



**Impacts of Advanced Processing Techniques on the Physicochemical,
Structural, and Allergenic Properties of Atlantic Cod**

Xin Dong

Department of Bioresource Engineering

McGill University, Montreal

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Abstract

Seafood, encompassing both fish and shellfish, is a nutrient-rich component crucial for human health. Despite its benefits, seafood allergies present a global health challenge, causing reactions that vary from mild discomfort to severe, life-threatening conditions in affected individuals. This allergy is mainly divided into two categories: fish allergy and shellfish allergy, which are part of the well-known "big nine" allergenic foods group, including fish and crustaceans. A key allergen in fish, identified by the WHO/IUIS, is parvalbumin, a protein responsible for allergic reactions. In recent years, the seafood industry has employed various processing methods to decrease allergenicity while preserving the nutritional and sensory qualities of food products. These processing techniques can modify the biochemical properties of allergenic proteins and induce physicochemical changes within the seafood matrix. The effectiveness of these methods in altering seafood allergenicity varies based on the type and conditions of the processing applied.

This study provides a comprehensive overview of seafood consumption and classification, the prevalence and symptoms of seafood allergies, and the biochemical characteristics of major seafood allergens. It delves into recent advancements in processing techniques, including thermal (oven, steam, microwave, wet and dry heat), non-thermal (ultrasound, cold plasma), and combined (thermal glycation) treatments. These methods have been critically analyzed for their impact on the nutritional, structural, and allergenic properties of Atlantic cod. The study also explores molecular dynamics simulations, offering a microscopic perspective of allergenic structures, which is helpful in refining and optimizing food processing parameters. We found combined treatment showed the best performance in reducing allergenicity and keeping nutrients, followed by non-thermal outperforming thermal methods. Specifically, thermal treatments reduced cod allergen content by 7.80%–46.33%, with a maximum reduction of allergenicity by 46.33% and a most enhanced protein digestibility by 55.29%. Changes to the secondary and tertiary structures were noted with higher β -sheets and lower α -helices. More structural destructions with fragments and micro holes were observed with increasing processing durations. High-intensity ultrasound lowered allergen content by up to 31.82% and

improved protein digestibility by up to 12.24%. Ultrasound also maximumly increased TAC, TFC, TPC, and peptide content before digestion by 7.28% (US60), 3.00% (US30), 32.43% (US10), and 18.93% (US60), respectively. Color attributes and electron microscopy reflected structural changes in the cod samples, suggesting the effectiveness of ultrasound in altering protein structures. Cold plasma notably reduced cod allergen content by 9.14% at CP10, accompanied by considerable changes in protein secondary structure and microstructure. Additionally, TAC, TFC, and TPC saw their maximum increases of 21.56% (CP10), 68.48% (CP10), and 14.00% (CP30), respectively. Protein content observed before and after a two-stage digestion process could be maximally enhanced by 10.76% and 30.20% at CP20. Meanwhile, the peptide content before and after this digestion process could see its maximum boost by 61.18% and 26.27% at CP30. The digestibility of most samples treated with cold plasma remained almost stable. For the thermal-glycation treatments, the combination of microwave (150 °C) and glucose could significantly decrease the cod allergen content by up to 16.16% and significantly increase IVPD to 69.05%. Glucose showed better performance than lactose when combined with microwave heating. In comparison to 100 °C, the treatments at 150 °C induced higher IVPD and peptides in cod. In the last study, molecular dynamic simulation results revealed significant structural changes in Gad m 1, including reversible molecule transformations. Additionally, the properties of the molecule's surface were influenced by various factors, such as the SASA and snapshots representing compactness within the protein structure. Notably, higher pressure (3 kbar) resulted in decreased values for the RMSD and Rg. Overall, the findings demonstrate that the processing techniques investigated significantly reduce the allergenicity and improve the nutritional values of cod. This research not only provides valuable insights for food industry professionals and consumers concerned with food allergies but also lays the groundwork for the future development of hypoallergenic food products, further advancing food safety and quality.

Résumé

Les fruits de mer, comprenant à la fois le poisson et les crustacés, sont un élément riche en nutriments essentiels à la santé humaine. Malgré leurs avantages, les allergies aux fruits de mer représentent un défi de santé mondial, provoquant des réactions allant d'un léger inconfort à des affections graves, potentiellement mortelles, chez les personnes touchées. Cette allergie se divise principalement en deux catégories: l'allergie au poisson et l'allergie aux crustacés, qui font partie du groupe bien connu des « neuf grands » aliments allergènes, dont font partie les poissons et les crustacés. Un allergène clé du poisson, identifié par l'OMS/IUIS, est la parvalbumine, une protéine responsable des réactions allergiques. Ces dernières années, l'industrie des fruits de mer a eu recours à diverses méthodes de transformation pour réduire l'allergénicité tout en préservant les qualités nutritionnelles et sensorielles des produits alimentaires. Ces techniques de transformation peuvent modifier les propriétés biochimiques des protéines allergènes et induire des changements physico-chimiques au sein de la matrice des fruits de mer. L'efficacité de ces méthodes pour modifier l'allergénicité des fruits de mer varie en fonction du type et des conditions de transformation appliquées.

Cette étude fournit un aperçu complet de la consommation et de la classification des fruits de mer, de la prévalence et des symptômes des allergies aux fruits de mer, ainsi que des caractéristiques biochimiques des principaux allergènes des fruits de mer. Il explore les progrès récents dans les techniques de traitement, notamment les traitements thermiques (four, vapeur, micro-ondes, chaleur humide et sèche), non thermiques (ultrasons, plasma froid) et combinés (glycation thermique). Ces méthodes ont été analysées de manière critique pour leur impact sur les propriétés nutritionnelles, structurelles et allergènes de la morue franche. L'étude explore également les simulations de dynamique moléculaire, offrant une perspective microscopique des structures allergènes, utile pour affiner et optimiser les paramètres de transformation des aliments. Nous avons constaté que le traitement combiné présentait les meilleures performances en matière de réduction de l'allergénicité et de conservation des nutriments, suivi par les méthodes non thermiques surpassant les méthodes thermiques. Plus précisément, les traitements thermiques ont réduit la teneur en allergènes de la morue de 7,80% à 46,33%, avec

une réduction maximale de l'allergénicité de 46,33% et une digestibilité maximale des protéines de 55,29%. Des modifications des structures secondaires et tertiaires ont été notées avec des feuillets β plus élevés et des hélices α inférieures. Des destructions structurelles plus nombreuses avec des fragments et des micro-trous ont été observées avec l'augmentation des durées de traitement. Les ultrasons de haute intensité ont réduit la teneur en allergènes jusqu'à 31,82% et amélioré la digestibilité des protéines jusqu'à 12,24%. Les ultrasons ont également augmenté au maximum la teneur en TAC, TFC, TPC et peptides avant digestion de 7,28% (US60), 3,00% (US30), 32,43% (US10) et 18,93% (US60), respectivement. Les attributs de couleur et la microscopie électronique reflétaient des changements structurels dans les échantillons de morue, suggérant l'efficacité des ultrasons pour modifier les structures protéiques. Le plasma froid a notamment réduit la teneur en allergènes de morue de 9,14% au CP10, accompagné de changements considérables dans la structure secondaire et la microstructure des protéines. De plus, TAC, TFC et TPC ont connu leurs augmentations maximales de 21,56% (CP10), 68,48% (CP10) et 14,00% (CP30), respectivement. La teneur en protéines observée avant et après un processus de digestion en deux étapes pourrait être améliorée au maximum de 10,76% et 30,20% au CP20. Pendant ce temps, la teneur en peptides avant et après ce processus de digestion pourrait voir son augmentation maximale de 61,18% et 26,27% au CP30. La digestibilité de la plupart des échantillons traités au plasma froid est restée quasiment stable. Pour les traitements de thermo-glycation, la combinaison de micro-ondes (150 °C) et de glucose pourrait diminuer considérablement la teneur en allergènes de morue jusqu'à 16,16% et augmenter considérablement l'IVPD jusqu'à 69,05%. Le glucose a montré de meilleures performances que le lactose lorsqu'il est combiné au chauffage aux micro-ondes. Par rapport à 100 °C, les traitements à 150 °C ont induit une IVPD et des peptides plus élevés chez la morue. Dans la dernière étude, les résultats de simulation de dynamique moléculaire ont révélé des changements structurels importants dans Gad m 1, notamment des transformations moléculaires réversibles. De plus, les propriétés de la surface de la molécule ont été influencées par divers facteurs, tels que le SASA et les instantanés représentant la compacité au sein de la structure protéique. Notamment, une pression plus élevée (3 kbar) a entraîné une diminution des valeurs du RMSD et du Rg. Dans l'ensemble, les résultats

démontrent que les techniques de transformation étudiées réduisent considérablement l'allergénicité et améliorent les valeurs nutritionnelles de la morue. Cette recherche fournit non seulement des informations précieuses aux professionnels de l'industrie alimentaire et aux consommateurs préoccupés par les allergies alimentaires, mais jette également les bases du développement futur de produits alimentaires hypoallergéniques, faisant ainsi progresser davantage la sécurité et la qualité des aliments.

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Contribution to original knowledge

In this work, an overview of recent advances in processing techniques (thermal, non-thermal, combined (hybrid) treatments) and main allergen detection methods for seafood products is initially reviewed. Besides, the seafood consumption and classification, the prevalence and symptoms of seafood allergy, and a description of biochemical characteristics of the major seafood allergens are also clearly introduced. Atlantic cod is recognized as highly nutritious seafood; however, it is also common allergenic food to induce food allergy. Focusing on Atlantic cod, this research employs a range of innovative processing methods, including various thermal treatments (oven, steam, microwave, wet heat, and dry heat), non-thermal treatments (ultrasound and cold plasma), and combined methods (thermal glycation). The research systematically assesses the impact of these processing techniques on the physicochemical characteristics and allergenic potential of cod. Moreover, this work involves molecular dynamics (MD) simulations to model the behavior of the primary cod allergen, parvalbumin Gad m 1, under specific external thermal forces at various temperatures and pressures. The major contributions of this study can be summarized as follows:

1. **Efficacy of Processing Techniques in Allergen Reduction:** The study provides a comprehensive evaluation of thermal, non-thermal, and hybrid processing techniques, emphasizing their effectiveness in reducing allergenicity. Notably, it quantifies the reduction in allergenicity for thermal (up to 46.33% with steam as the most effective method), non-thermal (up to 31.82% reduction using high-intensity ultrasound), and hybrid techniques (up to 16.16% reduction with microwave and glucose), offering valuable benchmarks for the food industry.
2. **Enhancement of Nutritional Quality through Optimized Processing:** Beyond allergen reduction, this research highlights the impact of processing techniques on improving the nutritional profile and digestibility of seafood. The study demonstrates that certain processing conditions not only reduce allergenicity but also enhance *in-vitro* protein digestibility (up to 55.29% with steam, 12.24% with ultrasound, and 69.05% with microwave and glucose), thereby contributing to the dual objectives of safety and nutrition in food processing.

3. Insights into Protein Structure and Functionality: The research provides in-depth insights into the structural and functional changes in seafood proteins post-processing. It explores the modifications in the parvalbumin molecule, a major seafood allergen, under various temperatures and pressures for a specific duration. The findings, particularly the decrease in RMSD and Rg values at higher pressures (3 kbar), add a molecular dimension to our understanding of allergenicity and its mitigation, paving the way for targeted interventions in food processing.

4. Recommendations for Processing Optimization: By correlating specific processing conditions with allergenicity reduction and nutritional enhancement, the study offers actionable recommendations for the food industry. It identifies optimal conditions for various processing techniques and underscores the superiority of certain methods (e.g., steam, high-intensity ultrasound, microwave with glucose) in achieving the desired outcomes, thus providing a framework for optimizing food processing techniques in practice.

These contributions are poised to have a substantial impact on the food industry, offering pathways to reduce the prevalence of seafood allergies and improve the nutritional quality of seafood products, ultimately enhancing consumer health and safety.

Contribution of authors

In accordance with the McGill Guidelines for a Manuscript Based Thesis, the contributions made by the candidate and the co-authors to the completion of this work are described here.

Xin Dong is the principal author of this work, supervised by Prof. Vijaya Raghavan from the Department of Bioresource Engineering, McGill University, Canada. Prof. Raghavan, as the thesis supervisor and director, co-authored all the manuscripts, offered scientific guidance, and contributed to co-editing and reviewing the manuscripts.

The following are the manuscripts for publication (only present my Ph.D. period):

1. **Dong, X., & Raghavan, V. (2022).** A comprehensive overview of emerging processing techniques and detection methods for seafood allergens. *Comprehensive Reviews in Food Science and Food Safety*, 21(4), 3540-3557.
2. **Dong, X., & Raghavan, V. (2024).** Effect of Varying Thermal Processing Techniques on Allergenicity and Structural Alterations in Atlantic Cod. *Food Chemistry* (Under revisions)
3. **Dong, X., & Raghavan, V. (2024).** Influence of High-Intensity Ultrasound on the Conformational Dynamics and Allergenic Properties of Atlantic Cod Parvalbumin. *Food Chemistry* (Under review)
4. **Dong, X., & Raghavan, V. (2024).** Influence of high-intensity ultrasound on physicochemical characteristics and *in-vitro* digestibility of Atlantic cod. *Food Research International* (Under revisions)
5. **Dong, X., & Raghavan, V. (2024).** Cold plasma treatment of Atlantic cod: Insights into the physicochemical, structural, and allergenic characteristics. *Food Chemistry* (Under review)
6. **Dong, X., & Raghavan, V. (2024).** Modifications of the Structure and Allergenic Characteristics of Atlantic Cod Induced by Thermal-Glycation Treatment. *International Journal of Biological Macromolecules* (Under review)
7. **Dong, X., & Raghavan, V. (2024).** Molecular modeling simulations of thermal and pressure processing in Atlantic cod major allergen parvalbumin Gad m 1. *Computers in Biology and Medicine* (Under review)

8. **Dong, X., & Raghavan, V. (2022).** Recent advances of selected novel processing techniques on shrimp allergenicity: A review. *Trends in Food Science & Technology*, 124, 334-344.

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1. **Dong, X., & Raghavan, V.** Thermal Processing-Induced Alterations in Fish Allergenicity and Protein Structure: A Comprehensive Study. JIRU-MicroMeNu International Conference, Quebec City, Canada, October 2023 (Poster presentation)

2. **Dong, X., & Raghavan, V.** Effect of Thermal Processing Techniques on Allergenicity and Structural Changes in Fish. PEOPLE 2023 International Conference, Montreal, Canada, August 2023 (Poster presentation)

3. **Dong, X., & Raghavan, V.** Molecular dynamic simulations of temperature and pressure processing in Atlantic cod major allergen parvalbumin Gad m 1. CSBE/SCGAB AGM and Technical Conference 2022, Charlottetown, Canada, July 2022 (Oral presentation)

4. **Dong, X., & Raghavan, V.** Effects of Ultrasound and Microwave Processing on Physiochemical and Allergenic Properties of Shrimp. The Canadian Chemical Engineering Conference (CCEC) 2021, Canada, October 2021 (Oral presentation)

5. **Dong, X., & Raghavan, V.** Recent Advances of Novel Food Processing to Shrimp Physiochemical and Allergenic Properties. 6th Biological & Biomedical Engineering (BBME) Symposium, Montreal, Canada, May 2021 (Oral presentation)

6. **Dong, X., & Raghavan, V.** Mini review: worldwide food allergy prevalence and the characterizations of “big eight” allergenic foods. Canadian Society for Bioengineering (CSBE-SCGAB) Webinars, Canada, September 2020 (Oral presentation)

Chapter 1: Introduction

Seafood plays a crucial role in human health due to its naturally functional and nutrient-rich qualities. It is widely regarded as a significant source of protein. However, the cases of seafood allergy are increasing around the world every year, which has reported that seafood can trigger a series of allergenic reactions ranging from mild to life-threatening (Lee et al., 2013). This increase in seafood allergies, encompassing both fish and shellfish allergies triggered by allergenic proteins in these foods, underscores the need for effective management strategies.

Atlantic cod (*Gadus morhua*) is a commercially significant fish species harvested from the North Atlantic Ocean (Sodeland et al., 2022). This groundfish is among the most popular globally because of its significant nutritional benefits. Additionally, it serves as a crucial source of protein in human diets (Jafarpour et al., 2020). However, like other fish, cod contains parvalbumin, which is the predominant allergen in fish products (Fu et al., 2019). It poses a risk to individuals with seafood allergies. Addressing this challenge, effective food processing techniques offer potential pathways to alter allergenic proteins and reduce the allergenicity of fish while preserving its nutritional and sensory qualities.

Food processing techniques, broadly categorized into thermal, non-thermal, and combinatory approaches, exert significant influence on the composition and chemical properties of foods. Recent advancements in these techniques have been particularly focused on modulating the allergenic potential of fish products, while minimizing nutritional and sensory quality losses. The biochemical profile of allergenic proteins may undergo substantial modifications post-processing, accompanied by chemical transformations within the fish matrix. Critically, the specific type and parameters of the employed processing techniques can differentially impact fish allergenicity (Jiménez-Saiz et al., 2015). This research investigates a spectrum of thermal (oven baking, steaming, dry heating, boiling, and microwave), non-thermal (ultrasound and cold plasma), and hybrid method (thermal glycation) processing techniques on Atlantic cod. The objective is to identify optimal processing conditions that significantly alter or eliminate the major allergen, parvalbumin, while concurrently preserving the maximal nutritional integrity of the fish.

Furthermore, the application of molecular dynamics (MD) simulations provides visual insights into understanding the molecular changes in allergenic proteins during processing. Normally, food processing techniques can be optimized to favor the creation of higher-quality, safer, more functional, and more nutritionally valuable food products. Modeling food processes through the application of MD simulations, namely, the Groningen Machine for Chemical Simulations (GROMACS) software package, is helpful in achieving a better understanding of the structural changes occurring at the molecular level to the biomolecules present in food products during processing. MD simulations can be applied to define the optimal processing conditions required for a given food product to achieve a desired function or state (Smith et al., 2022). This research aims to explore the structural alterations in the allergen parvalbumin, seeking to identify processing conditions that effectively minimize allergenicity without compromising the nutritional integrity of Atlantic cod. This structured and contextual approach not only aims to enhance the safety and nutritional quality of seafood but also contributes to the broader field of food science by offering insights into the molecular mechanisms behind allergen modification.

Contextual transition

The previous chapter established the goals of the project. In the next chapter, an overview of recent advances in processing techniques (thermal, non-thermal, combined (hybrid) treatments) and main allergen detection methods for seafood products are provided. The review starts with the seafood consumption and classification, proceeding with the prevalence and symptoms of seafood allergy, followed by a description of biochemical characteristics of the major seafood allergens. As the topic is multi-disciplinary in scope, it is intended to provide information for further research essential for food security and safety.

Chapter 2: Literature review

Manuscript I – A comprehensive overview on emerging processing techniques and detection methods of seafood allergens

Xin Dong *, Vijaya Raghavan

Department of Bioresource Engineering, Faculty of Agricultural and Environmental Sciences, McGill University, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

Corresponding Author: Xin Dong

Mailing address: Department of Bioresource Engineering, Faculty of Agricultural and Environmental Sciences, McGill University, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

2.1 Abstract

Seafood is rich in nutrients and plays a significant role in human health. However, seafood allergy is a worldwide health issue by inducing adverse reactions ranging from mild to life-threatening in seafood-allergic individuals. Seafood consists of fish and shellfish, with the major allergens such as parvalbumin and tropomyosin, respectively. In the food industry, effective processing techniques are applied to seafood allergens to lower the allergenicity of seafood products. Also, sensitive and rapid allergen detection methods are developed to identify and assess allergenic ingredients at varying times. This review paper provides an overview of recent advances in processing techniques (thermal, non-thermal, combined (hybrid) treatments) and main allergen detection methods for seafood products. The article starts with the seafood consumption and classification, proceeding with the prevalence and symptoms of seafood allergy, followed by a description of biochemical characteristics of the major seafood allergens. As the topic is multi-disciplinary in scope, it is intended to provide information for further research essential for food security and safety.

2.2 Introduction

Seafood is important in human health as it is naturally functional and highly nutritious food. Seafood is generally considered as an invaluable protein resource. It was reported that seafood proteins account for at least 20% of animal protein intake in over 3.1 billion people (FAO, 2016). Seafood compasses numerous essential micronutrients such as polyunsaturated fatty acids (PUFA), vitamins (A, B, and D), and trace minerals (calcium, iron, zinc, and selenium) (Dasanayaka et al., 2020). Furthermore, seafood contains abundant omega-3 fatty acids, which can decrease the risk for heart disease and ischemic stroke by regular intake of 1-2 times per week (Rimm et al., 2018). With great popularity among consumers, seafood products keep up with an increasing trend in international trade across various countries (Ruethers et al., 2018). In 2014, total seafood production was estimated at 167 million tonnes worldwide; per capita annual consumption of seafood products was more than two times in 2014 of over 20 kg compared with that in 1960 of at most 10 kg (FAO, 2016). Although seafood plays a significant role in the human diet, it is one of the key allergenic foods which has the capacity to trigger anaphylaxis among seafood-allergic individuals.

