrade other non-collagen proteins in the process. This process is known as the PSC process.

2.3.2.2 Pepsin/enzyme soluble collagen (PSC) extraction

The pepsin soluble collagen extraction process is the most used extraction method in the collagen extraction industry. The process is very similar to the ASC process however it uses protease enzymes to further increase the solubility of collagen in the acidic solution. A figure describing how pepsin augments the process to solubilize collagen is shown below.

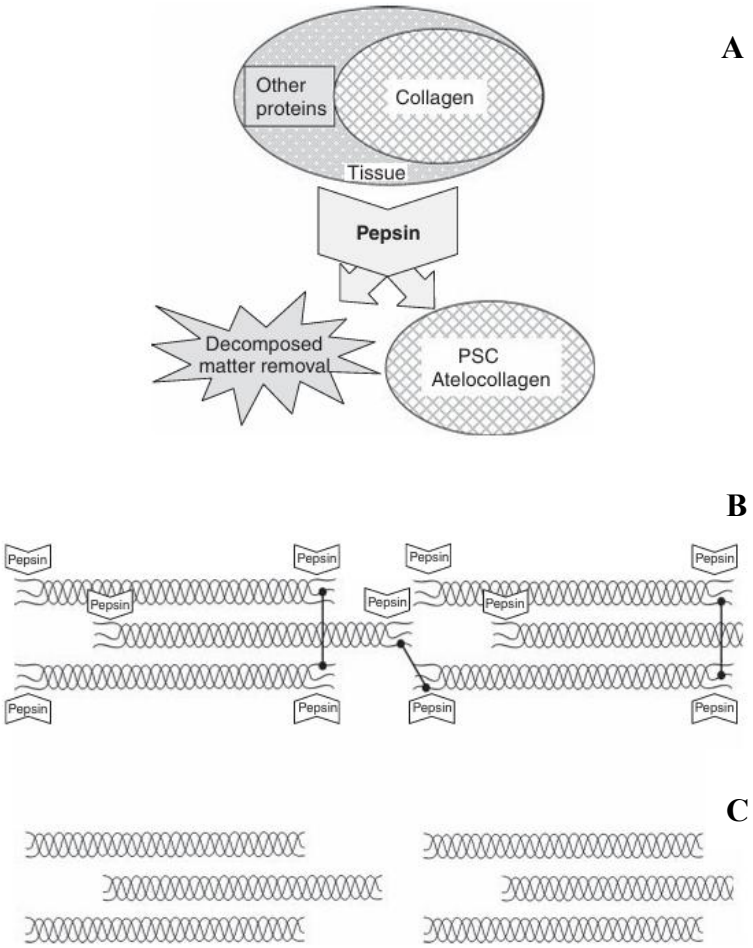

Figure 2.15. Schematic of (a) collagenous tissue degradation through pepsin treatment, (b) cleavage of the telopeptide region of collagen by pepsin and (c) atelocollagen after telopeptide cleavage by pepsin (Benjakul et al., 2012).

As depicted in Figure 2.15.a, when collagenous tissue is exposed to the proteolytic enzyme pepsin, it results in the formation of atelocollagen. This type of collagen has undergone modification, specifically the removal of the non-helical N-terminal and C-terminal segments, which are referred to as telopeptide regions. As illustrated in Figure 2.15.b, pepsin has the unique ability to identify and cleave these non-helical ends, ultimately yielding atelocollagen, as shown in Figure 2.15.c. The telopeptide region has strong intermolecular covalent crosslinks that cannot be cleaved, which decreases the solubility of the collagen (Felician et al., 2018). On top of increasing efficiency and product solubility, the protease also cleaves some non-collagenous proteins which increases the purity of the final product. These terminal ends present major antigenic effects as they have been

shown to form strong macromolecular interactions with antibodies to elicit antigenic responses (Lynn et al., 2004). Collagen contains additional antigenic sites in its core structure, and pepsin may not always perfectly digest its ends. As a result, collagen may still possess antigenic properties, albeit to a lesser extent (Lynn et al., 2004).

Various enzymes have been tested in enzyme aided-collagen extraction such as trypsin, alcalase, papain but pepsin is the most abundant enzyme used in industry (Felician et al., 2018). While the process remains relatively easy and the expense of adding the enzyme is relatively affordable when considering the increased yields, the process still requires a lot of solvents and is quite time consuming.

In terms of enzyme catalyzed reactions, there are parameters that need to be carefully monitored and controlled. For pepsin, the parameters include temperature and pH, and their activity curves are demonstrated in Figure 2.16 below.

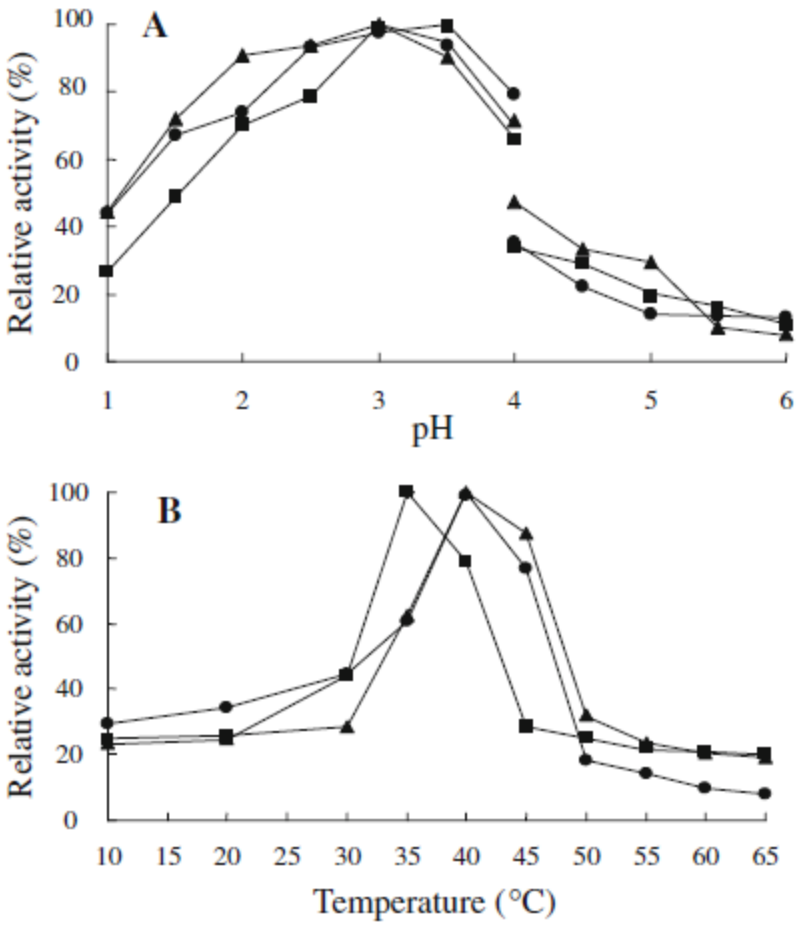

Figure 2.16. Examples of (a) pH versus activity curve of pepsin and (b) temperature versus activity curve of pepsin (Weng et al., 2011). Three different pepsins were purified from rice fish eel (*Monopterus albus* Zuiew) which are labelled as pepsin 1 (P1, circle), pepsin 2 (P2, triangle) and pepsin 3 (P3, square) in this figure.

In Figure 2.16.a describing pepsin activity with respect to pH, the optimal point is around 3 to 3.5 (Weng et al., 2011). From pH 2 to 4, the relative activity of pepsin 1 (P1, circle), 2 (P2, triangle) and 3 (P3, square) stayed be 70 % to 95 % and as it approaches pH of 6, it drops off to less than 20%. Above a pH of 5.5, the stability of pepsin begins to decrease and becomes nearly loses all activity (Piper & Fenton, 1965). These pH activity trends are applicable for pepsins 1, 2 and 3 which have been purified from the stomach of rice fish eel (*Monopterus albus* Zuiew)(Weng et al., 2011). Similar pH ranges and trends were also reported by a group who studied the activity

and stability of pepsin, for their clinical importance (Piper & Fenton, 1965). Increasing the solution's pH above 7 can be an effective way of permanently denaturing pepsin (Konno et al., 2000) once the extraction process is finished, which will prevent further collagen degradation. Given most pepsin protocols are in acetic acid solutions at 0.5 M, the pH is typically around 2.8 and therefore the pepsin will have good activity in this range. In Figure 2.16.b, the optimal temperature of pepsin is around 35 to 40 ℃ and begins to drop off before or after this point (Weng et al., 2011). This optimal temperature range was the case for each pepsin tested (P1, P2, P3) by Weng et al. (2011) as shown in Figure 2.16.b. Similar results were observed by a group who immobilized pepsin on chitosan-silica beads and they observed optimal temperatures around 40 °C (Cahyaningrum & Sianita, 2014). The norm for native collagen extraction procedures is typically done at 4 ℃, where the pepsin activity is far from its optimal point. This can suggest why complete digestion of the protein does not occur and why the process requires days of extraction time for pepsin to be effective. It is important to note with respect to Figure 2.16 above, that while pepsin 1, 2 and 3 were distinct, they were derived from the same source and have similar chemical traits such as molecular weight, pI and enzyme activity rates (Weng et al., 2011). Therefore, even though different variants of pepsin exist, they share similar optimal activity ranges in terms of pH which was 3 to 3.5 and temperature 35 to 40 ℃ (Weng et al., 2011). This demonstrates that the enzyme is predicable and can be controlled to an extent in a production process.

2.3.2.3 Ultrasound assisted extraction

Ultrasound assisted extraction (UAE) uses the creation of mechanical waves in a solution to produce compressive (high pressure) and rarefaction (compression) in molecules, which causes a cavitation force. A schematic of the process is shown below.

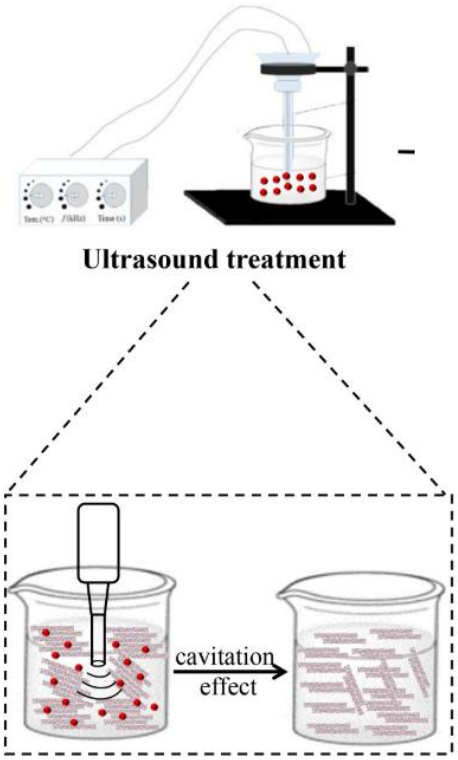

Figure 2.17. Schematic of ultrasound assisted extraction (UAE) of collagen (H. Liu et al., 2023). Image was apart of graphical abstract from the H.Liu et al. (2023) article.

As depicted in Figure 2.17, the force of cavitation led to the breakdown of collagen protein bundles in tissue into individual collagen molecules, thereby facilitating their exposure to acidic treatment (Kyung et al., 2013). Both covalent and non-covalent bonds are broken from this type of treatment (H. Liu et al., 2023). The production and extinction of microbubbles causes temperature increases up to 5000 K and 1200 bar in these defined turbulent locations (Song et al., 2018). This allows for the breakdown of cell walls, which ultimately decrease particle size and increase the mass transfer of the solvent into the solid allowing greater diffusion of soluble biomolecules into the liquid phase. In a study by Kyung et al. (2013), they showed that they could increase the collagen yield in sea bass from 20% using the traditional PSC process to nearly 90% and drop the extraction time from 3 days to less than a day.

The relationship between a solution's solvent-to-mass ratio, pH, salt concentration, temperature, and stirring speed, as well as the intensity of ultrasound, significantly influences both the rate and

efficiency of extraction. An experiment looked at the ultrasonic power applied to the system (between 0 to 200 W) and how it affected the extraction of collagen. What was observed was that the extraction yield increased as the power was increased to 150 W and then it began to decrease with increasing power inputs. This may be due to the fact that excessive ultrasonic power may decrease the collagen solubility or destroy the structure (Zou et al., 2020).

Ultrasound assisted extraction technologies aid with decreasing process time, waste, labour cost, cold storage equipment, energy, and the amount of chemicals consumed while maintain similar purities to commercially available type 1 collagen (Dahmoune et al., 2014; Song et al., 2018). The UAE does have some disadvantages. For example, if the frequency is set too high, the product may degrade. Additionally, if the probe sensitivity decreases, the extraction efficiency will also decline. Furthermore, the process requires very fine or small particles, which can only be achieved through specialized milling operations (Filgueiras et al., 2000).

2.3.2.4 Pressurized acidified water extraction

Pressurized acidified water extraction was proposed initially by Barros et al. (2015). A schematic of this extraction method is shown in the figure below.

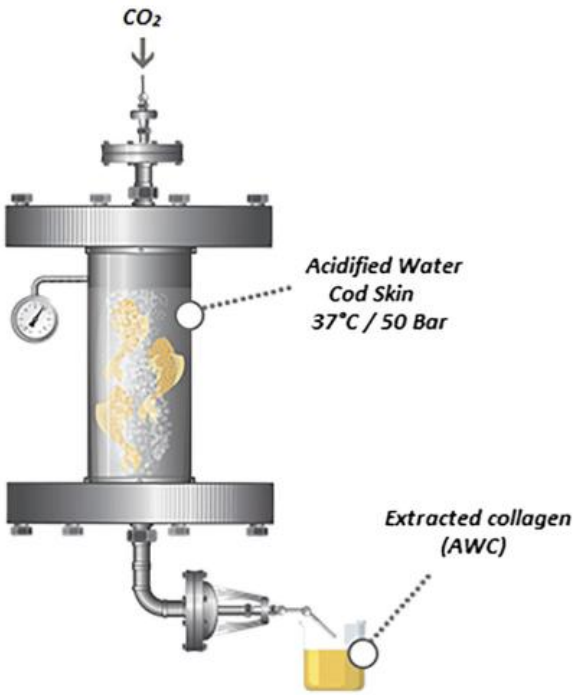

Figure 2.18. Schematic of pressurized acidified water extraction of collagen (Sousa et al., 2020).

As shown in Figure 2.18, this method combines water with carbon dioxide at a high pressure which causes the carbon dioxide to dissolve in water, forming carbonic acid and acidifying it to a pH between 3.0 and 3.4 (Silva et al., 2016). This condition promotes the dissolution of collagen, a substance that is soluble in acidic environments with a pH of around 3, causing it to diffuse from the solid tissue into the liquid phase (X. Lin et al., 2019). Temperature has an impact carbon dioxide solubility where a decrease in temperature increase CO_2 solubility (Zubal et al., 2018). This is due to the fact the dissolution of gases in water is an exothermic process and given the dissolved reaction of carbon dioxide is reversible, the lower the temperature increases the solubility of CO_2 as it favors the forward reaction. It is important to keep the temperature relatively low especially for marine collagen given it has a low thermal degradation temperature (Barros et al., 2015; Silva et al., 2016). This process does not use any organic solvents, which is great in terms of having an environmentally sustainable process. Silva et al. (2016) looked at optimizing this process for marine sponges where they varied variables such as extraction time and vessel pressure/concentration of CO_2. They optimized the reaction vessel to run at 10 bar for 3 hours where they obtained a yield of 11 %.

This method seems to be simple, relatively inexpensive and produces high yields. However, it works with highly pressurized equipment which presents a potential safety issue if proper care is not taken and the morphological features of the collagen have changed at temperature above 10 ℃ (Zubal et al., 2018). Zubal et al. (2018) suggested running the process at 4 ℃ and keeping it at high pressures at such low temperatures can be quite expensive.

2.3.2.5 Salt soluble collagen extraction

A simple and green method to extract collagen is known as salt soluble collagen (SSC) extraction. This method uses a salt bath (such as sodium chloride) for an extending period of time to extract the collagen proteins from the material (Zhou et al., 2016). With concentration of neutral salt less than 1 mol/L, the salt increases the solubility of the collagen by decreasing the electrostatic interactions between each protein, whereas when the concentration goes above 1 mol/L, the collagen begins precipitating out of the solution due to the competition with the salt for neighboring water molecules. The yield of collagen using SSC extraction (1.13 %) is much lower than ASC (14.49 %) or PSC (49.10 %) extraction (Zhou et al., 2016). This is expected as the purpose of the acid and enzymes is to expose amino acids that are cross-linked and further extract the collagen from the solid phase into the liquid, whereas the salt has a less of impact on these cross links and therefore leads to lower solubility (Yang & Shu, 2014). Typically, SSC extraction yields are not high in marine organisms (García-Sifuentes et al., 2016).

In general, each collagen shares similar chemical and physical properties with some slight differences. Compared to PSC extracted collagen, the Zhou et al. (2016) study showed the molecular order of the collagen produced from the SSC extraction method was higher than PSC as the enzymes in PSC method can alter the native form of collagen. SSC collagen had the lowest glycine content in the study by Zhou et al. (2016). This can be due to impurities of other proteins left in the salt extraction process and would need further purification to reduce the amount of non-collagen protein (Zhou et al., 2016). SSC collagen had the lowest thermal stability which could also be attributed to these impurities. In summary, while the salt method preserves many properties of the original structure of collagen, its yields make it difficult to scale for commercial applications.

2.3.2.6 Sodium hydroxide extraction method

The alkaline method soaks the collagen rich materials in alkaline solutions such as sodium hydroxide over a period. This technique eliminates all amino acids that have sulfhydryl and hydroxyl groups (Naomi et al., 2019). The method also has shown to easily denature the protein by cleaving the peptide chain (Suo-Lian et al., 2017). This reduces the proteins nativity and can affect the functionality of the protein. This method has been used to extract collagen type I, especially in the leather waste but has its drawbacks (Naomi et al., 2019). The alkaline liquid effectively breaks down the collagen in leather scraps, producing low-molecular-weight versions of the protein (Yang & Shu, 2014). The interaction of low-molecular-weight collagen with elevated levels of ash and chromium that was present in the starting source material will lead to the breakdown of amino acids that contain hydroxyl and sulfhydryl functional groups (Yang & Shu, 2014). This results in drastic property changes in the molecule, making it unsuitable for applications like those in the medical field (Yang & Shu, 2014). Alkali-solubilized collagen has been shown to lose its ability to form fibrils under physiological conditions (Suzuki et al., 1999). The alteration in the collagen structure caused by alkaline treatment disrupts the intermolecular fibril interactions, making the protein useless for fields of medicine such as tissue scaffolding (Suzuki et al., 1999). This method also is paired with extraction at high temperatures between 130 ℃ to 180 ℃ (Pal & Suresh, 2016). Given marine collagen denatures at higher temperatures, this dramatically impacts and alters the collagen to be studied in the following set of experiments. In terms of yields, alkaline collagen extraction rate is lower than other methods presented in this section (Suo-Lian et al., 2017).

2.3.2.7 Production of gelatin and collagen hydrolysates

In lower grade applications, collagen is further hydrolyzed to produce smaller fragments which can be as small as 500 Da. Many of the pretreatment steps as discussed above are applicable when extracting gelatin and collagen hydrolysates (J. Wang et al., 2010; M. Zeng et al., 2007). They are done at higher temperatures to speed up the process, as a native product is not desired. Degradation of the triple helix due to temperature increase does not matter for gelatin and hydrolysate products. There are methods, that also use hot distilled water at 40-45 ℃ for 8 - 24 hours to extract the

gelatin or hydrolysates from the material (J. Wang et al., 2010; M. Zeng et al., 2007). Other protocols use the pepsin-acid soluble procedure at higher temperatures (40-55 ℃) and shorter reaction times (1 - 5 hours) to get the hydrolyzed products (Abedin et al., 2015). The reaction will take place much more rapidly for pepsin, as it operates better a temperature near 37 ℃ compared to 4 ℃ when producing intact collagen. There are also protocols that produce collagen though the pepsin acid method then follow by protease hydrolysis using enzymes such as neutrase, which cleave specific sites on the collagen backbone to give these degraded products (Fawzya et al., 2020).

2.3.3 Post extraction and purification techniques

Upon collagen extraction, there remains impurities in the solution including solvents, salts and small molecular weight molecules that were not removed in the pre-treatment process. To enhance the quality and purity of the obtained collagen, it is necessary to eliminate undesirable substances and prevent deterioration during the final processing steps. This can be achieved through a series of post-treatment procedures, ultimately leading to collagen that is suitable for storage. These steps summarized in Figure 2.13 which include crude recovery, purification, and preservation. In literature, dialysis, chromatography, solvents, salting-out centrifugation and filtration have been studied extensively. The selected technique is highly dependent on the intermolecular interactions and the collagen solubility in the media after extraction. This task can be difficult, especially considering the distinct types of collagens and the variety of diverse tissues which they are derived from (Senadheera et al., 2020). Before any purification techniques, the collagen molecules need to be precipitated out of the acid solution. To perform this precipitation, two techniques are used which are (1) salting out method and (2) neutralization or isoelectric precipitation.

2.3.3.1 Salting out method

Salting out is the most common method used to perform the primary crude purification step. This method incorporates the principle of ionic strength of the solution to precipitate proteins and is showcased in the following figure.

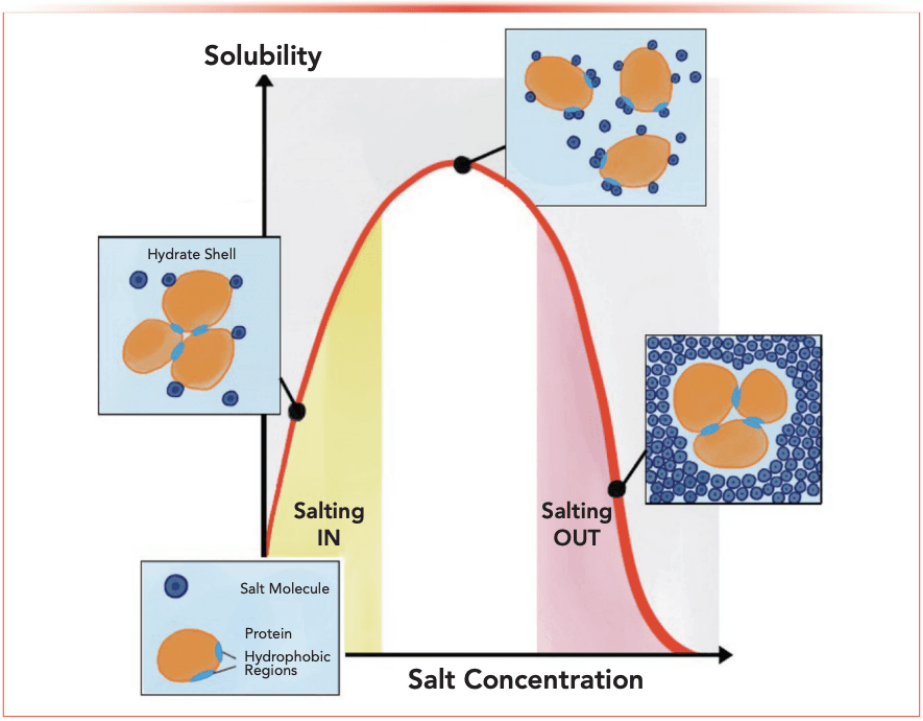

Figure 2.19. Summary of the effect of salt concentration on protein solubility. (Raynie, 2023).

In Figure 2.19, at low salt concentrations, the protein's solubility can actually increase as the salt concentration in the solution rises. This phenomenon, known as salting in, continues until it reaches a maximum protein solubility. Any further addition of salt will then cause the protein's solubility to decrease, a process referred to as salting out (Raynie, 2023). As shown in Figure 2.19, when the ionic strength of the solution exceeds the maximum solubility point, the solubility of proteins decreases. Neighboring solvent molecules begin to interact with the salt molecules instead of the proteins, causing them to aggregate through hydrophobic interactions. This is known as salting out. At low salt concentrations, the salts behave according to the Debye-Heckel theory and at higher concentrations, the effectiveness of each salt follows the Hofmeister series (Hyde et al., 2017; Okur et al., 2017). In terms of collagen, the solubility of collagen in solution significantly drops off when sodium chloride reaches 2-4 % (0.35 M-0.7 M) (Abedin et al., 2014; P. H. Li et al., 2020; M. Zhong et al., 2015). However, many protocols still use up to 0.8 M to ensure all collagen have exited the solution (Saito et al., 2002). According to Hyde et al. (2017), the Debye-

Heckel theory is only valid up to concentrations of 0.1 M without any additional empirically derived parameters. Therefore, the Hofmeister series is more suitable for post-treatment purification methods of collagen (Oechsle et al., 2015). The Hofmeister series is illustrated in Figure 2.20.

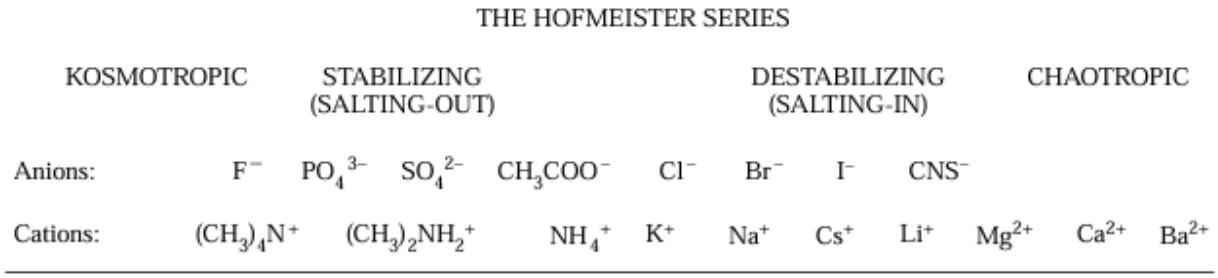

Figure 2.20. The Hofmeister series for anions and cations (Cacace et al., 1997).

According to Figure 2.20, cations and anions can be categorized as kosmotropes or chaotropes. Kosmotropes interact well with water molecules, causing the protein's solubility in solution to decrease and precipitate (Hyde et al., 2017; Kang et al., 2020). Chaotropes have a poor affinity for water, which can cause proteins to lose their structure and increase their solubility in the solution (Hyde et al., 2017; Kang et al., 2020). The ions and molecules to the left are more kosmotropic, while those to the right are more chaotropic. Sodium chloride (NaCl) is often used as a salting-out agent, but other salts, such as potassium thiocyanate (KSCN), have also been shown to be effective at different concentrations (Oechsle et al., 2015). This is due to its position in the Hofmeister series, which reflects the specific ion effect and ionic hydration strength (Tobias & Hemminger, 2008). In essence, each salt has a unique concentration and potency for extracting proteins. The choice of salt may also depend on availability, cost, downstream processing constraints, health effects, and environmental impacts when it is disposed.

2.3.3.2 Isoelectric precipitation/neutralization

Another property that can be exploited to precipitate collagen is pH and its isoelectric point (pI). Proteins that make up collagen have a minimum of two ionizable groups which are the terminal acidic carboxylic group and the basic amino group. There are other amino acids such as lysine, arginine, histidine, aspartic acid and glutamic acid which have an additional group that the charge

is affected by the environmental pH. Each amino acid has its own pH where the charges are balanced on a molecule, its individual isoelectric point. In a protein, all the individual amino acids interact together to form this overall isoelectric point. The following figure demonstrates the phenomena for this purification technique.

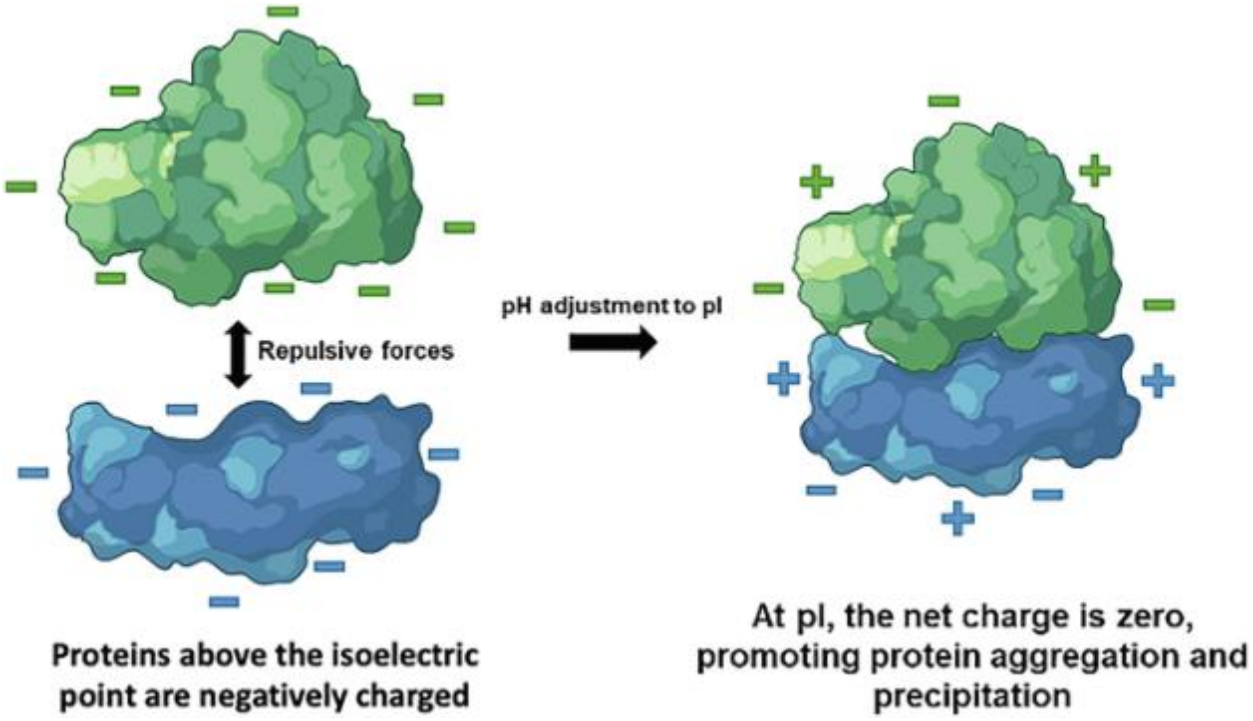

Figure 2.21. Example of isoelectric precipitation when the surrounding environmental pH is initially greater than the protein's pI (Cruz-Solis et al., 2023).

According to Figure 2.21, when the pH of the protein's environment is higher than the pI, the charges are predominantly negative and repel each other. In the same way, when the pH is below the pI, the proteins become positively charged and repel each other (Cruz-Solis et al., 2023). At neutral charge, the protein interacts with other proteins through hydrophobic interactions and aggregate together. A pH of 5-7 is the most common isoelectric point of collagen with the lowest solubility but this depends on species, collagen type and environment (X. Lin et al., 2019). This approach is preferable to salting out due to the reduced salt output, which necessitates less downstream purification and uses less water (X. Lin et al., 2019; Spigolon et al., 2022). Regarding final

output, Vate et al. (2023) demonstrated that yields remain consistent whether salting-out or isoelectric precipitation techniques are used, with the latter producing a purer product. The thermal stability and fibril formation did not change, no matter which technique was used. In an industrial setting isoelectric precipitation would be favored, given less processing time and material required to put into the process.

2.3.3.3 Dialysis

A common purification technique used, especially in lab-scale size extracts, is dialysis. Figure 2.22 provides an illustration of a collagen extract undergoing at a lab-scale.

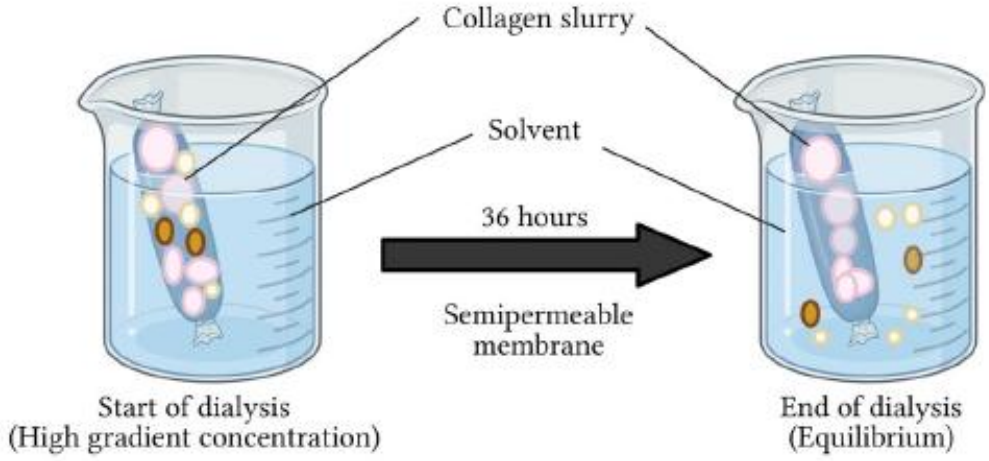

Figure 2.22. Schematic of lab-scale dialysis treatment of a collagen extract (Mohamad Razali et al., 2023).

As can be seen in Figure 2.22., this method allows small macromolecules to pass through the semi-permeable membrane, while keeping the larger collagen proteins. The driving force of the separation operation is the chemical potential difference between the feed stream/source and the dialysate stream or surrounding solvent. It continues to occur until an equilibrium is reached (Lightfoot, 2004). This process is primarily dictated by diffusive mass transport processes, while in more concentrated systems, bulk flow also has an effect (Lightfoot, 2004). Additionally, the interior solvent of a dialysis bag or feed stream typically has a higher concentration of solutes than the permeate stream or reservoir. An osmotic pressure gradient is created that causes the solvent to

flow into the dialysis bag in a batch process or into the feed/retentate streams in a continuous or semi-continuous process (Lightfoot, 2004). Na_2HPO_4 and distilled water are the primary agents used in dialysis, particularly in the deactivation of enzymes in PSC treatment and the preservation of collagen molecules, respectively. (Mizuta et al., 2022). The semipermeable membrane is made of polymer materials such as cellulose, PES and poly(ethylene-co-vinyl alcohol). Industrial dialyzers take configurations including the tank type, plate and frame type and tube type. However, commercialization of this is difficult (Noorzai & Verbeek, 2020). Dialysis is not as efficient as other separation unit operations. As shown in a study by Saallah et al. (2021), ultrafiltration techniques gave a yield of around 11.4% whereas the dialysis-salt out technique gave a yield of 5.2 %.