Patients suffering from seafood allergies may fail to identify the offending seafood species due to the diversity of seafood products and their different common names (Davis et al., 2020). Besides, some irregular phenomenon in marketing including fraudulent substitution and mislabeling have been noticed in many seafood products, especially in fish (Willette et al., 2017). Thus, it is important for patients to distinguish and identify their specific allergenic seafood for better avoidance (Davis et al., 2020). As shown in Figure 2.1, edible seafood can be generally classified into fish (vertebrates) and shellfish (invertebrates). Subsequently, edible fish species are mainly categorized as bony fish and cartilaginous fish, which are in the phylum of Chordata. Within fish allergy, bony fish (cod, salmon, and catfish) allergy has been reported more than cartilaginous fish (ray, shark, and ghost shark) allergy due to the higher allergenicity of bony fish (Kalic et al., 2019). Notably, the patient allergic to one fish species may have an almost 50% possibility to be cross-reactive to another fish species (Kobayashi et al., 2016). Edible shellfish consists of crustacean and mollusk, belonging to the phyla of Arthropoda and

Mollusca (Ruethers et al., 2018). Crustacean constitutes a diverse set of species such as prawns, shrimps, crabs, and lobsters, which are classified as arthropods together with dust mites, spiders, and insects like cockroaches (Kamath et al., 2017). Mollusk also contains a large group of species and can be broadly subdivided into three classes (bivalve, cephalopod, and gastropod). Among them, several main species are commonly consumed by humans, comprising squid (calamari), abalone, mussels, oysters, scallops, clams, and cockles. Crustacean and mollusk clinically implicate cross-reactivity in many species due to their panallergens (Ruethers et al., 2018).

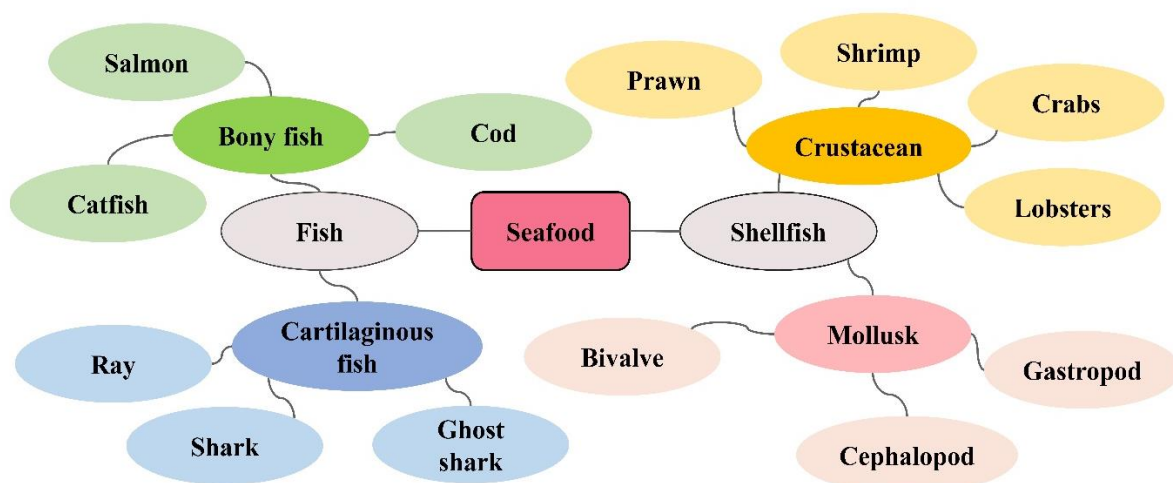


Figure 2.1. Classification of common edible seafood.

In this review, we will explain the global prevalence, immunological mechanism, and symptoms of seafood allergy. Through careful examination of the main allergens in seafood, current methods of seafood processing techniques (thermal and non-thermal) applied to reduce seafood allergenicity and seafood allergenicity detection methods will be comprehensively explored and discussed. Furthermore, clinical diagnosis and medical treatments of seafood allergy will also be summarized for the best management of seafood-allergic patients.

2.3 Seafood allergy prevalence and symptoms

Seafood allergy is comprised of fish allergy and shellfish allergy, which belongs to two of the “big eight” allergenic foods, fish and crustacean (Dong et al., 2021a). Fish allergy affects approximately 7% of pediatric populations worldwide, and crustacean ranks the third in order of the “big eight” allergenic food only after peanut and tree nut by affecting over 2% of the population in the world (Buyuktiryaki et al., 2021; Laly & Sankar, 2020). Moonesinghe et al. (2016) reported the global prevalence of fish allergy and shellfish allergy ranging from 0% to 7% and 0% to 10.3%, respectively. Studies suggest that fish allergy prevalence is similar worldwide, whereas shellfish allergy prevalence is significantly correlated to geography with higher prevalence in several regions, such as Australia, Asia, the United States, and Europe (Moonesinghe et al., 2016). Moreover, shellfish is identified as the major causative leading to anaphylaxis in Southeast Asia, Hongkong, and Taiwan, probably due to the high consumption of shellfish in the local diet of the population (Laly & Sankar, 2020; Moonesinghe et al., 2016).

Table 2.1. Common clinical manifestations of seafood allergy

Organ involved	Severity	Typical symptoms
Skin	Mild	Mild rashes, hives (urticaria), swelling (angioedema), palate itching, generalized pruritus
Gastrointestinal tract	Mild	Vomiting, nausea, abdominal cramping, diarrhea
Respiratory tract	Mild	Laryngospasm, wheezing, upper airway obstruction, asthma (might severe)
Cardiovascular system	Mild	Hypotension
Ocular	Mild	Conjunctivitis
Neurological system	Severe	Loss of consciousness
Multi-organ (probably)	Life-threatening	Anaphylaxis

Fish allergy and shellfish allergy are both thought to be persistent, although their natural history is still unknown. Fish allergy generally develops early in life, whereas shellfish allergy develops later, from adolescence onwards (Ebisawa et al., 2015). It has been concluded that both fish allergy and shellfish allergy are usually induced through 3 channels, including food intake, direct skin contact, and breathing of the odors (Laly & Sankar, 2020). In most cases, hyperallergic consumers suffer from seafood allergy by ingesting specific allergic seafood; the allergenic ingredients may trigger allergic responses from mild to life-threatening by entering the intestinal tract and contact with the mucosal immune system, which is similar to other food allergies (Dong et al., 2021a; Palomares, 2013). The clinical manifestations of seafood allergy may vary within an individual and between individuals (Khora, 2020) (Table 2.1). To date, the best way to avoid suffering from food allergies is to avoid the intake of the potential allergens for seafood-allergic patients (Dong et al., 2021a).

2.4 Seafood main allergens

So far, there are twelve allergens in different fish species which have been identified by the WHO/IUIS (Table 2.2). Parvalbumin and tropomyosin are the predominant allergens in fish and shellfish, respectively (Fu et al., 2019). They are highly heat-stable and biochemical-stable allergens, which may in part explain the persistence of seafood allergy (Ebisawa et al., 2015). The parvalbumin family comprises two isoform lineages, parvalbumin α and parvalbumin β , in which parvalbumin β is recognized as the major fin fish allergen. Parvalbumins are calcium-binding muscle proteins with low molecular weight ranging from 9 to 14 kDa (X. Zhang et al., 2021). They are highly thermostable and abundantly exist in fast twitching white muscle entail for calcium signaling. The Ca^{2+} coordinating amino acid residues are known for their contribution to the conformational IgE epitopes. The first identified fish parvalbumin is a Baltic cod allergen (Gad c 1) in the early 1970s, followed by other parvalbumins widely existing in commonly consumed fish species, such as cod, carp, and herring (Elsayed & Aas, 1971; Kuehn et al., 2013; Lim et al., 2008). The homology of amino acids among different fish species is very high, reaching 60% to 90%, such as in cod, carp, hake, chub, and Atlantic salmon (Bugajska-Schretter et al., 1998). Due to the cross-reactivity, people allergic to one fish species

have approximately a 50% chance to react adversely to another fish species (JF, 2003). Interestingly, among tilapia-allergic individuals, tropomyosin, a major shellfish allergen, was also characterized as a fish allergen (Liu et al., 2013). It has been reported that the tilapia allergen demonstrates high homology to human tropomyosin and potentially triggers inflammatory bowel diseases (Kuehn et al., 2014). As mentioned before, tropomyosin is the major allergen in shellfish and responsible for ingestion-related allergic reactions (Prester, 2016). Tropomyosin is a muscle protein with a molecular weight of 34–38 kDa. It belongs to the family of actin filament-binding proteins with distinguished isoforms to express in muscle and non-muscle tissues (Khora, 2016). Table 2.2 summarizes various tropomyosin identified in different shellfish species. Besides, tropomyosin is known as a pan-allergen with high cross-reactivity in related species, such as crustaceans, insects, and various classes of mollusks (Wild & Lehrer, 2005). The molecular homology of tropomyosin existing in shellfish species is very high, especially within the same species. It was reported that the homology between various crustacean species is approximate 98%, and the homology between molluscan is up to 68%–100% (Lee et al., 2012). It is estimated that 75% of patients allergic to one shellfish have risks to be allergic to another shellfish. Thus, all shellfish-allergic patients are suggested to avoid the intake of all shellfish products due to the highly cross-reactive allergens present in shellfish species. In addition, other main seafood allergens are also identified, such as beta-enolase, collagen, and gelatine in fish, and arginine kinase, myosin light chain, and sarcoplasmic calcium-binding protein in shellfish (Table 2.2) (Ayuso et al., 2008; Hamada et al., 2001).

Notably, up to 6% of seafood-allergic patients have allergic reactions to both shellfish and fish allergens, and hence it is generally unnecessary for the avoidance of both fish and shellfish due to no cross-reactivity between parvalbumin and tropomyosin (Khan et al., 2011; Lopata & Lehrer, 2009).

Table 2.2. Main allergens identified in fish and shellfish (data from www.allergen.org)

Category	Biomedical name	Molecular weight/ kDa	Allergen	Main species
Fish	Beta parvalbumin	11-12	Lep w 1	Megrim, whiff, turbot fish
			Pan h 1	Striped catfish
			Seb m 1	Ocean perch, redfish,
			Xip g 1	snapper
			Clu h 1	Swordfish
			Cyp c 1	Atlantic herring
			Gad c 1	Common carp
			Sal s 1	Baltic cod
			Thu a 1	Atlantic salmon
	Beta enolase	47-50		Yellowfin tuna
			Pan h 2	Striped catfish
			Cyp c 2	Common carp
			Gad m 2	Atlantic cod
			Sal s 2	Atlantic salmon
	Aldolase A	40	Thu a 2	Yellowfin tuna
			Pan h 3	Striped catfish
			Gad m 3	Atlantic cod
	Tropomyosin	35-36	Thu a 3	Yellowfin tuna
			Lep s 1	Silverfish
			Pro c 1	Red swamp crayfish
	Collagen alpha	130-140	Pan h 4	Striped catfish
			Lat c 6	Barramundi
			Sal s 6	Atlantic salmon
	Creatine kinase	43	Pan h 7	Striped catfish
			Sal s 7	Atlantic salmon
	Triosephosphate isomerase	25-28	Arc s 8	Crayfish
			Pan h 8	Striped catfish
			Pro c 8	Red swamp crayfish
	Pyruvate kinase PKM-like	65	Pan h 9	Striped catfish
	Beta-prime-component of vitellogenin	18	Onc k 5	Chum salmon
	L-lactate dehydrogenase	34	Pan h 10	Striped catfish
	Glucose 6-phosphate isomerase	60	Pan h 11	Striped catfish
	Glyceraldehyde-3-phosphate dehydrogenase	36	Pan h 13	Striped catfish
Shellfish	Tropomyosin	34-38	Pen i 1	Shrimp

			Cra c 1	North Sea shrimp
			Cra g 1	Pacific oyster
			Exo m 1	White legged freshwater shrimp
			Mel l 1	King prawn
			Scy p 1	Mud crab
Arginine kinase		40-45	Lit v 2	White shrimp
			Pen m 2	Black tiger shrimp
			Scy p 2	Mud crab
Myosin light chain		17.5-23	Cra c 5	North Sea shrimp
			Hom a 3	American lobster
			Lit v 3	White shrimp
			Pen m 3	Black tiger shrimp
			Scy p 3	Mud crab
Sarcoplasmic	calcium-	20-25	Cra c 4	North Sea shrimp
binding protein			Cra a 4	Pacific oyster
			Lit v 4	White shrimp
			Scy p 4	Mud crab

2.5 Effects of processing techniques on seafood allergenicity

In recent years, different processing (thermal and non-thermal) techniques are applied to seafood products to reduce their allergenicity with the least loss in nutritional value and sensory quality. After processing, biochemical characteristics of allergenic proteins may be altered and chemical reactions in the seafood matrix components occur. Importantly, the type and conditions of the processing techniques applied can variably affect seafood allergenicity (Jiménez-Saiz et al., 2015). Table 2.3 summarizes recent advances on the impacts of processing techniques (thermal/ non-thermal/ combined (hybrid) treatments) on seafood allergenicity. For various fish and shellfish products, processing might cause an increase, decrease, or no effect in their allergenicity.

Table 2.3. Effect of various processing techniques on seafood allergenicity

Processing technique		Seafood category	Sample	Treatment	Detection method		Result	References
Thermal processing	Wet-heat	Fish	37 southern hemisphere fish species	100 °C for 45 min	Western blot		Decreased the IgG binding in parvalbumins	(J. Liang et al., 2021)
	Heat		Muscle extracts from Pacific mackerel parvalbumin	20–140 °C	ELISA	using human sera	Reduced the IgE reactivity of the parvalbumin	(Kubota et al., 2016)
	Heat		Parvalbumin in diverse bony and cartilaginous fish	95 °C for 15 min	Molecular analysis	and Western blot	Reduced antibody reactivity in bony fish; a complete reduction in cartilaginous fish	(Saptarshi et al., 2014)
	Heat		purified cod parvalbumin	25-105 °C	Indirect competitive ELISA	non-	No alterations	(Somkuti et al., 2012b)
	Heat	Shellfish	Raw kuruma prawns (<i>Marsupenaeus japonicus</i>)	20–80 °C	SDS–PAGE	and Western blotting	No changes	(Usui et al., 2013)
	Boil		Fresh raw shrimp	100 °C for 15 min	SDS-PAGE		Increased IgE-binding activity and immunoreactivity	(Arámburo-Galvez et al., 2018; Peram et al., 2013)
	Steam		Shrimp (<i>Penaeus monodon</i>)	3 min	SDS-PAGE		An enhanced intensity of tropomyosin band	(Lasekan & Nayak, 2016)
	Bake		Shrimp (<i>Penaeus monodon</i>)	200 °C for 4 min	SDS-PAGE		an enhanced intensity of tropomyosin band	(Lasekan & Nayak, 2016)
	Fry		Shrimp (<i>Penaeus monodon</i>)	>200 °C for 1 min	SDS-PAGE		an enhanced intensity of tropomyosin band	(Lasekan & Nayak, 2016)
	Microwave roast		Shrimp (<i>Penaeus monodon</i>)	at power level 3 for 2 min	SDS-PAGE		an enhanced intensity of tropomyosin band	(Lasekan & Nayak, 2016)
	Grill		Shrimp (<i>Penaeus monodon</i>)	>250 °C for 7 min	SDS-PAGE		an enhanced intensity of tropomyosin band	(Lasekan & Nayak, 2016)
	Boil		Shrimp (<i>Penaeus monodon</i>)	5 min	SDS-PAGE		an enhanced intensity of tropomyosin band	(Lasekan & Nayak, 2016)
	Boil		Crab (<i>Scylla paramamosain</i>)	100 °C for 15 min	SDS-PAGE		no obvious distinction in immunoreactivity	(Liu et al., 2018)
	Steam		Crab (<i>Scylla paramamosain</i>)	100 °C for 20 min	SDS-PAGE		no obvious distinction in immunoreactivity	(Liu et al., 2018)
	Microwave		shrimp (<i>Litopenaeus vannamei</i>)	2.45 GHz, 1000 W, 75–125 °C for 5–15 min	Sandwich ELISA and SDS-PAGE		A maximum reduction of 75%	(Dong et al., 2021b)
Non-thermal processing	High hydrostatic pressure	Fish	Cultured large yellow croakers (<i>Larimichthys crocea</i>)	300-600 MPa for 10 min	SDS-PAGE		Reduced the allergenicity	(H. Zhang et al., 2020)
	Drying		Atlantic cod		Indirect competitive ELISA		Increased the IgE binding activity	(Sletten et al., 2010)
	Marinade		Moroccan fish species in Fez region (sardine, common pandora, and shrimp)	marinade with olive oil, lemon juice, salt, pepper and garlic for a day at 4 °C	ELISA		Reduced Immunoreactivity	(Mejrhith et al., 2018).
	Fermentation	Shellfish	Moroccan fish species in Fez region (sardine, common pandora, and shrimp)	Ferment crushed shrimp mixed with salt for several weeks	ELISA		Immunoreactivity all reduced; maximum reduction in IgG binding were marked in fermented sardine of 64.5%, and in fermented shrimp of 69.2%	(Mejrhith et al., 2018).
	High pressure		Tropomyosin in crab (<i>Scylla paramamosain</i>)	0.08 MPa for 15 min	Western blotting, SDS-PAGE and inhibition ELISA		Reduced the immunoreactivity of tropomyosin	(Liu et al., 2018)
	Extraction buffers		Pacific oyster (<i>Crassostrea gigas</i>)	with PH value of 3.0-10.3	SDS-PAGE		more IgE-reactive bands on immunoblotting in high pH buffers; less IgE-reactivities in low pH buffers	(Nugraha et al., 2021)

	Enzymatic hydrolysis		Tropomyosin in shrimp extracts	Pepsin and pancreatin	Western blot	Tropomyosin remains stable and immunoreactive	(Toomer et al., 2015)
	Ultrasound		shrimp (<i>Litopenaeus vannamei</i>)	5-20 min at room temperature	Sandwich ELISA and SDS-PAGE	A maximum reduction of 76%	(Dong et al., 2020b)
	Electron beam irradiation		Shrimp (<i>Solenocera melanthero</i>)	Doses of 0, 1, 3, 5, 7, 9 kGy, rate of 1 kGy/s	Indirect ELISA analysis and Western blot	Decrease the IgG binding capacity and immunoreactivity of tropomyosin	(Guan et al., 2018)
Combined processing	Thermal-glycation	Fish	Purified Alaska pollock Parvalbumin	Glycation with glucose, fructose, ribose, and galactose at 60 °C for 1 h	Indirect competitive ELISA	Enhanced IgE/IgG binding capacities in the parvalbumins glycated with glucose and fructose; while a loss of IgE/IgG binding capacities in the parvalbumins glycated with ribose, lactose, and galactose	(M. Zhang et al., 2021)
	Thermal-glycation		Recombinant silver carp parvalbumin	Glucose at 60 °C for 72 h	Dot blot	Reduced IgE binding	(Zhao et al., 2017)
	Thermal-glycation		Fish protein hydrolysates	Glycation with ribose at 121 °C for 30-90 min	Histamine release	Reduced the allergenicity	(Yang et al., 2015)
	Hot-smoked		Antigenic proteins in fish samples (carp, Atlantic herring, and Sockeye salmon)	70–80 °C for 16 min	Indirect non-competitive enzyme-linked immunosorbent assay and Dot blot	Slightly decreased immunoreactivity	(Zhu & Hsieh, 2021)
	High-temperature pressure	Shellfish	Edible portions in fresh raw shrimp	0.08 MPa, 115 °C, 15 min	Western blotting	Reduced the immunoreactivity	(Liu et al., 2019)
	High-temperature pressure combined with glycation		Shrimp (<i>Litopenaeus vannamei</i>)	0.08 MPa at 110-121°C for 6 min; glycation with galactose	SDS-APGE and Western blotting	A significant decline in the IgG/IgE-binding activity	(Liu, Huan, et al., 2021)
	High pressure steaming		Shrimp (<i>Penaeus monodon</i>)	0.14 MPa at 121 °C for 20 min	SDS-PAGE	lower IgE binding capacity	(Lasekan & Nayak, 2016).
	Pressure combined with soaking		Shrimp (<i>Metapenaeus dobsoni</i>)	Pressure for 5-20 min; soaking in salt, baking soda, papain and acetic acid)	SDS-PAGE and immunoblotting	significant reduction of IgE activity and a absent tropomyosin band in shrimp extracts; significant increase in IgE activity and retained tropomyosin band in peeled shrimp	(S J et al., 2021)
	High-temperature pressure		Crab (<i>Scylla paramamosain</i>)	0.08 MPa, 115 °C, 15 min	Basophil activation test	Decreased the allergenicity	(Liu, Han, et al., 2021)
	Thermal-glycation		Tropomyosin in Scallop (<i>Chlamys nobilis</i>)	Glycation with xylose at 30–100 °C	Dot blotting, western blotting, Inhibition ELISA	Reduced IgE binding activity and immunoreactivity	(Bai et al., 2021)

2.5.1 Thermal treatment

In fish products, wet-heat treatment (100 °C for 45 min) decreased the IgG binding in parvalbumins within 37 kinds of southern hemisphere fish species from Australia by a quantitative detection, Western blot (J. Liang et al., 2021). Moreover, heating (20–140 °C) muscle extracts from Pacific mackerel parvalbumin caused a reduction in the IgE reactivity of the parvalbumin determined by ELISA using human sera, with a complete decline of IgE reactivity observed at 140 °C heating (Kubota et al., 2016). In addition, heat processing (95 °C for 15 min) was applied to parvalbumin in a diversity of bony and cartilaginous fish from the Asia-Pacific region; the results showed that such thermal treatment caused a decline in the antibody reactivity to multimeric parvalbumins for most bony fish, and a complete reduction of reactivity for cartilaginous fish measured by molecular analysis and Western blot (Saptarshi et al., 2014). However, the allergenicity of purified cod parvalbumin treated by heat (25-105 °C) exhibited no alterations by utilizing an indirect non-competitive ELISA test with allergic-patient sera (Somkuti et al., 2012b).

In shellfish products, an enhanced intensity of tropomyosin band in shrimp (*Penaeus monodon*) was observed during SDS–PAGE analysis of extracts by heat treatments, including steaming (3 min), baking (200 °C for 4 min), frying (>200 °C for 1 min), microwave roasting (at power level 3 for 2 min), grilling (>250 °C for 7 min) and boiling (5 min) (Lasekan & Nayak, 2016). Besides, fresh raw shrimp after a thermal treatment (boiling at 100 °C for 15 min) showed higher IgE-binding activity and immunoreactivity by the results from SDS-PAGE. The increase in allergenicity is probably due to the molecular unfolding in allergens leading to the exposure of their conformational epitopes.

Oppositely, high-intensity microwave treatment (75–125 °C for 5–15 min) could significantly reduce the allergenicity of shrimp (*Litopenaeus vannamei*) by 75% determined by a sandwich ELISA test (Dong et al., 2021b). This is because higher temperatures might reduce allergenicity because irreversible aggregation with covalent and hydrophobic interactions may occur after an extremely high-intensity thermal processing (Arámburo-Galvez et al., 2018; Peram et al., 2013). Whereas in another study, crab (*Scylla paramamosain*) tropomyosin treated by boiling (100 °C for 15 min) and steaming (100 °C for 20 min) demonstrated no obvious distinction in immunoreactivity measured by SDS-PAGE (Liu et al., 2018). Similarly, no changes were observed in the antigenicity of purified tropomyosin (Pen j 1) from raw kuruma prawns (*Marsupenaeus*

japonicus) after heat treatment (20–80 °C) analyzed by SDS–PAGE and Western blotting (Usui et al., 2013).

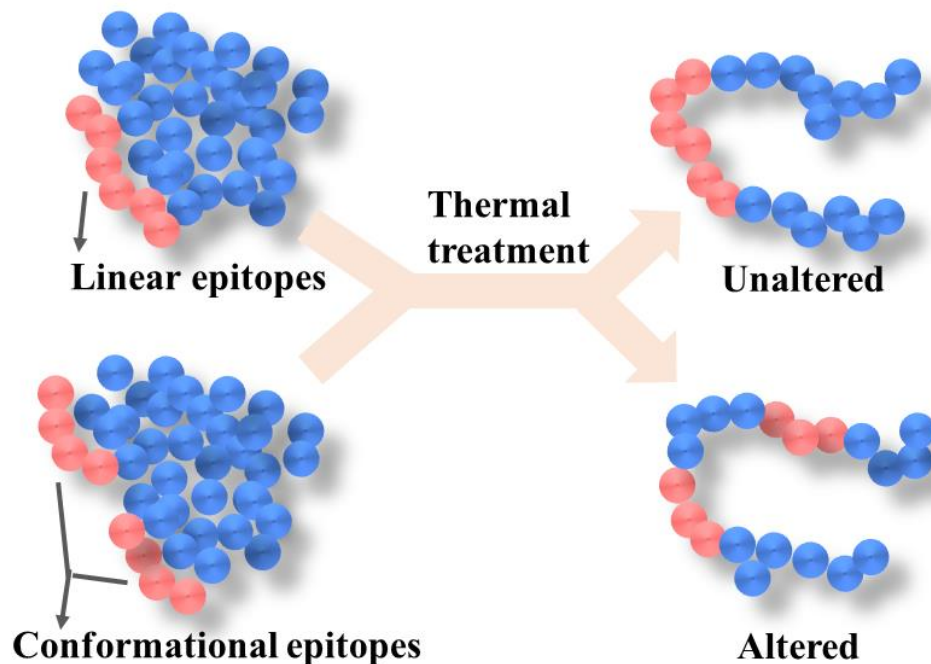


Figure 2.2. Effect of thermal treatment to epitopes in allergenic protein

As discussed above, thermal treatment is commonly used to process seafood products to both affect seafood allergenicity by conformational changes in allergens and influence the interactions in seafood ingredients. The reasons can be that allergenic protein molecules are unfolded causing a loss in their secondary and tertiary structures, which further leads to covalent and non-covalent interactions between intra and/or inter-molecules. Conformational epitopes in allergenic protein can be expressed or destroyed and thereby affecting allergenicity, whereas the linear epitopes remain unaltered (Figure 2.2) (Rahaman et al., 2016). Despite the wide application of thermal processing, non-thermal methods are being considered for reducing seafood allergenicity due to their better performance in preserving the original characteristics of seafood, such as maintaining the organoleptic properties, color, and nutrient contents (Shriver & Yang, 2011).

2.5.2 Non-thermal treatment

For fish products, high hydrostatic pressure (300- 600 MPa for 10 min) treatment could effectively reduce the allergenicity of cultured large yellow croakers (*Larimichthys crocea*) by altering the stability of the secondary and tertiary structure of parvalbumin (H. Zhang et al., 2020). Conversely, drying increased the IgE binding activity in Atlantic cod tested by an indirect competitive ELISA (Sletten et al., 2010). In another study, three commonly consumed Moroccan fish species in the Fez region (sardine, common pandora, and shrimp) were processed by canning, marinade, and fermentation; ELISA results showed that all the processing methods resulted in a loss of the immunoreactivity of human IgE to fish species, with a better performance after treatments by marinade and fermentation. The maximum reduction in IgG binding was marked in fermented sardine at 64.5% and fermented shrimp at 69.2% (Mejrhith et al., 2018).

For shellfish products, the allergenicity of shrimp (*Litopenaeus vannamei*) treated by ultrasound (5-20 min at room temperature) showed a significant decline of 76% in tropomyosin measured by a sandwich ELISA (Dong et al., 2020b). Electron beam irradiation (doses of 0, 1, 3, 5, 7, 9 kGy, rate of 1 kGy/s) could also decrease the IgG binding capacity and immunoreactivity of tropomyosin from *Solenocera melanthero* due to the changes in the secondary structure of shrimp tropomyosin (Guan et al., 2018). Similarly, the immunoreactivity of tropomyosin in crab (*Scylla paramamosain*) was obviously reduced after high-pressure treatment at 0.08 MPa for 15 min detected by Western blotting, inhibition ELISA, and SDS-PAGE (Liu et al., 2018).

Alternatively, eight different extraction buffers (with a PH value of 3.0-10.3) were applied to protein in Pacific oyster (*Crassostrea gigas*) using serum from five shellfish-allergic patients; the results revealed more IgE-reactive bands on immunoblotting in high pH buffers, and fewer IgE-reactivities in low pH buffers (Nugraha et al., 2021). In addition, tropomyosin in shrimp extracts remained stable and immunoreactive after *in-vitro* gastric digestion (pepsin and pancreatin) determined by Western blot, whereas epitope binding capacity predominately decreased after pancreatin digestion possibly due to the denatured and destroyed secondary and/or tertiary conformations (Toomer et al., 2015).

It has been assessed that non-thermal processing can effectively change the allergenicity or immunoreactivity of seafood proteins because irreversible alterations in secondary, tertiary, and quaternary structures of allergenic proteins occurred by influencing the covalent bonds. Such

alterations are mostly affected by treatment conditions, such as treatment power, frequency/rate, concentration and time duration, etc. Other than lowering seafood allergenicity, non-thermal processing can significantly inactivate microorganisms without heating to preserve sensory quality and nutritive value in seafood, and further assuring seafood safety and extending the shelf life (Olatunde et al., 2021). Therefore, non-thermal processing is regarded as a well-balanced technique between safety and minimal processing, between acceptable quality and economic constraints, and between novel processing resources and traditional processing techniques (Zhang et al., 2011).

2.5.3 Combined (hybrid) treatment

In fish products, purified Alaska pollock Parvalbumin was glycated with glucose, fructose, ribose, lactose, and galactose at 60 °C for 1 h; the enhanced IgE/ IgG binding capacities were observed in the parvalbumins glycated with glucose and fructose, while there was a loss of IgE/ IgG binding capacities in the parvalbumins glycated with ribose, lactose, and galactose analyzed by the indirect competitive ELISA. The converse results were probably because different reducing sugars variedly exhibited various glycation effects in modifying protein conformations in causing the differences in specific recognition of antigen and antibody (M. Zhang et al., 2021). Similarly, thermal-glycation treatment (glucose at 60 °C for 72 h) also led to a reduction of IgE binding in recombinant silver carp parvalbumin detected by Dot blot (Zhao et al., 2017). Also, the allergenicity in fish protein hydrolysates analyzed through a response surface methodology reduced after the treatment of glycation with ribose at 121 °C for 30-90 min (Yang et al., 2015). Interestingly, antigenic proteins in fish samples (carp, Atlantic herring, and Sockeye salmon) were retained even after single processing conditions of salting, smoking, and heating (boiling 0–8 min) treatment tested by both indirect ELISA and dot blot. However, a slight decrease in immunoreactivity was observed when hot smoked treatment was applied at 70–80 °C for an additional 8 min, revealing that repeated heating might degrade or aggregate the antigens (Zhu & Hsieh, 2021).

In shellfish products, high-temperature pressure (0.08 MPa, 115 °C, 15 min) could decrease the allergenicity in crab (*Scylla paramamosain*) tropomyosin by Basophil activation test due to the alteration in the protein structure such as denaturation (Liu, Han, et al., 2021). Tropomyosin from autoclaved shrimp (*Penaeus monodon*) had a lower IgE binding after high pressure steaming (0.14 MPa at 121 °C for 20 min) analyzed by SDS-PAGE (Lasekan & Nayak, 2016). Also, a high-

temperature pressure treatment (0.08 MPa, 115 °C, 15 min) in autoclaving can reduce the immunoreactivity of the edible portions in fresh raw shrimp by using Western blotting measurement. Such results were induced by the macrostructural and microstructural alterations in shrimp samples, including interactions between the multi-components and allergens in the food matrix (Liu et al., 2019).

Shrimp (*Litopenaeus vannamei*) processed by Maillard reaction (glycation with galactose) with high temperature–pressure (0.08 MPa at 110-121°C for 6 min) showed a significant decline in the IgG/IgE-binding activity due to the modified macrostructure of lysine, arginine, and cysteine residues in antigen epitopes (Liu, Huan, et al., 2021). A thermal-glycation method, Maillard-reacted Tropomyosin (TM epitopes with lysine and arginine), could significantly decrease the IgE binding activity and further result in lower immunoreactivity in Scallop (*Chlamys nobilis*) by destroying and masking saccharide residues on the tropomyosin surface (Bai et al., 2021). In another study, shrimp (*Metapenaeus dobsoni*) was pretreated into both shrimp extracts and peeled shrimps, and then treated by pressure (5-20 min) combined with soaking treatments (in salt, baking soda, papain, and acetic acid). The results of shrimp extracts indicated that there was a significant reduction of IgE activity and an absence of tropomyosin band in the immunoblot using pooled sera of the shrimp-allergic patient, whereas a converse result in peeled shrimp with significantly increasing IgE activity and retained tropomyosin band due to the retention of allergenicity in peeled shrimps. Such different results might be explained by increasing rigidity in shrimp tissue induced by the gestalt-binding interactions between tropomyosin and actin (Holmes & Lehman, 2008).

Physical food processing techniques are powerful in eliminating seafood allergens, but most combined (hybrid) treatments (combining thermal and non-thermal processing) show a better performance than single treatments (Fu et al., 2019). Therefore, based on current study findings in eliminating seafood allergens, there is a greater likelihood of making seafood accessible for hyperallergic consumers.

2.6 Detection methods of seafood allergens

As discussed above, the allergenicity of the same protein might exhibit differences in different matrices, which is probably affected by a number of factors such as matrix, antibody, and detection approaches (Griesmeier et al., 2010). To assess seafood allergen, novel detection measurements

with high sensitivity and efficiency are necessary (Fu et al., 2019). To date, detection and quantification methods applied to food allergen sources generally include enzyme-linked immune sorbent assay (ELISA), polymerase chain reaction (PCR), lateral flow device (LFD)/dipstick, lateral flow immunoassay (LFIA), liquid chromatography-mass spectrometry (LC-MS), and biosensors. Nevertheless, ELISA, LFD, and PCR are the only methods commercially available for detecting and quantifying allergens in fish (Ruethers et al., 2020).

2.6.1 Enzyme-linked immune sorbent assay (ELISA)

ELISA is the most common method to quantify allergenic protein in food products. It is based on monoclonal or polyclonal antibody recognition of one or more allergens in food products, with a detection limit of ~0.1–5 mg/kg and as parts per million (p.p.m.) (Monaci & Visconti, 2010). As shown in Figure 2.3A, four types of ELISA assays are usually used for allergen detection, including direct, indirect, sandwich, and competitive ELISA (Hidayat & Wulandari, 2021). In experimental protocol, ELISA analysis consists of a series of incubation and wash steps after protein extraction. It finally acquires sample concentration relying on a standard curve generated by a diluted allergenic protein standard (Karsonova et al., 2020). ELISA analysis has been widely used in the routine detection of food allergens, because it is mature, easy, and provides high sensitivity and specificity.

In a study focusing on epitope analysis, an indirect ELISA was used to characterize the IgE-binding and IgG₄-binding to a major fish allergen Lat c 1.01 (Sharp et al., 2021). Moreover, ELISA was also applied to investigate the IgE sensitization/cross-linking capacity of fish collagen among 101 fish-allergic individuals, which is a new finding for collagen in diagnostic tests and it helped further to improve patient safety (Kalic et al., 2020). In addition, the capacity of various ELISA tests might help demonstrate their usefulness through their varied results. Ruethers et al. (2020) reported that three commercial ELISA kits demonstrated a limited capacity and distinguished detection rates in the detection of both raw and heated extracts from bony fish (26–61%), canned bony fish (65–86%), and cartilaginous fish (0%). Besides, Dasanayaka et al. (2021) developed a newly sandwich ELISA method, which was based on goat IgG (capturing antibody) and rabbit IgG (detecting antibody) targeting soluble antigenic fish proteins (detection targets) to detect undeclared fish residues in foods and thereby to reduce the incidents of fish allergies. Recently, Zhao, Li, Li, et al. (2022) also developed a poly- and monoclonal antibody-based (PcAb and mAb)

sandwich ELISA method to detect crustacean tropomyosin in foods, and this novel method showed better matrix-tolerance and detectability in processed foods.

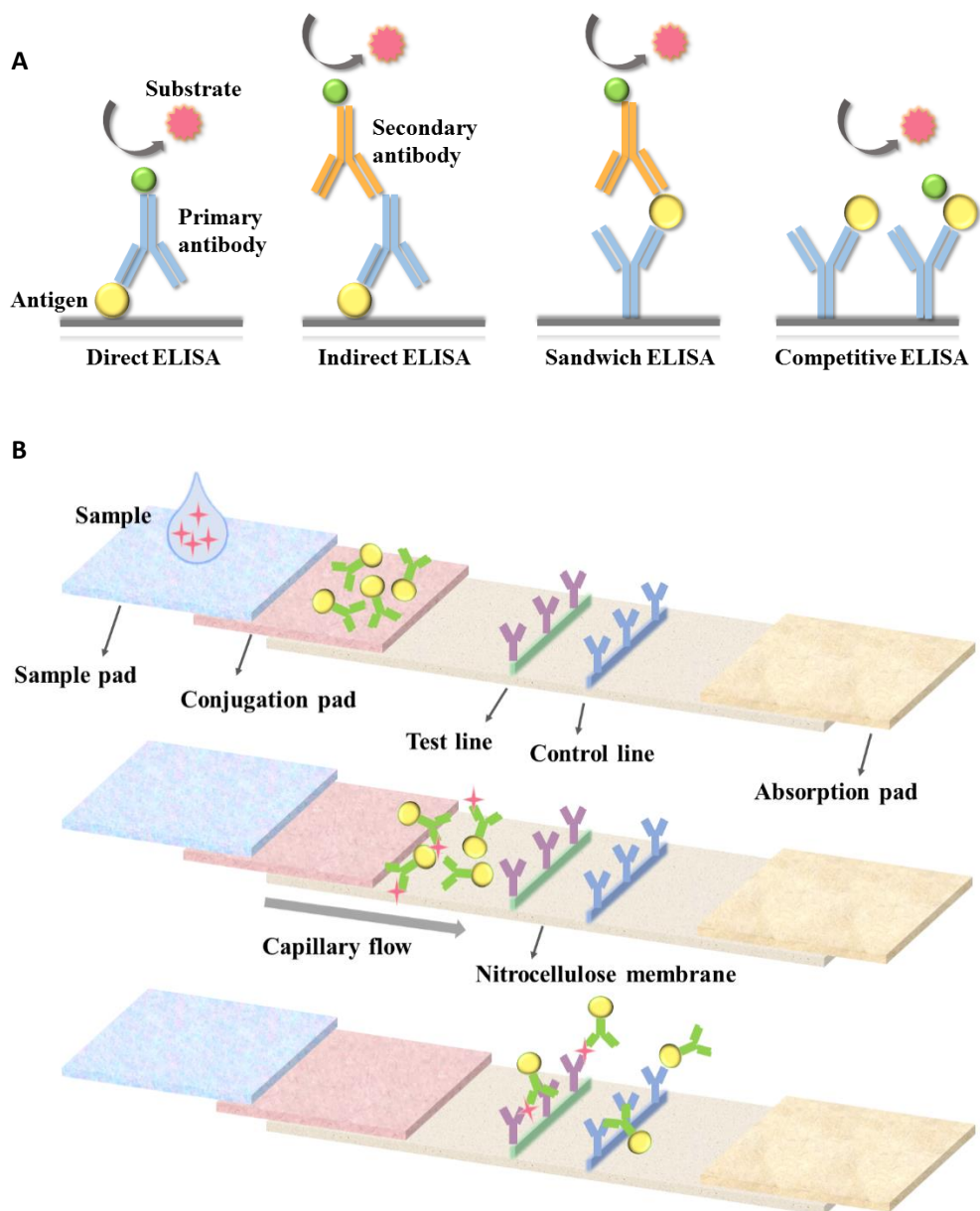


Figure 2.3. (A) Four types of ELISA assays used for allergen detection (B) Schematic diagram of lateral flow immunoassay.

As studies described, ELISA analysis, a protein-based assay, shows an overall sufficient sensitivity in the simultaneous quantification of seafood proteins. However, different processing methods, antibody composition, experimental operations, reagents, and standards may cause large

quantitative differences in allergenic proteins, and also false positive or false negative results (Asensio et al., 2008; Parker et al., 2015). Moreover, another inconvenience of the immunoassays is that protein solubility might be altered by processing techniques, which can affect the subsequent detection with protein-based approaches (Mattison et al., 2016). Instead, other detection methods, such as DNA-based assays, are necessary for seafood analytic tests since DNA molecules can maintain their integrity better than proteins (Kuehn et al., 2017).