2.3.3.4 Filtration

Filtration is another method to concentrate and purify collagen. Typically, ultrafiltration is used to purify collagen with membrane cutoffs less than100 kDa (Noorzai & Verbeek, 2020). A schematic of an overview of the process is presented below.

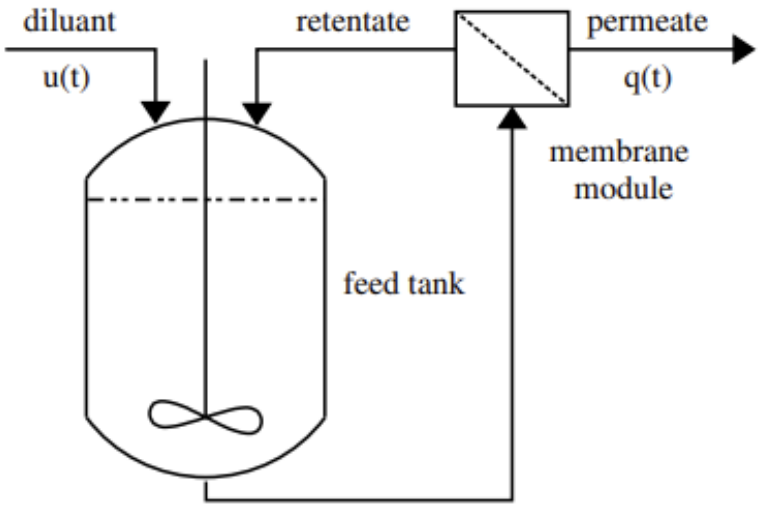

Figure 2.23. A simplified schematic detailing the ultrafiltration process (Jelemenský et al., 2016).

As can be seen in Figure 2.23, the sample or diluent is added to the feed tank. From there, it flows to the membrane module. In this module, the inlet stream is split into the retentate and permeate. A typical configuration is shown, where the retentate is recycled back to the feed tank, and the

permeate is collected in a separate reservoir. The process can operate in reverse, with the permeate being recirculated, while the retentate is collected separately, or neither of them needs to be recycled, but they are stored in separate containers. For our project, collagen is retained on the retentate side of the membrane while allowing the permeate to pass through with smaller non-collagenous protein, salts and other matter (Noorzai & Verbeek, 2020). The retentate is recycled back into the feed tank in our experiments so collagen can be collected. The driving force for this unit operation is pressure (typically 2 to 10 bar). Filtration separates molecules based on their size and incorporates both diffusive and bulk flow mass transport processes. Depending on the solute - membrane interactions, there are charge and shape factors that can affect the transport. Depending on the membrane, molecular weights between 300 to 500 000 Daltons can be separated (Lightfoot, 2004). Membranes are made of polymeric material such as polysulfone, cellulose and polyamides or inorganic materials such as pyrolyzed carbon (Lightfoot, 2004). In an experiment conducted by Scutaria et al. (2019), they observed the optimal transmembrane pressure difference to purify the collagen was at 5 bar They also noted that, as collagen proteins get larger, the rejection coefficient improves, but the membrane flux decreases. Confirming by scanning electron microscopy (SEM) experiments, they noticed collagen buildup on the membranes lead to decrease in the flux (Scutariu et al., 2019)

Ultrafiltration is commonly used on an industrial scale as it has high potential throughputs and is cost-effective (Noorzai & Verbeek, 2020). There are typically two configurations for ultrafiltration which are crossflow/tangential flow or dead-end filtration and are summarized in the figure below.

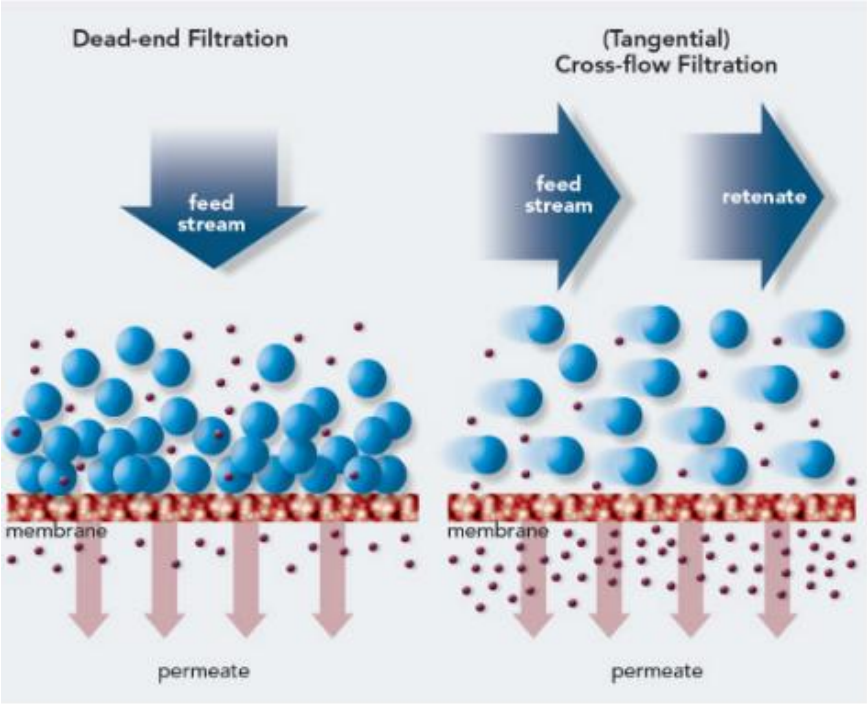

Figure 2.24. Comparison of dead-end filtration versus cross-flow (tangential) filtration (Paipuri, 2014).

As shown in Figure 2.24, in cross-flow filtration, the feed comes across the membrane parallel to it. The permeate passes through the membrane and collects in the center, then it is transported away. The existing feed stream can be mixed with the retentate or can be separated. Dead-end filtration passes through a filter and retentate product accumulates on the membrane whereas the permeate passes through.

While this method has been more effective in terms of yield compared to dialysis, it also has its limitations when purifying collagen. Membrane materials such as poly acrylonitrile are problematic since they are hydrophobic and cause severe fouling during the separation process (Awang et al., 2020). Mechanisms that cause this include chemical adsorption, salt precipitation and biofilm deposition. At an industrial level, this increases the amount of maintenance cost. Therefore, if ultrafiltration is desired, charged and hydrophilic materials are preferred. Even with this drawback, ultrafiltration is still considered an energy efficient and eco-friendly technique (Awang et al., 2020).

Compared to dialysis, the purification time can be significantly reduced, while maintaining the collagen's physical and chemical properties (Saallah et al., 2021). Collagen has a subtly distinct morphological structure when created through dialysis or ultrafiltration. This discrepancy can be attributed to the transportation method specific to each technique (Saallah et al., 2021). Dialysis relies more on diffusion whereas ultrafiltration relies on pressure gradient and the collagen becomes highly concentrated on the membrane surface, producing a flatter sheet structure.

2.3.3.5 Chromatography

Chromatography is a prevalent purification method in a wide range of industries, including pharmaceuticals, cosmetics and nutraceuticals, used in both laboratory and commercial settings. Chromatography is a broad ensemble of techniques which rely on different properties to purify the product. Some of these techniques include affinity chromatography, size-exclusion chromatography, ion exchange chromatography, and liquid chromatography. Each chromatographic technique separates the feed components by their affinities to the selected mobile and stationary phases. For protein separation to obtain highly pure products, these techniques are typically placed after size centrifugation and filtration operations (Senadheera et al., 2020).

In size exclusion chromatography (SEC), the liquid mobile phase passes through a porous polymeric stationary phase which separates the macromolecules based on their size. Cui et al. (2007) used gel filtration (a type of SEC) to further purify their collagen extracts from *S. japonicus* after initial dialysis purification (Cui et al., 2007). This technique is an attractive purification technique as it works based on physical properties of the protein and has minimal impact on its confirmational structure (Senadheera et al., 2020). The helical arrangement of collagen was maintained which is important to try to preserve the native functionality (Yan et al., 2017). Also, given its high recovery rate and compatibility with many physiological conditions, SEC is used quite extensively at the industrial scale (Senadheera et al., 2020).

Another chromatographic technique is ion exchange which utilizes the electrostatic interactions between charged proteins and the column matrix. Two types of matrices include cationic and anionic. Materials such cellulose, dextran, polystyrene and acrylic based products have been used as

matrices in these types of columns (Awang et al., 2020). For example, the Sato group used a diethyl aminoethyl cellulose packing material which allowed for a faster and higher yield purification of type V collagen (Sato et al., 2003). The packing resin interacts with charged side chains of the amino acids which are influenced by their respective acid dissociation constant (pKa). Collagen is an amphoteric molecule that has both positively and negatively charged sidechains. Its overall net charge is impacted by the solution it is in according to its isoelectric point. The pH of the buffer used in the separation process is also important as this could alter the charge of the compounds and matrix. The increase or decrease in the pH buffer can lead to protein contamination in the end. This method can create high concentrations of products without aggregation and regenerating the internal resin packing can save money. However, it is labor-intensive and there is a risk of contamination from bacterial growth within the resin (Awang et al., 2020).

2.3.4 End product processing and preservation

On the collagen market, products have been preserved in both liquid and powdered form. For powdered products, there are two common drying methods: spray drying and lyophilization. The most common method is lyophilization which dries materials through sublimation and therefore very effective in preserving properties of temperature sensitive products. While effective, they are expensive equipment to purchase, operate and maintain compared to other drying methods. Spray drying is used with collagen because the small particles dry very quickly in a hot gas. It is also effective with heat-sensitive products thanks to its short contact time. According to Latip et al. (2015), multiple chemical characteristics were found to be comparable between freeze-dried and spray-dried collagen using various characterization methods, including Fourier transform infrared spectroscopy. However, through microscopic analysis, it was discovered that the spray-dried collagen had contracted and was physically smaller than the lyophilized version. They deemed spray drying is still a viable technique depending on the final product and end application. Shaviklo et al. (2010) showed that freeze dried fish protein products had better functional and structural properties as opposed to spray dried. For example, the emulsifying capacity of collagen powder was 20 % greater in a freeze-dried product compared to spray dried product (Shaviklo et al., 2010).

The final product can also be in a liquid form which would be the retentate after the ultrafiltration or dialysis processes. The product can be concentrated to become viscous. While liquid collagen may be easier to incorporate into various products given it is already solubilized and ready to be absorbed into systems, its shelf life is shorter as it can be more easily oxidized in a solution by the formation of free radicals in water (Skibsted, 2010). Also, the increased moisture content allows for bacterial growth. Both products can be stored at 4 ℃ for short periods of time but for longer term storage, the products should be kept at least -4 ℃ (Noorzai & Verbeek, 2020).

2.4 Collagen characterization and quality control

To study the various chemical, structural and biological characteristics of collagen, a variety of methods can be used to verify its type, size, identity, purity, conformation, structure, thermal stability among other properties. A summary of methods is listed in the Table 2.2 below.

Table 2.2. Summary of quality control properties and test for collagen extraction.

Property	**Test**	**Indicator**	**Indicating values for type 1 collagen**
Collagen type, size, purity	SDS PAGE	Molecular weight	α_1 – 115 to 150 kDa α_2 – 115 to 150 kDa β – 200 to 250 kDa γ - 300 to 400 kDa (Jafari et al., 2020; Senadheera et al., 2020)
Identity, purity, size, nativity	Native PAGE	Molecular weight	300 to 400 kDa (Rajabimashhadi et al., 2023)
Structure, identification	Fourier transform infrared spectroscopy (FTIR)	Absorption wavelength in IR region	Amide I – 1600 – 1700 cm^{-1} Amide II - 1550 – 1600 cm^{-1} Amide III – 1220 -1320 cm^{-1} Amide A – 3400 – 3440 cm^{-1} Amide B –2900 -3100 cm^{-1} (Spigolon et al., 2022)
Purity, identification	Ultraviolet visible (UV-VIS) spectroscopy	Absorption wavelength in UV region	210 – 230nm (Senadheera et al., 2020)
Yield, function	Colorimetric hydroxyproline quantification assay	Concentration of hydroxyproline	Species dependent, 5-15 % (Senadheera et al., 2020)
Structure, thermal stability	Circular dichroism spectroscopy	Absorption polarized light	Positive band – 220 nm to 222 nm Negative band – 195 nm to 198 nm (Jafari et al., 2020)
Thermal stability	Differential scanning calorimetry	Denaturation temperature and heat flow	Species dependent < 45 °C (Z. Liu et al., 2010; Senadheera et al., 2020)
Morphology	Scanning electron microscopy	Electron scattering	Variable
Identity	Isoelectric point	Charge amino acids	4 – 7 (Benjakul et al., 2012; Senadheera et al., 2020)

2.4.1 Gel electrophoresis.

Gel electrophoresis separates proteins based off their molecular weight and charges. Gel electrophoresis can also be used to determine the isoelectric point (pI) of a protein by using pH gradient along the gel allowing proteins to migrate to determine when their overall net charge (zeta potential) is 0. In the case of collagen, this is the pH where the protein is the least soluble.

Two common types of gel electrophoresis methods are native polyacrylamide gel electrophoresis (Native PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). SDS PAGE analyzes the purity of the applied sample, and the type of collagen extracted. In the body wall of sea cucumber, typically type I is observed so distinct α1 and α2 helices would be expected. Certain groups claim that in various species of sea cucumber, they observe the conventional α1 and α2 helices while other groups have hypothesized that sea cucumber collagen consist solely of α1 (Saito et al., 2002). β (2 α helices) and γ (3 α helices) helices are also observed and the SDS treatment is not able to reduce protein linkages. The molecular weights of the α, β and γ chains are summarized in Table 2.2. Native gels are not used to identify the type but allow to see if the protein is still intact in its native form (between 300-400 kDa) or if it has broken apart into smaller fragments. Both Native PAGE and SDS PAGE provide insights into possible impurities in the samples under study.

2.4.2 UV-VIS spectroscopy

As mentioned in Table 2.2, UV-vis spectroscopy uses the principle of ultraviolet light absorbance by amino acids and proteins to monitor their purity. Typically, proteins absorb light between 260-280 nm due the amino acids tyrosine, tryptophan and phenylalanine (Cui et al., 2007; M. Zhong et al., 2015). The electronic transition occurring at these wavelengths is π-to-π* (meaning electron is excited from a π-orbital to a higher energy state π*-orbital) and is related to the conjugated double bonds of the aromatic functional groups that these amino acids have (Xiang et al., 2022). Collagen

contains very few of these amino acids, but they are long chains that contain many peptide bonds and their carbonyl groups. These groups from the peptide bonds absorb light at about 230 nm (Ju et al., 2020). The electronic transition occurring at 230 nm is specifically an n-to-π* transition (meaning electron is excited from a non-bonding state to a higher energy state π* - orbital). This transition is primarily influenced by the presence of carbonyl, carboxylic acid, and amide functional groups (Xiang et al., 2022; Zhang et al., 2023). The band at 280 nm (π → π *) is weaker than the 230 nm (n →π *) due to the higher concentration of functional groups that absorb light at 230 nm than at 280 nm (Lucas et al., 2006). The signals between 260 and 280 nm could mean non-collagen protein impurities. NaCl also absorbs light at around 210 nm and therefore peaks at this location could mean salt impurities (Tong et al., 2023).

2.4.3 FTIR spectroscopy

Fourier transform infrared spectroscopy gives key insights into the type of molecule and the secondary structure of the protein. When infrared radiation is applied to a molecule, it causes it to vibrate, bend, and stretch its various chemical bonds. An important chemical bond in proteins is the peptide bond, which is formed between the amino and carboxyl groups of two neighboring amino acids in their primary chain sequence. Table 2.2 shows 5 key bands that are particular to collagen. The amide A band corresponds to the N-H stretching vibrations (3400–3440 cm^{-1}) of this bond. The frequency can decrease if it is involved in hydrogen bonding with other protein chains (Benjakul et al., 2012; M. Zhong et al., 2015). Amide B signal is typically due to asymmetrical stretching of the C-H groups neighboring the peptide bond (2850 - 3000 cm^{-1}) (Benjakul et al., 2012). The amide I band's signal is associated with stretching of the C=O carbonyl group (1600-1700 cm^{-1}) of the peptide bond (M. Zhong et al., 2015). The amide II band's signal is created by the combined vibration of the N-H group (1580-1650 cm^{-1}) and C-N stretching (1000-1250 cm^{-1}) in the peptide bond (Jackson et al., 1995). The amide III band is the result of C-N stretching coupled with N-H in-plane bending, and CH_2 group wagging vibrations (1200-1350 cm^{-1}) found in the polypeptide backbone (Benjakul et al., 2012; Jackson et al., 1995). A strong sign of effective triple helix preservation is a ratio of approximately 1 between the amide III band and the peak at 1450 cm^{-1}. It has been shown that pepsin can have an effect slightly on this ratio, but the tertiary structure of collagen remained intact (Benjakul et al., 2012). Additionally, the closer the amide I

bands are to 1630 cm^{-1}, the more irregular the protein's secondary structure becomes. As this number increases, the protein's secondary structure begins to form its proper structure, which contains alpha helices (1645-1660 cm^{-1}) and beta-turns (1660-1700 cm^{-1}) (M. Zhong et al., 2015).

2.4.4 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is a method that measures the absorbance differences between left and right circularly polarized light of optically active or chiral molecules as a function of wavelength (Andrews & Tretton, 2020). CD spectroscopy gives key insights on the structure of, folding and binding properties of proteins (Oslan et al., 2022).

Light, a form of electromagnetic radiation, travels as a wave. Its electric and magnetic fields oscillate perpendicularly to each other (Jasco, 2020). Circular dichroism spectroscopy can operate at various points on the electromagnetic spectrum, including UV, visible, and sometimes infrared (Subadini et al., 2022). For the purposes of this project, however, we will refer only to CD signals in the UV-vis range (200 - 800 nm). Unpolarized light has oscillating fields in multiple directions. Linearly polarized light travels in a single plane, while circularly polarized light rotates and the electric field vector moves in a helical pattern around the axis of light propagation (Urbach, 2010). Circularly polarized light can be emitted in two directions: left-handed or right-handed. If the electric field oscillates counterclockwise, the light is left-handed polarized; if it oscillates clockwise, it is right-handed polarized (Urbach, 2010). Figure 2.25 shows how a CD spectrometer works.

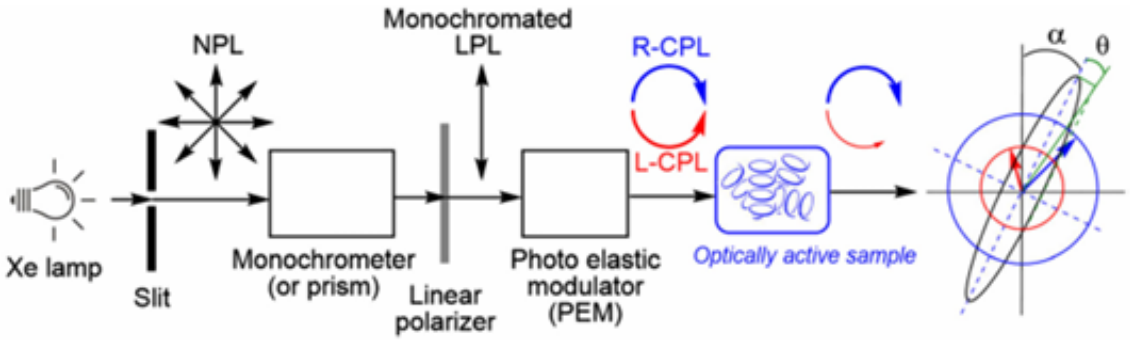

Figure 2.25. Schematic overview of circular dichroism spectrometry (Nakano et al., 2021).

According to Figure 2.25, the CD spectrometer emits naturally polarized light (NPL) that passes through a monochromator and a linear polarizer, producing linearly polarized light (LPL). Once the light is polarized, it is directed through a photoelastic modulator (PEM), producing both left-handed circularly polarized light (L-CPL) and right-handed circularly polarized light (R-CPL) (Jasco, 2020). When light passes through a chiral substance, its left- and right-handed circular components travel at different rates and are absorbed to varying degrees by the detector (Subadini et al., 2022). This enables the identification of the rotational angle α of a plane of polarization of LPL around the optical axis that intersects a chiral molecule. The spectrometer measures an elliptical angle or ellipticity θ, which is the tangent of the ratio of the minor to major axes of elliptically polarized light of a chiral sample (Nakano et al., 2021). The chirality of a molecule is the key characteristic of circular dichroism spectroscopy. The absorption difference between left-handed and right-handed molecules can only be observed with chiral molecules. This is because chiral molecules are asymmetric and preferentially absorb one type of light over another (Urbach, 2010). Proteins have many chiral centers along their polypeptide backbone, and their structure (such as secondary structure) can also affect the observed signal (Jasco, 2020). There are two absorption bands that are important for proteins: a strong π to π* transition around 190 nm, and a weaker n to π* transition around 210–220 nm (Jasco, 2020). Molecules that are achiral have a symmetrical structure and can absorb UV light, but they do not emit a signal that distinguishes between the two forms of circularly polarized light, making them undetectable by the apparatus (Presta & Stillman, 2005).

CD can be used to determine if the alpha helices are in a triple helix or denatured. It detects peptide linkage chromophores that absorbed powered radiation (Laasri et al., 2023). This method can be run at various temperatures which can give insight on what temperatures denature the protein. Before the analysis, the protein needs to be folded and in the case of collagen, this process takes quite some time therefore the protein should be placed on ice several hours to days before running the experiment. As mentioned in Table 2.2., marine collagen has a minimum at 198 nm and a maximum peak at 220 nm (Alves et al., 2017). The peak at 220 nm indicates the triple helix of collagen is intact (Alves et al., 2017).

2.4.5 Quantification of hydroxyproline

Hydroxyproline content gives not only an assessment of yield, but also purity and insight into thermal stability. There are HPLC methods that can separate the amino acid sizes based on chromatography principles, but common methods used are colorimetric (Senadheera et al., 2020). To convert from hydroxyproline concentration to total collagen, typically a factor of 7.57 is used (Senadheera et al., 2020). As depicted in Table 2.2, the yield can vary depending on the species (between 5 % to 15 %) (Senadheera et al., 2020). In this project, this assay was used to quantify collagen in the extracts as opposed to thermal stability which would look at determining the amount of hydroxyproline residues per 1000 amino acid residues.

2.4.6 Other

Other methods for studying protein characteristics include Differential Scanning Calorimetry (DSC), X-ray Diffraction (XRD), and Electron Scanning Microscopy (ESM). ESM examines collagen, pores, and other microstructures. XRD provides detailed information about protein structure and order, including secondary and tertiary structures. DSC is an irreversible process that measures the decrease in heat capacity as the collagen triple helix unfolds, while the primary structure remains intact (Senadheera et al., 2020). Table 2.2 provides more information on this technique, but it was not used in this project to monitor thermal effects of collagen, CD curves were used to estimate them instead. At the Centre de Recherche sur les Biotechnologies Marines (CRBM), we used various methods to study the extracts, carefully monitoring the effects of including, modifying, or excluding steps in the collagen extraction process.

Chapitre 3 - Materials and methods

3.1 Raw materials

Sea cucumber *Cucumaria frondosa* were harvested by Pêcheries Shipek (Ekuanitshit,QC) immediately placed on ice upon catch. Upon arrival at port, the sea cucumbers were frozen to -20 ℃. The material was kept frozen during transport to the CRBM laboratory by freezer shipping containers aboard the local vessel Bella Desgagné (Relais Nordik). Upon receiving the biomass at the lab, two (2) sea cucumbers were placed in a single vacuum sealed bag. The vacuum sealing was done by a commercial vacuum sealer model SD2022 from (HI-Tech Vacuum, Canada). This was all done while the *C. frondosa* remained frozen. The material was kept frozen at -20 ℃ until needed for the experiments described in this thesis.

Pepsin (EC 232-629-3), and calf skin collagen type 1 (CAS 9007-34-5) were purchased from MP biomedical (Santa Ana, CA, USA). The Native PAGE gels used were precast NativePAGE Bis-Tris 4-16% (Thermoscientific, USA). The native gel protein marker used was Native Mark Unstained Protein Marker, the Native PAGE Running Buffer (20X) and the NativePAGE Cathode buffer (20X) were from Thermoscientific. The SDS PAGE gels used were precast Mini-Protean polyacrylamide 7.5% Tris-Glycine from Bio-Rad (Hercules, CA, USA). Precision Plus All Blue Standard protein marker, SDS Tris-Glycine Running buffer (10X) and Laemmli Sample Buffer (4X) were also purchased from Bio-Rad. The pierce BCA Protein assay kit was purchased from Thermoscientific. Bovine Achilles tendon collagen (CAS 9007-34-5) collagen was procured from Sigma Aldrich (Burlington MA,USA). All other chemicals used in this study were purchased at an analytical grade from Sigma Aldrich (Burlington MA, USA) or VWR International (Radnor, PA, USA).

3.2 Collagen processing pre-treatment

3.2.1 Product milling

Sea cucumbers were removed from freezer at -20 ℃. The weight of each sea cucumber was collected using a Viper MBST30 balance (Metler Toledo, USA). If all subsequent procedures after milling were conducted at temperatures between 4 and 6 degrees Celsius, the milling process was performed at 4 degrees Celsius. Otherwise, it was done at ambient temperature. They were dissected along the longitudinal access, and all internal organs were removed from the *C. frondosa*, leaving only the body wall and its skin. The body wall was then cut into 2-inch x 2-inch chunks using a knife, and then they were passed through a meat grinder model 10-0801 (Weston, USA). The particle size produced from the mill was 7 mm. The product was then left at 4 ℃ if proceeded to next pre-treatment stage shortly after (within next 2 hours). If the next pre-treatment step was not completed within two hours after milling, the product was stored at a temperature of -20 °C until the subsequent pre-treatment step was initiated. According to Section 2.3.1.1, the more freeze-thaw cycles a sea cucumber experiences, the greater the risk that its collagen properties will change. The effects of freeze-thaw cycles on these properties were not studied in this project. The number of freeze-thaw cycles was limited to a maximum of two. Most specimens of *C. frondosa* experienced only one, minimizing the impact of sea cucumber and collagen breakdown, as detailed in section 2.3.1.1. It is important to note that, unless otherwise specified, each sea cucumber underwent only one freeze-thaw cycle before being used in the extraction processes outlined in this project. To distinguish between one and two cycles, we define a sample undergoing one cycle as having been harvested, frozen, and then used directly in the extraction process. The limited amounts that underwent a second freeze-thaw process meant that they were placed in the freezer again after the first cycle and thawed out before being used in the extraction process. This was necessary due to the limited amount of sea cucumber available for the experiments. As an example, the initial lab-scale biomass was 100 grams. The body wall contained more than this amount (greater than 150 g), so it was frozen for future experiments. Also, as mentioned above, all experiments were done at 4°C to keep the material cool and minimize the potential impact of thawing to room temperature (see various sections in Chapter 2).

For the disaggregation (3.2.2), deproteination (3.2.3), and extraction steps (3.3), there were two different methods/approaches taken to the extraction. The conventional extraction method (using acid soluble or pepsin soluble protocols) will be denoted as (a) and the adapted express method will be denoted as (b) in these sections. The methods are similar, but the main difference is in (a), all experiments are done at 4 ℃ and for longer durations (up to 72 hours) whereas (b) was done at temperatures between 20 ℃ to 40 ℃ for much shorter durations. The post-extraction steps were the same, just method (a) was done at 4 ℃ and (b) was done at room temperature unless stated otherwise for a specific step. In section 3.3, there is third protocol that was developed by CRBM for another marine species and was adapted in this project for sea cucumbers. It is denoted by (c).

3.2.2 Disaggregation

(a) Milled sea cucumber was added in a 10:1 liquid to solid ratio to one of the following solutions: a) 0.1 M Tris-HCl, 4 mM EDTA at pH 8.0; b) 0.1 M Tris-HCl, 50 mM EDTA,0.5 M NaCl, 0.2 M β-mercaptoethanol at pH 8.0; c) 0.1 M Tris-HCl, 50 mM EDTA, 0.5 M NaCl, at pH 8.0; and d) distilled water. The solutions were mixed using laboratory mixer RZR 2102 (Heidolph, USA) at 800 rpm for 24, 48 or 72 hours depending on the trial run. When solutions a or c were used, the solids were collected on a 425 µm sieve followed by a 150 µm sieve and washed with a 5:1 liquid to solid ratio with distilled water for 15 minutes at 400 rpm to remove excess salts. The solution was filtered again on a 425 µm sieve, followed by a 150 µm sieve. The solid was then placed in a 10:1 liquid-to-solid ratio of distilled water for 24 or 48 hours, depending on the trial run, and mixed at 800 rpm in the laboratory mixer. Solutions b and d did not need to undergo the 24-48 hours water washing step.

Next, the solutions were filtered using a 425 µm, where the supernatant was collected, and remaining pellet was discarded. The supernatant was deemed the free collagen fibril forming solution (Saito et al., 2002). The supernatant was centrifuged at 4 ℃ for 30 minutes at 11000 xg where the pellet was collected, and the supernatant was discarded. The pellet was rinsed with deionized water in a 10:1 liquid-to-solid ratio. For 15 minutes at 400 rpm, the pellet was redispersed using the laboratory mixer RZR 2102. The solution was centrifuged at 4 ℃ for 30 minutes at 11000 xg where the pellet was collected, and the supernatant was discarded. The pellet was brought to the

next step. Unless specified, the solution a disaggregation solution was used throughout the report at an incubation time of 24 hours and then in distilled water for 24 hours.

(b) Milled sea cucumber was added in a 10:1 liquid to solid ratio to one of the following solutions: a) 0.1 M Tris-HCl, 4 mM EDTA at pH 8.0; or b) distilled water. The solutions were mixed using laboratory mixer RZR 2102 (Heidolph, USA) at 800 rpm for 2 hours. The solutions were centrifuged at 20 ℃ for 30 minutes at 11000 xg where the pellet was collected, and the supernatant was discarded. The pellet was brought to the next step. Unless specified, the solution a disaggregation solution was used.

3.2.3 Non-collagenous deproteination

(a) The pellet from 3.2.2.a was combined at a ratio of either 10:1 or 20:1 liquid to solid ratio with 0.1 M NaOH. The mixture was mixed in a laboratory mixer at 600 rpm for 24 – 48 hours, depending on the experiment. For the purposes of this report, unless otherwise specified, the deproteination time will be assumed to be 24 hours at a liquid-to-solid ratio of 20:1. The solution was then centrifuged at 11000 xg for 30 minutes at 4 ℃ and the pellet was kept. To bring the pellet back to a pH of 7, two methods were used. The first method was to successively wash (at 10:1 liquid to solid ratio) the pellet with deionized water and centrifuging for 20 minutes at 11000 xg and 4 ℃. This step was repeated until pH of the pellet reached 7.0 monitoring with a pH meter SB90M5 (VWR, USA). The second method collected pellet after the NaOH treatment and was combined 2:1 solid to liquid ratio with deionized water, stirring at 400 rpm. Using 1 M HCl, the solution was brought to a pH of 7.0. This solution was centrifuged at 11000 xg for 45 minutes at 4 ℃ and the pellet was collected. Unless stated otherwise, the second method was used to bring the pellet to pH 7.0.

(b) The pellet from 3.2.2.b was combined at a ratio of 10:1 liquid to solid ratio with 0.1 M NaOH. Using a laboratory mixer, the mixture was mixed at 600 rpm for 2-4 hours depending on the experiment. Unless otherwise stated, a 3-hour deproteination time will be assumed. The solution was then centrifuged at 11000 xg for 30 minutes at 4 ℃ and the pellet was kept. To bring the pellet back to a pH of 7, the pellet was collected after the NaOH treatment and combined at a 2:1 liquid

to solid ratio with deionized water and stirred at 400 rpm. Using 1 M HCl, the solution was brought to a pH of 7.0. The solution was centrifuged at 11000 xg for 45 minutes at 4 ℃ and the pellet was collected.

3.3 Extraction of collagen

(a) The pellet collected from section 3.2.3.a was subjected to the following extraction procedure. The pellet was combined at liquid to solid ratios of 10:1 or 20:1 with 0.5 M acetic acid. While the acid soluble protocol was tested, the protocol that was used predominantly in this thesis was pepsin soluble protocol which in addition to the acetic acid, pepsin was added to the solution at a concentration of 0.45 g/L. The extraction was performed for 20 to 72 hours. Unless otherwise stated, an extraction time of 24 hours was used. The mixture was agitated between 600 rpm to 800 rpm depending on the system's viscosity using a laboratory mixer. The solution was collected and applied to purification steps described in section 3.4.

(b) The pellet collected from section 3.2.3.b was subjected to the following extraction procedure. The pellet was combined at liquid to solid ratio of 10:1 with 0.5 M acetic acid or 0.1 M HCl. While the acid soluble protocol was tested, the protocol that was used predominantly in this thesis was the pepsin soluble protocol. Consequently, in addition to 0.5 M acetic acid, pepsin was included in the solution at concentrations of 0.01, 0.1, 0.3, and 0.7 g/L. The extraction was performed for 2 to 4 hours. Unless stated, 3 hours extraction time was used. The mixture was agitated between 400 rpm depending on the system's viscosity using a laboratory mixer. The extraction temperatures used were 20 ℃, 30 ℃ and 40 ℃. The solution was collected and applied to purification steps described in section 3.4.

(c) The third extraction protocol tested was protocol developed by CRBM for the extraction of native fish collagen. While the details cannot be shared due to the confidentiality agreements in place for the developed process, it uses a non-enzymatic acid approach to liberate collagen from the biomass. The pretreatment uses a combination of solvents, acids and bases to prepare the material for extraction followed by conventional recovery techniques.