2.6.2 Polymerase chain reaction (PCR)

PCR technology is a highly specific and automatic DNA-based methodology used to monitor allergenic ingredients during seafood processing. DNA methodologies are indirect to detect seafood allergens as they detect the DNA sequences from the allergenic food components rather than detecting the allergenic protein itself (Broeders et al., 2012). With high sensitivity, even small amounts of DNA copies can be detected using specific PCR. Besides, DNA has a more stable performance than proteins, especially in thermal treatments. (Sun et al., 2009).

For the detection of potentially allergenic proteins in seafood, various PCR assays have been described. A study verified that both commercial real-time PCR (DNA-based) and ELISA kits (immunochemical approach) can recognize shrimp allergens in kimchi, saeu-jeot, and saeu-aekjeot, however, with variable sensitivities. The variations are caused by the detection limits and different reactivity to shrimp allergens, including tropomyosin, and sarcoplasmic calcium-binding protein (Jeong & Kim, 2020). In addition, quantitative real-time PCR with a set of specific primer pair, Tropo-F, and Tropo-R, could be successful for the identification and quantitative analysis of the presence of tropomyosin in shrimp (Phan et al., 2020). Besides, Eischeid (2019) also used a real-time PCR method to detect cod and pollock in complex food matrices (cooking oil, clam chowder, and hushpuppy mix) and achieved low detection limits at 1–10 mg/kg. Similarly, Fernandes et al. (2018) proposed and compared two real-time PCR systems based on a TaqMan probe and the EvaGreen dye. They found that both systems are precise, but the probe system demonstrated higher sensitivity and dynamic range (0.0001–50%) than the EvaGreen (0.05–50%). Except using PCR assay alone, multiplex PCR assay combined with capillary electrophoresis was developed to detect tropomyosin genes in oyster, mussel, abalone, and clam, and the 18S rRNA gene in eukaryotes. The results showed that this assay is very useful and efficient in detecting tropomyosin allergens from mollusk species in seafood (Suh et al., 2020).

Until now, plenty of useful PCR assays have been established in related research. Advantages of DNA-based assays compared with protein-based methodologies include that the target DNA can be efficiently extracted from uncooked or cooked products almost without being affected by thermal processing. This is because DNA exposed to the cooking temperatures can remain fragmented but detectable in seafood products (Linacero et al., 2020). Although some food components may interfere with the PCR assay to reduce the amplification efficiency, PCR inhibitors can be removed during DNA extraction and purification procedures with the help of suitable DNA clean-up steps (López-Calleja et al., 2007). In recent years, PCR technology is becoming popular to be used in detecting food allergens, but with some limitations, such as unsuitable for the identification of targeting allergenic proteins with unidentified genes in food matrices (van Hengel, 2007). Thus, more efforts in the development of detection methods are needed with further refinement.

2.6.3 Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) is regarded as a sensitive multiplexed quantification method for food allergens. In experiments, extracted proteins are reduced, alkylated, and then enzymatically hydrolyzed into peptides. The peptide mixtures generated will be within a specific range for LC-MS analysis. Subsequently, LC-MS separates these peptides based on differences in their relative affinity to the stationary phase (column) and the mobile phase (solvent). Electrospray ionization is conducted by eluting and ionizing, and it is consequently through LC-MS (Croote & Quake, 2016).

Lv et al. (2020) established the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with high sensitivity and selectivity for simultaneously quantifying biomarkers and validated it in a cell model during allergy-related research. Based on liquid chromatography-tandem mass spectrometry and multiple reaction monitoring mass spectrometry (LC-MRM-MS/MS), a new approach was developed with simple, sensitive, and accurate advantages. This method utilized a thermal purification procedure followed by a completely optimized tryptic digestion. In this study, parvalbumin beta in flounder (*Paralichthys olivaceus*) has been successfully determined at a low level to the extent of 0.10 µg/g with satisfactory accuracy and precision (Sun et al., 2019). Similarly, Stella et al. (2020) established a method coupling liquid chromatography with high-resolution tandem mass spectrometry (LC-HRMS/MS) to select

marker peptides in allergens and simultaneously detect crustaceans. The method has been validated on fish contaminated at 10 µg/g for crustaceans (Stella et al., 2020). Jianhua Wang et al. (2021) also developed an assay based on high-performance liquid chromatography-tandem mass spectrometry (LC–HRMS) to detect crustacean TM qualitatively and quantitatively. They found that this approach demonstrated high accuracy and reproducibility (recovery range of 91%-109%, standard deviations <15%, limit of quantification of 1.6 mg/kg) in various food matrices (chicken sausages, beef balls, and egg-milk biscuits).

Furthermore, LC-MS has been developed for both online and offline quantification of seafood allergens based on target peptides, which is especially suitable for the determination of seafood allergens during seafood processing due to its indifference to conformational protein epitopes. However, further studies are in need on the application of LC-MS for seafood allergen detection, since the high cost of professional mass spectrometry equipment and advanced operating techniques may lead to some limitations for wide application in the food industry (Bereszczak & Brancia, 2009).

2.6.4 Lateral flow device (LFD) and lateral flow immunoassay (LFIA)

The LFD/dipstick is a qualitative or semi-quantitative tool to be implemented in food allergen analysis. It is relatively easy to operate, portable, and inexpensive. The method involves the application of test antigen/analyte and antibody with a membrane, such as nitrocellulose, nylon, or polyvinylidene difluoride (Sharma et al., 2017). LFIA serves as a semiquantitative method using visual signals to indicate allergens detected. The principle of LFIA operation is based on allergen interaction with antibodies, demonstrating the test results through antibody-coated colored particles (Figure 2.3B) (Xu et al., 2021).

Koizumi et al. (2014) developed and validated a sensitive and visual lateral flow assay for detecting shrimp allergenic protein. The detection limit to shrimp protein extract was 25 µg/L, equivalent to 1 µg/g protein in a food sample.; the test results could be obtained within 20 min without sophisticated procedures or expensive equipment. The concordance was up to 97% between LFD and validated ELISA for commercially processed foods (Koizumi et al., 2014). Zheng et al. (2012) developed a quantitative LFIA based on a superparamagnetic nanoparticle (SPMNP) probe for fish major allergen parvalbumin. Comparative test results indicated that the relative consistency was 93.1% between the LFIA and Western Blot assay in 29 food extract samples. Besides, the detection

time of the LFIA approach was within 20 min whereas the Western Blot assay commonly takes about 5 h (Zheng et al., 2012). Afterward, Y. Wang et al. (2019) designed quantum-dot-based LFIA to detect crustacean tropomyosin with a limitation of 0.5 µg/mL visually and 0.05 µg/mL by instrument. The LFIA has been verified as a specific and reproducible assay with consistent and comparable test results to the validated ELISA kit. (Y. Wang et al., 2019).

LFD and LFIA usually demonstrate advantages, including quick response (within several minutes), high sensitivity, visible test results, and ease of usage, although some of them are mono-color and unstable (Y. Wang et al., 2019). Therefore, LFD/LFIA, as a novel-developed detection technology, is potentially anticipated to boost the applications for the on-site detection of tropomyosin (Galan-Malo et al., 2019).

2.6.5 Biosensors

Biosensors are considered as an alternative technology to analyze and track food allergens. It is rapid, sensitive, selective, and available for site analysis, such as surface plasmon resonance (SPR) biosensor, surface-enhanced Raman spectroscopy (SERS), amperometric biosensors, voltammetric biosensors, quartz crystal microbalance (QCM) biosensors, and molecularly imprinted polymers-based (MIP) biosensors. Nevertheless, biosensor techniques generally require expensive instruments and skilled operators (Zhou et al., 2019).

The surface plasmon resonance (SPR) biosensor is applied to the high-throughput analysis of food allergens. SPR biosensor aims to measure the refractive index changes based on the surface plasmon, which is induced by the biomolecules interacting with the surface of SPR biosensors. It is relatively simple, inexpensive, and easy to use compared to other biosensors (Šípová & Homola, 2013). SPR serves as a highly sensitive optical sensing approach and can realize the real-time monitoring of small changes in the effective refractive index of a metal-dielectric interface (Michel et al., 2017). Zhou et al. (2020) proposed an SPR biosensor method with gold patterned biochips to detect and quantify shellfish tropomyosin; they found that this developed methodology to be suitable for the determination of tropomyosin from various shellfish species in 3 min, which was a very accurate and rapid technology (Zhou et al., 2020).

Except for the SPR biosensor, Chinnappan et al. (2020) developed an aptamer biosensor for the detection of shrimp tropomyosin. This aptamer-based sensor successfully detected tropomyosin in 30 min with sensitive, selective, and specific advantages (Chinnappan et al., 2020). In addition, a

gold nanoparticle-based label-free colorimetric assay was proposed as a visual colorimetric detection technology to detect the shrimp tropomyosin. The aptasensor is highly reliable, selective, and sensitive with the observed detection limitation of 70 nmol/L in shrimp (Pavase et al., 2021). However, it was reported that the aptamer stability and function might be altered due to the instability and relative nonspecific binding of aptamers (Stoltenburg et al., 2007).

2.6.6 Comparison between allergen detection methods

Table 2.4 summarizes the advantages and disadvantages of current seafood allergen detection methods. According to the present studies, there is no doubt that immunoassays, including Indirect ELISA and Western blot, are the main detection methods in investigating the processing effect on fish allergens. The mechanisms are clarified that the binding between antibodies and processed allergenic proteins lead to a modified capacity to elicit allergic reactions (Verhoeckx et al., 2015). Nonetheless, immunoassay might be inaccurate, since it can present wrong results due to cross-reaction of the antibody with non-target allergenic proteins, and not efficient enough in allergen detection procedure. On the contrary, LFIA is more efficient and reliable and can be operated for on-site detection (Xu et al., 2021). Compared with protein-based methods, LC-MS and DNA-based methods gradually gain more attention due to their inherent independence from biomolecular interactions. However, they basically require unportable and expensive equipment with specialized and complex operations (Holzhauser & Röder, 2015). In recent years, biosensors are widely applied in allergen detection, such as multiplex detection, on-site quantitative detection, and even unknown allergens detection. Although it is a sensitive, rapid, and easy measurement method, the device format may lead to some restrictions in allergen detection applications (Xu et al., 2021).

Table 2.4. Summarization of common allergen detection methods for seafood products

Method	Advantages	Disadvantages
ELISA	• High sensitivity & specificity • Easy operation • The most common method and have a wide application	• Results are easily be affected by various factors • False positive or false negative results might be obtained • Protein can be altered after processing
PCR	• Stable to maintain the integrity even after thermal processing • High sensitivity & efficiency • Less prone to contamination	• Unsuitable to identify target allergenic proteins with unidentified genes • More expensive than protein-based methods
LC-MS	• High sensitivity & accuracy • Improved reproducibility • Dynamic range	• Expensive and unportable professional equipment • Requiring specialized and complex operating techniques
LFD/LFIA	• On-site detection method • High sensitivity & efficient • Visible test results • Ease of usage • Cheap	• Mono-color • Instability
Biosensors	• Rapid • Sensitive & selective • Available for site analysis • Ease of usage	• Instability • Low affinity

Therefore, reliable, high-throughput, and real-time detection methods with high accuracy and sensitivity are crucially required in detecting allergenic ingredients in seafood products. This is meaningful to monitor cross-contamination during seafood processing to further reduce the risks of allergen cross-contamination and also to avoid allergen intake accidentally to some extent.

2.7 Conclusion

Seafood allergy is a global health problem. Both fish and shellfish allergies may affect human life quality with mild or severe symptoms. Seafood processing techniques, including thermal, non-thermal, and combined (hybrid) treatments, are powerful in reducing allergenicity by modifying the allergen conformations. Compared to thermal treatments, non-thermal treatments usually show

better results in retaining the nutritional value and sensory quality of seafood products. Compared to the single treatments, combined (hybrid) treatments have a better performance with higher efficiency in eliminating seafood allergens. It is to be noted that the investigations of main allergen detection methods include ELISA, PCR, LFD/LFIA, LC-MS, and biosensors; all of them show their various advantages and disadvantages. The reliable, rapid, and on-site allergen detection methods with high accuracy and sensitivity are future research trends for seafood products. However, the avoidance of allergenic seafood intake is still the only standard way to clinically protect seafood-allergic patients. Therefore, in-depth research is necessary to decrease the risk of seafood allergy, and to create an accessible and safe environment for worldwide customers in the future.

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Author Contribution

Conceptualization, X.D.; Methodology, X.D.; Investigation, X.D.; Data curation, X.D.; Writing—original draft, X.D.; Writing—review and editing, X.D. and V.R.; Supervision, V.R.; Project administration, V.R.; Funding acquisition, V.R.

Conflicts of Interest

The authors declare no conflict of interest reported in this paper.

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Contextual transition

Chapter 2 summarized the seafood processing techniques, including thermal, non-thermal, and combined (hybrid) treatments, are powerful in reducing allergenicity by modifying the allergen conformations. In Chapter 3, we will investigate the effect of various thermal processing techniques, including oven, steam, microwave, wet heat, and dry heat, on the allergenicity, secondary structure, and *in-vitro* protein digestibility of Atlantic cod.

Chapter 3

Manuscript II – Effect of Varying Thermal Processing Techniques on Allergenicity and Structural Alterations in Atlantic Cod

Xin Dong * and Vijaya Raghavan

Department of Bioresource Engineering, Faculty of Agricultural and Environmental Sciences,
McGill University, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

*Corresponding Author- Email: xin.dong2@mail.mcgill.ca

ORCID: <https://orcid.org/0000-0002-9052-3563>

3.1 Abstract

Fish as one of “big nine” allergenic foods can trigger food allergy to threaten public health. In this study, Atlantic cod was treated with various thermal treatments (oven, steam, microwave, wet heat, and dry heat) at 100 °C for 10 min to assess the effect on allergenicity, secondary structure, and *in-vitro* protein digestibility. The soluble protein content, peptides, and microstructure were also evaluated. The results indicated thermal treatments effectively reduced the allergenicity by 7.80%–46.33%. Steam demonstrated the best performance leading to the lowest allergen content (46.33%) and the highest *in-vitro* protein digestibility (55.29%) in cod. Changes to the secondary and tertiary structures were noted with higher β -sheets and lower α -helices. More structural destructions with fragments and micro holes were observed with increasing processing durations.

Keywords: Thermal treatment; fish allergy; Atlantic cod; protein structures; allergenicity

3.2 Introduction

Atlantic cod (*Gadus morhua*) is a commercially significant fish species dominant in the North Atlantic Ocean (Sodeland et al., 2022). It is the most widely consumed groundfish worldwide, constituting 16% of the global groundfish supply (Yu et al., 2022). It is renowned for its exceptional nutritional value, providing ample proteins, vitamins, and omega-3 fatty acids essential for human health (Dave & Routray, 2018). Atlantic cod is classified as a low/moderate mercury fish, recommended by the U.S. Food & Drug Administration (FDA) as a healthy dietary choice for pregnant and lactating individuals (Næss et al., 2020). Additionally, it is recognized as a lean protein source with minimal carbohydrate content, making it lower in calories compared to chicken, red meat, and oily fish. Therefore, it can be the ideal meat choice for individuals with diabetes and those who follow low-carb, paleo, pescatarian, and gluten-free diets (FDA, 2020).

Fish allergy, however, is acknowledged by the Food Allergy and Research Education report as one of the “big nine” food allergies (Messina & Venter, 2020). Fish allergy manifests through mild-to-severe symptoms, including oral allergy syndrome, diarrhea, urticaria, dyspnea, and in severe cases even life-threatening anaphylactic shock (Shimojo et al., 2022). It affects 7% of the pediatric population worldwide suffering from lifelong severe allergic reactions. Until now, complete avoidance of intaking fish is the only way to prevent fish allergy (Dong & Raghavan, 2022a). To ensure the safety of fish-allergic consumers, it is crucial to label food products containing fish ingredients clearly and accurately on the food manufactured products (Campuzano et al., 2020).

World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org) has officially identified several proteins as fish allergens, such as parvalbumin, aldolase A, β -enolase, tropomyosin and vitellogenin (Ruethers et al., 2018). Parvalbumin is the primary fish allergen to trigger over 95% of fish allergies (Fernandes et al., 2015). It is a small muscle protein (10–13 kDa) with calcium-binding protein structure (Palupi et al., 2021). Fish parvalbumin is widely acknowledged as a relatively thermal-stable food source (Somkuti et al., 2012a). Previous studies applied heat treatments at 20–140 °C for up to 45 min, which found structural changes in parvalbumin which can be intensified with an increase in heating temperature and perhaps leading to a decrease in fish allergenicity (Kubota et al., 2016; J. Liang et al., 2021; Saptarshi et al., 2014; Somkuti et al., 2012a). However, long-time high-temperature processing ways fail to satisfy eco-friendly and cost-

effective attributes in finance and time, which contradicts the principles of sustainable development and carbon management for safer environment. To date, no research has proposed the optimal thermal processing technique for effectively reducing fish allergenicity. In this study, several common thermal processing techniques including oven, steam, microwave, wet heat, and dry heat, are compared to treat Atlantic cod at 100 °C for 10 min. The study findings provide clear insights into the impact of thermal processing on the allergenicity and structural changes of Atlantic cod. Additionally, the thermal processing technique that demonstrated the most favourable performance in our study could serve as a basis for future development and production of hypoallergenic foods in the food industry.

3.3 Materials and Methods

3.3.1 Thermally processed cod samples

Deceased Atlantic cod meat was purchased from a local seafood market (L'artisan de la mer, Montreal, Canada). As shown in Figure 3.1, fillets were cut into pieces for appropriate storage at -20 °C. Before the experiments, the cod was thawed to room temperature and then minced. The minced cod were subjected to various thermal treatments, including oven baking using an oven (Thermo Fisher Scientific, Canada), steam using a high-pressure reactor (Zhengzhou Beirun Instrument Co., China), microwave using the Mini WAVE Digestion Module (SCP Science, 115 V, 60 Hz, 15 A, 1000 W, Canada) at a frequency of 2.45 GHz, wet heat using a water bath (Shellab, 6 A, 110-120 V, 50/60 Hz, USA), and dry heating using a dry bath (Lab-line Instruments, 120 V, 50/60 Hz, 1000 W, USA) at 100 °C for 10 min. All treatments were performed in triplicates. After cooling to room temperature, the samples were freeze-dried using a freeze dryer (model 7420020, Labconco Corporation, Kansas City, USA) for 48 h. The obtained cod powder was stored at -20 °C for subsequent analysis.

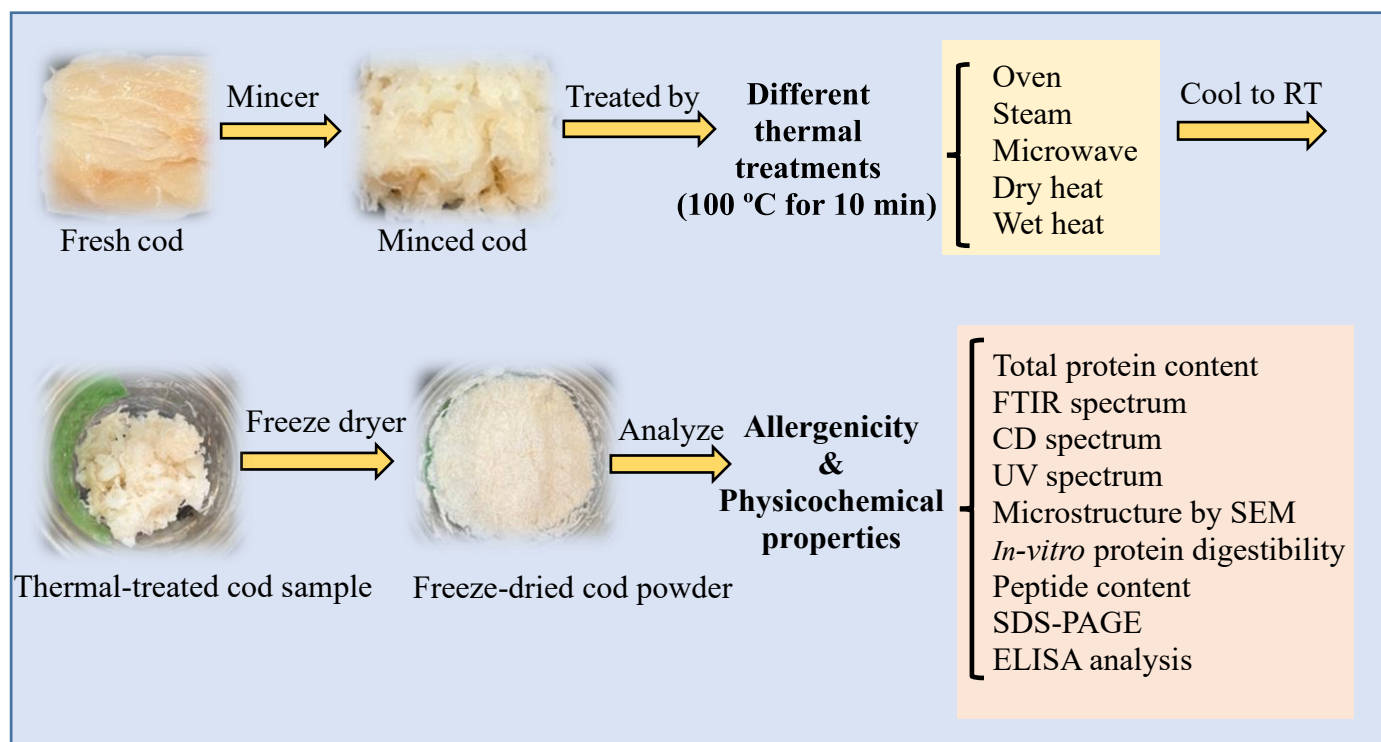


Figure 3.1. The schematic overview of the experimental setup and the pretreatment of Atlantic cod samples.