3.4 Collagen processing post-treatment

3.4.1 Product recovery

After the extraction step, the samples are centrifuged for 1 hour, 11000 xg at 4 ℃ using Avanti J-26X centrifuge (Beckman Coulter, USA). The supernatant was kept, and the pellet was discarded. From here, one of the three following steps were done: salting out, isoelectric-like precipitation with centrifugation, isoelectric-like precipitation only.

3.4.1.1 Salting out

Using a Thermoscientific Whatman MA 1395 magnetic stir plate (USA), the supernatant was mixed with 5 M NaCl until the final NaCl concentration was 1 M, all while being stirred at 200 rpm. The newly combined solution allowed to mix for 10 minutes and then agitation was stopped. The solution was left for 24 hours. The solution was then centrifuged for 30 minutes at 11000 xg and 4 ℃. The pellet was collected, and the supernatant was discarded.

3.4.1.2 Isoelectric-like precipitation and centrifugation

Using a magnetic stir plate, the solution was mixed at 400 rpm. Typical at this stage, the products pH ranged between 2.9-3.1. Using 3 M NaOH, the solution was neutralized to a pH of 6.5-7 using a pH meter. The stirring was stopped, and the solution was allowed to sit for 24 hours. After 24 hours, the solution was then centrifuged for 30 minutes at 11000 xg and 4 ℃. The pellet was collected, and the supernatant was discarded.

3.4.1.3 Isoelectric-like precipitation

Using a magnetic stir plate, the solution was mixed at 400 rpm. Typical at this stage, the products pH ranged between 2.9-3.1. Using 3 M NaOH, the solution was neutralized to a pH of 6.5-7. The pH was monitored using a pH meter. The stirring was stopped, and the solution was allowed to sit for 4 hours. The solution was then applied to the next step.

3.4.2 Product purification

To purify the final product, there were two methods that were used: ultrafiltration and dialysis.

3.4.2.1 Ultrafiltration

The pellet was resolubilized in 0.5 M acetic acid, at a 20:1 volume to weight ratio. This was done by mixing the solution at 400 rpm, for 30-120 minutes, depending on the solubilization rate of the material. Upon completion, any large particles that did not dissolve were removed by vacuum filtration on a 40 μm filter paper (VWR, USA).

The type of system used was the Minimate tangential flow filtration system (PALL, USA). The operating conditions were set at 2 bar for the feed pressure, the feed flow rate ranged between 25-50 mL/min, depending on the viscosity of the solution. The retentate was recirculated back to the feed chamber while the permeate was discarded. The type of filter used was a modified polyether-sulfone membrane with a molecular weight cutoff of 70 kDa (PALL, USA). The recirculated feed was mixed at 400 rpm to ensure the feed solution was homogenized. Depending on the sample, the mixture was diafiltered to reduce salt and unwanted small particles in the mixture with a diluting solvent. Typically, if the samples were salted out, the diluting solution used was acetic acid and if the solution underwent isoelectric-like precipitation, it was deionized water. For specific diafiltration and dilutions performed, refer to raw lab data (not shown). Times varied depending on each sample's salt and protein concentration but could take up to 24 to 48 hours if the sample was concentrated. The retentate was collected after the filtration was complete.

3.4.2.2 Dialysis

Samples from section 3.4.1 were added to a cellulose ester dialysis bag, having a molecular weight cutoff of 100-500 Da (Repligen, USA). The sample inside the tube was added at a ratio of 1:100 against the dialysate which was deionized water. The dialysate was stirred at 75 rpm. For the first 8 hours, the buffer was exchanged every 2 to 4 hours. Following this, the buffer was exchanged

every 8 to 24 hours until the measured total dissolved solids in the dialysate was 0 ppm. The dissolved solids were measured using a handheld total dissolved solids meter, model HI98311 DiST 5 (Hanna, USA). The process took between 48 to 72 hours.

3.4.3 Product preservation

For long term storage (>1 month), all samples were dried. Under a month, the solutions were stored in a refrigerator at 4 ℃. Two drying methods were tested which included lyophilization or spray drying. Unless stated, the drying method used was lyophilization.

3.4.3.1 Lyophilization

All samples were placed in glass dishes and placed at -20 ℃. The samples were placed in a VirTis Ultra 50 L freeze dryer (SP Scientific, USA). If required, the product was brought to pH 5.5 before being placed in the freeze dryer using 3 M NaOH to prevent any damage to the dryer and unexpected product boiling. The dryer began working at -46 ℃ and 37 milliTorr. The dryer was augmented by 5 ℃ increments every 30 minutes until 25 ℃ was reached. This was to ensure the frozen samples did not turn to liquid first which could cause issues of sample bubbling and loss of product. Once 25 ℃ was reached, the samples were left for 48 to 72 hours.

3.4.3.2 Spray drying

The solution was spray dried using a SD-micro Niro spray dryer (GEA, Germany). The feed flow rate of solution was set at 8 mL/min, the inlet temperature was kept at 180 ± 5℃ and outlet temperature was 90 ± 5℃. A 0.5 mm nozzle was used.

The powders were recovered and weighed, vacuum sealed and stored at -20 ℃ until further testing was required.

3.5 Pilot scale-up

The pilot run was scaled up from benchtop extractions which began with 100 g of sea cucumber body wall. In the pilot run, 4 kg of whole *C. frondosa* (body wall and internal organs) were used.

All steps were conducted at room temperature unless stated otherwise. The pilot scale experiment followed the section protocols for the pretreatment, extraction and post-treatment steps as listed in table 3.1.

Table 3.1. Summary of steps executed in the pilot-scale *C. frondosa* extraction protocol.

Stage Number	**Process Stage**	**Step description**	**Protocol refence section**
1	Pre-treatment	Product milling	3.2.1
2	Pre-treatment	Disaggregation	3.2.2.B
3	Pre-treatment	Non-collagen deproteination	3.2.3.B
4	Extraction	Extraction	3.3.B at conditions 30 °C, 0.1 g/L pepsin for 90 minutes
5	Post-treat-ment	Isoelectric-like precipitation	3.4.1.3
6	Post-treat-ment	Ultrafiltration (25%) No-treatment (75%)	3.4.2.1 No section, left as is
7	Post-treat-ment	Lyophilization (63%) Spray Drying (22%) No Treatment (15%)	3.4.3.1 3.4.3.2 No section, left as is

In process stages 6 and 7, the percentage associated with the description shows how much of the sample was allocated to the type of treatment as different scenarios wanted to be tested.

Next, Table 3.2 shows what equipment modifications were made to accommodate the pilot scale run as opposed to what was done at the lab-scale.

Table 3.2. Summary of the equipment scaled up from the laboratory to the pilot phase for the extraction of collagen from *C. frondosa.*

Processing operation	**Lab Scale set-up**	**Pilot Scale set-up**
Product milling	Residential meat grinder 10-0801 (Weston, USA).	Commercial meat grinder , Optimo 32 (MADO, Germany)
Disaggregation and deproteination steps	Mixed in 1 to 4 L breakers with paddle mixer	Mixed in 35 L stainless steel vessel with paddle mixer
Solid-liquid separations in pre-treatment step	12'' 425 µm and 12'' 150µm sieves and lab-scale centri-fuges 11000 xg,	12" 425 µm sieve with shak-ing at 200 rpm on vibratory shaker
Extraction	Hot plate with thermocouple magnetic stirrer, 4 L beakers	10 L pilot jacket reactor with ethylene glycol temperature control system 10L Glass Reactor (Chemglass Life Sciences, USA)
Post-extraction solid-liquid centrifugation	Lab-scale centrifuge 11000 xg , model Avanti J-26X (Beckman Coulter, USA)	Disc centrifuge, 10000 rpm model CTC 3-03-107, (GEA Westfalia, Germany)

3.6 Moisture content and mass analysis

Moisture content of samples was measured using a HB43 moisture analyzer (Mettler Toledo, USA). 1.0 g of solid or powder products were placed on the balance to be measured while 3.0 g of liquid were placed on the balance when analysis was done on these substrates. The precision of the balance is ± 0.1% of the mass to be dried.

In this project, analytical balances were used to determine the mass of various substances. The particular scale used was determined based on whether the item to be weighed fell within its oper-ational range, meaning that it could produce precise readings. A summary of the analytical scales used throughout this project, along with their operational limits and accuracy, can be found in Table 3.3.

Table 3.3. Summary of analytical balances used for measurements throughout the project.

Balance	Working range*	Precision
Mettler Toledo UMX2	82 µg to 2.1 g	0.1 % of mass being measured
Mettler Toledo XS205DU	8.2 mg to 220 g	0.1 % of mass being measured
Mettler Toledo AX504	82 mg to 510 g	0.1 % of mass being measured
Mettler Toledo Viper MB ST30	8.2 g to 30 kg	0.1 % of mass being measured

*Working range is based on USP standard 41 (United States Pharmacopedia, 2024).

3.7 SDS PAGE analysis

Using the gels and reagents described in section 3.1, SDS PAGE analysis was carried out using Mini Protean Gel Electrophoresis system from Bio-Rad. The protocol followed CRBM's standard operating procedure CRBM-EB-0014, which was developed from the manufacturer protocol (Bio-Rad Laboratories, 2012).

4X Laemmli buffer was combined with 2-mercaptoethanol (10:1 ratio). This was the sample buffer. The total amount of sample loaded on a gel was 20 µL. Based on the protein content of the specimen being analyzed, a specific amount of the sample was mixed with 5 µL of sample buffer. This combination was designed to load 20 µg of protein onto the gel. To reach 20 µL, distilled water was used to reach the final voulme if needed. The samples were incubated in a 70 ℃ bath for 10 minutes. 20 µL of sample and 5 µL of Precision Plus All Blue protein standard (Bio-Rad, USA) were loaded on the gels and were run in a PROTEAN electrophoresis cell (Bio-Rad) for 60-75 minutes at 120 V in 1X SDS-Tris-Glycine buffer 25 mM Tris, 192 mM glycine, 0.1 % [w/v] SDS, pH 8.3. The concentrated product in section 4.1 was diluted 10:1 with distilled water

Gels were stained on a rocking platform in 50 % [v/v] methanol, 10 % [v/v] acetic acid, 0.03 % [w/v] Coomassie Brilliant Blue for approximately 1 hour. The gel was then destained in 20 % [v/v] methanol, 10 % [v/v] acetic acid for approximately 1 hour. Gels were scanned and visualized using

Chemi-Doc (Bio-Rad, USA) imaging system. Figure 3.1.a demonstrates a standard calf skin type I collagen from Sigma Aldrich run with this sections SDS PAGE conditions.

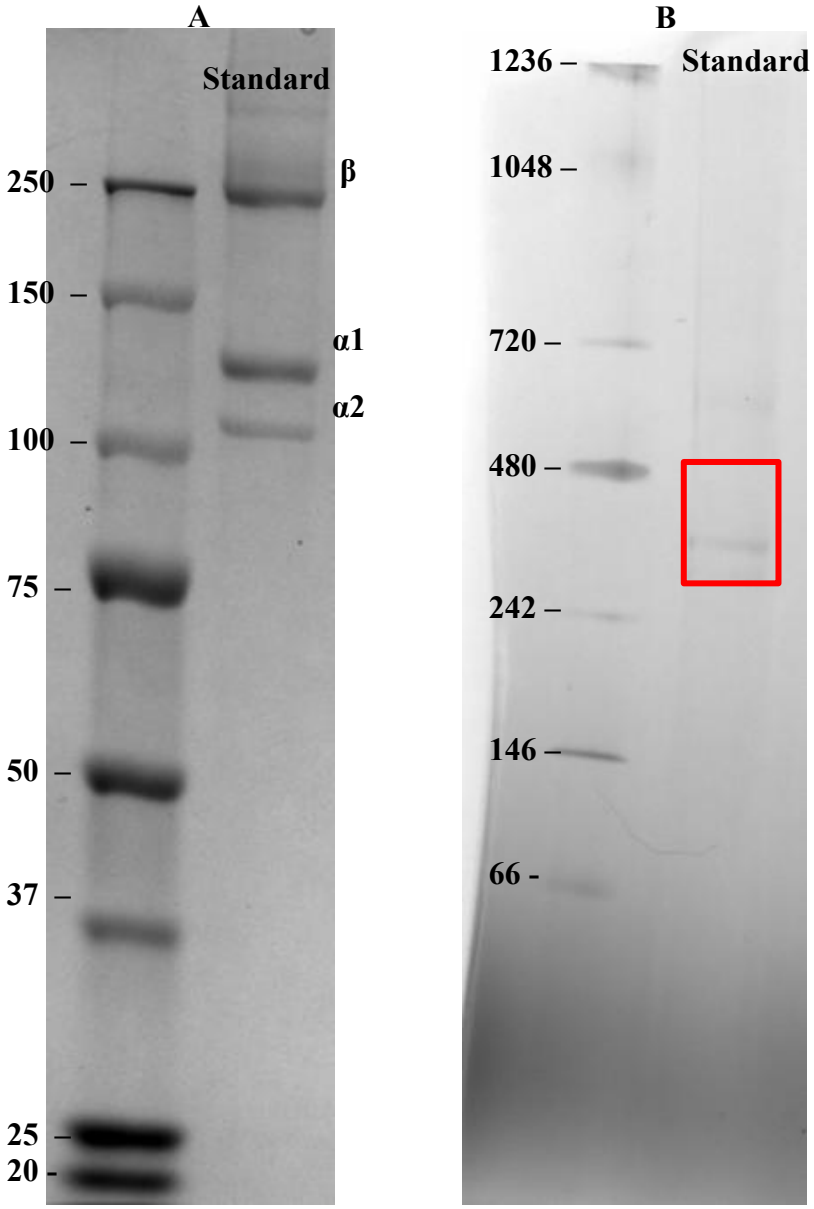

Figure 3.1. (a) SDS PAGE of calf skin type I collagen. (b) Native PAGE of calf skin type I collagen. The ladder is in the leftmost lane in each figure.

Figure 3.1.a shows the α1 and α2 chains at 120 kDa and 105 kDa respectively, while the β chain is at 240 kDa.

3.8 Native PAGE analysis

Using the gels and reagents described in section 3.1, Native PAGE analysis were carried out using Powerpack VWR (VWR, USA) system. The protocol followed CRBM's standard operating procedure CRBM-EB-0008, which was developed from the manufacturer protocol (Life Technologies, 2013).

2.5 µL of 4 X sample buffer was combined with a volume of sample and deionized water to reach a volume of 10 µL. The amount of sample added depended on its protein concentration. The desired protein to be loaded on the gel was 5 µg. 10 µL of sample and 5 µL of Native Marker (Thermoscientific, USA) were run in a Native PAGE electrophoresis apparatus for 105-120 minutes at 150 V. The anode running buffer 20 X was diluted to 1 X wither distilled water and the cathode buffer was prepared from 20X running buffer and 20 X cathode additive, both diluted to a concentration of 1 X. The concentrated product in section 3.1 was diluted 10:1 with distilled water

The gel was then destained in 40% [v/v] methanol, 10% [v/v] acetic acid for approximately 2 hours. Gels were scanned and visualized using Chemi-Doc (Bio-Rad) imaging system. Figure 3.1.b demonstrates a standard calf skin type I collagen from Sigma Aldrich run with the Native PAGE conditions described in this section. There are bands with molecular weights between 300 and 380 kDa (red box). These bands correspond to collagen in its native state as discussed in section 2.4.1. However, since there are two distinct bands present, it is possible that the collagen molecules in this sample have experienced different levels of hydrolysis, or alternatively, may possess varying tertiary/quaternary structural arrangements.

3.9 UV-Vis

UV-vis spectrometry were carried out on both liquid and powder extracts. Liquid samples were applied directly to a model 220 Evolution UV-vis spectrophotometer (Thermoscientific, USA) and blanked against its corresponding liquid media (typically 0.5M acetic acid, neutralized 0.5 M NaOH, 0.1 M Tris-HCl with 4 mM EDTA pH 8.0, 0.1 M NaOH or distilled water). The equipment error with respect to wavelength was ± 2 nm and with respect absorbance was ± 1.3%.

100 mg lyophilized powder samples and calf skin collagen standard were solubilized in 10 mL 0.5 M acetic acid and vortexed. If the signal was too strong, samples were diluted 10 X to obtain a concentration 1 mg powder/mL solution. If large particles remained in solution after solubilization, samples were filtered with a 5 µm nylon filter and syringe. Each were placed in quartz cuvette and the path length was 1 cm. The wavelength range measured was from 220 nm to 450 nm. The figure below demonstrates a UV spectra of calf skin type I collagen from Sigma Aldrich.

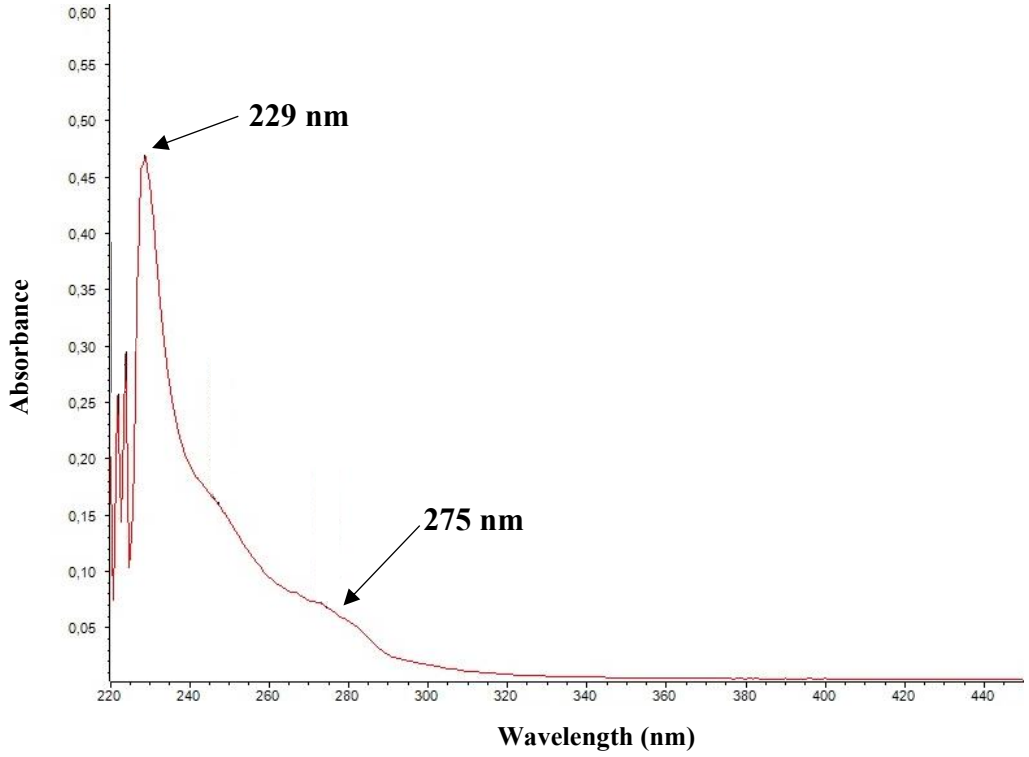

Figure 3.2. UV-vis spectra of calf skin type I collagen. The solvent used to dissolve the collagen powder and blank was 0.5 M acetic acid.

Figure 3.2. shows peaks at 229 nm and 275 nm and the theory of what these peaks mean and how they relate to collagen are described in section 2.4.2.

3.10 FTIR

Lyophilized samples (approximately 20 mg) and bovine Achilles tendon collagen standard was measured using a Cary 630 Fourier Transform Infrared spectrophotometer (Agilent, USA). The

spectra were analyzed from 4000 cm^{-1} to 650 cm^{-1} in transmittance mode at a resolution of 4 cm^{-1} intervals and 20 scans at a temperature of 20 °C. The equipment error with respect to wavenumber was ± 0.3 cm^{-1}. The sampling technique used for the projects FTIR measurements was a 68043-68310 attenuated total reflection (ATR) sampler with a diamond module (Agilent, USA). The figure below demonstrates a FTIR spectra of bovine Achilles tendon type I collagen from Sigma Aldrich.

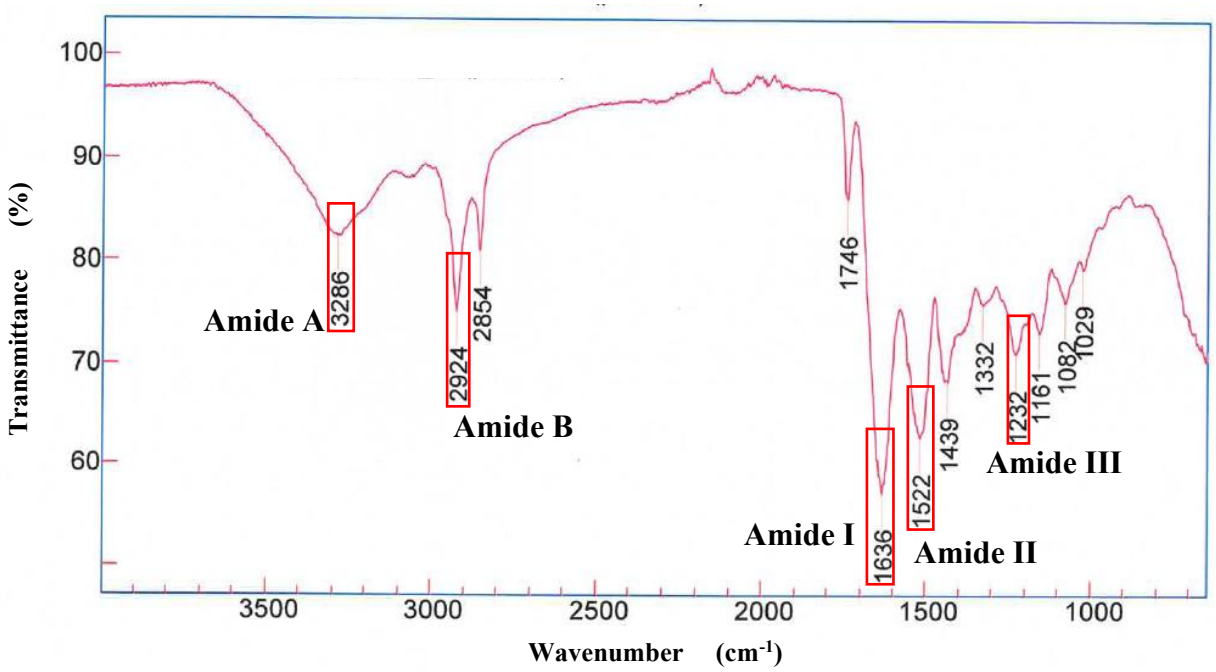

Figure 3.3. FTIR spectra of bovine Achilles tendon type I collagen.

Figure 3.3 contains all the peaks as discussed in section 2.4.3 which are amide A, B, I, II and III. There are other peaks that showed up on the figure but the peak at 1746 cm^{-1} is interesting. According to the literature, C=O can have frequencies up to 1800 cm^{-1}. In particular, peaks around 1750 cm^{-1} correspond to the vibrational stretch of C=O from ester bonds (Riaz et al., 2018). The peak at 1746 cm^{-1} may indicate the presence of a type I beta turn. Not all types of collagen have this. This will depend on the source and anatomical section of the animal (Juszczak, 2004; Xiao et al., 2007). Other articles have suggested this is a potential contamination from fat tissue (Sandt, 2024). This peak is not always seen in marine collagen (Abedin et al., 2013; S. Lin et al., 2017; F.

Liu et al., 2017). In the experiments conducted in chapter that used FTIR, no peaks around 1750 cm^{-1} were observed.

The FTIR curves presented in this thesis did not undergo spectral deconvolution to identify missing peaks. The goal was to standardize the analysis method to avoid the complexity of interpreting peaks from one sample to the next. Impurities in the sample can also affect the accuracy of this method, especially if they have similar peak frequencies to the protein of interest (Fellows et al., 2020). The samples have been purified in the project, but they have not undergone the level of purification in which one can guarantee that only the molecule of interest is present in the extract. Once a consistent product with high purity is achieved, spectral deconvolution can be used to amplify the signal and analyze all peaks with precision. Another reason for not using this technique is to make sure that the FTIR methods and analyses are consistent with those used by other groups in the available literature. This allows appropriate comparisons between the results. Other reports have not documented the use of spectral deconvolution when analyzing collagen extracted from different sea cucumber species (Abedin et al., 2013; Adibzadeh et al., 2014; S. Lin et al., 2017; F. Liu et al., 2017; M. Zhong et al., 2015).

3.11 Heavy metal and elemental mineral analysis

The analysis of heavy metals and other elemental minerals were executed according to the standard operating procedure CRBM-ICP-0013 using the triple quadrupole ICP-MS 8900 (Agilent, USA). The method has been adapted and follow AOAC official methods (Latimer Jr., 2023). To summarize, the samples were subjected to microwave-assisted acid digestion with concentrated sulfuric acid. Subsequently, they were loaded into the apparatus and ionized using inductively coupled argon plasma, with the mass spectrometer serving as the detection tool.. The elements analyzed included Be, Na, Mg, Al, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Cd, Sb, Ba, Hg, Ti, Pb. To determine the uncertainty of the concentrations for each sample and measured element, two methods were used. If data was available through multiple repeated experiments, the arithmetic mean, standard deviations, and number of replications (n) were used to calculate the measurement uncertainty. This will be explicitly stated in the table and/or text. In the absence of data indicating whether multiple trials were conducted to determine the average and standard deviation

of the measurement, it is assumed that equation 3.1 was used to calculate the error. The equation is summarized below.

$$u(x) = \sqrt{s_y^2\left(\frac{1}{m}+\frac{1}{n}+\frac{(y-\bar{y})^2}{\sum(y_i-\bar{y})^2}\right)} \times \frac{1}{|a|} \qquad \textit{Eq 3.1.}$$

Where u(x) is the error in the measured concentration, s_y^2 is the variance of the residual regression curve, m is the number of repeated measurements of the sample, n is the number of points of calibration, y is the measure signal of the sample, $\bar{y}$ is the average of y_i's of the calibration points, y_i are the individual calibration response values and a is the slope of the regression curve. The equation came from the CRBM protocol CRBM-ICP-0013. An example of a typical calibration curve is shown in Figure 3.4.

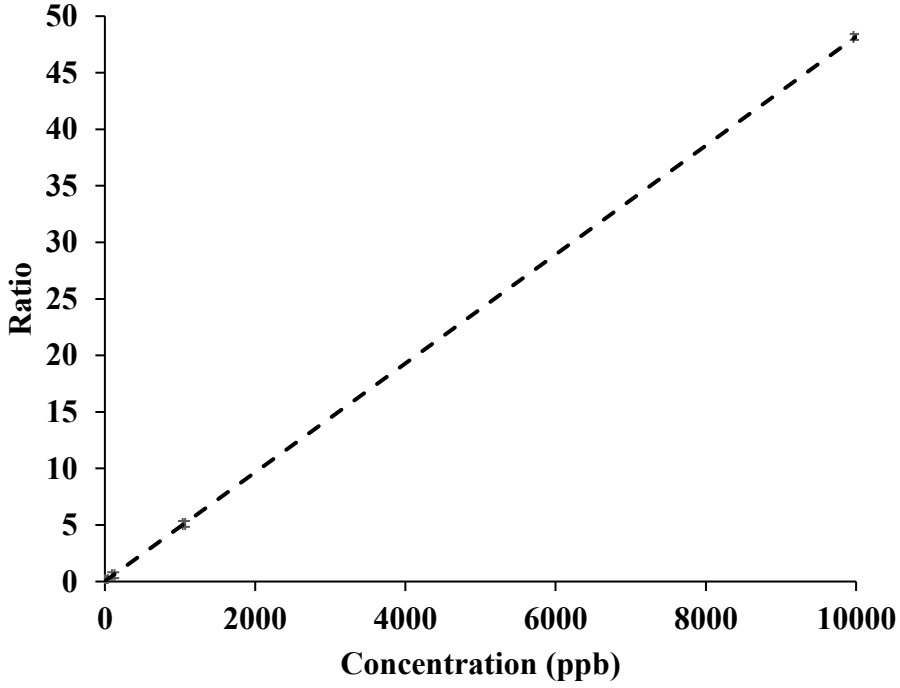

Figure 3.4. Representative standard calibration regression curve for sodium measured by ICP-MS. The linear regression analysis produced the following parameters: slope = 0.0048 ppb^{-1}, intercept = 0.0258, and R^2 = 1. The x-error bars were calculated using equation 3.1, while the y-error bars are based on the standard error of the regression residuals.

The calibration curve was done for each run during ICP-MS analysis. Other elements had their own specific calibration curves but were similar to what is observed in Figure 3.4

3.12 Collagen quantification assay

To quantify collagen in the developed extracts, a total collagen quantification kit that detected collagen based on the levels of hydroxyproline in a given sample was used. The kit was called Quickzyme Total Collagen Assay (Quickzyme Biosciences, Netherlands). The protocol executed followed what was provided by the manufacturer (Quickzyme Biosciences, 2022). Each lyophilized powdered sample was dissolved in 6 M HCl at a concentration of 300 mg powder/mL solution. 125µL of the kit's standard was combined with 125 µL of 12 M HCl. The samples and standard were left to digest on a heat block at 95 ℃ for 20 hours.

After 20 hours, the samples and standard were cooled to room temperature and centrifuged for 10 minutes at 13000 xg. The samples were then filtered using a 0.2 µm syringe filter and the liquid permeate was collected. 200 µL of each sample was then added to 100 µL of deionized water. The standard did not need to pass through a 0.2 µm syringe filter given no black particles (fat or carbohydrates) were present. Dilutions of the standard were made according to the manufacturer's recommendations of 0 µg/mL to 300 µg/mL in dilutions of 4 M HCl.

Next 35µL of standard and samples were placed on the assay microplate. The samples and standards were performed in duplicate. 2X serial dilutions were made to avoid samples being out of the range of standards. 75 µL of assay buffer was added to each well and mixed. For 20 minutes at 20 ℃, the plate was mixed using an orbital shaker at 75 rpm. Next, a detection reagent was prepared by adding a 2:3 ratio of reagent A to B from the collagen kit. 75 µl of the detection agent was added to each well. The plate was shaken by hand for 1 to 2 minutes and added to a 60 ℃ oven (Thermoscientific, USA) for 60 minutes. The plate was removed and allowed to cool to room temperature. Using the Spark plate reader (Tecan, Switzerland) the samples were measured at a wavelength of 570 nm. The reported margin of error for the absorbance measurements is as follows: ± 0.008 for readings between 0 and 0.8, 1% of the measured absorbance for readings between 0.8 and 2.5, and 1.5% of the measured absorbance for readings between 2.5 and 3. In this series of

experiments, the absorbances are typically below 0.8. Each sample and standard were blanked with the value from S8 and standard curve with line of best fit was developed with the standards. An example of the curve produced can be seen in Figure 3.5.

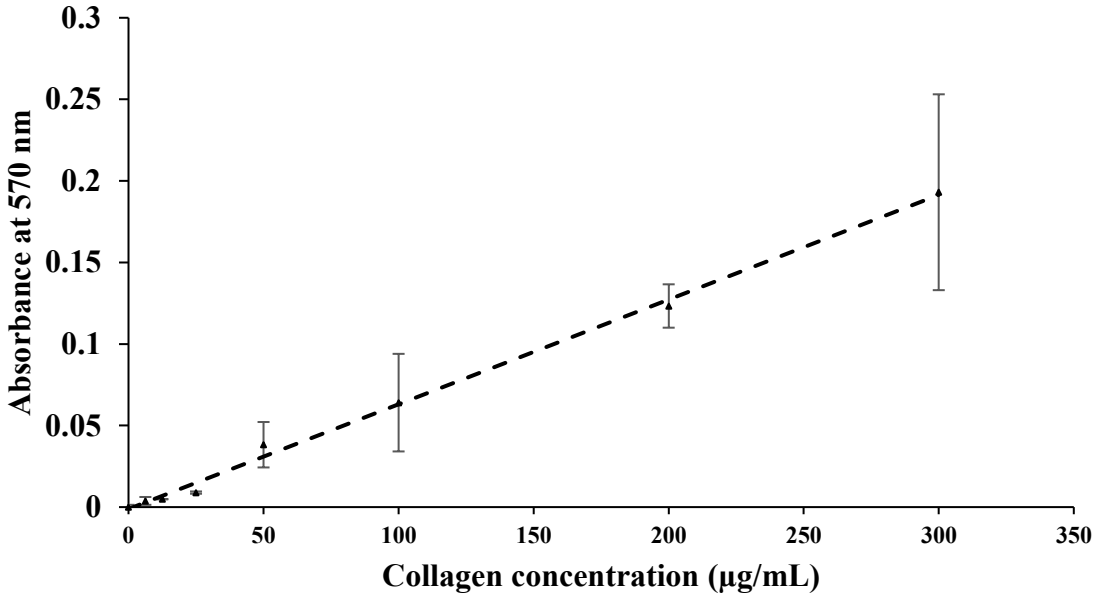

Figure 3.5. Representative collagen standard curve of total collagen assay. The linear regression analysis produced the following parameters: slope was 0.0064 mL/µg, intercept was -0.00113 and the R^2 value was 0.9967. Points are mean values and y-error bars are 1 standard deviation, n=2.

Using the curve in Figure 3.5, collagen concentrations in the samples were calculated based on their dilutions.

3.13 Circular dichroism spectroscopy and denaturation temperature

Circular dichroism spectra of extracts were recorded with Jasco-815 spectrometer (Jasco Inc, USA) at 190 nm to 260 nm using a quartz cuvette, having a path length of 0.1 mm. According to the manufacturer, the spectrometer has a precision of ± 0.1 nm and repeatability of ± 0.05 nm for wavelength measurements. It also has a precision of ± 0.02 mdeg for ellipticity measurements and a precision of ± 0.2 °C when reporting temperatures. Lyophilized collagen powder samples were dissolved in 0.5 M acetic acid and neutralized to pH 7.0 with 1 M NaOH. The samples were either

dialyzed or ultrafiltered according to protocols in sections 3.4.2.1 and 3.4.2.2. For CD experiments where temperatures were kept constant at either 4 ℃ or 60 ℃, the wavelengths were varied from 260 nm to 190 nm, at a scan speed 100 nm/min, data interval 0.1 nm, bandwidth 1.00 nm and 10 scans. To monitor changes in protein confirmation at different temperatures, the samples were tested from 4 ℃ to 60 ℃ with an increase of 1 ℃/min. For the variation of temperature experiments, wavelengths of 198 nm and 220 nm were used. The following wavelengths were chosen to measure the impact of temperature changes on the sample's structure for a few reasons. Firstly, these wavelengths correspond to the minimum (~198 nm) and maximum (~220 nm) peaks of collagen and its intact triple helix (Alves et al., 2017; Greenfield, 2007; Holmes et al., 2017; Jafari et al., 2020). Secondly, both wavelength measurements are well within the limits of the equipment (163 nm to 900 nm). Since the solvents (acetic acid and water) used are achiral, the CD apparatus will not detect any signals from these solutions, as they do not preferentially absorb right- or left-handed light. To minimize the risk of background noise, a blank was applied before each sample. In summary, the selected wavelengths can provide accurate measurements of conformational changes with respect to temperature, without being affected by detection limits of the spectrometer or impacts from background solvents.