3.3.2 Total soluble protein content

Total soluble protein content was determined through the extraction process using freeze-dried cod powder (0.2 g) with phosphate-buffered saline (PBS) solution (0.1 M, pH = 7). Following 30-min incubation at room temperature, the mixture was centrifuged at 5,000×g, 4 °C for 20 min. The supernatant was collected for subsequent analysis. The total soluble protein content in cod samples was determined using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Canada). Following the provided protocol, the diluted albumin (BSA) standards and BCA working reagent (WR) were prepared appropriately. Then, the prepared standards and samples (25 µL) were added to each microplate well. The WR (200 µL) was subsequently added to each well and mixed thoroughly on a plate shaker for 30 seconds. After an incubation (avoid light) at 37°C for 30 min, the absorbance at 562 nm was measured by a plate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada).

3.3.3 FTIR analysis

The investigation of cod protein secondary structures was conducted using Fourier transform infrared (FTIR) spectroscopy. Samples were placed on the diamond crystal of the FTIR spectrometer (Nicolet Magna 158,750 FTIR, Nicolet Instrument Corp., Madison, WI) and scanned in the infrared region ($600\text{--}4000\text{ cm}^{-1}$) with a resolution of 4 cm^{-1} . OMNIC software (Version 8, Thermo Nicolet Co., Madison, WI) was used for the spectral analysis, and OriginPro software (Version 9, Origin Lab Corporation, Northampton, MA, USA) was employed for curve-fitting to quantify secondary structural changes in the amide I band ($1700\text{--}1600\text{ cm}^{-1}$). The resulting peak areas would provide insights into the secondary structural composition of cod proteins.

3.3.4 CD spectra analysis

Circular dichroism (CD) spectroscopy was used to examine the secondary structures of cod protein. Circular dichroic measurements were determined by Chirascan (Applied Photophysics, USA) with the thermostat set at $23.4\text{ }^{\circ}\text{C}$. Each sample was prepared with PBS to a concentration of 6 mg/mL and filtered through a $0.22\text{ }\mu\text{m}$ membrane. All Samples were scanned from 190 to 260 nm with a bandwidth of 1 nm, time per point used was 0.5 s and a scanning time for once was 65 s. A cuvette with a path length of 1 mm was filled by 185 μL of sample for each sample measurement. The final spectrogram was averaged by five accumulated spectra.

3.3.5 UV absorption spectra measurement

Conformational alterations in cod protein can also be inferred by changes in UV absorption spectra. About 10 mg freeze-dried cod powder of each sample was extracted with PBS buffer ($10\times$, $\text{pH} = 7$) to a final concentration of 1 mg/mL . Following a 30-min incubation at $25\text{ }^{\circ}\text{C}$, the mixture was centrifuged at $4,000\times g$, $4\text{ }^{\circ}\text{C}$ for 10 min, and the resulting supernatant was collected. As described by Zhao, Li, Xu, Ji, et al. (2022), the UV spectrum data of cod soluble extracts (1 mg/mL) was recorded from 220 to 320 nm at 0.5-nm wavelength intervals by using a UV/Visible spectrophotometer (Ultrospec 2100 pro, Biochrom, USA) at room temperature. PBS buffer was used as the blank for the measurements and comparisons.

3.3.6 Microstructure

Microstructural changes in cod samples were observed by a Scanning Electron Microscope (SEM) (TM3000, Hitachi High-Technologies Corporation., Tokyo, Japan). The freeze-dried cod samples were thawed to room temperature and then transferred to the measuring platform. Images were captured at various magnifications (100× and 500×) to visualize the microstructural features.

3.3.7 SDS-PAGE analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the following protocol. The protein extract (10 µL) was mixed with β-mercaptoethanol and 2x Laemmli sample buffer and then heated at 95 °C for 5 min. Running buffer was prepared by 10× Tris/Glycine/SDS mixed with distilled water. Samples were loaded for 10 µL per lane, and a molecular weight marker (10–250 kDa) (Bio-Rad, Philadelphia, PA, USA) was loaded for 5 µL. Electrophoresis was performed in a vertical unit (Mini-PROTEAN® Tetra System, BIO-RAD, Philadelphia, PA, USA) at 200 V. After electrophoresis, gels were washed in water for 5 min. After removing water, the gel was stained by Bio-Safe™ Coomassie G-250 Stain (Bio-Rad, USA) for 1 hr and then was rinsed with water for 30 min. The figures were captured using a digital camera (Canon, EOS Rebel SL2 DSLR Camera, America).

3.3.8 ELISA analysis

A sandwich ELISA (Fish Parvalbumin ELISA Kit, Arigo Biolaboratories) was utilized to quantify the parvalbumin content in the cod samples. The ELISA kit consists of an antibody-coated microplate, HRP-antibody conjugate, standards, extraction and sample dilution buffer (10×), wash buffer (10×), TMB substrate, and STOP solution. All materials should be equilibrated to room temperature before use. According to protocol, the cod samples were homogenized and then mixed with 20 mL of pre-diluted Extraction and Sample Dilution Buffer. The suspension was incubated in a water bath at 60°C for 15 min with a slight shake to ensure good homogeneity every two minutes. Afterwards, the samples were centrifuged for 10 min at 2000 g to separate the supernatant from the precipitate completely. The microplate was added particle-free solution (100µL) of standards and samples in each well in triplicates. After a 20-min incubation at RT, 1X wash buffer (300 µl) was used to wash each well for 3 times. HRP-Antibody Conjugate (100 µl) was added into each well, which was followed by the repeated 20-min incubation at RT and wash well. TMB

mixture (100 µl) was added to each well, and then the plate was incubated for 20 min at RT in the dark. After the Stop Solution (100 µl) adding to each well, the color of the solution changed from blue to yellow. The microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada) was used to read absorbances at 450 nm. The additional dilution has to be considered when calculating the concentration.

3.3.9 *In-vitro* protein digestibility

The *in-vitro* protein digestibility assessment was conducted in accordance with a two-stage procedure. Freeze-dried cod powder (0.2 g) was mixed with a PBS buffer (0.1 M, 10 mL, pH = 7) to a final mixture concentration of 20 mg/mL. After 0.1 M HCl solution adjusting pH to 1.5, pepsin solution (10 mg/mL, 50 µL) was added to start the first-stage digestion at 37 °C for 30 mins. Subsequently, after adding 1.0 M NaOH solution adjusting pH to 7.8, pancreatin solution (10 mg/mL, 200 µL) was added to start the second-stage digestion at 40 °C for 60 mins. The digestion was stopped by adding Na₂CO₃ solution (150 mM, 50 µL). The BCA method was used to determine the initial protein and final undigested protein content of cod samples. The *in-vitro* protein digestibility (IVPD%) was calculated by using the following equation:

$$\text{IVPD}\% = \frac{\text{initial protein} - \text{final undigested protein}}{\text{initial protein}} \times 100$$

3.3.10 Peptide content

The o-Phthaldialdehyde (OPA) Reagent was used to assess the peptide content. The OPA reagent (50 mL) was obtained by mixing sodium tetraborate (25 mL, 100 mM), sodium dodecyl sulfate (2.5 mL, 20% w/v), OPA (40 mg) dissolved in methanol (1 mL), β-mercaptoethanol (100 µL), and ddH₂O. The digestion mixture (10 µL) was mixed with OPA reagent (190 µL) for a reaction time of 2 min. The microplate reader (Synergy HTX Multi-Mode Reader, BioTek, Canada) was used to read the absorbances at 340 nm. The standard curve was obtained by using Leucine-glycine.

3.3.11 Statistical analysis

The analysis of variance (ANOVA) feature of the SPSS program (IBM SPSS Statistic, Ver. 29.0.0.0) was used to examine the experimental data. The means were separated using the Duncan, multiple range test, and significance was established at $p \leq 0.05$.

3.4 Results and Discussion

3.4.1 Total soluble protein, protein digestibility and peptide content

The total soluble protein of the cod after various thermal treatments and after each *in-vitro* digestion stage are shown in Figure 3.2a. Before digestion, total soluble (initial) protein content demonstrated an overall reduction in the treated cod samples. The steamed sample showed the lowest protein content by decreasing 70.71% in comparison to that in control. After the first-stage digestion, the protein content decreased to a lower value in all treatments. Except for steam and wet heat, a significant reduction was observed in other samples, especially in control decreasing by 63.59%. After two-stage digestion, total soluble (undigested) protein content significantly decreased to the lowest value, especially in steam by a reduction of 26.80% (Figure 3.2a). In a previous study by Dong et al. (2021b), it was observed that the total soluble protein content in shrimp (*Litopenaeus vannamei*) significantly decreased by 50–75% upon exposure to microwave heating (75–125 °C for 5–15 min). This decline in protein content was attributed to the degradation and hydrolysis of proteins caused by the frictional force generated in the electromagnetic field during microwave treatment (Iuliana et al., 2015). Smida et al. (2014) reported that the protein content decreased from 18.3% in fresh fish to 14.2% in sun-heated fish, which implied thermal treatment can lead to the destruction of cell membranes and the aggregation of sarcoplasmic protein, further resulting in protein deterioration at higher temperatures.

Peptides are considered as a significant category of taste-active compounds that can enhance flavor attributes, such as mildness, continuity, and mouthfeel (Kirimura et al., 1969). The peptide content of the cod after different thermal treatments and after each *in-vitro* digestion stage are shown in Figure 3.2b. The peptide content after digestion showed a generally increasing trend in each treatment. Although the peptides increased slightly following the first-stage digestion, there were no significant differences between the treatments in comparison to the initial level. However, the peptide content was significantly enhanced in all treatments after the two-stage digestion, especially in dry heat increasing by 15.52%. With the exception of the dry-heated sample, the treated samples showed a reduction in peptides compared to the control group, which could be linked to protein denaturation and polymerization. Similarly, Dong et al. (2021b) reported microwave heating (75–125 °C for 5–15 min) caused decreased peptides in shrimp compared to that in the untreated sample. The soluble proteins were digested into the peptides with low

molecular weight, which probably resulted in lower allergenicity compared to the control group (Rao et al., 2018).

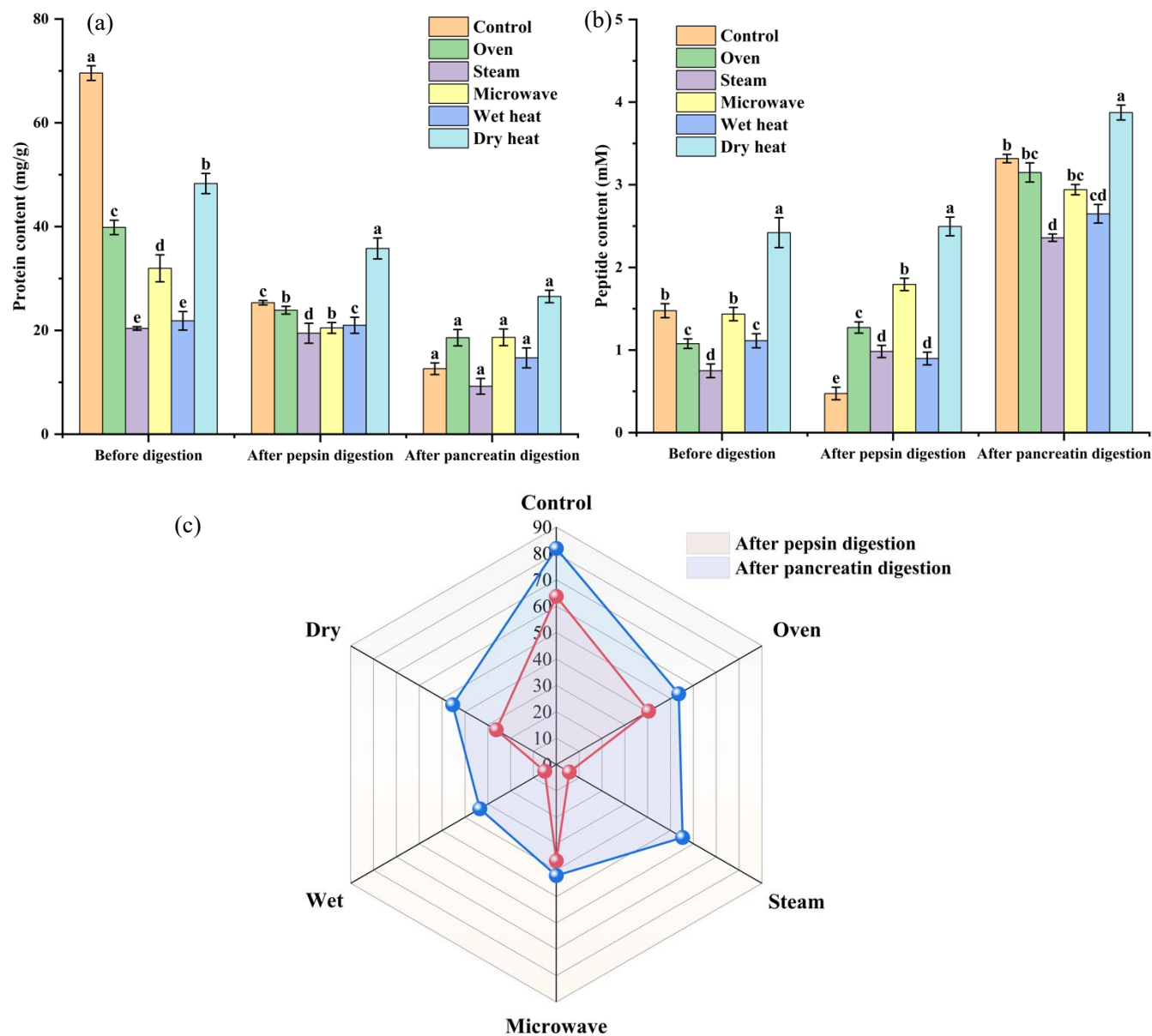


Figure 3.2. Changes in the (a) protein content, (b) peptide content, and (c) *in-vitro* protein digestibility (%) of the cod after various thermal treatments.

The *in-vitro* protein digestibility (IVPD) of cod samples is shown in Figure 3.2c. After two-stage digestion, the final digestibility of the control was 81.94%, while the digestibility of the treated samples was significantly decreased, with the lowest IVPD at 33.51% in the wet-heated sample. In treated samples, the steamed sample showed the highest value (55.29%) of IVPD, followed by the oven-baked sample (53.62%), dry-heated sample (45.35%), and microwaved sample (42.09%).

Similar results were shown in the previous study. Dong et al. (2021b) reported IVPD in shrimp (*Litopenaeus vannamei*) significantly decreased after microwave heating (75–125 °C for 5–15 min). However, Tavares et al. (2018) studied thermal treatments (boiling at 98 °C for 5–15 min, baking at 220°C for 20–30 min, and deep-frying at 160 °C for 5–15 min) led to significantly higher IVPD than that in raw samples during both pepsin and pepsin/trypsin digestion in hairtail fillets. Jiang et al. (2022) found various cooking approaches (steam, boil and roast) increased the IVPD (1.60–8.28%) in channel fish (*Ictalurus punctatus*) muscles, and the highest IVPD (66.78%) was observed in the steamed sample. The alterations in protein digestibility are highly correlated with their structural modifications, particularly in the protein secondary structures, such as alpha-helix and beta-sheet, which subsequently induce changes in their functional properties (Jin Wang et al., 2023).

3.4.2 FTIR spectra analysis of changes in the secondary structure

Changes to the secondary structure of cod protein during the various thermal process was determined by FTIR. The absorbance and transmittance were shown in Figure 3.3a and 3.3b, respectively. The amide I (C=O stretching) ranges from 1700 to 1600 cm^{-1} , amide II (C–N stretching and N–H bending) from 1600 to 1500 cm^{-1} , and amide III (C–N stretching and N–H bending) from 1360 to 1200 cm^{-1} . In FTIR spectra, amide I, II, and III peaks appear at ~1655, 1536 and 1238 cm^{-1} . Amide I frequencies closely correlate with protein secondary structures (Barth, 2007). digestibility (IVPD) of the cod after various thermal treatments. In Figure 3.3c, the amide I of the cod protein was analyzed to evaluate the structural changes that occurred during the thermal process. The absorption showed an initial increase peaking at ~1625 cm^{-1} , followed by a gradual decrease with increasing wavenumbers. Compared with the control, all treated samples showed a decreasing trend of absorption, with the steamed sample followed by dry-heated, wet-heated, oven-baked, and microwaved samples. This indicated that microwaves resulted in a maximum structure loss in cod proteins among the above thermal treatments. In Figure 3.3d, the secondary structure in control was made up of 48.31% β -sheet, 40.52% α -helix, 2.61% turns, and 8.56% unordered structures. After thermal treatments, an increase of β -sheet content was observed in treated samples (49.92%–63.80%) other than the microwaved sample with a little reduction (40.99%). However, the α -helix content decreased in all treated samples ranging from 8.17% (wet heat) to 37.72% (microwave). In comparison to the control, a higher percentage of unordered

structures (10.00%–34.63%) and turns (3.85%–8.47%) were generated in treated samples.

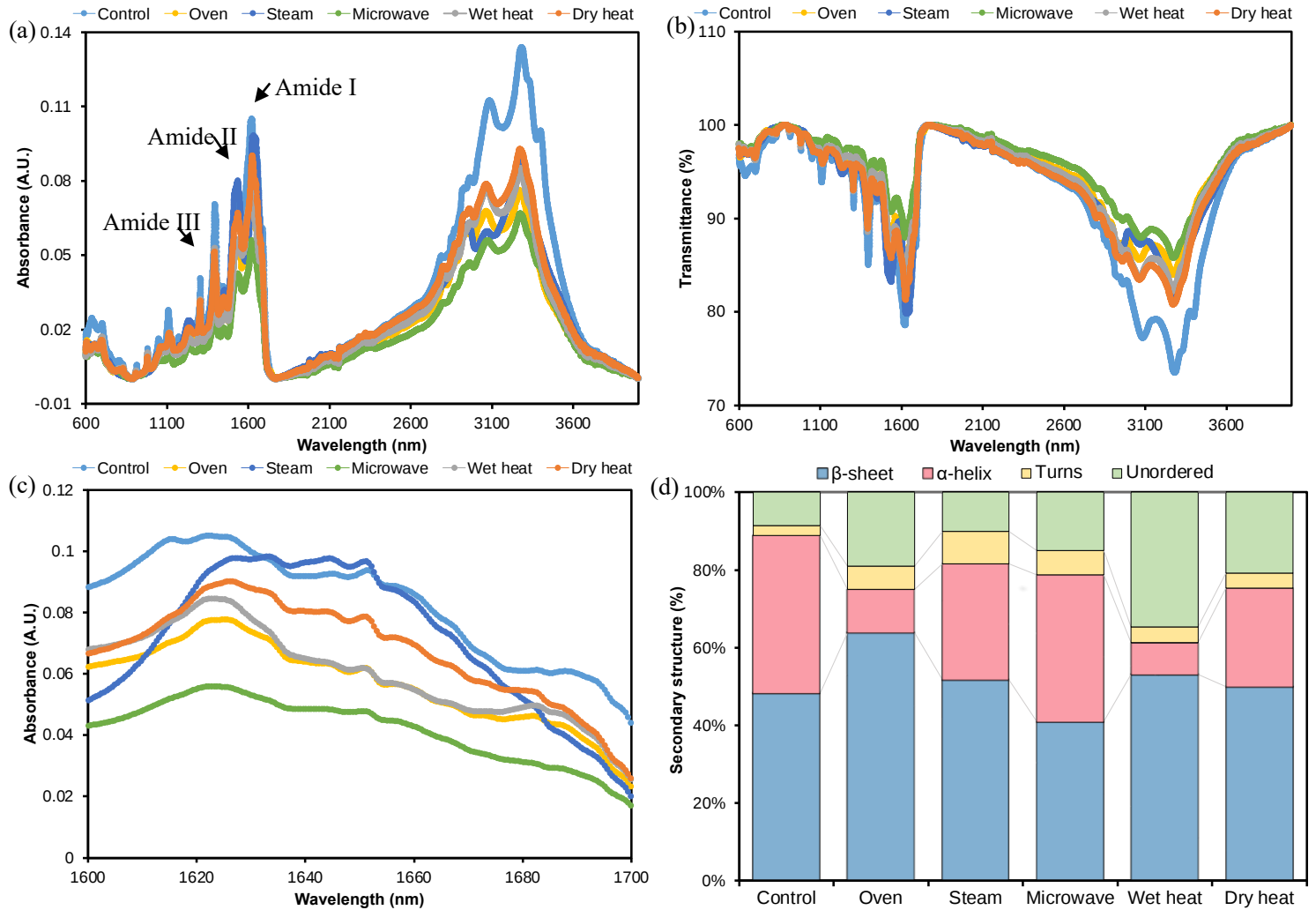


Figure 3.3. FTIR spectrum of Atlantic cod samples treated by different thermal treatments: (a) Absorbance (b) Transmittance (c) Amide I region ($1700\text{--}1600\text{ cm}^{-1}$); and (d) variations in relative areas of the four secondary structural bands fitted to the normalized FTIR spectra of the Amide I region.

Jiang et al. (2022) reported various cooking ways (steam, roast and boil) caused a significant increase in β -sheet structure and a decrease in α -helix observed in channel fish (*Ictalurus punctatus*) muscles, particularly in the roasted sample with the β -sheet content increasing by 104.14%. The steamed group had the lowest β -sheet and highest α -helix structures, suggesting that both the protein structure and its unfolding were more stable than in the other two groups. Besides, the

steamed group with the lowest β -sheet content also showed the highest IVPD. Previous research has yielded similar findings, where a highly linear and negative correlation was observed between the β -sheet structure content and protein digestibility of raw legumes (Carbonaro et al., 2012). Hes (2017) has highlighted the influence of the protein's secondary structure on the binding sites for its digestive enzymes and the consequent digestion procedure. Of the various secondary structures, α -helix and β -sheet structures are the most relevant. While intermolecular hydrogen interactions keep the β -sheet structure stable, intramolecular hydrogen bonds are responsible for the great stability of the α -helix structure. The unfolding of protein secondary structure, which exposes hydrophobic amino acids and improves the protein's capacity to bind to aldehydes, is typically shown by a decrease in the α -helix structure. Conversely, a rise in β -sheet structure usually signifies aggregated proteins. Understanding the impact of protein secondary structure on digestive enzyme binding can aid in developing better food processing techniques and products with improved nutritional properties (Jiang et al., 2022).

3.4.3 CD spectra analysis of changes in the secondary structure

CD analysis was conducted to monitor changes in the secondary structures of the cod protein and the normalized results are shown in Figure 3.4a. Analysis of CD spectra revealed peaks with the typical properties of a α -helical structure, including one distinct positive peak near 194 nm, two negative bands at 208 and 225 nm, and a negative peak at 204 nm that was attributed to the presence of β -sheets. All thermally treated samples showed lower intensity in the CD spectra (smaller negative peak values) when compared to the control. These modifications revealed that a rise in protein's disorder, e.g., random coil resulted from thermal unfolding. The intensity of peaks in the 195–210 nm band increased because of both the instability of α -helices and an increase in the content of random coils. The spectra revealed a structural change from typical secondary structure elements to twists and the random coil as the temperature rose. The conformational changes showed that denaturation during the heat procedure caused the cod protein's secondary structure to relax or diminish. The cod protein's hydrophobic core, which is typically hidden, is exposed as a result of this change (Han et al., 2015).

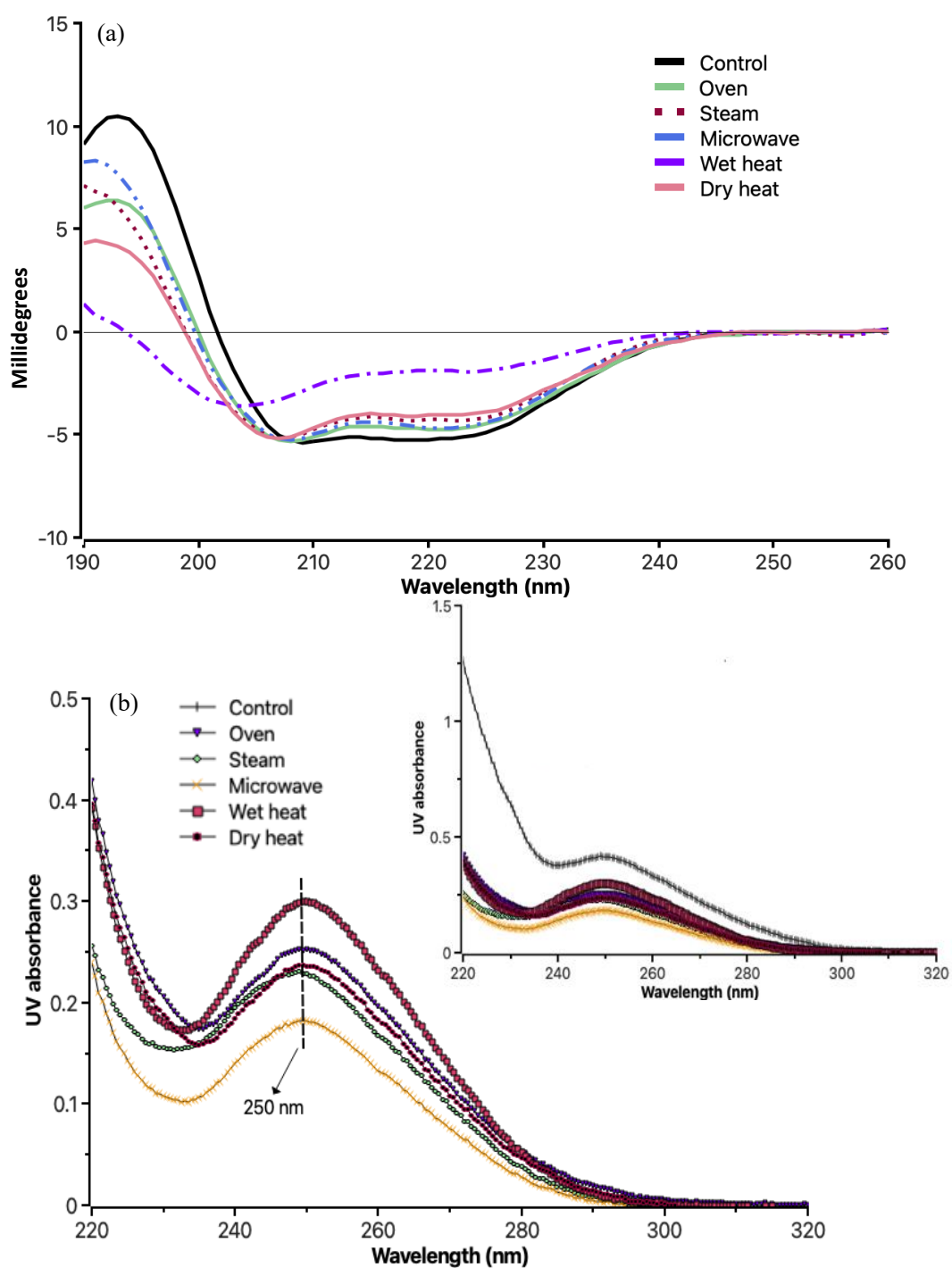


Figure 3.4. Changes in the CD spectra (a) and UV absorbance intensity (b) of the Atlantic cod samples.

3.4.4 UV absorption spectra analysis

The determination of the direction and magnitude of the UV spectra absorption peak is an effective method for evaluating tertiary structural changes in proteins, as it provides insights into alterations in the hydrophilic and hydrophobic environment (Zhao, Li, Xu, Zeng, et al., 2022). As shown in Figure 3.4b, a significant reduction of UV absorbance intensity was observed in thermal-treated samples compared with that in the control. The cod sample's highest UV absorbance peak was identified at a wavelength of 250 nm. In treated samples, the wet-heated sample showed the highest absorbance intensity, followed by oven-baked, dry-heated, and steamed samples, respectively. Meanwhile, the absorption intensity from the microwaved sample was always the lowest with a similar trend with others. In treated samples, an evident shift of the absorbance peak was observed along with the decreased absorption intensity, indicating that more hydrophobic amino acids were exposed on the protein surface triggering the unfolding of the proteins as observed by other researchers (Zhao, Li, Xu, Ji, et al., 2022). In their study, a similar shift obtained by UV spectra was observed in shrimp (*Litopenaeus vannamei*) soluble extracts (Zhao, Li, Xu, Ji, et al., 2022). They heated shrimp by boiling, frying, baking, steaming, and microwaving, which also implied thermal processing altered and rearranged the secondary structure of proteins because of the breakdown and generation of hydrogen bonds (Secundo & Guerrieri, 2005).

3.4.5 SDS-PAGE and ELISA analysis of changes in the allergenicity

This study examined the changes in the cod allergen following various thermal treatments using an SDS-PAGE measurement as a qualifying method. As represented in Figure 3.5a, the protein band intensity located at 12 kDa in the gel corresponds to the cod major allergen, parvalbumin. The lowest intensity of cod protein bands was found in the steamed sample, followed by the wet-heated and microwaved samples. However, increased intensity of the cod protein band was observed in the dry-heated sample, with almost no significant changes in the oven-baked sample in comparison to the control. Kubota et al. (2016) reported parvalbumin band decrease as a function of heating temperature and time in pacific mackerel protein extracts heated between 60–140 °C (60–80 °C using a dry bath, 100 °C in boiling water, and 120–140 °C using a Joule heating apparatus) for 5–30 min, and the decrease was induced by conformational changes (release of calcium) during heating. Saptarshi et al. (2014) found the parvalbumin band intensity from heated Atlantic cod to decrease but still maintain immunodetectable parameters after 95 °C for 15 min in

a water bath. The immunoreactive parvalbumin in the elephant shark was even not visible, whereas a stronger parvalbumin band in yellowfin tuna was found in comparison to unheated extracts. Therefore, heat processing had different effects on each species' antibody-antigen interaction.

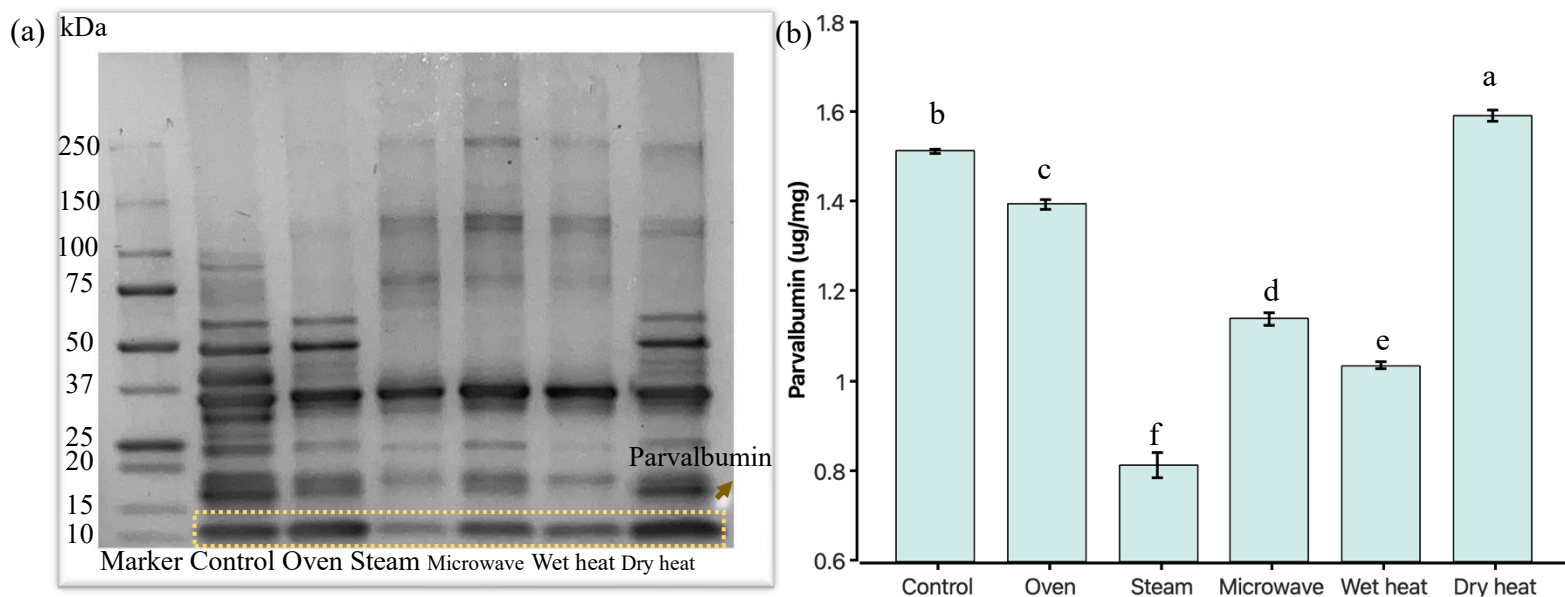


Figure 3.5. SDS-PAGE (a) and allergenicity (b) changes of cod samples before and after various thermal treatments.

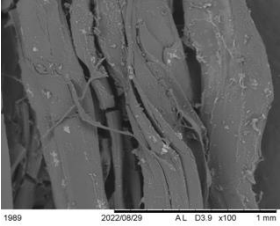
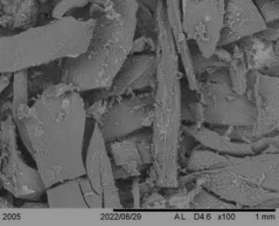
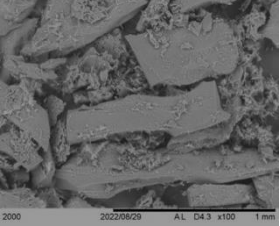
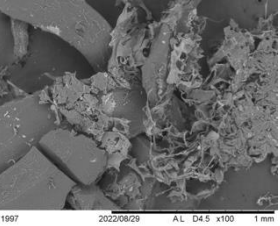
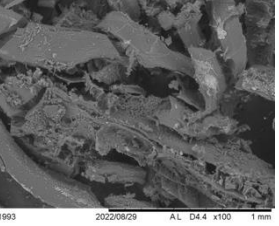
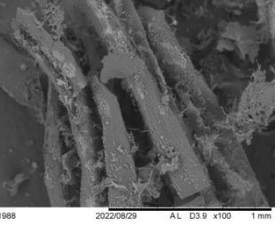
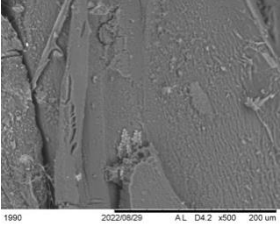
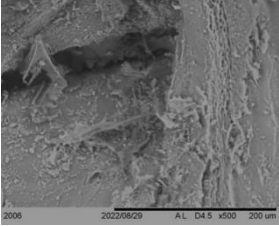
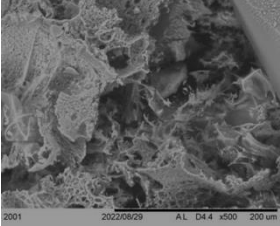
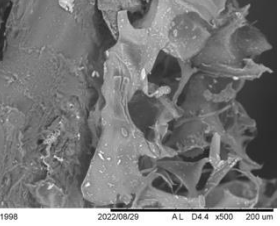
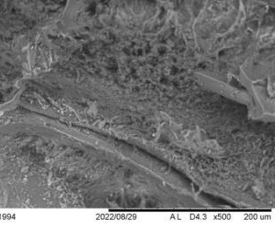
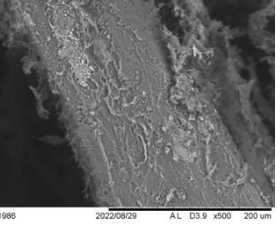
To determine how the thermal treatment of cod samples affected their allergen content, the Sandwich ELISA assay was used as a quantification method. As shown in Figure 3.5b, the results indicated that the allergen content of the most thermally treated cod proteins significantly decreased in comparison to the control, whereas the dry-heated sample they increased. The lowest allergen content was observed from the steamed sample (0.81 ug/mg) with a 46.33% decrease compared with the control (1.51 ug/mg). There were reductions for wet-heated (1.03 ug/mg) of 31.64%, microwaved (1.14 ug/mg) of 24.71%, and oven-baked samples (1.39 ug/mg) of 7.80%. Oppositely, an enhancement of 5.31% was found in the dry-heated sample (1.59 ug/mg). This might be because the thermal processing of cod proteins dramatically altered their structure, which destroyed IgE epitopes (Dong et al., 2021a). Somkuti et al. (2012a) reported that IgE binding was not affected in purified cod parvalbumin treated at 80 °C for 30 min determined by indirect non-competitive ELISA, which is due to the heat-induced secondary structure and calcium-binding ability changes which were insufficient to reduce antigenicity.

3.4.6 SEM observation for microstructure

The SEM images of freeze-dried cod samples after various thermal treatments are presented in Table 3.1. Results showed that the control group exhibited a smooth and solid surface, whereas the different levels of damaged microstructure were observed after thermal treatments. The most significant disruption in cod microstructures was detected in the steamed sample with the most alveolate holes and fragments observed, which is probably due to the destroyed intramolecular hydrogen bonds and disulfide bonds after treatment (Khem et al., 2016). Followed by the wet-heated sample, small particles and numerous fragments were clearly visible, especially in image magnification at 500 $\times$. The damaged tissues with disordered structures and irregular fragments at a moderate level were observed in the microwaved structure. For the dry-heated and ovened samples, they displayed a minor degree of microstructure disruption with rough surfaces.

Similar findings were observed in a previous study on shrimp, where considerable microstructural destruction occurred after microwave heating (75–125 °C for 5–15 min) (Dong et al., 2021b). Besides, Zhao et al. (2020) reported that whey protein in goat milk exhibited serious morphological damage after heat treatments (85–105 °C) by a pasteurizer and UHT sterilizer. These results revealed that thermal processing effectively boosts protein degradation and cell distortions (Kamble et al., 2020). The thermal energy can modify protein microstructures, and meanwhile affected the secondary structures (e.g., α -helix and β -sheet). This resulted in the breaking down of peptide chains and hydrogen bonds in the proteins, which ultimately enhanced the in-vitro digestibility and decreased the allergenicity of food products (Kamble et al., 2020; Jin Wang et al., 2021).

Table 3.1. SEM photomicrographs of freeze-dried cod samples after various thermal treatments (magnification at 100× and 500×).

	Control	Oven	Steam	Microwave	Wet heat	Dry heat
100×						
500×						

3.5 Conclusion

The study followed a systematic comparison of various thermal techniques treating Atlantic cod, involving allergenicity and a series of physicochemical properties. The relationship between the protein structure and allergenic properties in Atlantic cod was clarified. Steam was regarded as the most effective way which maximumly lowered the allergen content by 46.33% in Atlantic cod, corresponding to the lightest SDS-PAGE band intensity observed. Meanwhile, the steamed sample also showed the highest *in-vitro* protein digestibility of 55.29%. Microstructural holes and destruct pieces were generated after thermal treatments. The secondary and tertiary structures also changed after thermal processing. These results demonstrated thermal treatment has the potential to promote the process of eliminating fish allergenicity while causing the least damage to fish's original physicochemical properties. At present, steam could be concluded as the best thermal processing technology to reduce the allergenicity in fish products. However, only *in-vitro* experiments were used to obtain the results. To confirm whether thermal treatments can considerably lower allergenicity in Atlantic cod, additional research in future involving animal or clinical tests must be carried out.

Conflict of Interest

The authors declare no conflict of interest reported in this paper.

Acknowledgment

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Contextual transition

Chapter 3 revealed that various thermal treatments (oven, steam, microwave, wet heat, and dry heat) at 100 °C could change the allergenicity, secondary structure, and *in-vitro* protein digestibility of Atlantic cod. Steam was regarded as the most effective way which caused the lowest allergenicity and the highest IVPD of cod samples. In Chapter 4, high-intensity ultrasound (HIU) treatment, a novel non-thermal treatment will be investigated. The changes in protein structure and allergenicity of cod samples will be focused.