For each CD test, it is important that the high tension (HT) voltage value stayed below 600 V, so that the detector can monitor any differences between the left-handed and right-handed circularly polarized light (Rodger & Marshall, 2021). Below these values, the HT voltage is proportional to the sample's absorbance (Miles & Wallace, 2016). If the value exceeds this, the signal will start to become noisy, as too few photons reach the detector (Anon 2021). This often occurs when the sample are too concentrated (Miles et al., 2021). The HT voltage data associated with varying wavelengths, while maintaining a constant temperature, are presented in Figures 7.2 and 7.3 of Appendix A. Similarly, HT voltage data corresponding to fluctuations in temperature, with a fixed wavelength, are displayed in Figures 7.4 (198 nm) and 7.5 (220 nm) of appendix A.

All CD curves were normalized to 1 mg/mL and the concentration of the sample solutions were determined using a Nanodrop 1000 UV-vis spectrophotometer (Thermoscientific, USA), measuring the peak absorbance at 226 nm and using a standard collagen curve between 0 mg/mL to 4

mg/mL to calculate the collagen concentration. The instrument had a ± 1 nm uncertainty in wavelength measurement and a ± 0.003 uncertainty in absorbance. An example of the collagen standard curve is shown in appendix A.

To calculate the denaturation temperature at either wavelength, the equation proposed below by Greenfield et al. (2005) was used to first calculate ellipticity of the sample where 50% of the protein is denatured and 50% is in its folded confirmation (Greenfield, 2007).

$$\theta_t = (\theta_F - \theta_U) \cdot \alpha + \theta_U \qquad \textit{Eq 3.2.}$$

where Θ_t is the observed ellipticity at a given temperature, α represents the fraction folded which in the following analysis was 0.5, Θ_F is the ellipticity of the of the fully folded form and Θ_U is the ellipticity of the fully unfolded form. Θ_F and Θ_U are determined based on the average plateau values of ellipticity before the transition occurs and after the transition occurs respectively. To calculate the denaturation temperature, a linear regression using Excel was performed on the transition portion of the curve, yielding an equation where Θ_t was used to calculate the thermal denaturation temperature. At 198 nm and 220 nm, a first order linear regression model was used. A minimum R^2 of 0.9 or greater was used over the complete transition curve. If this minimum value was not met, no temperature was calculated.

3.14 Total protein quantification

To study the total protein in a powdered sample, the Kjeldahl method was used. 0.5 g of sample was combined with 5 g of potassium sulfate, 0.5 g of copper sulfate pentahydrate and 20 mL of 98% sulfuric acid. Using a Vapodest 30s Kjeldahl digestion block (C. Gerhardt GmbH & Co., Germany), the samples were progressively heated to 350 ℃ over a 28-hour period or until the digestion was complete (solution turned into a blue green colour). Each sample was left to cool overnight to room temperature. To each sample, 50 mL of deionized water was added, and the sample was shaken. 50 mL of 4 % w/v boric acid was combined with each sample. The sample was then set up on a steam distillation apparatus. To each sample, 40 % w/v NaOH was added

until the solution turned from the blue green to brown. A slight excess of sodium hydroxide was added. Through steam distillation, the azote was collected in a recovery flask and titrated with 0.1 N sulfuric acid until the equivalence point was reached where the solution went from yellow to a fluorescent pink. From this, the total percentage of nitrogen was calculated using the following equation:

$$\%Az = \frac{(V_s - V_b) \cdot N \cdot 1.401 \times 100}{m_s \cdot 0.1} \qquad \textit{Eq. 3.3}$$

where %Az, is the percentage nitrogen in the sample, V_s is the volume of sulfuric acid used to titrate the sample in mL, V_b is the volume of sulfuric acid used to titrate the blank in mL, N is the molarity of the sulfuric acid solution in normality and m_s is the mass of the sample in mg.

To calculate the percentage of total proteins, the following equation was used:

$$\%P = cf \cdot \%Az \qquad \textit{Eq. 3.4}$$

where %P is the total protein in the sample and cf is the correction factor which typically is 6.25 (Nishanthan et al., 2018; Truong & Le, 2019). Knowing the mass of powder collected, the total mass of protein, concentrations and yields were calculated.

3.15 Yield

In the following project, there were three different yield calculations used to analyze various experimental runs. The yields studied included total protein yield, total collagen yield and total solids extracted yield, and the equations and definitions are defined in this section.

Equation 3.5 describes the total protein yield and is represented as

$$Y_{p,i} = \frac{m_{p,i}}{m_{s,i} \cdot (1 - x_{w,i})} \qquad \textit{Eq. 3.5}$$

where $Y_{p,i}$ is the protein yield of the extract produced from run i in grams of protein per gram of dried sea cucumber, $m_{p,i}$ is the total protein extracted from run i measure in grams of protein, $m_{s,i}$ is the total amount of wet sea cucumber used in run i in grams of wet sea cucumber and $x_{w,i}$ is the moisture content of the sea cucumber used in run i in grams of water per grams of wet sea cucumber. The definition of i can be any run from 1 to 19.

Equation 3.6 describes the total collagen yield and is represented as:

$$Y_{c,i} = \frac{m_{c,i}}{m_{s,i} \cdot (1 - x_{w,i})} \qquad \textit{Eq. 3.6}$$

where $Y_{c,i}$ is the collagen yield of the extract produced from run i in milligrams of collagen per gram of dried sea cucumber, $m_{c,i}$ is the total collagen extracted from run i measured in milligrams of collagen, $m_{s,i}$ is the total amount of wet sea cucumber used in run i in grams of wet sea cucumber and $x_{w,i}$ is the moisture content of the sea cucumber measured in run i in grams of water per gram of wet sea cucumber. The definition of i can be any run from 1 to 19.

Equation 3.7 describes the total solids extracted yield and is represented as:

$$Y_{e,i} = \frac{m_{e,i}}{m_{s,i}} \qquad \textit{Eq. 3.7}$$

$Y_{e,i}$ is the yield of extracted solids for a particular step of run i in grams of extracted solids per gram of wet sea cucumber, $m_{e,i}$ is the total extracted solids extracted from run i for a particular step, $m_{s,i}$ is the total amount of wet sea cucumber added in run i in grams of wet sea cucumber. Equations 3.5 and 3.6 represent overall process yields, whereas equation 3.7 is a step-specific yield calculation, used to track the solid extraction during the disaggregation phase. The definition of i can be any run from 1 to 19.

3.16 BCA protein assay

Throughout the project, the bicinchoninic acid (BCA) protein assay was used and executed in two different ways, one being a qualitative check before executing a Native or SDS electrophoresis to visually determine if proteins were present in the sample. The other method allowed for a quantitative dosing tool to load an exact amount of protein on a Native or SDS gel. Unless stated, the qualitative check method was used as opposed to dosing method.

For the qualitative check method, 25 µL of sample was loaded was combined with 200 µL of BCA solution (50:1 ratio reactive A:B). The samples were agitated for 30 seconds and allowed to incubate for 30 minutes at 37 ℃ in an incubator (Thermoscientific, USA). The samples were visually inspected as the more purple the solution became, the more concentrated the protein sample and the more greenish blue the sample, the lower amounts of proteins were present in the sample.

For the quantitative method, the standard operating procedure CRBM-EB-00007 was used.

3.17 Error analysis

As mentioned in section 1.6, limited time and resources often prevented the execution of repeated tests. When multiple trials/iterations are conducted to measure specific result, the average result, standard deviation, and number of repetitions (n) will be explicitly indicated in the figure, table, or text. In cases where there are results accompanied by no indications that they were obtained via averaging, standard deviations and applying descriptive statistical analysis across multiple trials for a specific result, such error outcomes shall be presumed to have been calculated only by equipment-related uncertainties and propagation-of-error analysis. Absolute error describes the magnitude of an error, whereas relative error measures it as a proportion of the true value. Derived from principles of differential calculus, a set of rules was applied to the various measurements in this project to develop formulas for propagating errors through a series of calculations (Taylor, 2022). Equation 3.8 states the addition/subtraction rule, equation 3.9 states the multiplication/division rule, and the constant rule is given by equation 3.10. The equations are summarized below:

Operation	Uncertainty formula	
$z = Ax \pm By$	$\partial z = \sqrt{A^2 \cdot \partial x^2 + B^2 \cdot \partial y^2}$	*Eq. 3.8*
$z = x \cdot y \; or \; z = \frac{x}{y}$	$\partial z = z \cdot \sqrt{\left(\frac{\partial x}{x}\right)^2 + \left(\frac{\partial y}{y}\right)^2}$	*Eq. 3.9*
$z = C \cdot x$	$\partial z = \lvert C \rvert \cdot \partial x$	*Eq. 3.10*

where x, y, and z are arbitrary variables and A, B, and C are constants. Other formulas and rules exist for propagating errors of various functions, but only those mentioned above were used in this thesis.

Chapitre 4 - Résultats

There were many different extraction iterations executed throughout the project, all striving toward the main objective of delivering a viable collagen extraction process to the Lower North Shore, using *C. frondosa*. Table 4.1 will highlight the important parameters and information about each run. For the purpose of table 4.1, the conventional process is defined as:

1. Milling with meat grinder
2. Disaggregation - Tris-HCl with EDTA solution
3. Deproteination - NaOH
4. Extraction - Acid soluble collagen (ASC) or pepsin soluble collagen (PSC). The acid used was 0.5 M acetic acid
5. Recovery - Salting out with centrifugation until iteration 6 as then isoelectric like precipitation method became the standard (iteration 7 to 19)
6. Purification – Ultrafiltration (iterations 1 to 8, 18 and 19), no treatment (iterations 9 to 17)
7. Drying and preservation - Lyophilization and store -20 ℃

Table 4.1. Summary of *C. frondosa* collagen extraction conditions and experiments executed in the project.

Run Number	Process	Extraction Temperature (°C)	Time (hr)	Extraction Pepsin Concentration (g/L)	Test parameter or modification
1	CRBM protocol	4	48	0	Conventional
2	ASC	4	48	0	Conventional
3	PSC	4	24	0.45	Conventional
4	PSC	4	24	0.45	Added NaCl to disaggregation solution
5	PSC	4	70	0.45	Replaced salting out with isoelectric-like precipitation with centrifugation
6	PSC	4	24	0.45	Eliminated deproteination step
7	PSC	4	24	0.45	Reduce disaggregation solution to solid ratio from 10:1 to 4:1
8	PSC	4	24	0.45	Same as 7
9	PSC	4	24	0.45	Conventional
10	PSC	4	24	0.45	Addition of 2ME and NaCl to disaggregation solution
11	PSC	4	24	0.45	Same as 10
12	Pretreatment	N/A	24-72	N/A	Tested various conditions
13	1-ASC with acetic acid 2- PSC 3- ASC with HCl 4- Water	40	17-18	1/3/4 - 0 2 - 0.7	60 % ethanol treatment replaced disaggregation solution Ethanol and NaOH wash only performed for 2 hours Pre and post treatment steps done at 20 °C
14	1-ASC with acetic acid 2- PSC 3- ASC with HCl 4- Water	40	17-18	1/3/4 - 0 2 - 0.7	Water replaced disaggregation solution Water and NaOH steps only performed for 2 hours Pre and post treatment steps done at 20 °C
15	PSC	40	6	0.7	Water wash after disaggregation step only 2 hours Pre and post treatment steps done at 20 °C
16	PSC	40	6	0.7	No Tris-HCl step Only 2-hour water wash Pre and post treatment steps done at 20 °C

Run Number	Process	Extraction Temperature (°C)	Time (hr)	Extraction Pepsin Concentration (g/L)	Test parameter or modification
17	PSC	a- 40 b- 20 c- 20	a- 4 b-4 c-3	a- 0.1 b- 0.7 c- 0.1	Water pretreatment and NaOH pretreatment steps were done for 2 hours Pre-treatment and post treatment steps done at 20 °C
18	PSC	a- 40 b- 30 c- 20	a-3.5 b-2 c-2	a- 0.01 b- 0.1 c- 0.3	Conventional, except pretreatment and post treatment steps were done for 2 hours at 20 °C Dialysis and ultrafiltration purification methods were tested
19	PSC	30	1.5	0.1	Pilot scale-up run of 18 b, both lyophilization and spray drying were tested

The results will be broken down into three sections which include (i) Biochemical and chemical properties of the biomass, (ii) *C. frondosa* process synthesis and design, and (iii) Optimized collagen extract scale-up analysis.

4.1 Biochemical and chemical properties of *Cucumaria frondosa*

To accurately measure and contrast with previous research on sea cucumber collagen extraction methods, a range of biochemical and physical properties were examined. This will provide a starting point to understand how a process can be developed on the Lower North Shore of Quebec. These properties are summarized in table 4.2.

Table 4.2. Summary of biochemical and physical properties of the *Cucumaria frondosa* body wall from this study compared to other sea cucumbers.

Property	Moisture Content (%)	Protein Content (mg protein per gram dry sea cumber)	Collagen content (% - wet basis)
Cucumaria frondosa (This study)	84 ± 3 n = 11	590 ± 40 n = 2	1.6 ± 0.2 * n = 2
Cucumaria frondosa	87.4 **	661 **	<1 ***
Australostichopus mollis (F. Liu et al., 2017)	93	Not available	1.4
Parastichopus californicus (Z. Liu et al., 2010)	86-91	500-650	2.1-3.5

* Estimated with Quickzyme total collagen assay.
** Reference (Y. Zhong et al., 2007).
*** Reference (Hossain et al., 2020).

The moisture content and protein contents were similar to previous studies done on *C. frondosa* and other species. As for collagen content, the sea cucumber used in this study showed to have similar collagen content as *Australosticopus mollis* but less than the *Parastichopus californicus.* When compared to previous studies that were done on *C. frondosa*, the sea cucumber in this study

had higher amounts of collagen content. Next, various heavy metal and mineral concentrations were analyzed and are summarized in Table 4.3.

Table 4.3. Summary of elements and minerals in the body wall of the *Cucumaria frondosa* compared to other sea cucumbers.

Element (µg/g dry biomass)	***Cucumaria frondosa* (This study) n = 2**	***Acaudina leucoprocta*** (S. Lin et al., 2017)	***Holothuria tubulosa*** (Künili & Çolakoğlu, 2019)
Lead	0.14 ± 0.08	4.81	0
Mercury	0.011±0.003	0.97	1.13
Arsenic	3.7 ± 0.2	18.89	3.01
Chromium	40 ± 10	7.13	N/a
Cadmium	0.5 ± 0.3	0.91	0.44
Beryllium	0.002 ± 0.002	N/a	N/a
Nickel	15 ± 2	N/a	0.38
Sodium	80 000 ± 20 000	N/a	24 494
Potassium	14 800 ± 100	N/a	2781
Calcium	6 000 ± 1 000	63778	17 620
Magnesium	12 000 ± 3 000	12070	2655

The sea cucumber *C. frondosa*, the subject of this study, had physical, chemical and biochemical properties that were comparable to those observed by other groups working on different species of sea cucumbers. Compared to the species *A. lecuprocta* and *H. tubulosa*, *C. frondosa* utilized in this study had lower or the same levels of heavy metals except chromium and nickel. Chromium concentrations were 5.6 times higher and nickel levels were 40 times higher in *C. frondosa* in this study than what was observed in *A. leucoprocta*. Regarding the prevalent minerals and salts, the *C. frondosa* in this study contained more sodium and potassium than the other two species in Table 4.3, with moderate levels of calcium and magnesium. The results for *C. frondosa* in table 4.2 used descriptive statistics from two (2) samples. Further experiments are required to validate the potential environmental and physiological stress impacts that can explain the difference of mineral levels in *C. frondosa* compared to other species.

4.2 *Cucumaria frondosa* process synthesis and design

This project focused on the synthesis and design of a protocol that would deliver a product and protocol that could be easily implemented on the Lower North Shore. This section will go into detail on the experiments and results of those experiments to demonstrate how the final protocol was decided upon.

4.2.1 Performance of different collagen extraction processes

To begin the process synthesis, three protocols were tested which included (i) CRBM protocol developed for fish native collagen, (ii) conventional acid soluble collagen process and (iii) conventional pepsin soluble collagen. Images of salted out products of each process are shown below.

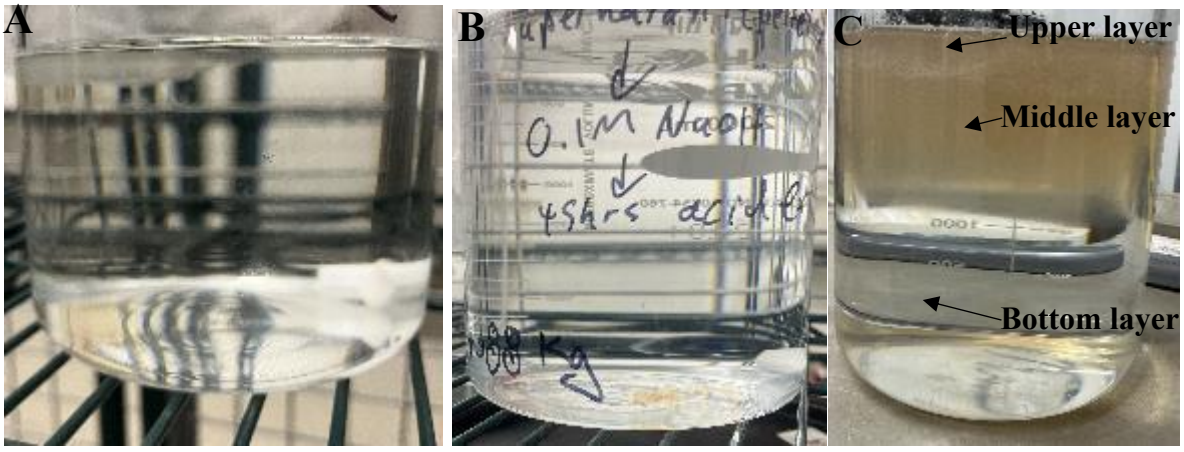

Figure 4.1. Formation of precipitates after salting out step for (a) CRBM protocol, (b) acid soluble collagen protocol and (c) pepsin soluble protocol to a final NaCl concentration of 1.1 M.

By visual inspection of Figure 4.1, something has precipitated in the pepsin soluble extract where nothing occurred in the other two extract products. In Figure 4.1.a, the CRBM protocol solution is clear with no precipitation. With the acid protocol in Figure 4.1.b, the solution is clear with a trace of insoluble particles on the bottom of the beaker. In Figure 4.1.c for the pepsin soluble protocol, there is layer formation at the top of the solution where a molecule has precipitated out. This visual result is like what was observed by Spigolon et al. (2022) when working with lump fish when the salting out step was executed. The solid seen in Figure 4.1.b is not how the precipitation should

look like. These results suggest that the pepsin soluble protocol extracted collagen from the biomass while the acid soluble and CRBM protocol did not. To reconfirm this observation, Native PAGE was done on the acid soluble and pepsin soluble processes and is illustrated in Figure 4.2.

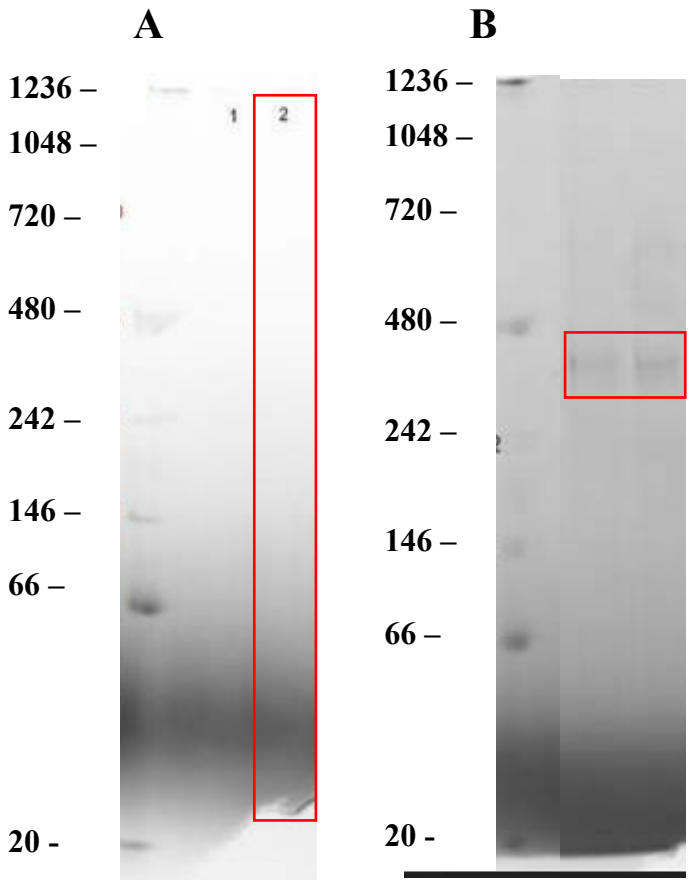

Figure 4.2. Native gel electrophoresis results of extract produced using (a) acid soluble collagen (lane 2) and (b) pepsin soluble collagen protocol. All molecular weights are in kDa. The ladder is in the leftmost lane in each figure.

The pepsin soluble extract was more effective in extracting collagen than the acid soluble protocol. In Figure 4.2.a lane 2 which was the salted-out acid soluble fraction, there are no protein bands present. In Figure 4.2.b, lanes 6 (middle layer in Figure 4.1.c) and 7 (top layer in Figure 4.1.c), a protein band was visible at about 380–400 kDa. This corresponds to the size that native collagen would migrate on these gels. The pepsin-soluble method was therefore selected for further experimentation.

4.2.2 Investigation of various extraction pre-treatment and post treatment steps

The pepsin-acid soluble extraction step is the core of the process. However, various pre- and post-treatment steps are essential to properly prepare the material. The subsequent sections will discuss various experiments related to these steps.

4.2.2.1 Comparison of recovery techniques after extraction

Two common recovery techniques that are used after the pepsin-soluble extraction step are salting out and isoelectric precipitation. In this thesis, the isoelectric point was not determined. Based on the literature review, collagen's lowest solubility in solution was observed between pH 5.5 and 7. Since pepsin is inactive at pH 7, the isoelectric precipitated extracts were brought to a pH of 7. Figure 4.3 shows the visual result of this.

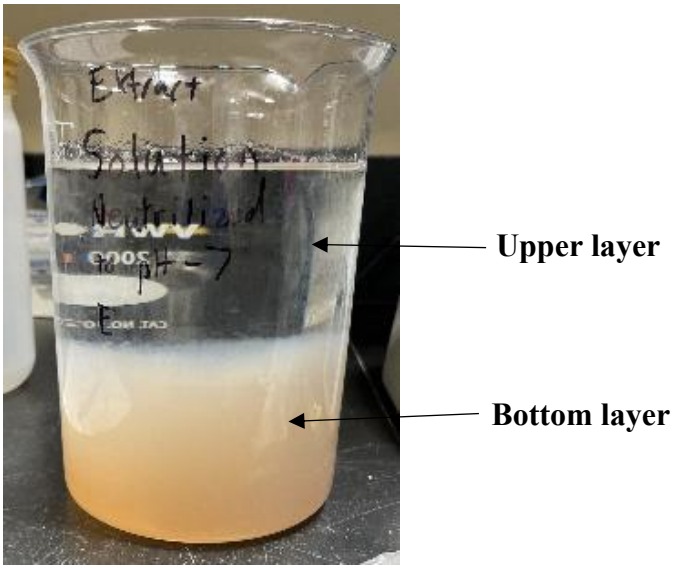

Figure 4.3. Phase separation of pepsin soluble extract after the solution was neutralized to a pH of 7.03.

In the case of Figure 4.3, the solid precipitates to the bottom of the mixture, opposite what was seen for salting out. Again, this visual result aligns with what Spigolon et al. (2022) observed when they performed isoelectric precipitation on their extracts. Therefore, this result confirms that the protein was precipitated out at pH 7.0. Once the precipitate is formed, they undergo centrifugation

to recover the pellet. Figure 4.4 looks at the Native PAGE of the salted out and isoelectric-like pellet.

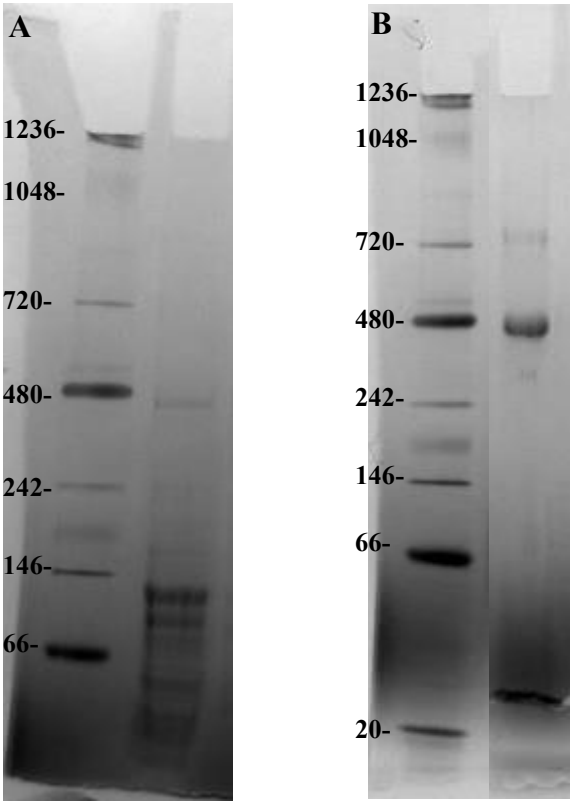

Figure 4.4. Pellets collected from centrifugation through (a) salting out recovery and (b) isoelectric-like recovery. All molecular weights are in kDa. The ladder is in the leftmost lane in each figure.

According to Figure 4.4, the isoelectric-like recovery method had fewer protein impurities than the salting-out method. Both the salted-out pellet (Figure 4.4.a) and isoelectric-like pellet (Figure 4.4.b) has bands that are slightly below the 480 kDa marker. In the salted-out protocol, there were more low-molecular-weight impurities or potential collagen degradation than the isoelectric product. The latter had a band at 720 kDa that did not appear in the salted-out extract. To further study the purity and structure, Figure 4.5 summarizes UV-vis and FTIR curves of each methods product.

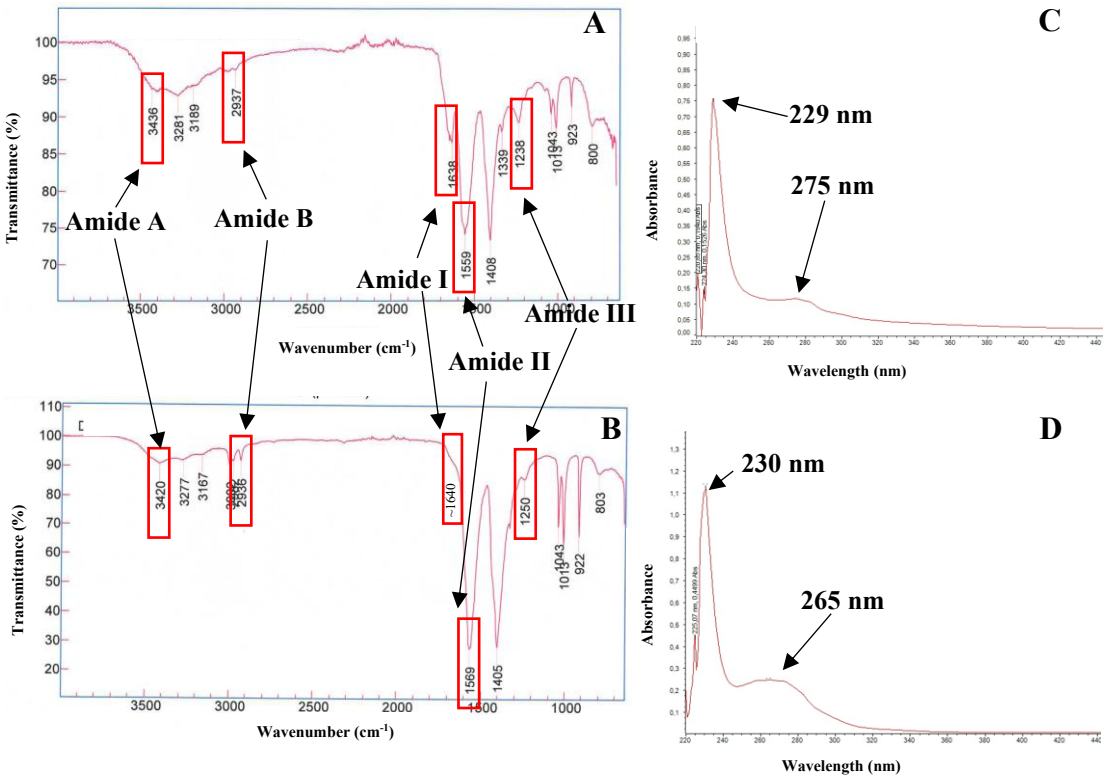

Figure 4.5. FTIR spectra of (a) isoelectric like precipitated product and (b) salted out product and UV-vis spectra of (c) isoelectric like precipitated product and (d) salted out product. The extract products used to develop these figures also included a centrifugation step to recover the pellet. For the UV-Vis experiment, the solvent used to dissolve the powdered samples and blank was 0.5 M acetic acid.

The FTIR were analyzed in each extract, and the isoelectric product contained all five essential peaks as seen in Figure 4.5.a. The salted-out recovery product had only four established peaks, and a weak peak that was the amide I band, which had a wavenumber of approximately 1640 cm^{-1} (Figure 4.5.b). Similarly, the isoelectric-like product had an amide I peak at 1638 cm^{-1} (Figure 4.5.a). Therefore, both samples collagen secondary structure demonstrated intermolecular cross-linking but was a mix between irregular shape and α-helices. The ratio of the 1450 cm^{-1} and the amide III could not be calculated because no signal was present at 1450 cm^{-1} in either extract. No conclusion could be made if the collagen extracted in this sample kept its triple helical because of the missing band. Both UV-vis spectra showed that the product had absorbance peaks near 230 nm, which corresponds to carbonyl groups and amide groups of peptide bonds that are highly abundant in collagen. There were also peaks near 280 nm. The FTIR spectra and UV-vis spectra of both

fractions are like the bovine tendon collagen standard's FTIR (Figure 3.3) and calf skin collagen's UV-vis spectra (Figure 3.2). The ratio of the absorbance at 280 nm to 230 nm directly measures the concentration of aromatic acids like tyrosine, tryptophan and phenylalanine in the sample, compared to the functional groups that are more prevalent in collagen. By extension, it provides an indirect measure of the collagen extract's purity relative to other proteins. Higher levels of amide groups (peptide bonds) are present in collagen than in aromatic amino acids, which can also be found in various non-collagen proteins, as measured at 280 nm. However, the specific wavelength may vary slightly based on the concentration of tyrosine and tryptophan. The corresponding ratios for each fraction are outlined in Table 4.4.

Table 4.4. A280/A230 UV-vis ratios of salted out versus isoelectric-like precipitation. Both methods also used centrifugation to recover the pellets.

Parameter	**Salting out precipitation with centrifugation recovery**	**Isoelectric-like precipitation with centrifugation recovery**
A280/A230	0.177 ± 0.003	0.140 ± 0.003

The isoelectric like recovery product had a slightly lower A280/A230 ratio, indicating that it had less aromatic amino acid and indirectly, less non-collagenous proteins. The protein content and sodium content were analyzed and summarized in table 4.5.

Table 4.5. Final total protein yield and sodium content collected by salting out versus isoelectric-like precipitation.

Parameter	**Salting Out with centrifugation recovery**	**Isoelectric-like precipitation with centrifugation recovery**
Protein content (mg protein per g of dry biomass)	71 ± 8	100 ± 10
Sodium content * (%)	0.8794 ± 0.0001	0.5544 ± 0.0004

*The sodium concentration measurement was immediately carried out following the completion of each precipitation recovery technique, before any subsequent post-processing steps, such as centrifugation or refinement.

The total protein content was slightly higher when isoelectric-like precipitation was used, and the sodium content was less. However, it is difficult to define a conclusion on the yield, since the extraction time was tripled for the isoelectric precipitation reaction. This could skew the result and interpretation. However, with respect to purity, time should have a minimal impact on the parameter. It is important to note that in Table 4.5, the protein content is measured from the final lyophilized product, whereas the sodium content was measured after each specific recovery step (salting-out or isoelectric-like precipitation). For instance, in the case of salting-out, the sample was collected from the upper layer in Figure 4.1, while for the isoelectric-like method, the sample was taken from the bottom layer in Figure 4.3 when determining the sodium content in Table 4.5. While the characterization techniques clearly show collagen was extracted and recovered using either product, the results in this section demonstrate that the isoelectric-like precipitate product was able to recover just as much of the protein product as the salted-out product but contained less non-collagenous proteins and lower amounts of salt, leading to its higher purity. The salted-out fraction had higher levels of impurities as shown in Tables 4.4 and 4.5. However, structurally, the molecules recovered are similar, even if some signals, such as the amide I band, are more pronounced depending on the type of post-treatment. According to literature, the signal intensity is related to the absorbance of the molecule(s) being analyzed by FTIR (Kaur et al., 2021). In this study, the signal of the amide I band in Figure 4.2.b (salted-out fraction) may be weaker than the amide I band in Figure 4.2.a (isoelectric-like precipitation fraction), as there are more impurities masking the amide I signal in the salted-out sample. The specific outcomes are not presented, but rather discussed in the procedural sections of each approach described in Chapter 3. The isoelectric-like precipitation technique is faster to perform than the salting-out method. Therefore, this method was used throughout the rest of the project

4.2.2.2 Importance of deproteination prior to extraction

In efforts to reduce processing time, a test was conducted to determine if the deproteination step was necessary prior to pepsin-acid extraction. Therefore, after the disaggregation step, the product that normally was passed to the deproteination step went directly to the pepsin acid step. After the salting out step was done, the resulting solution in Figure 4.6 was observed.