Chapter 4

Manuscript III – Influence of High-Intensity Ultrasound on the Conformational Dynamics and Allergenic Properties of Atlantic Cod Parvalbumin

Xin Dong * and Vijaya Raghavan

Department of Bioresource Engineering, Faculty of Agricultural and Environmental Sciences,
McGill University, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

*Corresponding Author- Email: xin.dong2@mail.mcgill.ca

ORCID: <https://orcid.org/0000-0002-9052-3563>

4.1 Abstract

The structural integrity and allergenic potential of cod proteins present significant challenges in the development of seafood products. This study was aimed to investigate the effects of high-intensity ultrasound (HIU) treatment on cod samples, focusing on changes in protein structure and allergenicity. Ultrasound treatments were applied at varying durations (0-60 min) to determine their impact on protein properties. Key findings include a significant reduction in total soluble protein content and a shift in protein secondary structure from α -helices to β -sheets and unordered structures, as evidenced by FTIR and CD spectroscopy. UV spectroscopy and SEM further confirmed these structural changes, indicating protein denaturation and potential Maillard reactions. SDS-PAGE results showed protein degradation and aggregation, while ELISA results revealed a notable decrease in allergen content by up to 31.82%, particularly in samples treated for longer durations (60 min). These modifications suggest that ultrasound treatment effectively alters protein structures and reduces allergenicity. The study concludes that HIU is a promising technique for improving the quality and safety of seafood products, offering new opportunities for innovation in the food industry.

Keywords: Ultrasound; Fish allergy; Cod protein; Allergenicity; Secondary structure

4.2 Introduction

Fish is an essential component of human diets worldwide and provides a high-quality source of protein. Atlantic cod, in particular, is one of the most widely consumed species of fish (Jarosz-Krzemińska et al., 2021). However, fish allergenicity has become a matter of growing concern since fish is one of the "big nine" allergenic food (Dong et al., 2021a). Fish allergy prevalence is more often seen in countries with higher fish consumption, such as Norway (3%) and Philippines (2.29%) (X. Zhang et al., 2021). Fish consumption has been associated with adverse reactions in individuals who are sensitive to this allergen. These adverse reactions can range from mild symptoms, such as itching and skin rashes, to severe symptoms, such as anaphylaxis, which can be life-threatening (Onyimba et al., 2021).

Parvalbumin is a commonly recognized major allergen in Atlantic cod and is responsible for many fish allergies (Mukherjee et al., 2023). It belongs to the class of calcium-binding proteins, and it has a low molecular weight of about 11-12 kDa with highly stable properties (M. Zhang et al., 2021). However, the allergenicity of parvalbumin can be reduced through degradation or modification of the protein, resulting in a form that is less immunogenic and less likely to trigger an immune response (Dong & Raghavan, 2022a). This is accomplished by utilizing effective food processing methods that alter the structure of parvalbumin, thereby reducing its allergenicity (Tsai et al., 2023). Thus, food processing methods play a significant role in reducing the allergenicity of fish and minimizing the risk of adverse reactions in sensitive individuals.

Thermal and non-thermal processing techniques can affect food from micro to macroscopic levels, including appearance, texture, flavor, and stability (Barbhuiya et al., 2021). Thermal treatment may negatively influence food properties and lead to physicochemical changes, such as breaking down vitamins and bioactive compounds or generating potentially toxic components (Dong & Raghavan, 2022b). In recent years, non-thermal technologies have been popular and gradually replaced thermal processing, because they are effective in retaining food original properties with minimal or no changes (Dong et al., 2021a). Novel nonthermal processing techniques commonly involve ultrasound, cold plasma, pulsed electric field, pulsed light, and fermentation (Dong & Raghavan, 2023).

Ultrasound as a non-thermal processing method has gained significant attention due to its numerous advantages such as environmental friendliness, less energy consumption, higher mass

transfer rate, and original flavor maintenance of food products during the processing (Chen et al., 2020). Moreover, ultrasound processing shows a potential application in producing hypoallergenic food products. Ultrasound can disrupt the conformational allergenicity epitopes of protein and loosen the protein structures, leading to lower allergenicity in foods (Dong et al., 2020b). Several studies have demonstrated that ultrasound effectively decreases the allergenicity in soy, milk, peanut, shrimp, and crayfish (Dong et al., 2021a). However, few studies have reported the application of ultrasound to fish to reduce allergenicity to date. Therefore, in the present study, the effect of ultrasound on fish allergenicity and structural changes was investigated and their relationship was further clarified.

4.3 Materials and Methods

4.3.1 Ultrasound-treated Atlantic cod samples

Atlantic cod meat (bought from a local seafood market, Montreal, Canada) was cut into pieces and then minced to homogenized status by using a mincer (GM 200, Retsch) at 4000 rpm for 30 s. The minced cod was immersed in water by a ratio of 1:1 (per gram/per milliliter) in an ultrasonic cleanser (FS30, Fisher Scientific, USA) for different time durations (0, 5, 10, 20, 30, and 60 mins) as shown in Table 4.1. After ultrasound treatments, samples were processed in a freeze dryer (model 7420020, Labconco Corporation, Kansas City, USA) for 48 h. When the samples were turned into powder, they were kept in the fridge at -20 °C. The sample containers were sealed well to prevent the moisture exchange with the environment.

4.3.2 Total soluble protein content

Total soluble protein content was determined by extracting freeze-dried cod powder (0.2 g) with phosphate-buffered saline (PBS) solution (0.1 M, pH = 7). Following 30-min incubation at room temperature, the mixture was centrifuged at 5,000×g, 4 °C for 20 min. The supernatant was collected for subsequent analysis. The total soluble protein content in cod samples was determined using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Canada) following the provided protocol.

4.3.3 FTIR analysis

Fourier transform infrared (Nicolet Magna 158,750 FTIR, Nicolet Instrument Corp., Madison, WI) spectroscopy was used to investigate secondary structures in cod protein. The absorbance was scanned in the spectra region ($1200\text{--}1800\text{ cm}^{-1}$) with a resolution of 4 cm^{-1} by OMNIC software (Version 8, Thermo Nicolet Co., Madison, WI). The software OriginPro 2022 was used to fit secondary structural peaks in the amide I band ($1700\text{--}1600\text{ cm}^{-1}$). During peak-fitting progress, the second derivative spectrum was used to identify the component bands. Following the appropriate wavelength and peak areas obtained, the proportion of each secondary structure in cod proteins can be estimated.

4.3.4 CD spectra analysis

Circular dichroism (CD) spectroscopy was used to examine the secondary structures of cod protein. Circular dichroic measurements were determined by Chirascan (Applied Photophysics, USA) with the thermostat set at $23.4\text{ }^{\circ}\text{C}$. Each sample was prepared with PBS to a concentration of 6 mg/mL and filtered through a $0.22\text{ }\mu\text{m}$ membrane. All Samples were scanned from 190 to 260 nm with a bandwidth of 1 nm , time per point used was 0.5 s and the scanning time for once was 65 s . A cuvette with a path length of 1 mm was filled by $185\text{ }\mu\text{L}$ of sample for each sample measurement. The final spectrogram was averaged by five accumulated spectra. The DichroWeb (<http://dichroweb.cryst.bbk.ac.uk>) was used for the analysis of protein secondary structures.

4.3.5 UV absorption spectra measurement

According to Zhao, Li, Xu, Ji, et al. (2022), a UV spectrophotometer (Ultrospec 2100 pro, Biochrom, USA) was employed to record the UV spectrum data of cod soluble extracts (1 mg/mL) from 190 to 400 nm at a wavelength intervals of 0.5-nm at room temperature. PBS buffer was used as the blank for the measurements and comparisons.

4.3.6 SEM observation

A Scanning Electron Microscope (SEM) (TM3000, Hitachi High-Technologies Corporation., Tokyo, Japan) was applied to observe microstructural changes in cod samples. A piece of freeze-dried cod sample was transferred to the measuring platform for observation. For microstructural visualization, the images were captured when they were magnified 100 times.

4.3.7 SDS-PAGE analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the following protocol. The protein extract (10 µL) was mixed with β -mercaptoethanol and 2x Laemmli sample buffer and then heated at 95 °C for 5 min. Running buffer was prepared by 10× Tris/Glycine/SDS mixed with distilled water. Samples were loaded for 10 µL per lane, and a molecular weight marker (10–250 kDa) (Bio-Rad, Philadelphia, PA, USA) was loaded for 5 µL. Electrophoresis was performed in a vertical unit (Mini-PROTEAN® Tetra System, BIO-RAD, Philadelphia, PA, USA) at 200 V. After electrophoresis, gels were washed in water for 5 min. After removing water, the gel was stained by Bio-Safe™ Coomassie G-250 Stain (Bio-Rad, USA) for 1 hr and then was rinsed with water for 30 min. The image was captured using a digital camera (Canon, EOS Rebel SL2 DSLR Camera, America).

4.3.8 ELISA analysis

A sandwich ELISA (Fish Parvalbumin ELISA Kit, Arigo Biolaboratories) was utilized to quantify the parvalbumin content in the cod samples according to protocol. After incubation and washing following microplate procedures several times, the solution color changed from blue to yellow. The absorbances were read at 450 nm using a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada).

4.3.9 Statistical analysis

The analysis of variance (ANOVA) feature of the SPSS program (IBM SPSS Statistic, Ver. 29.0.0.0) was used to examine the experimental data. The means were separated using the Duncan, multiple range test, and significance was established at $p \leq 0.05$.

4.4 Results and Discussion

4.4.1 Total soluble protein content

The effect of ultrasound processing method on total soluble protein content in cod is summarized in Table 4.1. The untreated sample (US0) exhibited the highest protein concentration at 1988.15 ± 12.42 µg/mL. In the case of ultrasonic processing, the protein content of all treated samples was significantly decreased by 23%-49%. The observed decrease in total soluble protein content with increasing ultrasound treatment duration suggests that ultrasound has a denaturing effect on cod

proteins (X. Liu et al., 2022). The mechanical forces and local heating effects generated by ultrasound likely cause protein unfolding, leading to a reduction in their solubility (J. Zhang et al., 2022). Liu et al. (2020) over-treated myofibrillar proteins with ultrasound (600 W) producing flocculation, resulting in reduced solubility. Moreover, the intense mechanical shear forces, temperature, and pressure generated by ultrasonic transient cavitation further destroyed the protein structure and inhibited the protein refolding (Wang et al., 2022). This effect is more pronounced with longer treatment durations, as evidenced by the progressive decrease in soluble protein content of cod samples. Similar results were also noted in the previous study, Dong et al. (2020b) reported that ultrasound (5-20 min) reduced total soluble protein content by up to 10.58-28.26% in shrimp. From previous research data it is known that, by prolonging the treatment period of ultrasonic processing, myofibrillar protein solubility increased and more soluble proteins will be formed (Amiri et al., 2018). Therefore, ultrasound treatment could have a significant effect on soluble protein content, and further analysis of protein structures might be necessary.

Table 4.1. Parameters of ultrasound treatment, total soluble protein content, and secondary structural proportion of ultrasound-treated cod protein estimated from CD spectra in the wavelength of 190-260 nm.

Ultrasound treatments	Durations (min)	Frequency (kHz)	Power (W)	Total soluble protein concentration ($\mu\text{g/mL}$)	Secondary structures			
					α -Helix (%)	β -Sheet (%)	Turns (%)	Unordered (%)
US0	0	40	130	1988.15 $\pm$ 12.42 ^a	33.23 $\pm$ 0.20 ^a	15.67 $\pm$ 0.18 ^d	21.36 $\pm$ 0.25 ^d	29.74 $\pm$ 0.11 ^{cd}
US5	5	40	130	1529.82 $\pm$ 16.11 ^b	32.97 $\pm$ 0.13 ^a	16.04 $\pm$ 0.10 ^d	21.51 $\pm$ 0.23 ^d	29.48 $\pm$ 0.05 ^d
US10	10	40	130	1251.49 $\pm$ 8.72 ^c	31.16 $\pm$ 0.06 ^b	17.39 $\pm$ 0.23 ^c	22.51 $\pm$ 0.07 ^{ab}	28.94 $\pm$ 0.19 ^e
US20	20	40	130	1131.21 $\pm$ 18.6 ^{cd}	26.76 $\pm$ 0.22 ^d	20.84 $\pm$ 0.18 ^b	22.27 $\pm$ 0.14 ^{bc}	30.13 $\pm$ 0.24 ^b
US30	30	40	130	1056.76 $\pm$ 8.76 ^d	27.33 $\pm$ 0.17 ^c	20.57 $\pm$ 0.02 ^b	22.11 $\pm$ 0.09 ^c	29.99 $\pm$ 0.08 ^{bc}
US60	60	40	130	1009.82 $\pm$ 9.01 ^d	14.34 $\pm$ 0.01 ^e	29.31 $\pm$ 0.16 ^a	22.60 $\pm$ 0.15 ^a	33.75 $\pm$ 0.26 ^a

4.4.2 FTIR spectroscopy

The secondary structure conformations of ultrasound-treated cod samples were examined using FTIR spectroscopy (Figure 4.1). Key absorbance peaks corresponding to different protein structures were analyzed, including amide I, II, and III bands. Amide I region, a sensitive marker for protein secondary structure, corresponds to the wavelength of 1700–1600 cm^{-1} . It involves the most prominent vibrational bands (C=O bonds) of the protein backbone structure as noted by Vanga et al. (2020). Amide II (1500-1550 cm^{-1}) is associated with the bending vibrations of N-H bonds and part of the C–N stretching (Chang & Tanaka, 2002). Amide III (1200-1300 cm^{-1}) encompasses both C-N stretching and N-H bending vibrations (Chang & Tanaka, 2002). In Figure 4.1a, the results revealed that ultrasound treatment induces considerable changes in the secondary structure of cod proteins. Higher FTIR absorbance in Amides I, II and III was observed when ultrasound time durations increased. The shift in the amide I band suggests a rearrangement of the protein backbone, potentially leading to a decrease in α -helix and an increase in β -sheet structures. This phenomenon is consistent with the notion that ultrasound induces protein unfolding and refolding due to its mechanical forces as recognized by many researchers including Yang et al. (2017). A shift in the peak positions within the Amide I region was observed following ultrasound treatment. An increase in peak intensity with longer ultrasound time involved a transformation from α -helices to β -sheets as noted by Zhao et al. (2019). Similarly, Cai et al. (2021) found that all peak widths in Amides I, II and III significantly enhanced in silk fibroin fibers after ultrasound processing (0-30 min). This indicates the structure degradation occurred for ultrasound-treated cod samples as recognized by Cai et al. (2021). Additionally, it was noted that following ultrasonication, the peak position in the amide-I region had shifted. This suggests that ultrasonication changed the secondary structure of the sunflower protein as noted in other studies (Li, Yang, Zhang, Ma, Qu, et al., 2016). The ultra-sonication process breaks down many interactions in protein molecules, leading to changes in secondary structure. These interactions could be between distinct regions of the protein molecule like disulphide bonds, and the local sequence of amino acids (Jin et al., 2015).

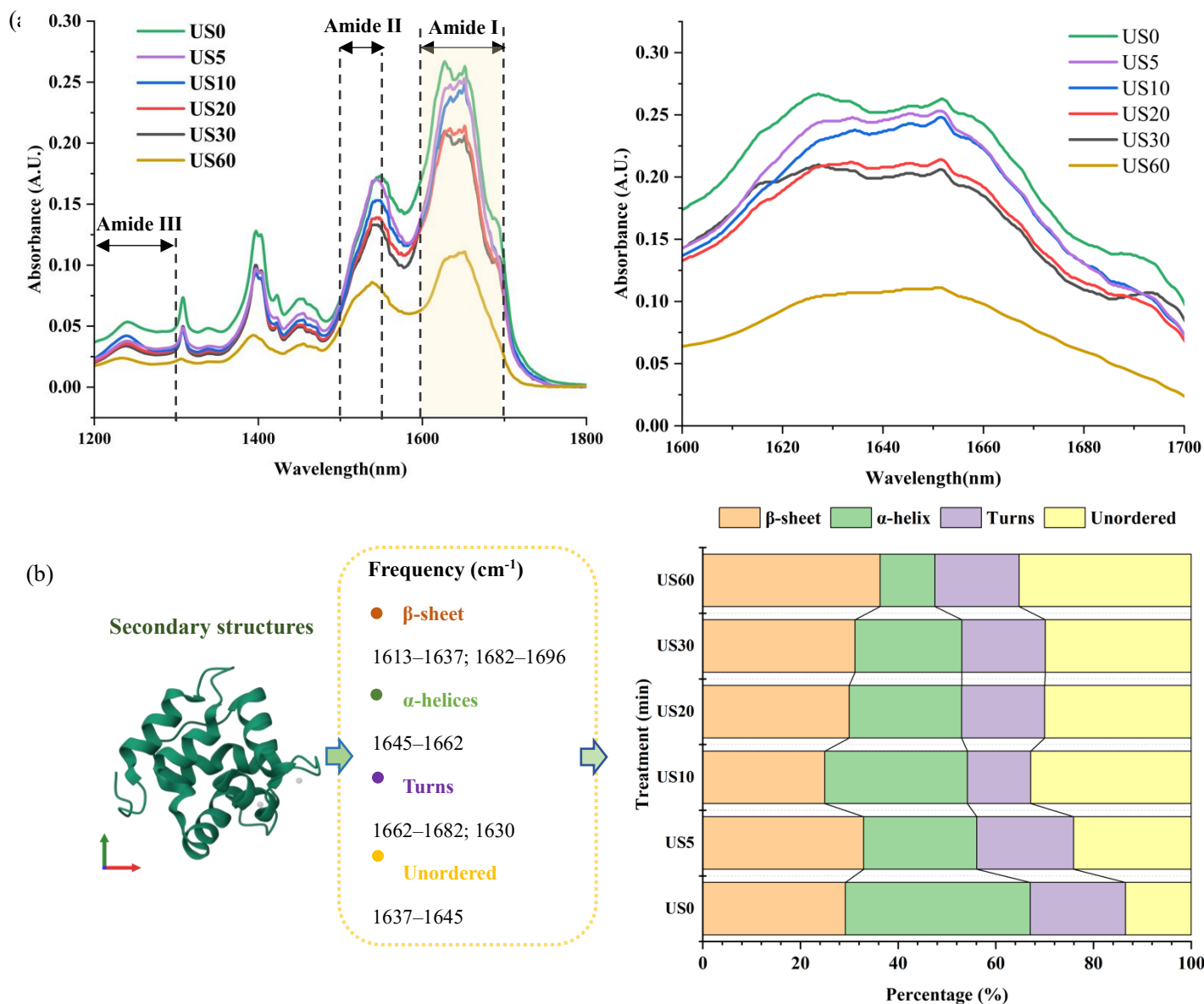


Figure 4.1. FTIR spectra, especially Amide I (a), Parvalbumin beta in PDB entry 2MBX front view and secondary structure percentage of Amide I (b) of cod samples during ultrasonic processing.

The percentages of β -sheet, α -helix, turn, and unordered conformation of cod samples estimated by FTIR results are shown in Figure 4.1b. With longer ultrasound treatment durations, β -sheets and unordered structure increased, and α -helix decreased while turns remain almost unchanged. This is because prolonged exposure to sonication can stretch the α -helix and β -sheets, inducing a shift in the protein's structure and further leading to the unordered structure within the molecule

(Yang et al., 2017). Protein molecules unfolded when the α -helix content decreased, but protein aggregation occurred when the β -content increased as recognized by Zhang et al. (2017). The involvement of β -sheets in the secondary structure of protein aggregates might be attributed to the relatively large surface areas for orderly hydrogen bonding (Wang et al., 2014). However, Vera et al. (2019) reported a drop in β -sheet and an increase in α -helix percentage which occurred in quinoa protein after US processing (5-30 min with on-off pulses of 10 s/10 s and 1 s/5 s). When acoustic cavitation lasts for a longer time, the temperature, pressure, and shear stresses may all increase, which could also cause additional protein structural alterations as recognized by Cheng et al. (2019). Changes in α -helix and β -sheet content may also affect the functional properties of proteins, such as allergenic potential.

4.4.3 CD spectroscopy

CD analysis was employed to evaluate cod samples to further explore secondary conformational changes induced by ultrasonic effect. In Figure 4.2a, the mean residue ellipticity ($[\theta]$) at wavelengths from 190 to 260 nm indicative of α -helices and β -sheets was quantitatively analyzed. After ultrasonic treatment, there was a significant decrease in the $[\theta]$ values at 222 nm and 208 nm, suggesting a reduction in α -helical content. Conversely, an increase in $[\theta]$ at around 218 nm was observed, indicating an increase in β -sheet structures. This phenomenon can be attributed to the mechanical forces generated by ultrasound, leading to the disruption of hydrogen bonds (Li, Yang, Zhang, Ma, Liang, et al., 2016). Besides, the disruption of the interaction between molecules and the relative position of the local sequence of the amino acids might occur during ultrasound processing (Jin et al., 2015). These finally resulted in stabilizing α -helices, thereby promoting the formation of β -sheets.