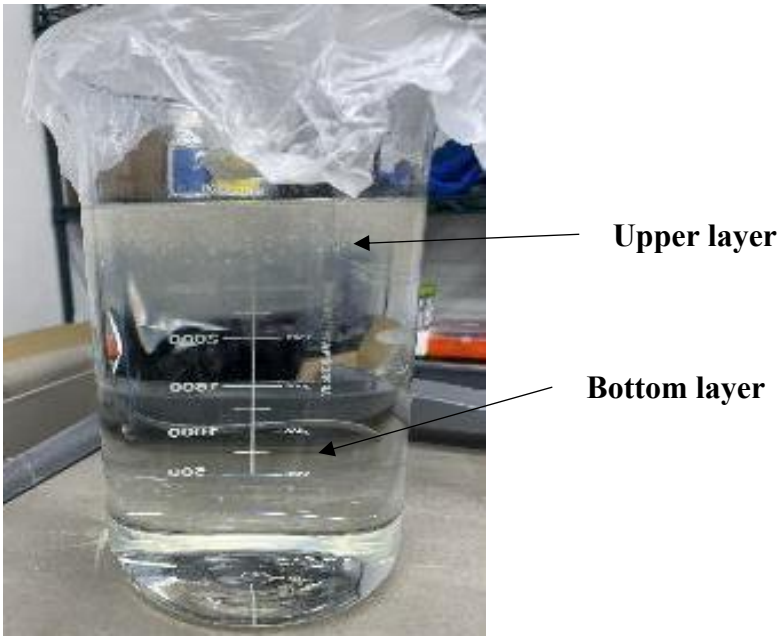

Figure 4.6. Salted out extract from run 6 which did not undergo a deproteination step before extraction.

When compared to Figure 4.1.c, a similar effect was observed but the amount of precipitation seemed to be less. To gain a deeper understanding of the factors involved, Figures 4.7 and 4.8 provide an analysis of the Native PAGE and SDS-PAGE of this sample, respectively.

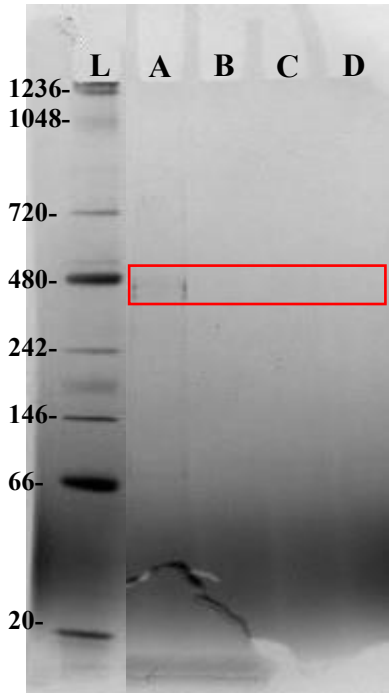

Figure 4.7. Native PAGE of fractions from the experimental run with no deproteination step (a) pellet from salted out fraction, (b) supernatant from salted out fraction, (c) solubilized pellet prior to 40 µm filtration, (d) solubilized pellet prior to 40 µm filtration (feed to ultrafiltration) and molecular weights are in kDa. The ladder is denoted by (l).

In Figure 4.7 lane a, which was the pellet produced from the upper layer in Figure 4.6, had two bands around 420 kDa, meaning protein was collected from the extraction process. In lane b, which was the supernatant from the salted-out extract and as expected, no collagen bands were present. Lanes c and d contained the pellet resolubilized prior to 40 µm filtration and after 40 µm filtration. There were no bands in each lane meaning the pellet was degraded somewhere in the extract or the concentration was not high enough to observe on the gel.

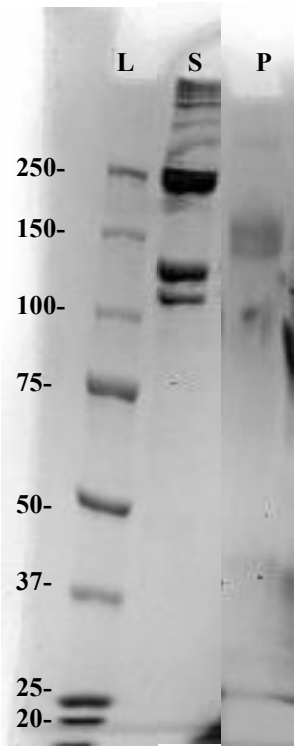

Figure 4.8. SDS Gel of purified product from extract run that did not undergo the deproteination step denoted by (p) and calf-skin type 1 collagen standard denoted by (s). The ladder was denoted by (l) and molecular weights are in kDa.

In Figure 4.8, two protein bands of the product were detected at 157 kDa and 100 kDa. The standard had bands at 120 kDa and 105 kDa, which corresponded to α1 and α2, respectively. Additionally, the standard had a band indicating the presence of β (250 kDa) and γ chains (>300 kDa). In the product lane, no bands were observed, suggesting that some degradation may have taken place. Given the low quantity of collagen extracted and the potential of protein degradation, it was concluded that the deproteination step was necessary in the extraction process.

4.2.2.3 Disaggregation liquid: solid ratio effects

The next pre-treatment parameter studied was the solid to liquid ratio with the initial sea cucumber (wet basis) and the Tris – EDTA disaggregation solution. The idea of studying this parameter was the hope of reducing the amount of chemicals needed and reducing the size of equipment, saving capital cost. Typically, the ratio was 10:1 disaggregation solution to solid ratio. However, in run 7, the ratio was lowered to 4:1 and the SDS PAGE gel is summarized in Figure 4.9.

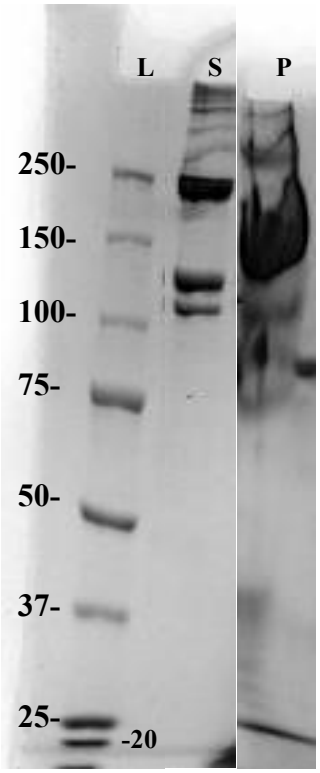

Figure 4.9. SDS PAGE of purified product from run 7 (PSC extraction with Tris-EDTA to sea cucumber ratio 4:1) denoted by lane (p), and calf-skin type 1 collagen standard denoted by (s). The ladder was denoted by (l) and molecular weights are in kDa.

As shown in Figure 4.9, there was a band present around 130-140 kDa and another around 105-115 kDa, indicating type I collagen was extracted. There were also bands present around 250 kDa and greater, demonstrating there were higher order native structures present in the solution. Several characteristics of the final product from run 7 were studied and summarized in Table 4.6. The results in the table are also compared to run 5, which had a disaggregation solution to solid ratio of 10:1.

Table 4.6. Summary of process extraction data when varying 0.1 M Tris-HCl/ 4 mM EDTA pH 8.0 to sea cucumber ratios (wet weight).

Ratio	Run Number	Yield (mg powder per g of dry sea cucumber)	Sodium content final product (%)	Adjusted yield excluding sodium (mg powder per g sea cucumber dry mass)
10:1	5	200 ± 20	12.99 ± 0.01	170 ± 20
4:1	7	48 ± 5	23.95 ± 0.01	36 ± 5

The yield was calculated based on total powder mass per gram of dry sea cucumber. There was a lot of sodium added throughout the process, and it is the most concentrated mineral in the initial biomass by 8-fold (see table 4.1). To get an approximation of the yield of extracted solids, the values were corrected based on sodium content percentages in the final product. The adjusted yield of the 10:1 ratio was approximately 5-fold greater than the 4:1 ratio. This result concluded that this ratio had an impact on the final extraction yield and that the standard value used in the literature worked well.

To date, the chemicals used were selected following a clean chemical approach knowing the goal of the project was to adapt in a region where the handling and disposal of these chemicals will be problematic. While collagen was successfully extracted, there was still collagen left in the biomass that could be extracted. The average yield from the previous set of experiments until this point was around 8.6 % protein content (dry basis). Knowing that collagen represents approximately 20 % of the total protein in body tissues, the dry weight yield would be 1.72 %. For various sea cucumber species, this number was greater than 20 % (S. Lin et al., 2017; Z. Liu et al., 2010).

Therefore, to further disaggregate the biomass, the Matsumura method was applied which included 2-mercaptoethanol and sodium chloride in hopes of disaggregating further the initial biomass to promote higher yields. The 2-mercapoethanol would not be scalable and applicable to be used in the process on the Lower North Shore but it was important to test if more molecular bonds could be disrupted in the biomass' macrostructure to see if more collagen could be extracted.

4.2.2.4 Impacts of the addition of 2-mercaptoethanol and sodium chloride to dis-aggregation solution

The addition of 2-mercaptoethnaol (2ME) and sodium chloride to the disaggregation solution with Tris-HCl and EDTA were evaluated using several methods. The first method used was SDS PAGE that compared the final products that were disaggregated with Tris-EDTA and Tris-EDTA-NaCl-2ME. The results are observed in Figure 4.10.

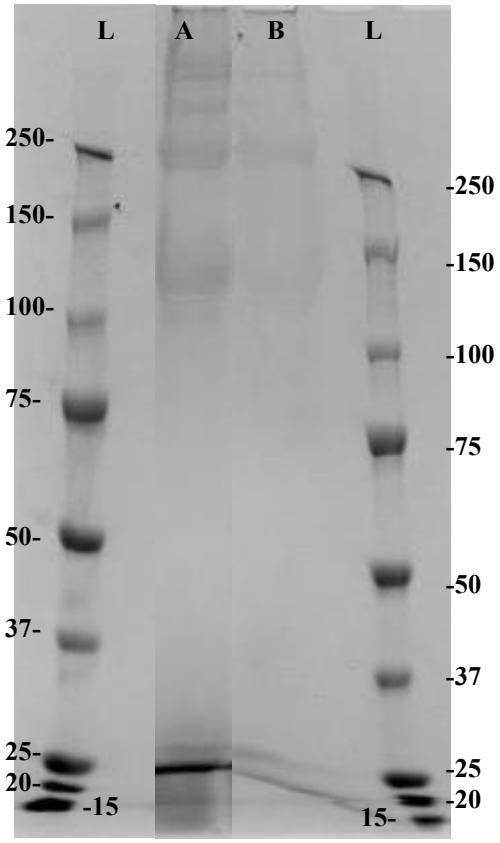

Figure 4.10. SDS Page gels of final PSC products when (a) Tris-HCl/EDTA and (b) Tris-HCl/EDTA/NaCl/2-mercaptoethanol was used for the disaggregation solution. L is the ladder and molecular weight are in kDa.

In Figure 4.10, both extracts had bands that were present around 120 kDa and had presence of β and γ helical forms. Therefore, it was concluded that collagen was extracted with higher order collagen structures present in the extract. To reconfirm the presence of collagen and its impurities, UV-vis plots are presented in Figure 4.11.

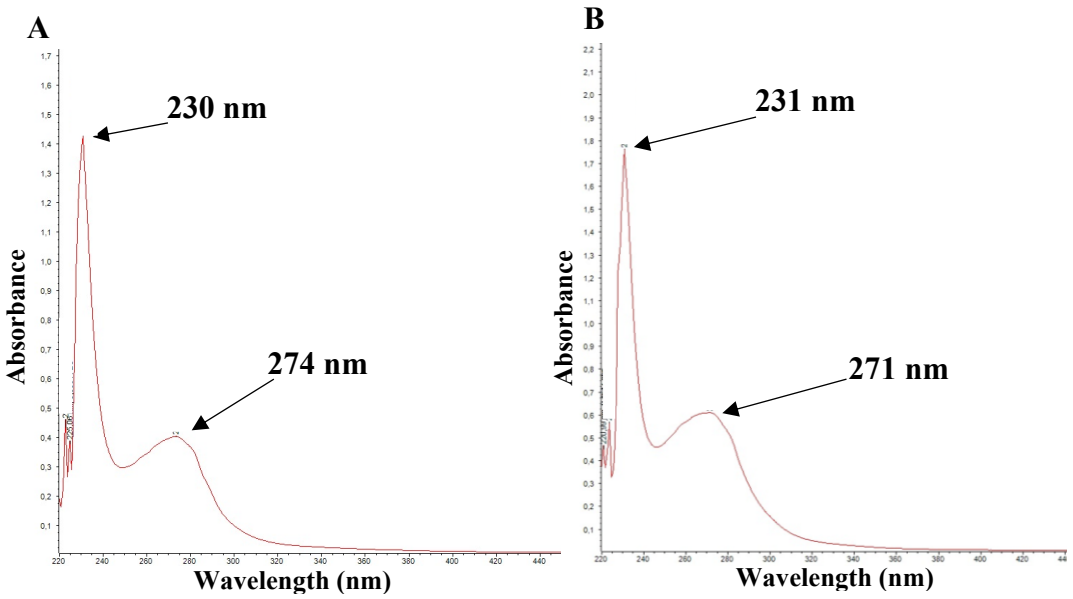

Figure 4.11. UV-vis spectra of final product when (a) Tris-HCl and EDTA and (b) Tris-HCl, EDTA, NaCl and 2-mercaptoethanol was used for the disaggregation solution. The solvent used to dissolve the powdered samples and blank was 0.5 M acetic acid.

According to Figure 4.11, the primary signal is at 230 nm for both extracts, with a secondary peak near 280 nm. This suggests the presence of aromatic amino acid residues in the sample, potentially related to the collagen extracted or from other proteins (Cui et al., 2007). Given the SDS PAGE consist of bands mainly related to collagen, one can infer that the band intensity is predominantly from collagen and not from non-collagenous proteins. Similar peaks were observed in sea cucumber species *A. mollis* and *H. cinerascens* (P. H. Li et al., 2020; F. Liu et al., 2017) The A280/230 ratios are evaluated in Table 4.7.

Table 4.7. Yield and purity information of final PSC products when (a) Tris-HCl/EDTA and (b) Tris-HCl/EDTA/NaCl/2-mercaptoethanol was used for the disaggregation pre-treatment step.

Disaggregation solution	Run Number	Total Collagen yield (mg collagen per gram dry biomass) n = 2	A280/A230
Tris-HCl and EDTA	9	8 ± 1	0.308 ± 0.006
Tris-HCl, EDTA, NaCl and 2ME	10	30 ± 1	0.257 ± 0.005

In Table 4.7, run 9 and run 10 had similar A280/A230 ratios but run 10 had higher collagen yields. In other words, the application of 2ME and NaCl led to an increase in collagen yield while maintaining a consistent A280/A230 ratio. This is significant because, as discussed earlier in this chapter, it indirectly indicates that the level of contamination or impurity of the collagen sample with respect to other proteins did not change or even decrease slightly but the yield increased. Compared to the literature, the collagen yields were still lower, as typically yields reached as high as 20 % in various species (S. Lin et al., 2017; Z. Liu et al., 2010). The results for runs 9 and 10 in table 47 used descriptive statistics from two (2) samples.

The collagen products from runs 9 and 10 were analyzed using CD spectroscopy to determine their thermal denaturation temperatures (T_d), as shown in Table 4.8.

Table 4.8. Thermal denaturation temperatures of collagen extract products from runs 9 and 10 at wavelengths 198 nm and 220 nm.

Run number	Thermal denaturation temperature (T_d) at wavelength 198 nm (°C)	Thermal denaturation temperature (T_d) at wavelength 220 nm
9	21 ± 2	N/A*
10	20 ± 2	20 ± 3

*Correlation of CD versus temperature did not have R^2 greater than 0.9, therefore calculation not possible.

In Table 4.8, the thermal denaturation temperature was similar in both runs, ranging between 20 ± 3 °C to 20 ± 2 °C. These values are like what has been observed in literature. Z. Liu et al.

(2010) reported a T_d between 17.9℃ to 18.5 ℃ for *P.californicus*, while lower than the T_d of *A.leucoprocta* which was 25.4 ℃ (S. Lin et al., 2017). All of these are lower than porcine collagen which was 37 ℃ (Abedin et al., 2013). No value was reported the thermal denaturation temperature of run 9 at 220 nm as a strong correlation was not able to be developed between temperature and ellipticity. Figure 4.12 provides further insight into this observation.

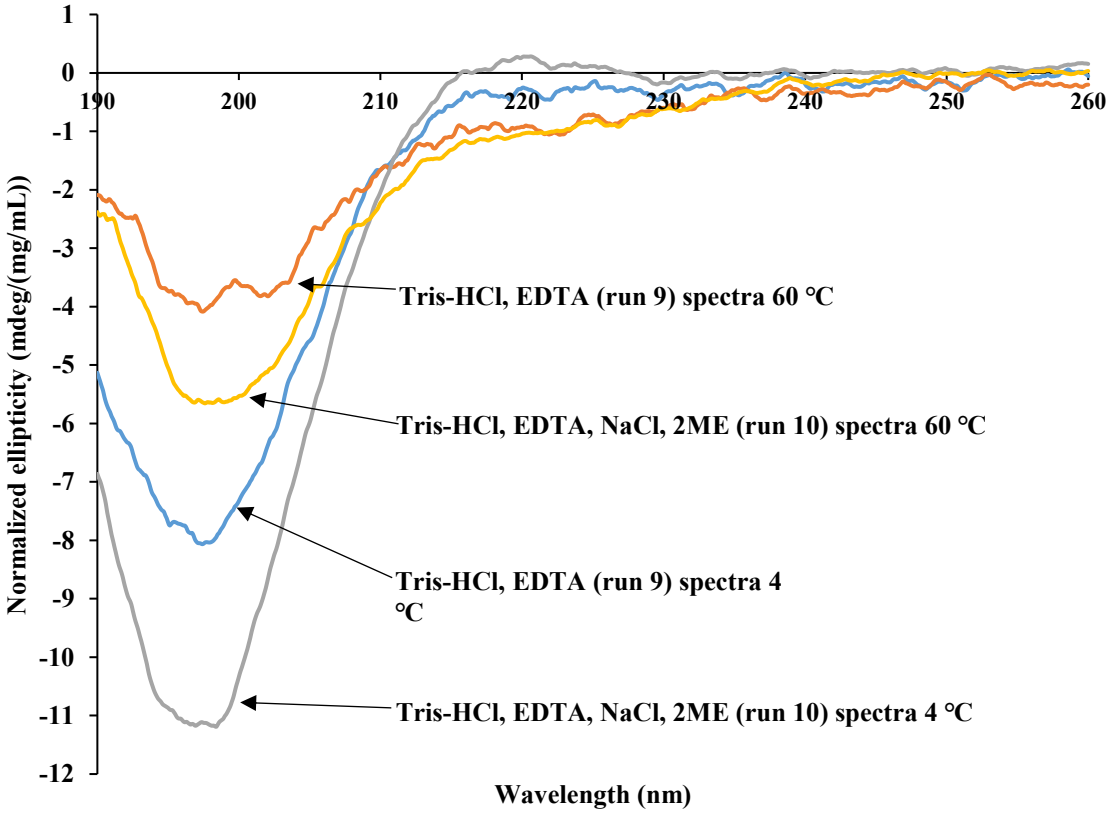

Figure 4.12. CD spectra of collagen product from the Tris-HCl EDTA disaggregation solution extract (run 9) where spectra was tested at 4 ℃ (blue) and at 60 ℃ (orange), and the Tris-HCl, EDTA, NaCl, 2ME disaggregation solution extract (run 10) was tested at 4 ℃ (grey) and at 60 ℃ (yellow). Each solution and result were normalized to 1 mg/mL.

In Figure 4.12 for run 10, a minimum peak was observed at 198 nm and a maximum was observed at 220 nm when the CD was run at 4 ℃. At 60 ℃, the 220 nm peak disappeared, and the 198 nm peak signal reduced which is expected when collagen denatures. At 220 nm, this peak is correlated with collagen in the triple helix confirmation (Alves et al., 2017). For run 9, while a minimum

peak is observed at 198 nm, the peak at 220 nm was never observed. Also, the curves are normalized to 1 mg/mL of total protein and the peaks in run 10 are more intense, suggesting a more concentrated sample.

This demonstrates that the disaggregation solution used in run 10 which used 2ME in the disaggregation solution was able to increase the extraction yield of the extract and confirm that more native collagen could be liberated from the biomass. With these findings, more studies were conducted on this disaggregation step as there were some key findings and results that came in changing parameters of this step. At this point, the strategy changed as instead of doing the complete process linearly, multiple tests were done on the same step to optimize a particular point in the process and in this case, it was the disaggregation step.

4.2.2.5 Impact of conditions on the disaggregation treatment of the sea cucumber's body wall

Table 4.9 describes the effects of modifying various operating conditions related to the disaggregation step. As mentioned previously and important to reiterate for this table, these results were not done in duplicates and triplicates and therefore general conclusions will be extrapolated from the table instead of direct comparisons of the values presented.

Table 4.9. Summary of the impacts of varying disaggregation solution conditions on the total suspended solids released in solution. The initial mass of the sea cucumber's body wall used in each test was 25 g.

Disaggregation Solution	Time (hours)	pH	Temperature 6 ℃	Temperature 20 ℃
			Solids extraction yield (mg suspended solids per gram sea cucumber wet basis)	
Tris-HCl, 2ME, NaCl and EDTA	4	8	29.0 ± 0.1	Not available
	20	8	33.0 ± 0.1	49.8 ± 0.2
	46	8	127.0 ± 0.5	Not available
	72	8	102.3 ± 0.4	Not available
	20	3	0*	Not available
	20	5.5	16.2 ± 0.1	63.2 ± 0.3
	20	12.5	0*	Not available
Tris-HCl, NaCl, EDTA	20	8	91.7 ± 0.4	Not available
Distilled water	72	6.5	53.6 ± 0.3	Not available

*Blank had higher dry weight percentage than solution, considered 0.

Before discussing any results from Table 4.9, the yield in this case was not determined through a quantification assay or protocol, it is simply determined based on the solids content of the permeate that passed through a 425 μm sieve after a specific set of disaggregation conditions was applied. Each sample, depending on its constituents, were blanked with the appropriate solutions to give the values in the table above. The first observation is the impact of time. At 46 hours, there seems to be an increase in the number of suspended solids that have entered the solution. Secondly, pH's between 5.5 and 8 were able to extract solids from the starting biomass and extreme pH's either acid or basic, the disaggregation step did not perform as well. For pH 3, the solid did not even disperse in the solution, it remained aggregated together, even when agitation was applied (results not shown). Thirdly, solution temperature has little to no impact on the liberation of solid from

biomass into solution between 6 ℃ and 20 ℃. The increase in value is not as pronounced as that of time and pH. Finally, having a disaggregation solution with just Tris-HCl, EDTA and NaCl may have similar benefits of liberating crude collagen fibrils in solution if the 2-mercaptoethanol was present. With further experimentation and repetition more concrete cause and effect can be determined.

After completing this optimization studies with 2ME, it was clear that additional chemicals and parameters could be added or modified to extract more collagen in its native form. As mentioned, it is clear 2ME cannot be used to implement the process on the Lower North Shore. To date all extractions were done at 4 ℃. Since collagen extraction is a thermodynamic process, it was proposed that adding heat would improve the extraction rate by enhancing pepsin activity and the reaction is inherently endothermic, meaning that any increase in temperature would increase the yield. This method would eliminate the need for hazardous chemicals, ultimately increasing the overall extraction yield. There are also other commercial processing benefits that will be discussed later. The main constraint when utilizing heat to extract native marine collagen is that it often undergoes denaturation at temperatures ranging from 15 to 20 °C in cold water species. As a result, the quantity of heat applied or the extraction temperature cannot be raised too much, as this would severely degrade the final product. This is the reason why there are few to no examples of this approach in the literature. However, it was determined that even if an extract could be produced with a proportion of native collagen mixed with denatured forms collagen and gelatin, it would still create a high value product and process to benefit the population of the Lower North Shore. Therefore, in the next set of studies, the use of additional and/or harsh chemicals would be replaced with heat.

4.2.3 Lab scale extraction optimization

Now that it was decided to work at higher temperatures, various extractions conditions were tested and summarized in Table 4.10.

Table 4.10. Summary of the total protein yields and UV-vis A280/A230 ratios obtained during collagen extraction at a temperature of 40 °C. For UV-vis experiments, all samples were dissolved and blanked with 0.5M acetic acid.

Run number	Disaggregation solution parameters	Extraction conditions	Total protein yield (mg protein / g dry biomass)	UV-vis A280/A230 ratio
13.2	60% Ethanol , time: 2 hours	0.5 M Acetic Acid, 40 ℃, 0.7 g/L pepsin Time: 17 hours	130 ± 10	0.151 ± 0.003
13.3	60% Ethanol, time: 2 hours	0.1 M HCl Time: 17 hours	35 ± 4	0.195 ± 0.003
14.2	Water, time: 2 hours	0.5 M Acetic Acid, 40 ℃, 0.7 g/L pepsin Time 18 hours	170 ± 20	0.175 ± 0.004
14.3	Water, time: 2 hours	0.1 M HCl Time: 18 hours	42 ± 4	0.147 ± 0.003
15	Tris-HCl, time: 21 hours Water, time : 2 hours	0.5 M Acetic Acid, 40 ℃, 0.7 g/L pepsin Time: 6 hours	230 ± 20	0.128 ± 0.002
16	Water, time: 2 hours	0.5 M PSC – 40 ℃, 0.7 g/L pepsin Time: 6 hours	180 ± 20	0.141 ± 0.003

Table 4.10 summarizes the A280/A230 ratios, an indirect measure of the ratio of non-collagenous to collagenous proteins, based on their amino acid and functional group compositions (as described in section 4.2.2). For all samples, the ratios remain fairly consistent, indicating that the collagen extract's purity was not significantly impacted by the specified extraction conditions. When comparing the runs 13.2 to 13.3 and 14.2 to 14.3, it observed that the PSC process extracted more protein than an ASC process with HCl, by approximately 4-fold. As was observed when the extraction was done at 4 ℃, the pepsin soluble process is still the most effective method. If we compare 13.2 (ethanol pre-treatment) to 14.2 (water pretreatment) of the PSC process, the protein yield of run 13.2 is 130 ± 10 mg protein per mg dry sea cucumber and 14.2 has a yield of 170 ± 20 mg

protein per g of dry sea cucumber. These results indicate that ethanol provided little to no augmentation in protein yield compared to pre-treating the sea cucumber body wall with water, and therefore ethanol was eliminated from a being a pre-treatment option. When comparing runs 15 which had an additional Tris-HCl pretreatment step compared to run 16 which was just water, run 15 extracted 230 ± 20 mg of protein per gram of dry sea cucumber compared to 180 ± 20 mg of protein per gram of dry sea cucumber for run 16. This shows that the use of Tris-HCl solution adds slight benefits compared to using solely water for the disaggregation step, allowing more protein material being extracted from the biomass and was incorporated into protocol being developed. Throughout the project, it was determined that of 30.0 ± 0.2 % (n = 3 samples) of the total protein is collagen following the extraction step regardless of any process parameter variations. Therefore, the protein values give insight into extracted collagen values and similar trends are expected.

After tuning the pre-treatment steps, the temperature and pepsin concentration were varied, and the results are summarized in Table 4.11. To note, the n = 2 samples in Table 4.11 is not that the extraction experiment was done twice, it is the collagen quantification assay was done in duplicate on the same extraction run. Similarly for n = 4 samples, the experiment was done on two separate occasions/trials and on each occasion, the assay was done in duplicate on the same sample.

Table 4.11. Effects of temperatures and pepsin concentration on collagen extraction at 10:1 0.5 M acetic acid to solid ratio. 40 g of deproteinized crude collagen solid was applied to each run setting. The extraction duration was 120 minutes.

Run Number	Temperature (°C)	Pepsin concentration (g/L)	Total collagen yield (mg collagen per gram dry sea cucumber), mean ± 1 standard deviation
17c*	20	0.1	42 ± 6 (n = 4)
18b	30	0.1	46 ± 3 (n = 2)
17a*	40	0.1	60 ± 1 (n = 2)
18c	20	0.3	49 ± 9 (n = 4)
17b*	20	0.7	57 ± 2 (n = 2)

*Values were corrected to if reaction took place for 120 minutes.

As temperature was increased from 20 ℃ to 40 ℃, there was an increase in total collagen yield from 42 ± 6 mg collagen/g dry sea cucumber to 60 ± 1 mg collagen/g dry sea cucumber. Compared to the literature, the yields were still lower, as typically yields reached as high as 20 % in various species (S. Lin et al., 2017; Z. Liu et al., 2010). Compared to the values in Table 4.6, the collagen yields were higher than the conventional extraction method by 5.6-fold, and 1.6-fold higher compared to when 2ME was added. The difference between 20 °C and 30 °C is not so pronounced as the differences compared to 40 °C. For each of these extraction temperature settings, the extraction was monitored over a period and samples were taken every 20 minutes, and the results were shown on SDS PAGE in Figure 4.13.

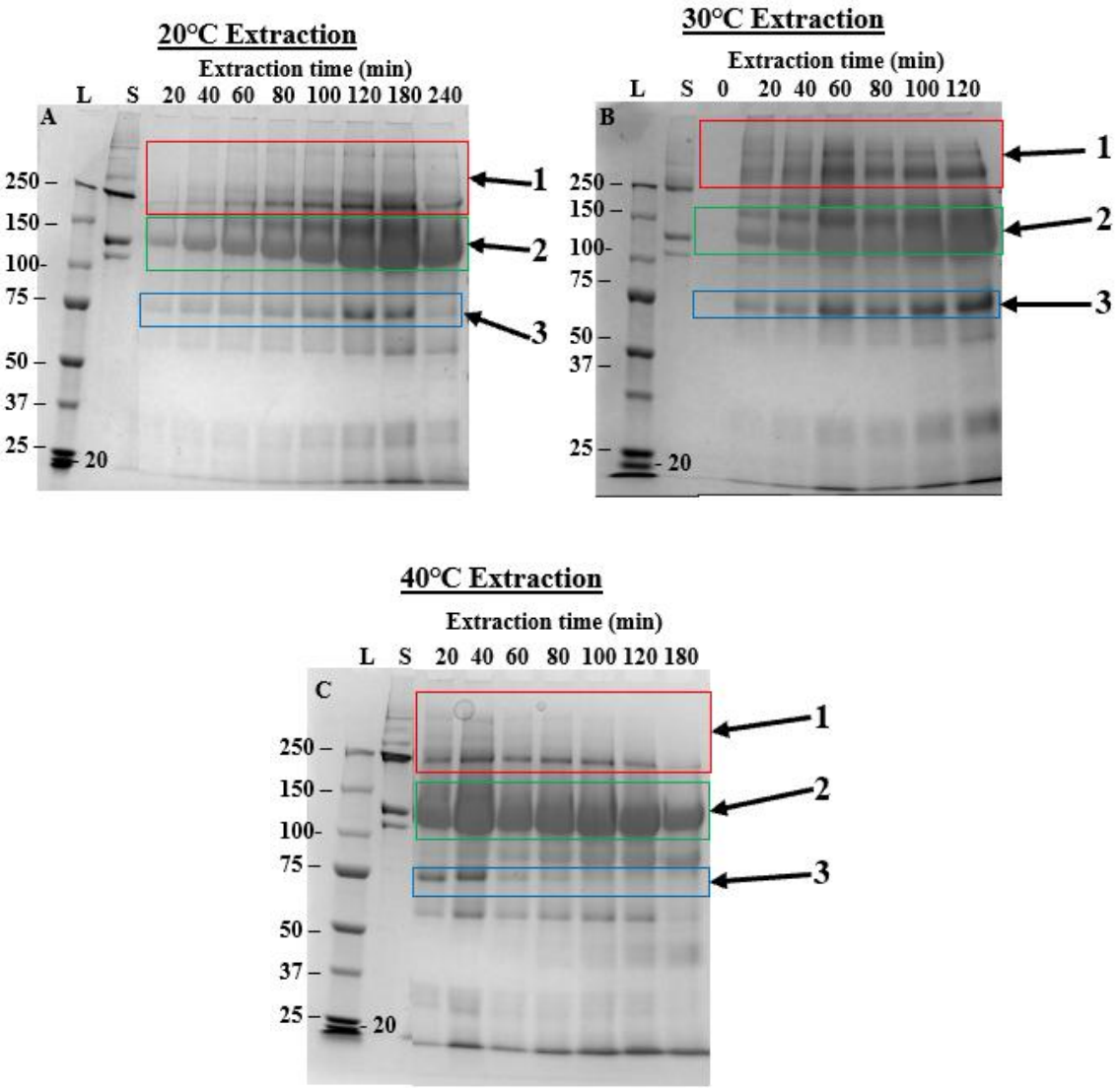

Figure 4.13. SDS-PAGE analysis of collagen extracts obtained at different temperatures (a) 20 °C, (b) 30 °C, and (c) 40 °C over a specified time period. The experiments were conducted using a pepsin concentration of 0.1 g/L and a 10:1 ratio of 0.5 M acetic acid to solid. For the lanes of each gel, (l) represents the molecular weight ladder in kDa, and (s) represents the calfskin type 1 collagen standard.

Figure 4.13 shows that, when pepsin operates near its optimal temperature of 40 °C, it causes more protein degradation and releases collagen from the biomass faster than extractions occurring at 30 °C and 20 °C. However, at 30 °C, it quickly liberates collagen without degraded products, which makes it an interesting operating environment for collagen extraction. Considering box 1 (red), which displays proteins with a molecular weight of 250 kDa or more, including the β and γ

forms of collagen, at temperatures of 20 °C (Figure 4.13.a) and 30 °C (Figure 4.13b), the band remains unchanged in intensity or becomes darker over time during the extraction process. As for the 40 °C run (Figure 4.13.c), at around 60 minutes, the protein band greater than 250 kDa begins to reduce in intensity while the band at 250 kDa begins to decrease in intensity at 120 minutes, indicating degradation. As for proteins between 100 and 150 kDa (box 2- green) which contain the α1 and α2, helices, at 20 °C the bands increase in intensity gradually up to 240 minutes. A similar observation can be seen for the run at 30 °C but occurs more rapidly. For the 30 °C temperature setting, once the time hits 60 to 80 minutes, the band around 120 kDa does not change intensity. At 40 °C, the band is at its maximum intensity 20 minutes into the reaction. Finally, box 3 (blue) looks at proteins near 75 kDa. It is plausible that a type of gelatin can take upon this molecular weight before being further degraded. Similar to the 100 kDa to 150 kDa proteins, the protein band's intensity increased from 0 to 120 minutes for 20 °C and 30 °C. However, the band in the 30 °C run darkened more rapidly. As for an extraction temperature of 40°C, the band is present until 40 minutes and then begins to dissipate and a band around 40 kDa to 50 kDa begins to appear at 60 minutes. This suggests that higher temperatures cause already deteriorated items to decompose even further. A comparable outcome was observed in a study on clown featherback fish (*Chitala ornata*), whereby as time and temperature increased, band intensities at 45 kDa and 83 kDa began to increase. The researchers inferred that this was a result of thermal degradation (Kittiphattanabawon et al., 2016). Native PAGE of these extraction conditions and periods of time was executed and can be observed in appendix A in chapter 7 (figure 7.6). Most importantly, this shows that a fraction of what is extracted holds its native molecular weight throughout the procces. Over time, more of the product is liberated from the biomass, while some potentially impure or degraded products build up.

According to Table 4.11, the effect of a pepsin concentration increases at a constant temperature of 20 °C on the overall collagen yield is not so evident. Based on the limited repetitions and data, the values between 0.1 g/L and 0.3 g/L of pepsin show a slight increase. However, their standard deviations overlap, so it is possible that the increase in pepsin concentration could report the same total collagen yield. Similarly, when analyzing the increase from 0.3 g/L to 0.7 g/L, the overlap of the standard deviations makes it difficult to differentiate if there is an increase when the pepsin

concentration is increased from 0.3 g/L to 0.7 g/L. However, there is a difference between 0.1 g/L and 0.7 g/L of pepsin. Although an association may exist whereby higher pepsin concentrations in a reaction might correlate with increased yields, additional experiments are necessary for confirmation.

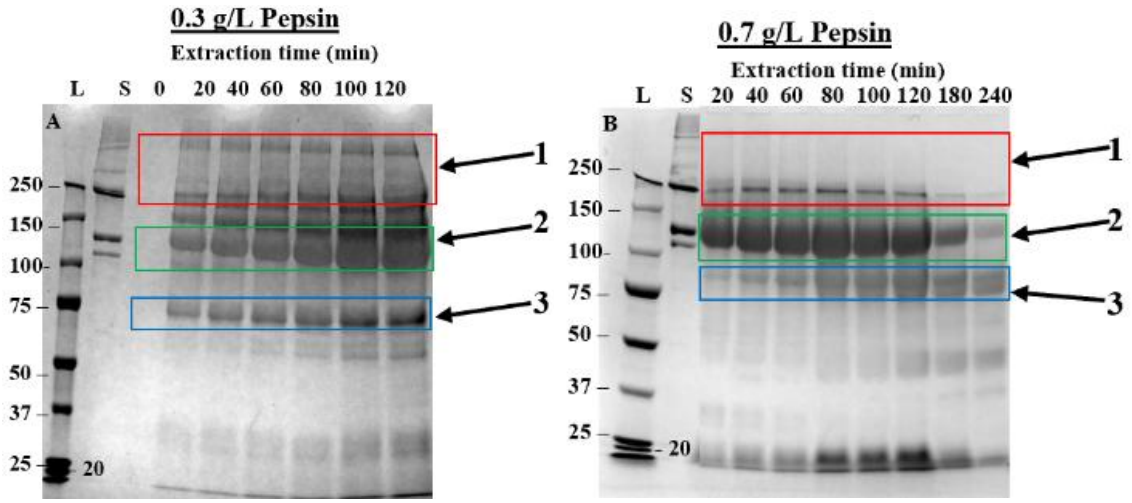

Figure 4.14. SDS PAGE analysis of collagen extractions over a period of time for varying extraction temperatures (a) 0.3 g/L and (b) 0.7 g/L when the temperature was kept at 20 ℃ and 0.5 M acetic acid to solid ratio was 10:1. For the lanes on each gel, (l) represents the molecular weight ladder in kDa, (s) represents the calf skin type 1 collagen standard.

According to Figures 4.13 and 4.14, as the pepsin concentration rises from 0.1 g/L to 0.7 g/L while maintaining a constant temperature of 20 °C, the extraction rate of collagen (α 1, 120 kDa and α 2, 105 kDa) increases. At higher concentrations, larger molecular weight structures, such as β and γ collagen, start to break down more rapidly. As stated in box 1 (red), proteins greater than 250 kDa exhibit a gradual increase in band intensity over time at 20 °C and 0.1 g/L (figure 4.14). At 0.3 g/L and 20 °C (figure 4.13), there is a gradual increase in proteins and bands greater than 250 kDa until 60 minutes, then the band intensity does not change. At a concentration of 0.7 g/L and a temperature of 20 °C, proteins above 250 kDa initially exhibit a decrease in band intensity for the first 80 minutes and then disappears. For proteins at 250 kDa, the band intensity decreases starting at 80–100 minutes and continues until the reaction is stopped.

At 105–130 kDa, the molecular weight of proteins range indicated by the box 2 (green) on each gel, the reaction was still producing more proteins with these molecular weights. The bands intensified over time, indicating the release of more collagen alpha helices. However, when the pepsin concentration was at 0.7 g/L, the band intensity began to decrease at 180 minutes.

Lastly, when examining box 3 (blue), which corresponds to proteins around 75 kDa. Interestingly, when the pepsin concentration was at 0.1 g/L, the band intensity increased throughout the reaction period. At concentrations of 0.3 g/L and 0.7 g/L, the band intensity also increased over time, but with the appearance of streaks at 60 minutes, suggesting that low molecular weight proteins were being produced.

Working at many different temperatures and concentrations, the next step was to look at if the conditions had an impact on the thermal denaturation temperatures which were characterized in Table 4.12 and the CD spectra at 4 ℃ and 60 ℃ were characterized in Figure 4.15.

Table 4.12. Thermal denaturation temperature of collagen extract products from run 18 at wavelengths of 198 nm and 220 nm.

Run *	**Thermal denaturation temperature (T_d) at wavelength 198 nm (°C)**	**Thermal denaturation temperature (T_d) at wavelength 220 nm (°C)**
18.a	20 ± 1	21 ± 2
18.b	17 ± 2	18 ± 2
18.c	18 ± 1	22 ± 2

*Run 18.a had extraction conditions of 40 °C, 0.01 g/L of pepsin and an extraction time of 207 minutes. For run 18b, the extraction conditions were 30 °C, 0.1 g/L of pepsin and an extraction time of 120 minutes. Finally, for run 18c, the extraction conditions were 20 °C, 0.3 g/L of pepsin and an extraction time of 120 minutes.

In Table 4.12, runs 18.a and 18.c had similar denaturation temperatures between 18 ± 1 ℃ and 22 ± 2 ℃, whereas 18 b had a slightly lower T_d which was between 17 ± 2 ℃ and 18 ± 2 ℃. All the calculated temperatures were within or very close to each other in terms of uncertainty ranges, which were calculated using equipment uncertainties and the error of propagation principles as described in section 3.17. Like runs 9 and 10, they were similar to the values of *P. Californicus* but less than *A. Leucopetra* (S. Lin et al., 2017; Z. Liu et al., 2010). To confirm each extract's

protein confirmation at 4 ℃ and 60 ℃, circular dichroism spectrums were developed in Figure 4.15.

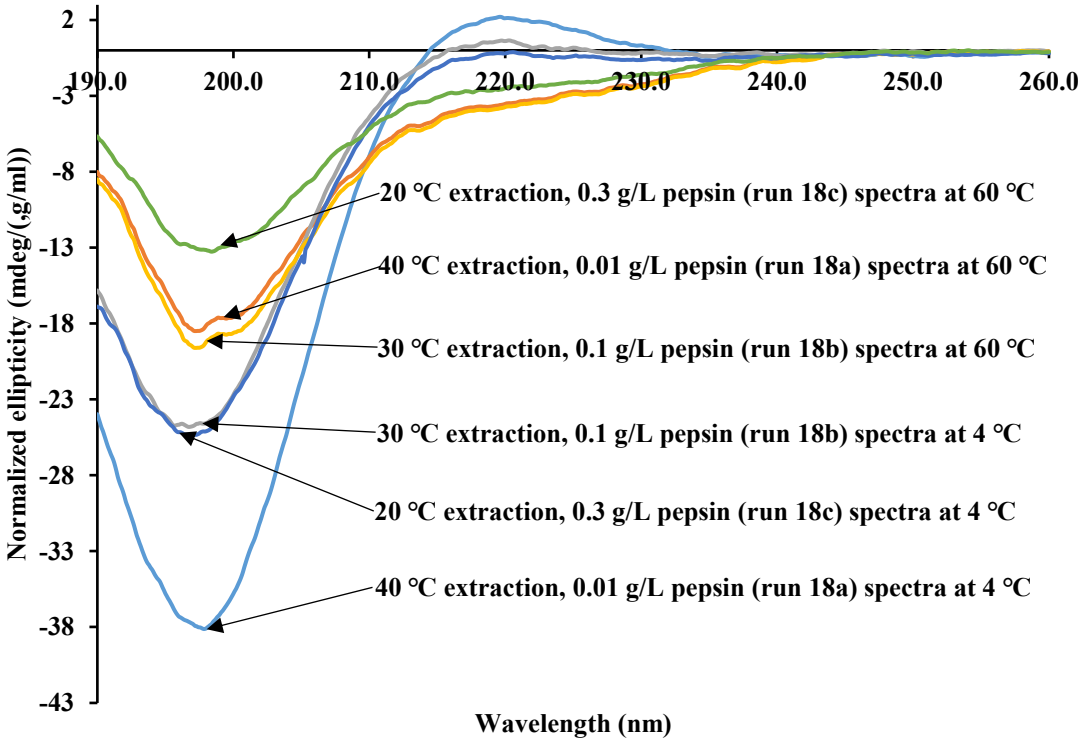

Figure 4.15. CD spectra of a collagen product obtained through extraction with 40 °C, 0.01 g/L pepsin for 207 minutes (18a) were analyzed at both 4°C (light blue) and 60 °C (red). Similarly, the solutions of 30 °C, 0.1 g/L pepsin for 120 minutes (18b) were examined at 4°C (gray) and 60 °C (yellow), while those of 20 °C, 0.3 g/L pepsin for 120 minutes (18c) were studied at 4°C (dark blue) and 60 °C (green). Each solution and result were normalized to 1 mg/mL.

As illustrated in Figure 4.15, each run, including 18.a, 18.b, and 18.c, reached its maximum at 220 nm. However, 18.a, with an extraction temperature of 40 °C and a pepsin concentration of 0.01 g/L, had the strongest peak. The second-strongest peak belonged to 18.b, which had an extraction temperature of 30 °C and a pepsin concentration of 0.1 g/L. The weakest peak was observed in the extract from 18.c, which was taken at 20 °C with a pepsin concentration of 0.3 g/L. When the CD was run at 60 ℃, none of the extracts had a peak at 220 nm meaning the triple helix was not formed at these temperatures. At 198 nm, 18.a had the most intense signal when the

CD was run at 4 ℃ whereas 18.b and 18.c had similar signals. At 60 ℃, 18.a and 18.b had similar signals whereas 18.c had the least intense signal. Given the nature of the samples selected for CD spectroscopy, it is difficult to depict whether the increase in extraction temperature or a decrease in pepsin concentration positively impact 18a's result but both parameters show to have impact on the CD curves and nativity of the collagen being extracted.

4.3 Optimized collagen extract scale-up analysis

To properly develop a process that can be implemented commercially on the Lower North Shore, studies and runs need to be done on a larger scale. This is not only to see how the product reacts but also to get an idea of how the equipment can be scaled. In this section, the performance and characteristics of the lab and pilot scale will be studied.

4.3.1 Performance

Run 18b, which had the extraction conditions of 0.1 g/L pepsin, 0.5 M acetic acid at a solid to liquid ratio of 1:10 and an extraction temperature of 30 °C, was scaled up from a 200 g batch to a 4032.6 g batch size (20-fold increase). In the case of the scale-up only, the whole sea cucumber was used (viscera & body wall) and the moisture content measured 89.89 %. During the pilot run, various products were created using different purification and drying techniques, and the results were compared to those of the lab-scale runs, as shown in Table 4.13.

Table 4.13. Summary of the performance results of the optimized collagen extraction process, conducted at both pilot and laboratory scales. The extraction step was performed at 30 °C, with 0.1 g/L pepsin concentration, 0.5 M acetic acid, and a liquid-to-solid ratio of 10:1 for 120 minutes at the laboratory scale and 90 minutes at the pilot scale.

Treatment and run	**Total powder yield (mg powder per g dry sea cucumber)**	**Total protein yield (mg protein per g dry sea cucumber)**	**Purification processing performance indicators to reach result**	**Sodium removal efficiency (%)**
Lyophilization and ultrafiltration, 1 diafiltration - pilot	31 ± 1	21 ± 1	Starting volume - 2032 mL Time to reach reduction – 19.5 hours	61.22 ± 0.01
Lyophilization only - pilot	449 ± 1	44 ± 1	Not applicable	Not applicable
Spray dryer only - pilot	194 ± 1	16 ± 1	Not applicable	Not applicable
Lyophilization only - lab	626 ± 5	156 ± 2	Not applicable	Not applicable
Lyophilization and Ultrafiltration, 7 diafiltrations - lab	26.8 ± 0.1	Information not available	Starting volume – 230 mL Time to reach reduction – 9 hours	>97.66 ± 0.04
Lyophilization and dialysis - lab	45.1 ± 0.1	Information not available	Starting volume – 38 mL Time to reach reduction – 71 hours	98.56 ± 0.06

In light of the results in Table 4.13, the powder yields obtained through dialysis and ultrafiltration purification were found to be comparable. However, dialysis reduced the sodium content by 98.56 ± 0.06, whereas ultrafiltration with 7 diafiltrations reduced the salt concentration by more than 97.66 ± 0.04. The ultrafiltration method was able to process 230 mL in 9 hours whereas to purify 38 mL using the dialysis method took 71 hours. Ultrafiltration proved to be more effective than dialysis in removing sodium to the same degree of reduction, a significant result from an industrial perspective.

When comparing the lab scale run to the commercial scale run, the powder yield and protein yields both decreased during the scale-up process as compared to its lab counterpart. The overall powder yield of the lab-scale and pilot-scale items was compared when ultrafiltration was used as the purification method. The pilot product had a slightly higher yield than the lab-scale product. It is important to point out that the sodium removal efficiency is lower in pilot process. This is expected given less diafiltration were performed at the pilot scale and more salt would be present in the pilot scale extract, producing more powder. When subjecting the extract to ultrafiltration, the difference in yield between lab and pilot-scale operations narrows significantly. The membrane consistently removes impurities and standardizes yield characteristics of the extract, regardless of the scale of the operation. This ensures that the extracts can meet a specific yield target, which is crucial when scaling up processes.

Finally, the lyophilized product had higher protein yields and powder yields than the spray dried product. Although the data are not shown, the UV-vis, FTIR, SDS PAGE and Native PAGE techniques were very similar for each product, regardless of whether it was dried by lyophilization or spray drying. The spray dried product had similar FTIR peaks to what was observed by Latip et al. (2015) and their sprayed dried collagen product.

4.3.2 Product characterization

The following section looked at key characteristics of the lab and pilot scale products, in hope of describing any significant changes in the scale up process. Table 4.14 looked at the amount of minerals and heavy metals that were in the remaining purified products.

Table 4.14. Summary of the optimized collagen product's mineral and heavy metal contents at lab scale and pilot scale runs after ultrafiltration. Extraction was performed at 30 °C, 0.1g/L pepsin concentration, 0.5 M acetic acid, and a liquid to solid ratio of 10:1 for 120 minutes at the lab scale and 90 minutes at the pilot scale.

Element	Lab scale extract (µg/g dry powder)	Pilot scale extract (µg/g dry powder)
Lead	0.4 ± 0.01	1.1 ± 0.5
Mercury	< 0.01*	0.092 ± 0.003
Arsenic	< 0.5*	0.5 ± 0.1
Chromium	2.5 ± 0.2	13 ± 1
Cadmium	< 0.08*	< 0.5*
Beryllium	< 0.2*	0.5 ± 0.5
Nickel	6.3 ± 0.6	7.5 ± 0.3
Sodium	4 930 ± 10	99 177 ± 5
Potassium	28 ± 6	380 ± 2
Calcium	103 ± 3	342 ± 7
Magnesium	11 ± 3	90 ± 10

*Measured value is less than calculated uncertainty of the measurement.

In terms of sodium concentrations before ultrafiltration, the lab scale powder had a value was 211 g of sodium per kg powder and the pilot scale powder had a value of 256 g of sodium per kg powder.

As shown in Table 4.14, the concentration values of the heavy metals and minerals were much lower in the lab powder product as opposed to the pilot scale. This is because there were more diafiltrations done on the lab scale product. In terms of sodium concentrations before ultrafiltration, the lab scale powder had a value was 211 080 ± 60 ppm and the pilot scale powder had a value of 255 731 ± 5 ppm, and the concentrations reduced to 4 930 ± 10 ppm and 99 177 ± 5 ppm respectively. For the elements mercury, arsenic, cadmium and beryllium from the lab-scale extract and cadmium from the pilot-scale extract, the measured concentration was less than the calculated uncertainty, and the theoretical concentration range would contain negative concentrations, which are not physically possible. Consequently, the results for these five instances were reported as being lower than their respective uncertainty thresholds.

To confirm the presence of collagen, its state and potential contaminants, SDS PAGE was conducted on both extracts and the results are seen in Figure 4.16.

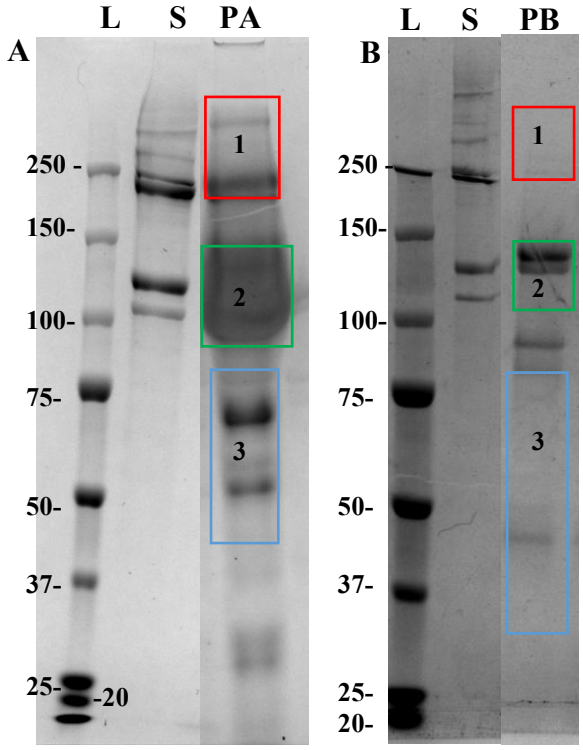

Figure 4.16. SDS-PAGE gel of the optimized pepsin-soluble collagen extract. The lab-scale extract is shown in (a), while the pilot-scale extract is in (b). Lane (l) is the molecular ladder, lane (s) is the standard, lane PA is the lab sample extracted product and lane PB is the product at the pilot scale. Box 1 (red) indicates proteins or equal to 250 kDa, box 2 (green) indicates proteins between 100 kDa to 140 kDa, and box 3 (blue) indicates proteins between 45 to 90 kDa.

Box 2 (green) in Figure 4.16.a has a band at 120 kDa with a smear above. This makes it unclear if there are two distinct α helices or if they are all the same length. Despite this, research supports both outcomes for type I collagen in sea cucumbers. (S. Lin et al., 2017; F. Liu et al., 2017). The pilot extract in Figure 4.16.b shows two bands, one at 135 kDa and the other at 120 kDa. Compared to the standard, the α helices are larger: the α1 chain is 120 kDa and the α2 chain is 105 kDa, while the pilot band is about 15 kDa larger for each chain. This explains why the native collagen form on the native gels are between 360 to 400 kDa, not the 340-350 kDa that is expected with calf skin standard. Several articles have reported these two chains as α1 and α2 at, confirming this is type 1 collagen (P. H. Li et al., 2020; Saito et al., 2002). In box 3, which represents proteins from 45 kDa

to 90 kDa, the bands could be degraded forms of α, β, or γ chain products, as shown in the literature when the extraction times and temperatures of collagen and gelatin increase (Gaspar-Pintiliescu et al., 2019; Kittiphattanabawon et al., 2016). In box 1 on the lab scale gel, there are β chain and trace amounts γ chain formations of collagen, whereas only β chains are located on the pilot scale gel which could be a sign of product degradation. When compared to the products in Figure 4.10, which were collagen extracts developed at 4 °C and had few or no bands below 100 kDa, both the lab-scale and pilot-scale runs, when extracted at 30 °C, had more proteins with molecular weights less than 100 kDa. This temperature change leads to the formation of degradation products. To confirm the presence of collagen's triple helix, Native PAGE was used in Figure 4.17.

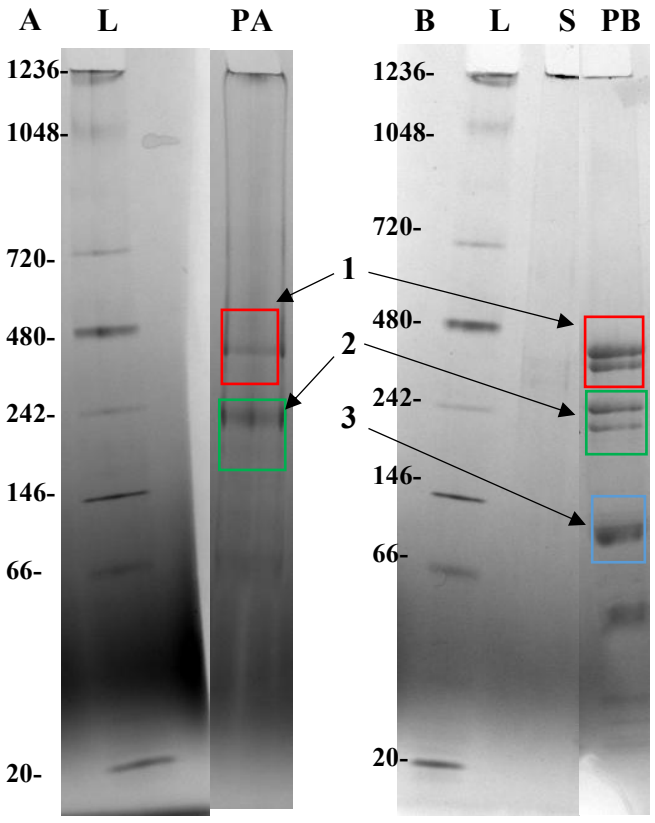

Figure 4.17. Native PAGE of optimized pepsin-soluble collagen extract. Lab-scale extract is in (a), pilot-scale in (b). Lane (l) is the molecular ladder, lane (s) is the standard, lane PA is the lab sample, and lane PB is the pilot-scale product. Box 1(red) indicates proteins between 340 kDa to 380 kDa, box 2 (green) indicates proteins between 210 kDa to 240 kDa, and box 3 (blue) indicates proteins at 100 kDa.

In box 1 for the lab and pilot extract gels from Figure 4.17, there are bands between 340-380 kDa which is typically of sea cucumber collagen. However, in the pilot extract, it is interesting that there is a double band whereas in the native extract, there is only 1. Box 2 contains two bands at 240 kDa, one on the pilot gel and one on the lab gel. This indicates that there are impurities with a very similar molecular weight. Alternatively, something in the pilot extract may have caused a slight change in some of the native collagen and its characteristics. The latter scenario seems more likely, since it corresponds to the triple helical molecular weight and the β chain form. In box 3, there are clearly bands around 100 kDa where there is none present in the lab scale extract. In the pilot extract there are smaller proteins around 25 kDa, 30 kDa and 40 kDa which are not present in the lab extract. The presence of proteins below the 340 kDa mark in both extracts indicates some sort of degradation occurring on both scales. As shown in Figure 4.17, the product extracted at 30 °C contained significantly more smaller proteins below 340 kDa than the Native PAGE in figure 4.4.b, which was extracted at 4°C.

Another test used to confirm identity of collagen, and its purity was UV-vis spectroscopy which is described in Figure 4.18.

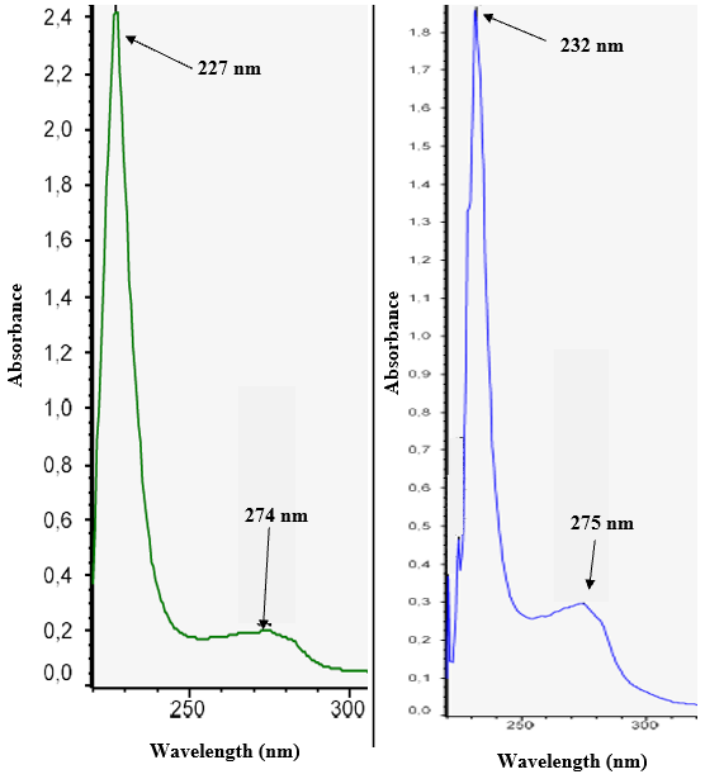

Figure 4.18. UV-vis spectra of optimized pepsin-soluble collagen extract. (a) Lab-scale extract and (b) pilot-scale extract. The solvent for all samples and blanks was 0.5 M acetic acid.

Both lab scale (Figure 4.18.a) and pilot scale (Figure 4.18.b) had signals near 230 nm which is a common characteristic of marine collagen since protein peptide bonds and in particular their amino acids are detected at this wavelength (Ju et al., 2020; P. H. Li et al., 2020; F. Liu et al., 2017). There are also peaks near 275 nm which are common in UV spectra as well, indicating presence of aromatic residues such as tyrosine in the collagen extracted or from other types of proteins in the extract. Given there are unattributed bands in the SDS PAGE and the Native PAGE of these samples, the peaks at 280 nm are likely from both non collagen and collagen proteins. The A280/A230 ratio was slightly less in the lab extract which was 0.091 ± 0.001 as compared to the pilot scale extract which was 0.164 ± 0.003. When analyzing this result and combining it with the observations in Figure 4.16.b, the lower molecular bands present in the pilot extract more likely collagen protein/amino acid degradation as compared to non-collagen protein impurities. To evaluate the quality of the two extracts, FTIR was used, as shown in Figure 4.19.

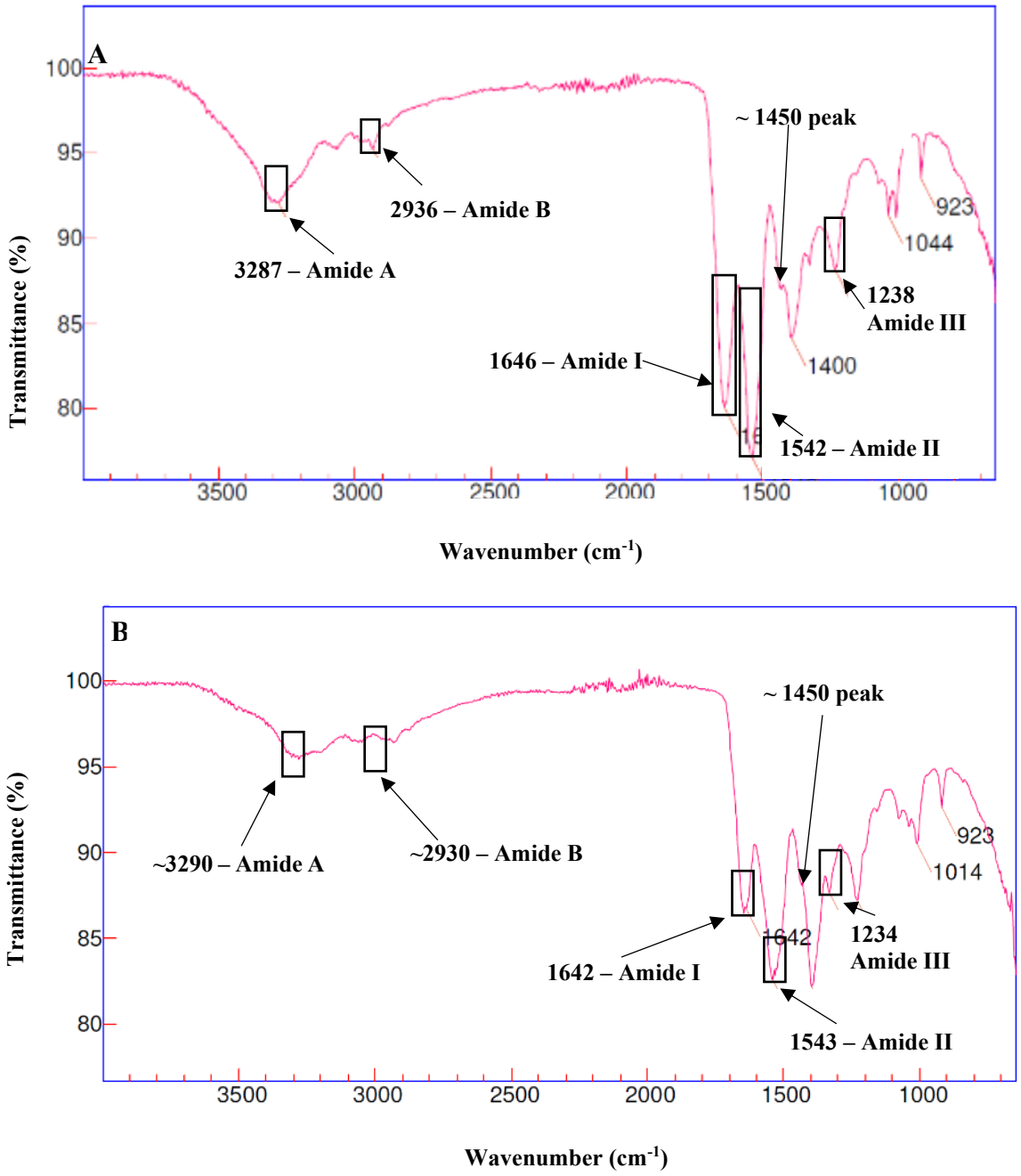

Figure 4.19. FTIR spectra of optimized pepsin-soluble collagen extract. (a) is the lab-scale extract and (b) is the pilot-scale lab.

As observed in Figure 4.19.a for the FTIR of the lab scale extract, all amide peaks associated with collagen were observed (amide A, amide B, amide I, amide II and amide III) at the expected locations and it looks similar to the standard in Figure 3.3 and other sea cucumber collagen curves

in the literature (Adibzadeh et al., 2014; S. Lin et al., 2017). The amide I peak in particular is measured at 1646 cm^{-1} meaning the α helices are still intact. Also, the amide III to the 1450 cm^{-1} band peak ratio is roughly 1.18. Values near 1 indicate that the integrity of the triple helix is intact and, in the lab scale extract it is. Also, with the wavenumber of the amide A bond being below 3300 cm^{-1}, this suggest than many of the N-H groups on the peptide back bone is heavily involved with hydrogen bonding to neighboring molecules, helping form the triple helix collagen (Spigolon et al., 2022). This result confirms with the CD spectra in Figure 7.7 of appendix A for run 18b spectra at 4 ℃ where there is a peak at 220 nm suggesting there is collagen present in its triple helical form.

As for the pilot scale extract, the peaks were similar to that of the lab scale extract. As observed in Figure 4.19.b, the amide I peak was slightly less than its lab counterpart, measuring 1642 cm^{-1}, however the 1450 to amide III peak ratio was slightly less valuing at 1.08. The amide A bond also measured below 3300 cm^{-1}, indicating an intact hydrogen bonding network. Therefore, the lab scale and pilot scale products measured by FTIR are the same.

Another important property that was studied was how the collagens helix changed over a variety of temperatures. To monitor this, circular dichroism was used over a temperature range of 4 ℃ to 60 ℃ at 198 nm and 220 nm, both characteristic peaks of collagen in its native form. Figure 4.20 describes this relationship.

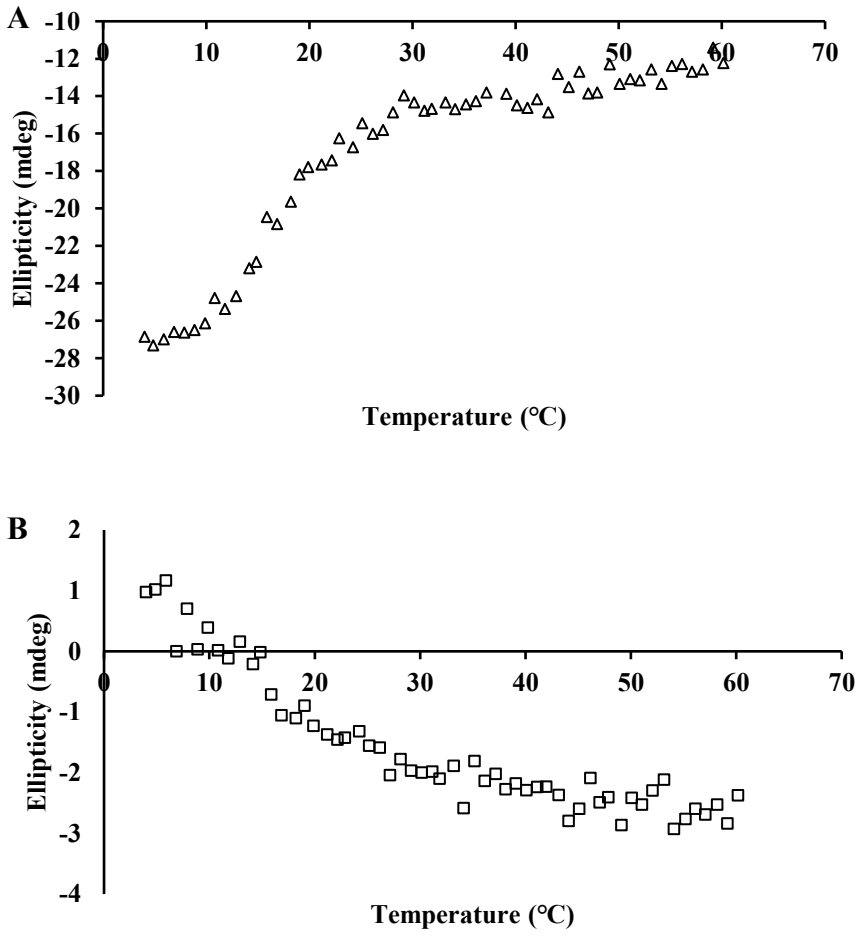

Figure 4.20. CD spectra of the lab-scale optimized pepsin-soluble collagen extract at wavelengths (a) 198 nm and (b) 220 nm, measured over a temperature range of 4°C to 60 °C.

In Figure 4.20.a, the ellipticity value stayed between -27 to -26 ellipticity until 10 ℃, holding its typical helical state. After 10 ℃, the curve began to increase until around 29 ℃ where the curve began to plateau, reaching the complete denatured state. By observation, the inflection point was roughly 18.5℃ which is similar to the thermal denaturation temperature of 18 ± 2 ℃ in Table 4.11. At 220 nm, the decrease almost began instantly increasing the temperature above 4 ℃ until reaching 30 ℃ where ellipticity reached a minimum and stayed at this value. By observation, the inflection point temperature is approximately 19 ℃ which is similar to the thermal denaturation calculated in Table 4.11 of 17 ± 2 ℃. Based on both curves, the product begins to significantly denature above room temperature.

Chapitre 5 - Discussion

Commercial processing of any item in a northern isolated community is never an easy task. The complexity increases when implementing extensive, time-consuming procedures that demand significant resources and rigorous quality assurance measures to guarantee that the final product conforms to the client's expected specifications. Typically, these types of processes also have higher economic returns. Specifically, native collagen meet the requirements described in the scenario above. The Lower North Shore and its residents can benefit from new industries that ensure the long-term vitality of the region. However, the question remains can a collagen process that produces high concentrations of native collagen be one of these commercial processing industries to fill this void. This thesis has shown that it is possible to produce a pilot-scale collagen extract containing both the native and denatured forms of the protein, which can be successfully implemented and scaled in the Lower North Shore.

Throughout the project, a decision was made to limit the number of replications to answer more process synthesis questions to have a clearer picture of the final collagen process to be implemented on the Lower North Shore. There were significant time constraints of less than 6 months to execute all the lab work and get from a lab-scale process to a pilot run. On top of this, the project only had access to a limited number of sea cucumbers. Fine tuning and repetitions could be done on site once the process design was delivered to the end-user of the Lower North Shore. A systematic approach was used to get concrete answers of particular pre-treatment, extraction and post-treatment steps that kept the main objective in mind of getting a pilot scale process which can be transferred to the communities of the Lower North Shore.

Like most processes, the three critical steps include the pre-treatment, extraction, and post-treatment of the material to deliver the desired process. From the outset, the pre-treatment procedures, especially the disaggregation stage, proved crucial for preparing *C. frondosa* for the extraction process. This ensured the effective release of soluble collagen into the solution. The first method used was a protocol developed by CRBM to liberate collagen from fish skins with mild acids and bases for short durations. Compared to fish skins, the body wall of *C. frondosa* is more rigid as it contains a network of complex interactions where many proteins, glycoproteins, carbohydrates

and proteoglycans interact (Thurmond & Trotter, 1996). These predominantly ionic and hydrogen-bonded interactions were disrupted by the addition of Tris-HCl and EDTA, allowing the collagen molecules in these fibers to dissolve (Thurmond & Trotter, 1996). Whether the pre-treatment steps were done at 4 ℃ as observed in results sections 4.2.2.4, 4.2.2.5, or at 20 ℃ in section 4.2.3, the use of this disaggregation solution provided benefits in terms of overall yield compared to other pre-treatment mediums such as water and ethanol, which rely more on solubility/miscibility to condition and extract molecules. While ethanol did not increase collagen yields as investigated in 4.2.2, ethanol can be an interesting pre-treatment step in developing phenolic acid coproducts (Hossain et al., 2020). This would maximize the utilization of the resource and its molecules to create secondary high-value products and increase the revenue of the processing facility. This thesis did not evaluate phenolic acids and coproducts but can be analyzed in future studies.

Another result demonstrating the existence of ionic effects rather than solubility-miscibility separation properties is Table 4.8, which shows the change in pH. At extreme acidic or basic pH, no extraction occurred. Given collagen has many amino acids and are polyprotic molecules, their charge is significantly affected by pH which can affect how they interact with neighboring molecules. It was shown that the change in pH causes the zeta-potential to change, which affected its solubility in solution (Abedin et al., 2014). In a solution, pure collagen can be insoluble at a pH range of 5 to 7 and form a precipitate that can be collected. However, when the fibril is not extracted from the body wall, the interaction of collagen with other molecules can change, leading to a different behavior and alteration in its physiochemical characteristics. While preliminary results suggest that the optimal pH range for disaggregation is 5.5-8, further investigations need to be performed to study the interactions and molecules collagen is interacting with to refine this step.

The deproteination step is essential in the process as removing it impacted the overall process yield. To minimize processing time, an experiment was done skipping the deproteination step. The rationale was that while decreasing the processing time by removing this step, all the impurities could be removed at the same time in the post-treatment steps, later in the process. Pepsin is not an enzyme specific solely to collagen, as it is an aspartic protease where its binding site recognizes hydrophobic amino acids such as tryptophan, phenylalanine and tyrosine (Castañeda-Valbuena et al., 2022; Fruton, 1970). If there are more proteins in the extract, pepsin can bind and hydrolyze

these other proteins which could impact the solubilization of collagen. Non-collagenous proteins are also a broad spectrum of proteins that are not collagen and could be involved in structural, signaling and mechanical roles (Licini et al., 2019). As mentioned by Licini et al. (2019), osteoblasts contain around 180 to 200 non-collagenous proteins. It is highly likely that this protein diversity exists in the extracellular matrix of cells and tissues from different species, including the sea cucumber's body wall. Therefore, this group of proteins can be enzymes that may degrade collagen or antagonist that can reduce the activity of pepsin, both providing explanations to why the reduced yield was observed when the deproteination step was removed.

Besides ionic charges and pH changes, there are also covalent disulfide interactions preventing collagen dissociation from the body wall which can be broken down using 2-mercaptoethanol. In the addition of 2-mercaptoethanol, the protein yield in section 4.2.2.4 increased. The crude collagen fibrils are held together by disulfide bonds (Shoulders & Raines, 2009). Incorporating 2ME would break the bonds, causing the fibrils to loosen and allow solvents and enzymes to reach and act on the necessary sites, resulting in the release of collagen into the solution. The 2ME should have no impact on the triple helical structure of type I collagen as there are no intermolecular disulfide bonds as confirmed by SDS gels and CD spectroscopy, since there are still proportions of the triple helical form after treatment. 2ME presents challenges at a commercial level and the challenge to manage this chemical is elevated in remote areas such as the Lower North Shore. The chemical is considered toxic and has hazard identifications for acute oral, dermal, and inhalation (ThermoFisher Scientific, 2010). Given many cosmetic and nutraceutical products are consumed through these passageways, this presents a challenge even if it is used as a minor solvent in the process. There are also concerns in terms of the product's storage, manipulation, and transport. It will be difficult to manage in an area that will be unable to treat or neutralize the molecule onsite. There were tests done using other chemicals such as using solely Tris-HCl, EDTA, and NaCl, or replacing 2ME with dithiothreitol (DTT) (data not shown) which is less toxic than 2ME (Mommaerts et al., 2015). To find a replacement for 2ME, other formulations of the disaggregation were used. When adding NaCl to the solution with Tris-HCl and EDTA, a similar total solid mass extraction result was observed when 2ME was in the disaggregation step. The NaCl would further intensify the ionic disruption in the disaggregation step allowing the body wall matrix to be more

susceptible to extraction. Given this was only completed once, further investigation into its true impact needs to be studied, along with other reducing agents. While both had effects on liberating collagen from the body wall, it was rapidly decided to go to a complete elimination of the chemical and use of heat which will be discussed in the next paragraph.

Another approach to replace 2ME was through the addition of heat to the process, helping solubilize the collagen without such a strong disaggregation step. While the complex body wall network would still be largely intact, pepsin operates much more efficiently at temperatures between 20 ℃ to 50℃ compared to operating at 4 ℃. However, the trade off with this is marine collagen is not as stable at elevated temperatures compared to mammalian species. The overarching goal of the project was to develop a sustainable process for the residents of the Lower North Shore to work at and open a new industry for years to come. Given poor yields were observed at 4 ℃, by adding heat to extract more collagen, one could benefit from extracting more product faster. A thought process that even performing the extraction at warmer temperatures, a portion of the extract would remain native and provide added value over 100% hydrolysate or 100% gelatin products on the market. Even though a mixed product having hydrolyzed and native collagen would be a lower value than pure native collagen, it was believed that augmentation in yield would offset the price difference between a mixed native/hydrolyzed product and pure native collagen. For applications such as nutraceuticals face cream or lotion cosmetics, hydrolyzed products are still commonly used given their smaller molecular weight form is more easily absorbed by the skin (D. Liu, Nikoo, et al., 2015). The switch from a purely native product to a mixed conformation would make it more suitable for these applications.

Regardless of the extraction temperature used between 20 ℃ to 40 ℃, the extracts still contained a fraction of collagen in its triple helical state. The higher extraction temperatures have increased the efficacy of pepsin causing it to cleave recognizable active sites more rapidly and liberate more collagen and increase the overall yield, which can be seen in section 4.2.3. The increase in temperature also increased the amount of degradation of the γ, β, and α forms of collagen, which was also observed when studying clown featherback fish (Kittiphattanabawon et al., 2016). The denaturation of the triple helix at elevated temperatures is likely due to thermal denaturation as opposed to enzyme denaturation. While more degradation was present at the higher temperatures, there

remained triple helical collagen in each fraction and this was also seen in other species such as tilapia and clownfish (Kittiphattanabawon et al., 2016; Kuwahara, 2021). Given that all the collagen is not completely degrading, there is a possibility that interactions with another molecule or itself causes a portion of the collagen molecules to remain in its triple helix at the elevated temperature. These bonds are likely covalent but not a disulfide bond since 2-mercaptoethanol is used and not ionic given the SDS detergent that is used with the electrophoresis experiments. The activity properties in the triple helical collagen in this project were not compared to what was extracted at 4 ℃. However, given the structure and function relationship of proteins and the numerous chemical characterization experiments done to validate that the structure of collagen has not changed much (other than the degradation products), there is no reason to assume the function of the triple helical collagen has changed. From the results in this case, extracts at and below 30 ℃ seemed to maximize yield and purity while minimizing product degradation and time.

Regarding the partially degraded triple-stranded collagen products, this may not be a detrimental aspect of the extract. Gelatin and collagen hydrolysates are more readily soluble in water and their smaller molecular weights make them easier to absorb in our body (Lopez et al., 2019). Having products with concentrations of the hydrolyzed product will spark interest from cosmetic industries, especially those involved in skin and topical products. Collagen hydrolysates also have great antioxidant properties (Abedin et al., 2015). Therefore, having an extract with different forms and sizes of collagen/gelatin can still provide a unique product to the marketplace with added value.

From a commercial processing standpoint, adding heat at the extraction step has significantly reduced the processing time. When working at 4 ℃, the minimum extraction time was 24 hours, whereas working at temperatures between 20 ℃ to 40 ℃ took under 2 hours. In a commercial plant, this dramatically reduces costs. Also, when working at 4 ℃, to ensure no product degradation, the steps need to be done in a cold room. Whereas working with heated extracts, the energy would only need to be applied to the reactor. An energy balance is not required to determine that much less energy and ultimately cost would be needed to heat the extraction reactor as opposed to cooling the room. Therefore, there is an incentive for industries to work at slightly above room temperature values when extracting collagen from the material.

The rate of collagen extraction increases as pepsin concentration is increased but the rate of degradation also increases. Literature has shown that collagen and its triple helix are fairly resistant to degradation by pepsin (Kadler et al., 2007). Therefore, it was surprising to see increases in the accumulation of degraded products as the concentration of pepsin increased from 0.1 g/L to 0.7 g/L. Most studies on native collagen are carried out at 4 ℃, far from pepsin's optimal activating point. Since pepsin attacks collagen preferentially at its telopeptide ends, it is conceivable that this enzyme does not cut any internal portions of the collagen. It was shown by Duan et al. (2012) that at temperatures above 30 ℃, the γ forms of collagen began to decrease. Upon letting PSC collagen remain in solution, with and without a pepsin inhibitor, they observed that pepsin was causing the protein to denature at these higher temperatures (Duan et al., 2012). This is likely because the pepsin is much more active. The pepsin concentration needs to be tightly controlled at higher temperatures to ensure the enzyme preferential attacks the telopeptide areas. Based on the results of this experiment, concentrations below 0.3 g/L at 20 ℃ seem to limit the attack to the telopeptide areas.

Regarding the post-treatment recovery phase, isoelectric-like precipitation offers comparable or even better yields and product quality (with fewer impurities) than salting out. Also, it is more beneficial in industrial applications due to its shorter processing time. Another benefit of using the isoelectric precipitation method is that it eliminates another stock solution to prepare (NaCl) given NaOH is already prepared for the deproteination step, simplifying the preparatory work in a processing plant. As seen in the literature, the yield was slightly higher with isoelectric precipitation with relatively similar purity when comparing to salting out (X. Lin et al., 2019). Both methods use a non-specific precipitation technique that requires high-speed centrifugation steps to recover the solids. If each precipitation method is subject to the same purification process, the isoelectric precipitation step can be more efficiently streamlined by incorporating a series of filtration or chromatography steps. This method involves precipitating the product to its isoelectric point and directly moving on to filtration or chromatography, eliminating the need for intermediary steps and equipment. Despite the different precipitation methods used, the underlying structures remained unaltered, as evidenced by consistent results in FTIR, UV-vis, and gel electrophoresis, mirroring findings from previous studies, suggesting no significant structural modifications were

observed (Spigolon et al., 2022; Vate et al., 2023). As expected, the precipitation methods do not react or modify the structure or sequence of the collagen; they only utilize its properties to separate it from other molecules in the solution. This will further simplify the process, making it easier to implement in the remote location of the Lower North Shore. With proper design of the filters and/or chromatography units, the units can be set up in a modular or parallel configuration to minimize processing downtime due to membrane fouling or maintenance.

For the most part, the dry weight yields of collagen were lower than other sea cucumber species which are on average 26 %, ranging from 7 % up to 80 % (Farooq et al., 2024). As shown in section 4.1, *Cucumaria frondosa* has mediocre collagen levels compared to other species. In this case, treatments with and without heat using the conventional disaggregation solution yielded approximately 4.5% and 1% respectively. Upon 2-mercaptoethanol being used without heating, the yields go as high as 3 %. While all are still under what is expected in literature by nearly 5-fold, the use of 2-mercaptoethanol or heat are disrupting bonds that allow pepsin to hydrolyze the material more effectively. Stiparin is one of the most predominant glycoproteins in a sea cucumber dermis and it interacts directly with collagen (Trotter et al., 1996). It also can be reduced to two subunits upon the addition of 2-mercaptoethanol, therefore making enzymes and solvents more accessible to collagen (Mizuta et al., 2013). Stiparin is tightly packed with collagen. While pepsin can break down the telomeres of collagen or free glycoproteins, all the collagen, along with other biomolecules, are initially packed in condensed tissues and fibrils. (Trotter et al., 1996). At low temperatures, since pepsin is not at its optimal operating temperature, the enzyme may have difficulty recognizing and cleaving the appropriate proteins in undissolved tissue. However, when the extract is heated to around 37 °C, pepsin can break down proteins more effectively and overcome some of the challenges posed by the sea cucumber's tight-packed, fibrous body wall. Many molecules such as carbohydrates and proteoglycans help make up the *C. frondosa* body wall (Hossain et al., 2020). It was also noted that in *C. frondosa*, there is a stiparin inhibitor known as tensilin which binds to stiparin blocking its ability to bind collagen fibers (Tipper et al., 2002). Given that cells need to be lysed to be able to extract tensilin from tissues, significant concentrations may have been still inside the cells during these extractions (Tipper et al., 2002). To liberate the mole-

cule and further disaggregate stiparin - collagen interactions, higher shear mixing should be incorporated in addition to grinding done in these experiments. It would be interesting to try, and theoretically, this should increase collagen yields. Also, research could be done into looking into similar molecules or adjuvants that can induce stiparin inhibition which by Tipper et al. (2002) is mediated through protein-protein ionic interactions. Trotter et al. (1996) had to do multiple pepsin treatments and disaggregation runs in order to extract adequate amounts of collagen, as after liberating the fibrils and then applying a single round of pepsin digestion, they were able to liberate approximately 1% of the total available hydroxyproline. Some of these molecules resist pepsin breakdown, potentially hindering tissue penetration and interacting with collagen (Thurmond & Trotter, 1996). This may explain the lower yield in this project.

A combination of heat and a fibril chemical bond-breaking reducing agent could liberate even more collagen rather than using one treatment method or the other. However, 2-mercaptoethanol needs to be replaced by a less aggressive reducing agent such as urea. Also, studies of what bonds are being broken in the disaggregation need to be studied in more detail to provide a better understanding of the disaggregation step.

An important part of the project was the scale-up of the process. To save processing time at a commercial scale, the dissection step of the sea cucumber was eliminated, and the whole sea cucumber was used. To maximize efficiency and minimize waste, it is advantageous to attempt to use up as much of the available resource as possible. The viscera could be used to develop other coproducts in a biomolecule refinery concept (Van Dyck et al., 2010) but this was outside the scope of this thesis and was not investigated. The data revealed that the total protein content in the pilot-scale yield was lower than that of the lab-scale product, which utilized only the body wall. One plausible explanation is that enzymes in the internal organs may have broken down proteins, including collagen, leading to a lower yield. Cathepsin B, D, E and matrix metalloproteinase-2 (MMP-2) are proteins that break down collagen, and they are released during autolysis by internal organs and the digestive system (Fan et al., 2024). Their organs were not present in the lab extract. The idea of further digestion is plausible, as small molecular weight proteins in the SDS PAGE could represent degraded proteins. The lack of β and γ forms suggests that they were degraded by proteolytic enzymes.

During the optimization process, efficiencies were implemented that led to the previously mentioned increased yields, as well as a reduction in processing times. A pilot mill was used instead of the lab scale mill. The time required to prepare the biomass remained the same, but more of it could be milled in a shorter period. The purification and drying steps were longer in the optimized pilot step as opposed to the conventional native collagen process at a lab scale. This was because more raw material or products were prepared and processed with the same equipment, they were not scaled for industrial batches. If pilot scale equipment was used, similar observations would have been made compared to the pilot mill versus its lab mill counterpart. In the conventional lab protocol, from disaggregation to extraction, the processing time was 72 to 144 hours. In the developed pilot-scale protocol, the time to cover these same steps was less than 24 hours. As pepsin was operating near its optimal temperature, the pre-treatment and extraction steps did not need to be carried out for as long to begin extracting collagen from the matrix. Knowing the importance of the disaggregation step is critical in liberating collagen from the extracellular matrix, it would be important to test if yields could be increased by using the conventional disaggregation time (24-48 hrs) with the developed temperature assisted extraction protocol. To reduce the bottleneck time of the disaggregation step, staggered batches could be used to minimize the time between disaggregation steps.

In the recovery step, the common method is salting out or bringing the collagen to its isoelectric point, causing it to precipitate. This precipitation is recuperated by high-speed centrifugation and redissolved in acetic acid, the solvent the collagen was in before the recovery. The purpose of the recovery step is to remove concentrated impurities. Adding acetic acid back in after it has been removed defies the usual principles of process design, which dictate that once streams are separated, they should not be recombined (Turton et al., 2002). To be more efficient and use fewer chemicals, a filtration train can be used to produce the same result. Sieves can be used to remove large particles and ultrafiltration, or chromatography techniques can be used to remove proteins and fine impurities. Along this wavelength to reduce the amount of liquid used in these steps saving time and cost, water washes in the pre-treatment steps were reduced and the one after the deproteination step to bring the solids to a pH of 7 was replaced by neutralizing with HCl. Successive water washes of biomass require a lot of time and water.

Other than the disaggregation step where the liquid to solid ratio could not be reduced from 10:1 to 4:1, there was not much optimization of this ratio. In general, increasing this ratio has a positive effect based on mass transfer principles. The driving force behind extraction processes is their concentration gradient. Due to the tightly packed body wall, solvents and salts are likely to face resistance in penetrating the material. To effectively disrupt the bonds between collagen molecules in neighboring molecules, the disaggregation solution requires high concentrations of solvents, salts and chemicals to diffuse into the material. This effect increases when the solid body wall is prepared in large chunks, since there is less surface area for the solvent or solution to come into contact with. In order for the solvent to disrupt the electrostatic and chemical bonds, it has to penetrate the dense network. If this process is limited by diffusion in the solid phase, then it will take longer (Geankoplis, 2003). From an efficiency and economic perspective, it is desirable to reduce this ratio as much as possible without affecting yield. This will minimize the amount of chemicals used, as well as minimizing the size of the equipment. In the case of the disaggregation solution, the yield was significantly impacted. Increased volumes of solvents or solutions will result in more interactions and deeper penetration of liquid into the solid phase, leading to greater solubilization and mass transfer into the solution (Jafari et al., 2020). However, when looking at the acid extraction step, excessive acetic acid should not be used as it creates higher concentrations of lower molecular weight peptides (Jafari et al., 2020).

In the pilot batch, two types of drying methods were used which were spray drying and freeze drying. While spray drying has reduced capital and operating costs as opposed to freeze drying, the high operating temperatures can denature proteins and change the properties of the collagen (Shaviklo et al., 2010). The powder produced from spray drying was more powder like where the lyophilized product formed a fibrous sheet and would require milling to bring back into a powdered form. Lower operating and capital costs, as well as the elimination of the milling step, make spray drying an interesting option for use on the Lower North Shore. The lower powder yield in spray drying could be explained by some particles getting past the filters and cyclone, exiting the system's exhaust, because the nozzle size used was very small to ensure good drying. Optimizations for atomization characteristics can easily be done according to the industrial setup that will be

selected and used. The protein content in the powdered specimens was slightly higher in the lyophilized product. This could indicate that the chosen sample for drying varied, or that there was possibly irreversible damage to the protein in the spray-dried sample due to the high temperatures. More studies such as electron microscopy, and circular dichroism to look at any impact on the protein's structure which will ultimately dictate its functionality. Various groups have noticed that water holding and emulsifying capabilities are superior in freeze dried products as opposed to spray dried products (Latip et al., 2015; Shaviklo et al., 2010; Q. ran Zeng et al., 2013). Although the information isn't shown, Native PAGE, SDS-PAGE, and UV-vis analyses yielded comparable results, suggesting that the two drying techniques produce powders that may share similar characteristics.

In the studies conducted in this project, dialysis took much longer given it is a passive technique compared to ultrafiltration, which is pressure driven. In an industrial setting, ultrafiltration would be more beneficial than passive dialysis. Dialysis could still be used, but it would have to be scaled using hemodialysis techniques which can handle larger process volumes but still rely on the same chemical potential separation principles and diffusive transport (Lightfoot, 2004). Chromatography columns would also be interesting at an industrial scale, especially when looking at extremely pure products. The purity would need to be defined by the client and from that a decision could be made on the post-extraction purification treatment scheme.

Collagen denaturation temperature gives information on the molecule's stability at various temperature. For the selected product, the denaturation temperature was between 17 ± 2 ℃ to 18 ± 2 ℃ which is common for cold water species. For various cold-water species, Akita et al. (2020) discussed that the average denaturation temperature is between 18 ℃ to 20 ℃. Similar to many cold-water marine organisms, the low hydroxyproline content is thought to contribute to their low denaturation temperature. This reduction in intermolecular hydrogen bonds makes the proteins more prone to structural modifications. A captivating investigation to conduct is to assess the hydroxyproline content of collagen derived from this *C. frondosa*. This will allow us to determine whether the hypothesis that low hydroxyproline content correlates with a lower denaturation temperature also applies to collagen from this particular species. It was interesting to see that while the products were extracted above the thermal denaturation temperatures at 20 ℃ to 40 ℃, circular

dichroism, FTIR, and Native PAGE results all confirm there are portions of the extract still in its triple helical form. It could be possible that the collagen is renaturing when reduced to appropriate temperature (Ohyabu et al., 2013). If the temperature does not begin to degrade the helices to the point where the helices are disrupted, this remains a possibility. Another explanation for this phenomenon is that a bridge molecule may still be bound to the molecule. While the intramolecular hydrogen bonding network is destroyed, these molecules attach themselves to the collagen molecule, keeping it stable. To further understand this, CD experiments could be conducted on the same extracts as those tested in this project, between 60 °C and 4°C. This would show whether the collagen can reform into its native state or whether it will remain unfolded.

Despite being subjected to high temperatures during the extraction process, the extract partially succeeded in producing triple-stranded collagen, following several chemical and biochemical quality control tests. Kittiphattanbawon et al. (2016) reported that triple helical fibril formation occurred when temperatures were near 45 ℃ in clown featherback fish. The group also showed that when the mixed product of degraded and intact triple helical collagen was formed, the products still had good gelling properties. Other labs looking at different species have observed CD spectra with positive results for native intact collagen as high as 65 ℃ (Sinthusamran et al., 2018). Gelatin and collagen share similar chemical and biochemical diagnostic signals, it is the variation in signals that allow one to differentiate one form over the other. The extracts also have gelatin or hydrolysate forms of collagen as well and therefore will have great bioactive properties as well (M. Zeng et al., 2007). The developed product is expected to inherit mechanical and functional characteristics from both gelatin and native collagen. However, these attributes may be less pronounced than those of a purified extract that contains only one form or the other. To validate the properties and effectiveness in different applications, these properties will need to be studied in more detail.

The levels of heavy metals found in the pilot-scale and laboratory-scale powders are below the maximum permitted concentrations set by Health Canada and the Food and Drug Administration (FDA) for use in cosmetics (Food and Drug Administration Agency, 2022; Health Canada, 2012). For example, the Health Canada limit of lead in cosmetic products is 10 ppm, arsenic is 3 ppm, mercury is 1 ppm and cadmium is 3 ppm (Health Canada, 2012). Both powders have lower con-

centrations than standard set for each heavy metal. However, in Europe, in countries such as Germany, the lab-scale powder is well below the limits, and the pilot-scale powder is borderline, as lead concentrations in cosmetic products must not exceed 2 ppm, arsenic 0.5 ppm, mercury 0.1 ppm, and cadmium 0.1 ppm. (German Federal Office of Consumer Protection, 2017).

From a technical perspective, the process is transferable to the Lower North Shore. From an economic perspective, the implementation is a bit more challenging. The following pricing information is based industrial scale chemical prices and information on sea cucumber pricing from the fisherman. The *C. frondosa* cost is roughly 6.18 $/kg of its wet weight. The chemical cost required to process collagen in based on the data from this project is 1.37 $/kg of wet *C. frondosa*. The price of collagen ranges quite significantly depending on its application. Laasri et al. (2023) mentioned it can go as high as 5000 - 50000 $/kg (Laasri et al., 2023), but a more reasonable range based on Shore Grow's inquiries with its stakeholder is between 100 - 2000 $/kg. Based on the yields in this project, comparing potential revenue versus raw material cost only, the process would generate a net loss of -1.80$ per kg processed sea cucumber assuming one could sell collagen for 2000 $/kg collagen. The following calculations do not include any equipment, building, or land purchases and operating costs such as labour, waste treatment, utilities. Based on the results of this study, the potential yield of collagen from *C. frondosa* is estimated to be around 114 milligrams per gram of dry sea cucumber. Assuming one could sell collagen for 2000 $/kg collagen, the process would generate a net gain of 5.56 $/kg wet sea cucumber processed. Other species can achieve greater yields as shown in Farooq et al. (2024), and therefore would achieve higher margins. The sea cucumber species may have more collagen initially in the body wall. Therefore, from an economic perspective, more work needed to be done to increase the yield and explore the alternative of developing coproducts to maximize resource value or work with clients to achieve the prices mentioned by Laasri et al. (2023).

By making improvements and refinements, the project developed a collagen extraction method that minimizes time, chemicals, and resources, making it practical for use in the Lower North Shore region. However, to make this project successful, logistics and transport within the area is a key part of the equation. In terms of fishing and transport of the resource, there is already a well-developed fishing industry and fishermen with most of the infrastructure to harvest. However,

there are not many fishermen with licenses with sea cucumber fish in the area. Also, the location of *C. frondosa*, between Kegaska and Blanc-Sablon, has not been well studied. Current studies only extend as far as the Havre-Saint-Pierre area (DFO, 2019). Therefore, more zoning and population studies must be conducted, as well as recruitment efforts, to identify those who will harvest the resource. In terms of resources, transportation, and exports, the Strait of Belle Isle and the northern coast of Newfoundland serve as a popular maritime corridor for ships bound for Europe or the Arctic Circle. Additionally, a local freighter makes a weekly trip from Rimouski to the Lower North Shore from April to January, which coincides with the sea cucumber harvest season. More research and planning are required in this sector to maximize the project's financial viability. However, as discussed, commercial transportation is already available in the region. Additionally, the processing plant can be designed to convert collagen from alternative sources, such as fish byproducts, to generate additional income streams and maintain production during the off-season. This can help offset the cost of building such a facility and provide jobs to residents for longer periods.

Chapitre 6 - Conclusion

6.1 Return to the goal and results

In the work presented in this thesis, a process was synthesized to extract collagen *Cucumaria frondosa* that could be harvested but industrially processed on the Lower North Shore. The area needs economic diversification and jobs, as from 2016 to 2021, the population has declined by 13% (Statistics Canada, 2021). The process proposed in this project would work in synergy with the existing fishing industry that would be able to supply the resource.

The initial focus of the project was characterizing *Cucumaria frondosa* based on its physical and biochemical properties. Parameters such as moisture and protein contents were like other sea cucumber species. The heavy metal contents were similar and if not lower than other available species from other countries which may be attributed to the pristine waters of the Lower North Shore. The collagen content of this *C. frondosa* is 1.6 ± 0.2 %, which falls within the average range compared to other species on the market (Z. Liu et al., 2010). In other words, if we consider the initial availability of collagen in the original source, suppliers who use different species in other nations with higher initial collagen levels may have a slight edge from an industrial perspective.

Secondly, the pepsin soluble extraction process was the most effective protocol to extract collagen from *C. frondosa*. Other, predominantly acid-based protocols were also tested. Yields were negligible, as has been observed in existing literature (Felician et al., 2018; Schmidy et al., 2016). Pepsin allows for further solubilization of collagen into solution by hydrolyzing its telopeptide ends. A common interpretation repeated throughout the results and discussion was how rigid and complex the sea cucumber body wall was, so acids alone were not enough to properly solubilize the material into solution. The process was viable for the Lower North Shore, as all the chemicals are simple and should be easily transported to the area.

Thirdly, the pre-treatment, extraction, and post-treatments steps were optimized, reducing the processing time by at least 48 hours to produce a mixed hydrolyzed and native collagen extract that is feasible and relatively simple to implement on the Lower North Shore. While performing all

extractions at 4 ℃, the yields being produced with a Tris-HCl and EDTA disaggregation solution were low. This was due to the fact of the complex nature of the *C. frondosa* body wall. While some ionic links were being disrupted with solely Tris-HCl and EDTA, the addition of 2-mercaptoethanol obtained a 3-fold increase in yield. It is likely that 2ME was also breaking disulfide bonds (Shoulders & Raines, 2009). This result showed that the degree of body wall fiber disaggregation had an important impact on the pepsin hydrolysis reaction performed later in the process. It was not desired to keep 2ME in the process due to its toxic nature which would cause implementation obstacles on the Lower North Shore. Therefore, in a quick decision, it was decided to work at room temperature for the pre-treatment and post-treatment steps and the extraction step was carried out with small amounts of heat, which ultimately reduced the overall processing time of the process. The higher temperature was thought to further disaggregate fibers and the body wall matrix. The optimal point selected was 30 ℃ and 0.1 g/L, extraction for 90 minutes as this would maximize the yield and native structure and minimize time and the amount of collagen disaggregation. However, the yield was approximately 46 ± 3 mg collagen per gram of dry *C. frondosa* body wall, which compared to other sea cucumbers is on the low end (Farooq et al., 2024).

For the fourth sub-objective, the optimized lab-scale extract of 200 g of wet sea cucumber was successfully scaled up to 4000 g batch of wet sea cucumber to continue along the commercialization path and deliver a viable process to the residents of the Lower North Shore. As a part of the scale-up, the whole sea cucumber was used to eliminate a processing step (dissection). The protein yields were lower which could have been due to enzymes, inhibitors, or protein competitors of the pepsin cleavage site in the viscera, further degrading proteins over time (Fan et al., 2024). Steps were simplified such as minimizing water washing steps in the pre-treatment stage and eliminating the centrifugation after the isoelectric-like neutralization post-treatment step, saving time and minimizing water usage. Preliminary investigations were made into spray drying as a final drying method and while a visually pleasing powder was produced, the yields were lower than lyophilization. Since only one trial of the spray-drying method was performed, there is potential for improvement in yield through optimization.

Finally, the final products at the lab scale and pilot scale were characterized and the results demonstrated at both scales, the products resemble other sea cucumber extracts existing in the present

literature. SDS gels in both extracts showed protein bands between 120-130 kDa, which are the α helices in type I sea cucumber collagen. The lab extract showed β (~200 kDa) and γ (>250 kDa) forms but in the pilot scale the β chain was observed, not the γ. As for the Native PAGE experiments, proteins around 380 kDa were indicated which based on the size of the three α helices was expected. There are degradation bands on each gel, indicating that the final product is a mix of both native, gelatin, and hydrolyzed products. With the UV-vis spectrums, a maximum signal was observed at 230 nm, which is typical for marine collagen since they contain high concentrations amide groups and carbonyl groups from many peptide bonds along each alpha chain (Ju et al., 2020). FTIR spectroscopy revealed the expected five amide peaks, with the amide I peak centering around 1645 cm^{-1}. This suggests that the collagen chains are not in a random coil but in ordered alpha helical structures. Even with extraction temperatures of 30 °C, the circular dichroism spectrum of the laboratory-scale product had a minimum ellipticity at 198 nm and a maximum ellipticity at 220 nm, indicating that at least part of the collagen is still in its triple helical form (Alves et al., 2017). The thermal denaturation temperature of the protein was between 17 ± 2°C and 18 ± 2°C which is as expected for cold water marine species (Akita et al., 2020). Both the lab and pilot scale extracts had heavy metal contents lower than the limits designated by Health Canada and the Food and Drug Administration Agency (Food and Drug Administration Agency, 2022; Health Canada, 2012). Given the resource comes from a pristine environment with low industrial activities, concentrations such as lead, and mercury are little to none in the initial resource and final product. All these results show that the product conforms to the typical structure and characteristics of native collagen, while also meeting the quality control requirements of various organizations. This makes the product and process a viable opportunity for marketing and exploitation by the Lower North Shore.

6.2 Limitations

A limitation as previously stated in the project was the limited number of repetitions of each run. The results should be seen as trends rather than significantly concrete results. Given the number of steps involved in the collagen extraction project, its complexity, the limited time (less than six months) and resources available, it would not have been possible to go through each step and

develop a transferable protocol for the Lower North Shore. To further strengthen the observations and results obtained in this paper, it would be essential to test their repeatability to develop statistically significant conclusions. However, for the private stakeholders in the project, the information allows for the development of a protocol that can be transferred to a remote area and in the scope of the project, served its purpose. The limited time and resources also played part in only doing one pilot test which would require more time and resources. This was another reason why the entire sea cucumber was used in the pilot run, as there was limited body wall left. This potentially could have affected the yields of the product at the pilot scale.

The amount of material produced at the end of the process was a limiting factor. To carry out various characterization tests, there was a minimum amount of product that was needed. Starting with batch sizes less than what was performed in the project would risk not having enough material to perform the necessary characterizations needed for each run.

Cucumaria frondosa protein content is highest in the summer months because the body wall is accumulating protein for reserves during lower feeding times i.e. winter months (David & Macdonald, 2020). The resource harvested in this experiment was in June. Therefore, in the actual fishing season, which is from April to October, with specific dates depending on the fishing zone, could affect the total yield of collagen. There were no comparisons between seasons. Given the tight time constraints and the challenges of obtaining sea cucumbers, the researchers did not examine the seasonal fluctuations in resource content or the impact on yield. The results could reconfirm existing data on the resource and help set realistic expectations of processing throughout months in the year where the yield may go down due to various environmental factors that may impact protein synthesis, or the end-user could better plan the appropriate times of harvest to ensure they are maximizing their harvesting periods. Before beginning commercial production, the topic of seasonal variation in protein content in *C. frondosa* should be carried out.

A factor that was not monitored was the number of freezing-thawing cycles experienced by the sea cucumber's body. As mentioned in section 3.1, the resource has undergone freezing and thawing cycle(s) before being processed. This was due to the amount of sea cucumber used at the lab scale (100 g) and the fact that the body wall of an individual weighed more (upwards of 150 g).

Discarding this biomass would have reduced the number of experiments and tests possible in the project. To reduce the impact, the milling process was carried out at 4°C to minimize the break-down of sea cucumber and its protein. Also, the number of freeze-thaw cycles was also limited to just two, but the majority of sea cucumbers processed underwent only one cycle. It's important to note that there may still be some variation in product quality between different batches.

Another limitation was associated with the collagen dosing methodology used for this project. After discussing with the manufacturer of the Quickzyme Total Collagen Assay, they mentioned that the results obtained with the cold-water *C. frondosa* may be lower than its actual value presenting a limitation. This is because of the low hydroxyproline content in cold water species and the standard used in this experiment is rat tail collagen, which has higher hydroxyproline content. To accurately get the proper dosage of sea cucumber collagen, a cold-water sea cucumber native collagen standard should be used which can be difficult to obtain or swap the collagen standard with a hydroxyproline standard and determine the hydroxyproline content in the sea cucumber body wall and/or powder products to get an accurate value.

Building on the previous point, the total collagen assay was not used on each extract, due to the time it took to develop and test various kits available, and budgetary reasons. Other kits such as the MAK322 collagen assay kit (Sigma Aldrich, USA) did not work so it took a few iterations to find the right one. These kits are expensive, the amount of samples produced would need many kits and the cost of this would be high. Other protocols could have been used, such as hydroxyproline testing with HPLC to dose the collagen but the equipment and protocols are not set up at CRBM so it would have to be sent externally which was not ideal. If the Quickzyme collagen assay was not used, total protein assays were used to infer trends in protein extraction. The results suggest that one would likely see similar observations for collagen, as it is the predominant protein.

Finally, given the limited amount of data and time conducted with the spray drying experiments, the results from the spray drying versus lyophilization are difficult to suggest whether spray drying is comparable to freeze drying. More product characterization and optimizations of the technology to the produced collagen need to be performed to ensure it is running properly, as compared to freeze drying.

6.3 Future experiments

Now that a product has been developed, testing functionality properties will be critical before commercializing the product. In terms of the cosmetic industry, tests looking at antimicrobial, anti-inflammatory, antiaging, and antioxidant activity are interesting information to have when trying to sell ingredients to be used in creams, lotions, gels, bars *etc*. In terms of the biomedical industry, wound healing, and tensile strength, among other properties would be interesting to see how the molecule can be implemented in biomaterials and tissue scaffolds.

Two important aspects of the effects of extraction were noted in this paper were use of 2-mercaptoethanol to disaggregate the initial fibers from other extracellular matrix of molecules and the use of heat at the extraction step to further solubilize the collagen. In terms of the disaggregation step, other reducing chemicals could be tested to promote the liberation of these molecules that are nontoxic. The precise mechanism of the disaggregation process and step is not well described, but it is crucial in the extraction of sea cucumber collagen. To monitor the effects of altered variables on collagen and collagen fiber three-dimensional structures, nuclear magnetic resonance (NMR) or X-ray crystallography techniques can be used to observe the changes. As for the topic of the added heat to the extraction step, it would be interesting to see if the collagen can be refolded, which can be monitored with CD spectroscopy. Additionally, analyzing the protein breakdown patterns of the elevated extraction temperature samples can verify that the altered structures seen on SDS-PAGE and Native PAGE gels are indeed degraded collagen, rather than contaminants.

Finally, there is a need for more iterations and optimization on a pilot scale, as well as working with companies to further develop research and development during the scaling-up process. At the pilot scale, factors such as heat transfer, mass transfer, and mixing become even more critical to ensure the reaction is as uniform as possible. Also, understanding product handling and processing times will be important to understand process economics. Within these tests, other common industrial processing techniques like spray drying can be tested to see if the collagen powder still holds interesting properties that can be applied to consumers.

6.4 Closing Remarks

In summary, a collagen extraction process was developed to be industrially implemented on the Lower North Shore. Through optimizations and learnings about each collagen extraction step, a process was developed and tested at a pilot scale that can be transferred to the population of the Lower North Shore to further the project and continue the steps along the commercialization pathway. While there are iterations and tests to come, a foundational process and product was delivered in the following master's project.

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Chapitre 7 - Appendices

7.1 Appendix A – Supplemental information

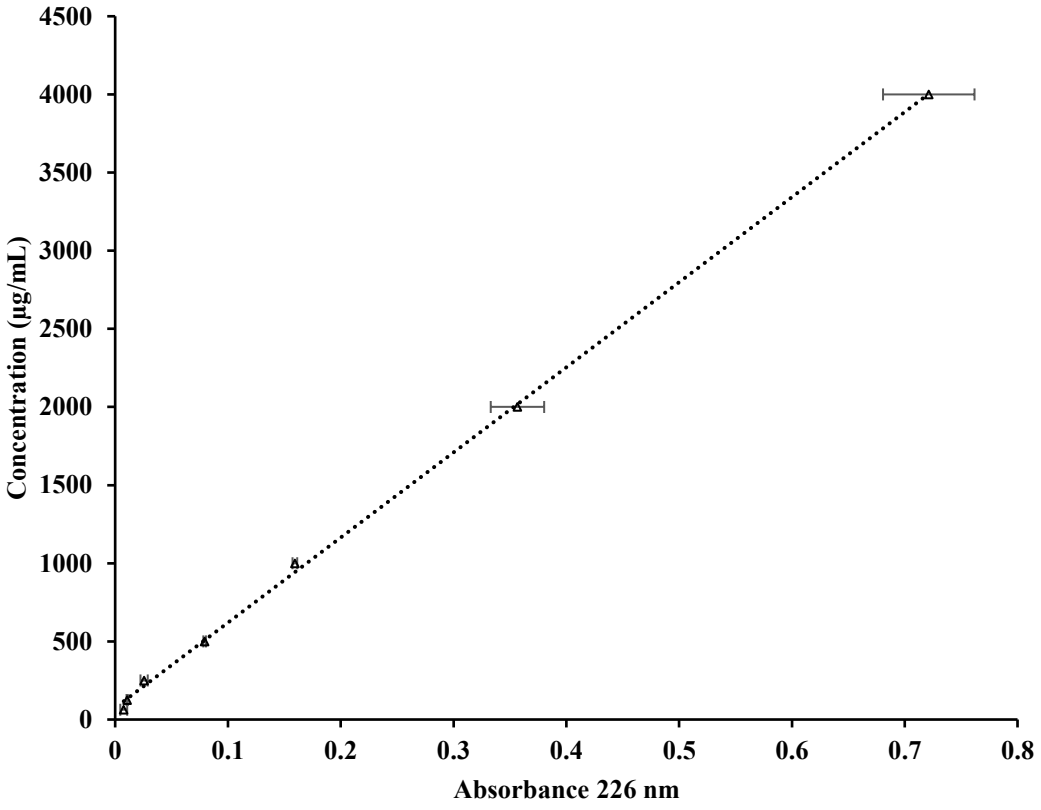

Figure 7.1. Standard collagen concentration curve produced using a nanodrop UV-vis spectrophotometer at a wavelength of 226 nm. The linear regression analysis produced the following parameters: slope was 5445.6 mL/µg, intercept was 75.456 µg/mL and the R^2 value was 0.9994. The points are average values and error bars are 1 standard deviation for n=2 samples.

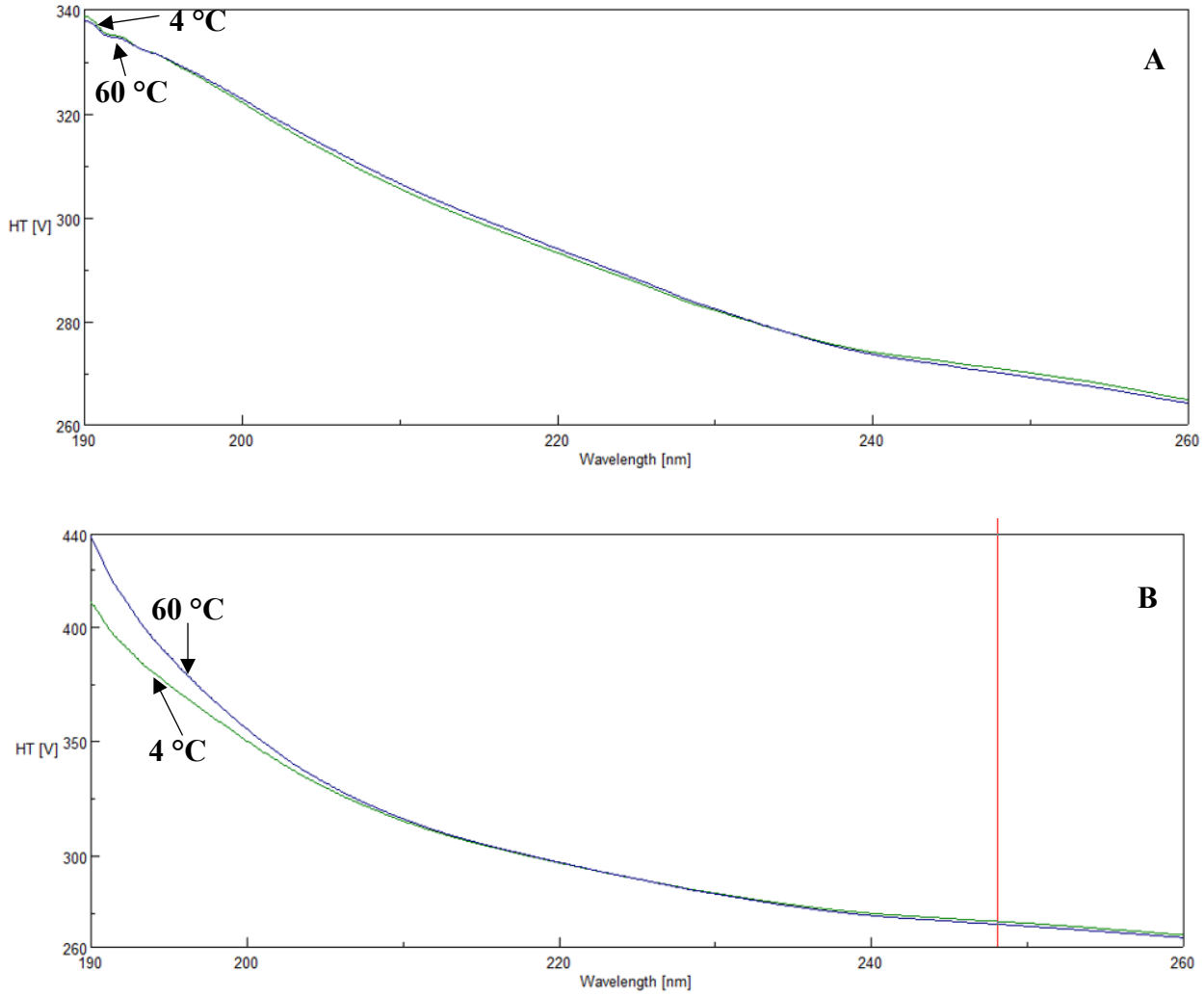

Figure 7.2. High-tension (HT) voltage versus wavelength plots, based on the results of circular dichroism spectroscopy experiments with collagen products obtained under different conditions: (a) the Tris-HCl, EDTA solution used for the disaggregation step during the pepsin soluble collagen (PSC) process (run 9); and (b) the Tris-HCl, EDTA, NaCl, 2ME solution used the disaggregation step during the PSC process (run 10); while varying the wavelength.

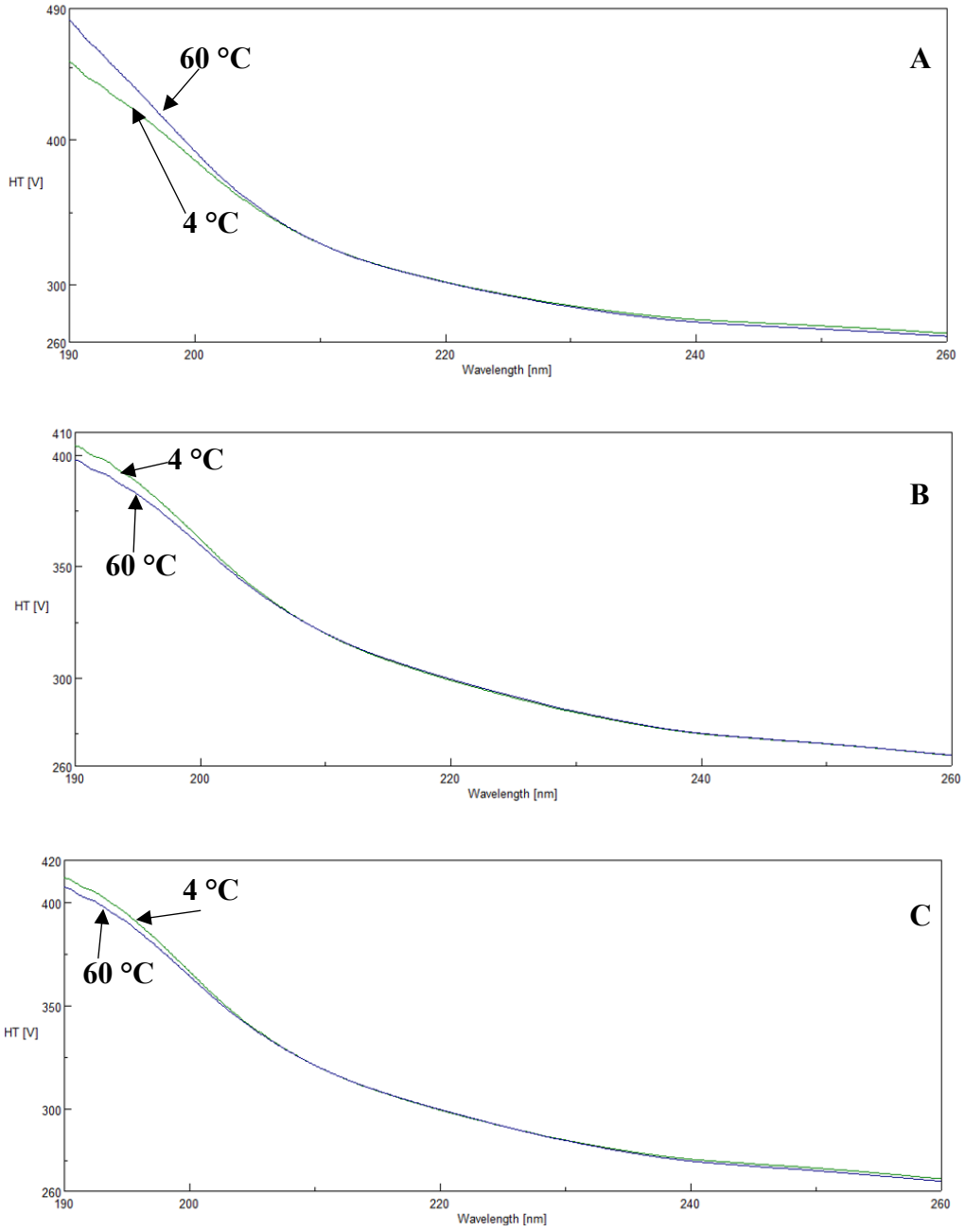

Figure 7.3. High-tension (HT) voltage versus wavelength plots, based on the results of circular dichroism spectroscopy experiments with collagen products obtained under different conditions: (a) pepsin soluble collagen (PSC) extracted at 40 °C, 0.01 g/L, for 207 minutes (18a); (b) PSC extracted at 30 °C, 0.1 g/L, for 120 minutes; and (c) PSC extracted at 20 °C, 0.3 g/L, for 120 minutes (18c). Each solution and result were normalized to 1 mg/mL. The CD spectra were obtained at a constant temperature of 4 °C (green) and 60 °C (blue), while varying the wavelength.

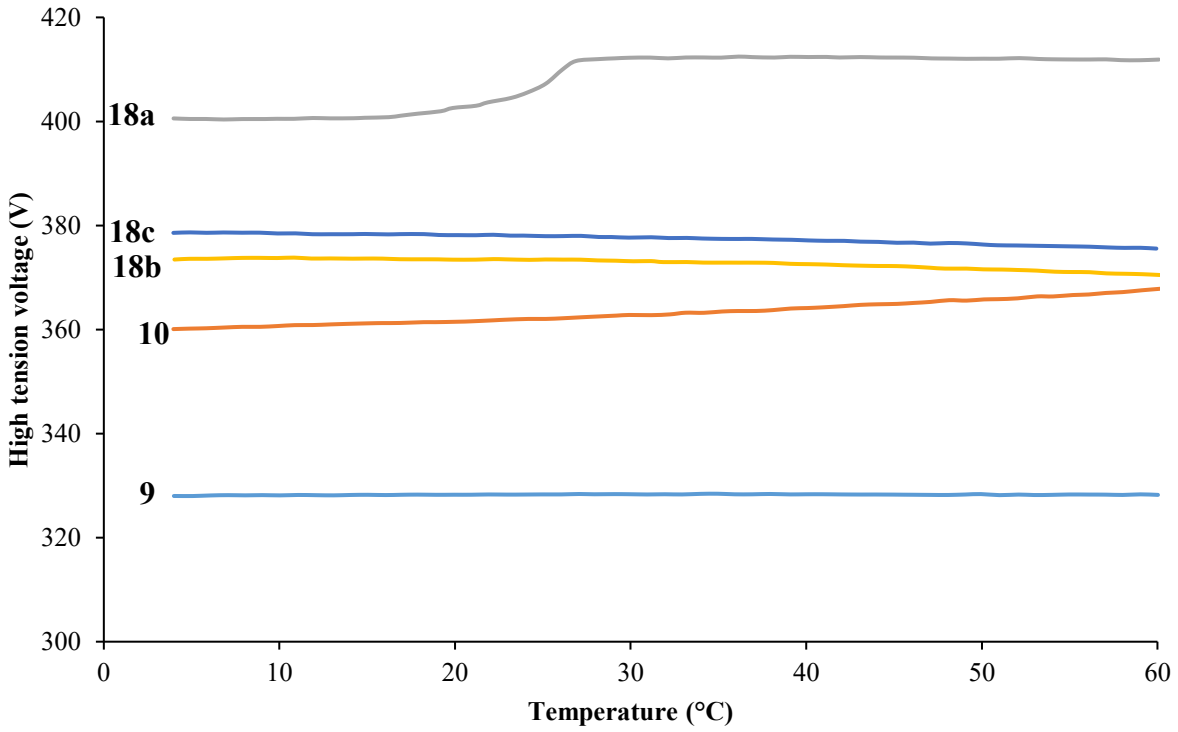

Figure 7.4. High-tension (HT) voltage versus temperature plots, based on the results of circular dichroism spectroscopy experiments with collagen products obtained under different conditions: Tris-HCl, EDTA solution used for the disaggregation step during the pepsin soluble collagen (PSC) process (run 9 – light blue); Tris-HCl, EDTA, NaCl, 2 βME solution used for the disaggregation step during the PSC process (run 10 - orange); the PSC extracted at 40 °C, 0.01 g/L, for 207 minutes (18a - grey); the PSC extracted at 30 °C, 0.1 g/L, for 120 minutes (18 b – yellow); the PSC extracted at 20 °C, 0.3 g/L, for 120 minutes (18c – dark blue). Each solution and result were normalized to 1 mg/mL. Wavelength was kept constant at 198 nm.

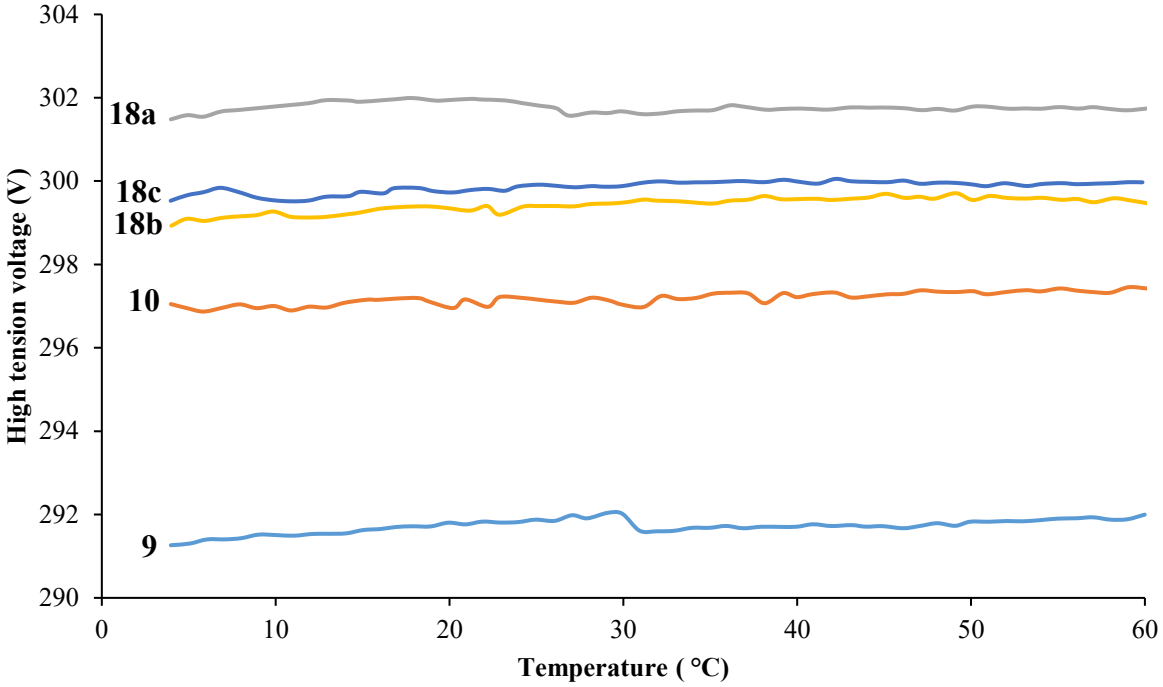

Figure 7.5. High-tension (HT) voltage versus temperature plots are based on the results of circular dichroism spectroscopy experiments with collagen products obtained under different conditions: Tris-HCl, EDTA solution used for the disaggregation step during the pepsin soluble collagen (PSC) process (run 9 – light blue); Tris-HCl, EDTA, NaCl, 2 βME solution used for the disaggregation solution step the PSC process (run 10 - orange); PSC extracted at 40 °C, 0.01 g/L, for 207 minutes (18a - grey); PSC extracted at 30 °C, 0.1 g/L, for 120 minutes (18 b – yellow); the PSC extracted at 20 °C, 0.3 g/L, for 120 minutes (18c – dark blue). Each solution and result were normalized to 1 mg/mL. Wavelength was kept constant at 220 nm.

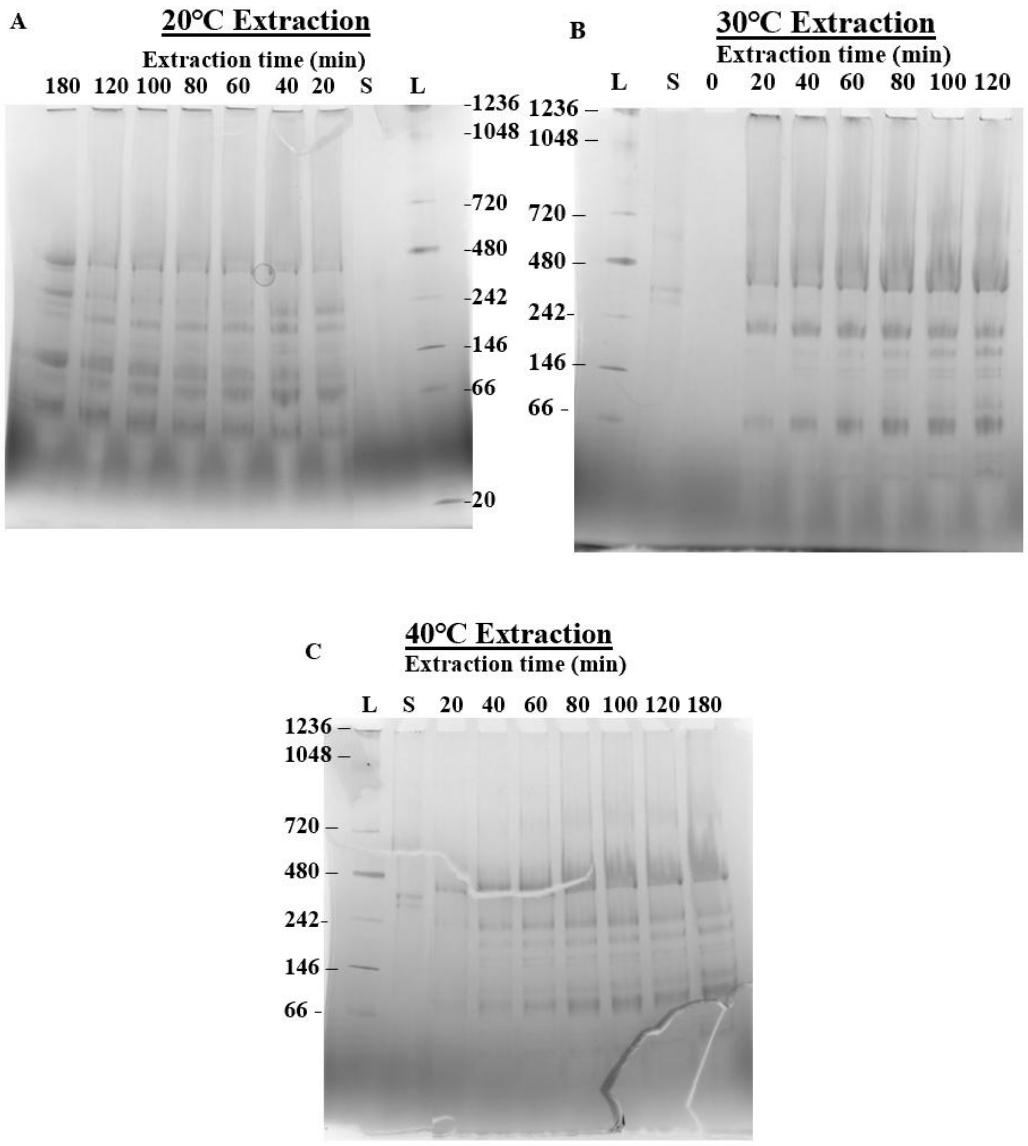

Figure 7.6. Native PAGE analysis of collagen extractions over a period of time for three different extraction temperatures: 20 °C (a), 30 °C (b), and 40 °C (b). The pepsin concentration was kept constant at 0.1g/L, while the acetic acid-to-solid ratio was maintained at 10:1. The following lanes are represented in each gel: (l) denotes the molecular weight marker, and (s) corresponds to the calf skin type 1 collagen standard.

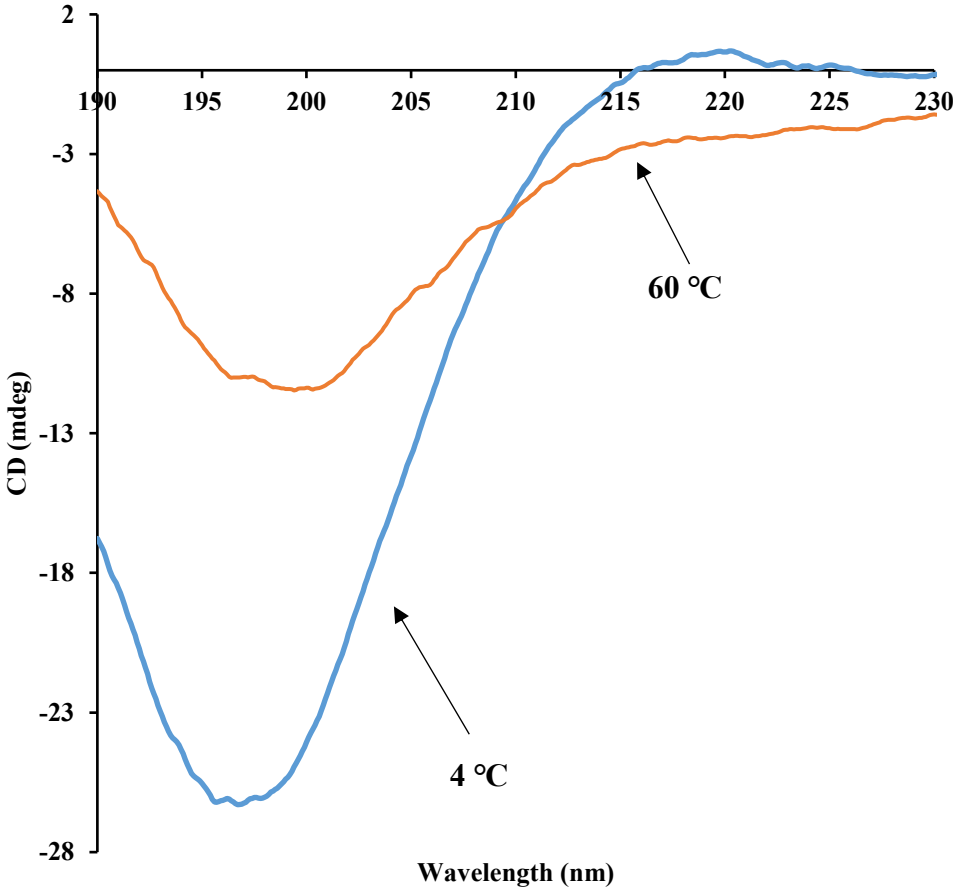

Figure 7.7. CD spectra of optimized pepsin soluble collagen extract at 4 ℃ (blue) and 60 ℃ (orange) at various wavelengths. Increments of wavelengths used were 0.1 nm from 190 nm to 260 nm