Moreover, the estimated percentages of α -helices, β -sheets, turns, and unordered structures were calculated (Figure 4.2b and Table 4.1). Ultrasonic treatment resulted in a decrease in α -helix content from 33.23% to 14.34%, while β -sheet content increased from 15.67% to 29.31%. The content of turns is almost stable with 21.36-22.60%; the unordered structure showed a slight increase from 29.74% to 33.75%. This is because turns are regarded as a highly ordered protein structure, while the α -helix, β -sheet, and unordered structures are classified as typical flexible and open protein (Siddique et al., 2016). After a 60-min ultrasound duration, the α -helix content considerably decreased by 56.84% compared to the untreated sample, whereas the β -sheet and the

unordered content increased by 46.54% and 11.88%, respectively. The trend of each secondary structure obtained by CD spectra is similar to that of the FTIR results. These results were probably caused by the protein's exposed molecular chain, which further caused the α -helix's ordered structure to be destroyed and transformed into β -sheets and unordered structures (K. Li et al., 2020). Furthermore, increased β -sheet content was also correlated with more protein-protein interactions within the exposed hydrophobic regions, leading to more intermolecular β -sheet generated (Macdonald et al., 2015). K. Li et al. (2020) reported similar results that the ultrasound (20 kHz, 450 W, 3-6 min) could significantly decrease α -helix and increase β -sheet and unordered structures of chicken myofibrillar protein. Jiang et al. (2014) also found that ultrasonic treatment (20 kHz, 150-450 W, 12/24 min) caused a drop in the α -helix proportion and a rise in the β -sheets content in the black-bean protein isolate by using CD analysis. However, ultrasonic treatment (20 kHz, 450 W) could increase the α -helix component and decrease the β -sheet component of whey protein concentrate (Vivian & Callis, 2001). According to this study, the alteration may have been brought about by a breakdown in the interactions that occur after sonication between different regions of the molecule and between local sequence lengths of amino acids, potentially leading to the aggregation of protein molecules (Jiang et al., 2014). Thus, ultrasonic treatment can effectively change the secondary structure of cod proteins.

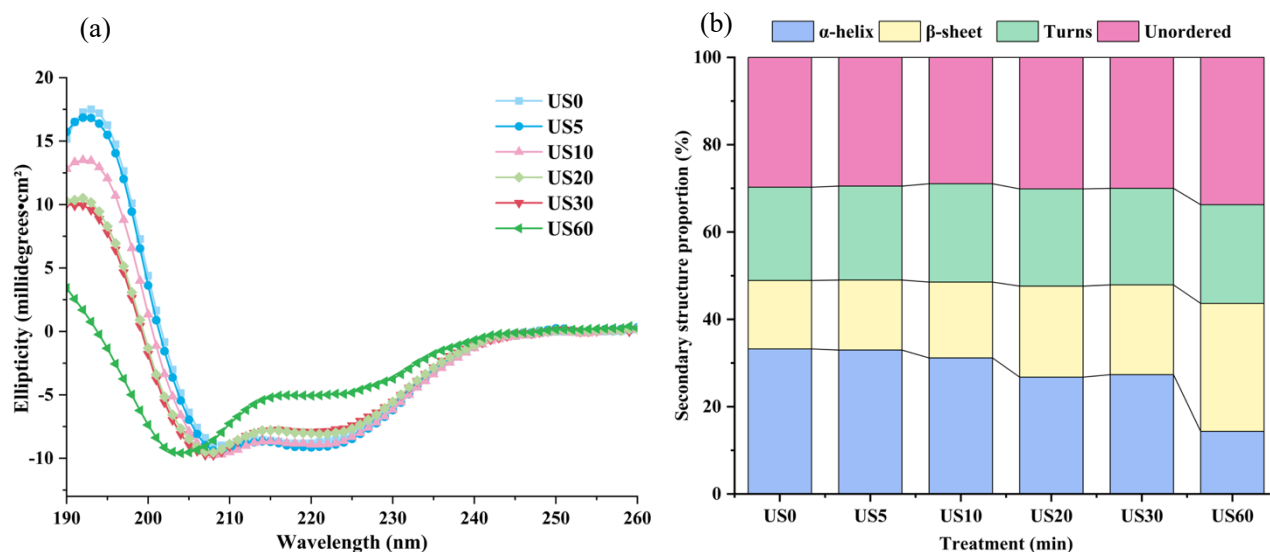


Figure 4.2. CD spectrum (a) and the percentage of secondary structure (b) in cod samples with ultrasonic processing.

Furthermore, some differences exist between the results obtained from FTIR and CD, particularly regarding the contents of α -helix, β -sheet and unordered structures. The sample status may be one of the reasons between different FTIR and CD readings. Lyophilized powder was employed in FTIR analysis, while aqueous solution was used in CD analysis. Additionally, different definitions for various secondary structure types are applied to FTIR and CD. The use of empirical basis sets as standards in some computations which is a different factor contributing to the differences as recognized by Zhu et al. (2018b).

4.4.4 UV spectroscopy

The alteration in the tertiary structure of cod proteins can be characterized on the basis of UV absorption spectra (Figure 4.3). The ultraviolet (UV) spectrum from 200 to 400 nm is a crucial range for analyzing the structural properties of cod proteins (Figure 4.3a). It can be divided into three bands, UVA (315–400 nm), UVB (280–315 nm) and UVC (200–280 nm) as recognized by Bajgar et al. (2021). The region around 200–220 nm is sensitive to the peptide bond itself. Absorbance in this range can provide information about the backbone conformation of proteins (Yang et al., 2023). The region between 250 and 280 nm is particularly important for detecting aromatic amino acids like tryptophan, tyrosine, and phenylalanine (Reinmuth-Selzle et al., 2022). In Figure 4.3b, the UV absorbance of cod extracts was observed at a wavelength of 190–400 nm with a maximum absorbance at 250 nm. Compared with the untreated sample, the ultrasound-treated samples showed an increase in UV absorbance. The results exhibited a greater blue shift at the absorption peak as longer durations.

Biter et al. (2019) reported proteins primarily absorb UV light due to the presence of tryptophan, tyrosine, and phenylalanine residues, with absorbance maxima at 280, 275, and 258 nm, respectively. In this study, the absorbance peak at 250 nm probably could be influenced by the presence of phenylalanine and some possible structural elements of the protein (Mach et al., 1991). Similarly, Ma et al. (2018) revealed that ultrasonic durations (20 kHz, 10–30 min, amplitude by 20–40%, 40–50 °C) reduced the UV absorbance in β -lactoglobulin. It might be brought about by modifications in the distribution of certain amino acids (Ma et al., 2018). Li and Xiong (2021) reported that a UV absorbance peak at around 260 nm was observed due to the phenylalanine that existed in oat proteins after ultrasound treatment of 0 to 5 minutes. This can be explained that protein denaturation and molecular unfolding might have caused by ultrasound. It may also break

down hydrophobic connections between protein molecules, causing hydrophobic groups to migrate in opposite directions and reducing the intensity as observation made by Zou et al. (2017).

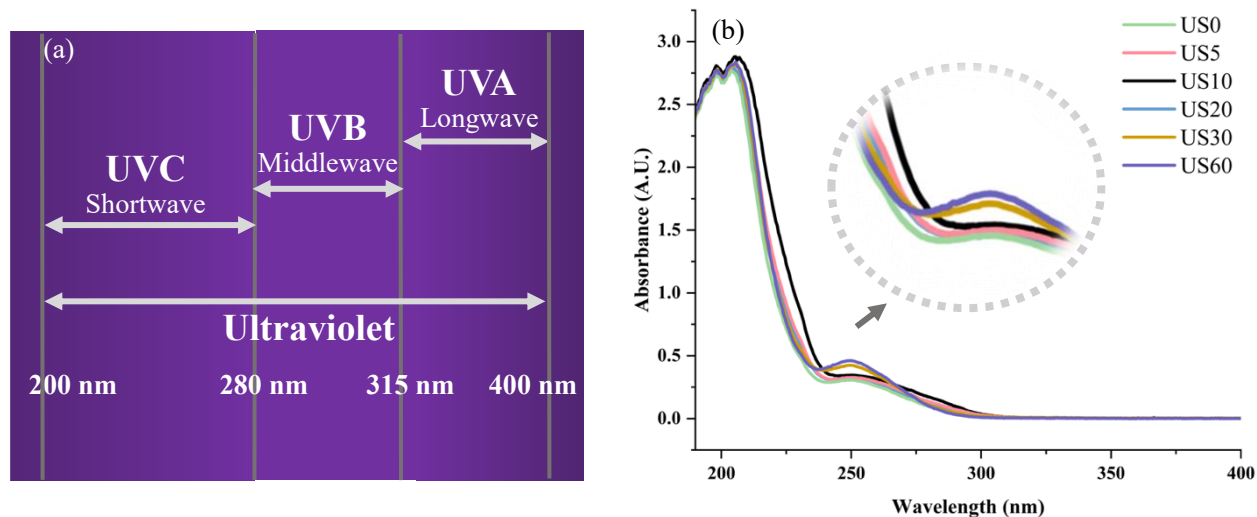


Figure 4.3. UV spectrum (a) and UV absorbance of cod samples before and after ultrasound treatments (b).

4.4.5 Scanning electron microscopy (SEM)

SEM was carried out to observe the microstructure changes in cod samples under different ultrasound treatments. A series of SEM images with a magnification of 100 times is shown in Figure 4.4. It was observed that the untreated cod sample (US0) presented the highest density and the most complete flake with few cracks. As the enhanced ultrasound durations, more and more fragments appeared in ultrasound-treated cod samples. It was more evident that more microstructural holes and strips were generated in US30 and US60. Among these differences, the most prominent were the faveolate holes observed on the ultrasound-treated sample, in contrast to the smooth surface of the untreated sample. These were because ultrasonic cavitation and mechanical effects caused tiny debris and irregular pores as observation made by Sheng et al. (2018). Ultrasonic treatment could cause the non-covalent interactions between protein molecules and changes in the spatial structure of cod proteins, and further increased the surface roughness of the protein (Q. Liang et al., 2021). Since protein often resides in aqueous solutions as soluble aggregates, the average hydrodynamic size of the aggregate is the size of the protein. Aggregates were agitated violently and collided during sonication, producing smaller fragmented particles

with a wider size dispersion (Arzeni et al., 2012). Similar results were reported in ultrasound-treated soy (Tian et al., 2020), okara fibers (Fan et al., 2020), and beef muscle (Barekat & Soltanizadeh, 2018). The findings in microstructural changes are consistent with the analysis of secondary structure. Therefore, the structural changes that occurred in cod protein may finally affect the alterations of cod allergenicity.

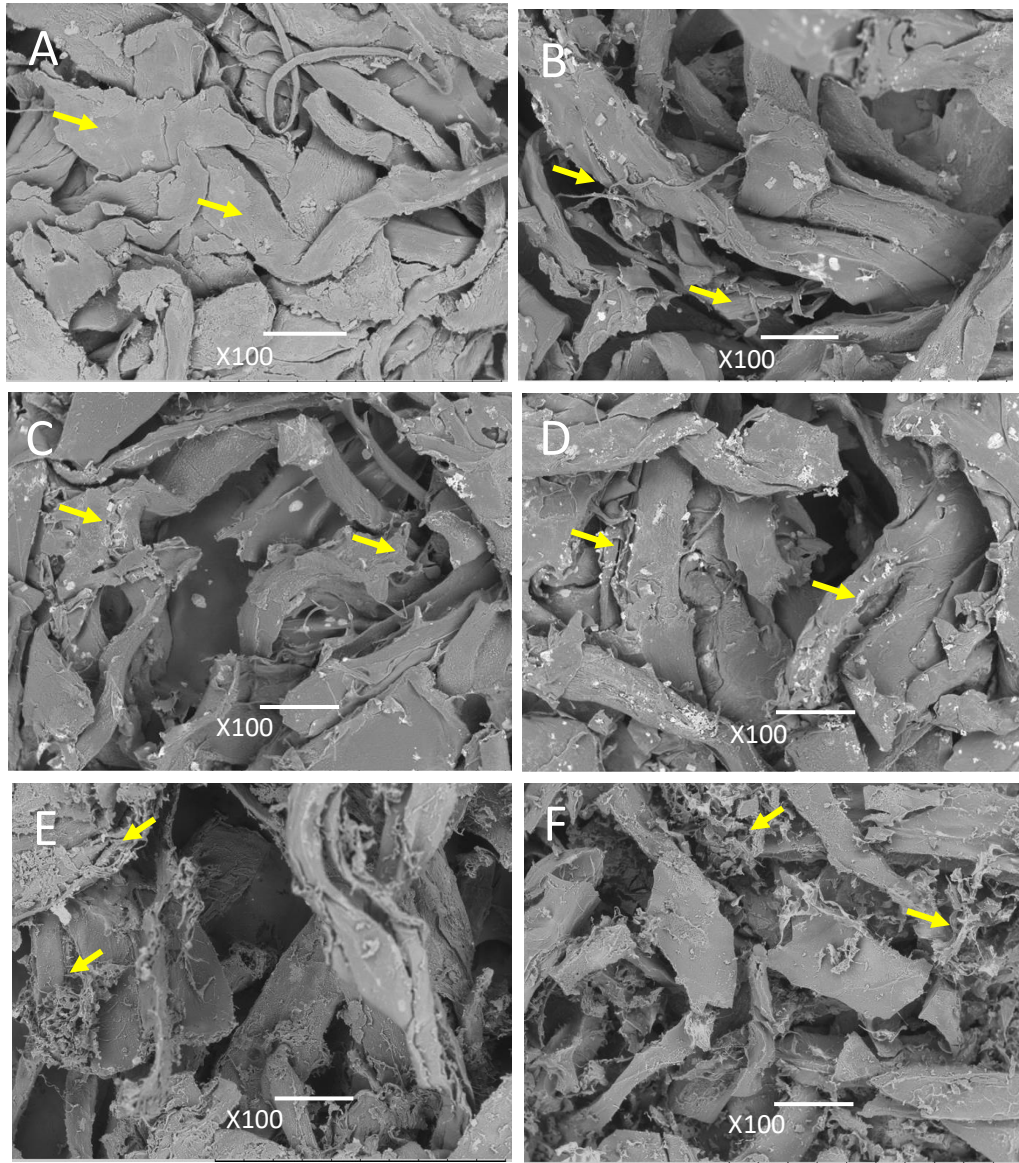


Figure 4.4. Microstructure of cod samples determined by scanning electron microscopy with the magnification of 100 times. US0 (A); US5 (B); US10 (C); US20 (D); US30 (E); US60 (F).

4.4.6 SDS-PAGE

SDS-PAGE was performed to analyze the protein profile of cod samples subjected to ultrasound treatment (Figure 4.5a). The protein bands showed a decreasing intensity with an increasing processing duration. The bands in US0 showed the highest intensity. After 5-10 min, the band intensity was lower slightly but visibly; after 20-60 min, the band intensity was lower greatly and was noticeable, especially in the US60 treatment with the lightest bands. The band intensity changes are consistent with the trend in the total protein content during ultrasound treatment. According to US0, more disappeared bands were observed in the ultrasound-treated samples, indicating the degradation of existing proteins. Changes in the apparent molecular weight of certain protein bands were observed, which might reflect protein aggregation or fragmentation due to the ultrasound treatment (Liu et al., 2020).

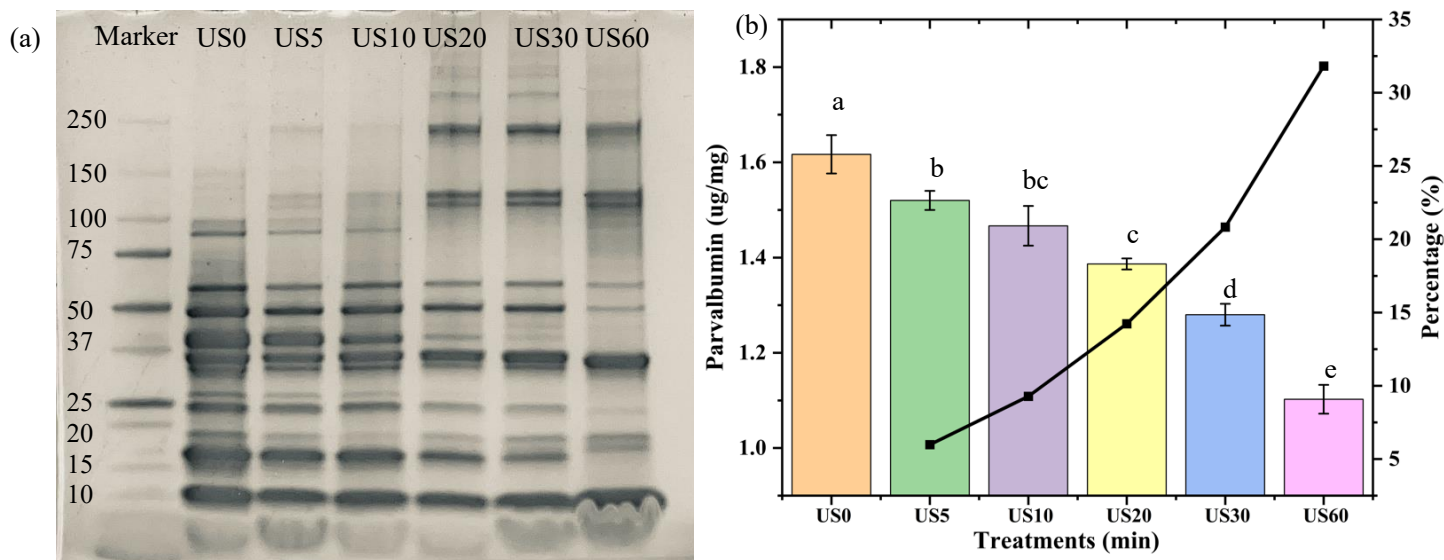


Figure 4.5. SDS-PAGE protein band (a) and ELISA analysis (b) of cod samples before and after ultrasound treatments.

Similar results have been reported in the shrimp, milk, and kiwifruit juice after ultrasound processing up to 0-60 min (Dong et al., 2020b; C. Wang et al., 2020; Jin Wang et al., 2021). However, Tammineedi et al. (2013) revealed that high-intensity ultrasound conditions (20 kHz, 500 W, 10-30 min) were not effective in lowering the allergenicity of major milk proteins. There was no visible change in the band intensities of SDS-PAGE gel, so higher intensities and longer treatment time were needed (Tammineedi et al., 2013). In this study, high-intensity ultrasonic

energy has altered the cod structure and further reduced the immunoactivity of cod allergens. To validate this result, the quantification procedure by ELISA test was conducted in the following work.

4.4.7 ELISA analysis

A sandwich ELISA assay was utilized to measure the variations in parvalbumin content of cod samples subjected to ultrasound treatment. As presented in Figure 4.5b, there was a steady decline in parvalbumin as the treatment duration increased. The lowest allergen content was observed in US60 with a decrease of 31.82%, followed in sequence by US30 (20.83%), US20 (14.23%), US10 (9.28%) and US5 (5.98%). Notably, the parvalbumin concentration in ultrasound-treated samples exhibited a significant reduction ($p < 0.05$) compared to the US0. In a similar vein, the parvalbumin levels during 20-60 min were significantly diminished ($p < 0.05$) in relation to samples with shorter time. However, the decrease between 5-10 min or 10-20 min was not of statistical significance. There is a possible correlation between the duration of ultrasound treatment and the denaturation or reduction of parvalbumin. Similarly, C. Wang et al. (2020) found that allergenicity reduction of milk casein was tightly correlated with the ultrasound treatment (25kHz, 900 W for 30-60 min). Dong et al. (2020b) reported that a maximum allergenicity reduction of 76% in shrimp was observed after a high-intensity ultrasound (5-20 min). The allergenicity reductions were strongly associated with changed structures due to cavitation effects occurring during the ultrasound processing (Dong et al., 2021a). It is evident that US60 demonstrated the most pronounced decrease in allergenicity, which suggests that an extended ultrasound treatment can be highly effective in reducing cod parvalbumin. Although ultrasound is an effective approach to mitigate allergenicity in cod samples, longer durations may affect other quality parameters of the cod.

4.5 Conclusion

This study has revealed that ultrasound treatment substantially alters the structural and allergenic properties of cod proteins. The primary effect observed is a decrease in total soluble protein content, likely due to ultrasound-induced protein denaturation. This outcome is further supported by FTIR and CD spectroscopy results, which indicate a transition in protein structures from α -helix to β -sheet and unordered forms, suggesting significant conformational changes due to the mechanical impact of ultrasound. Additionally, UV spectroscopy, SEM, and electron microscopy analyses corroborate these findings, showing changes in tertiary structure, microstructure, and color

indicative of protein denaturation and potential Maillard reactions. SDS-PAGE results align with these observations, demonstrating protein degradation and aggregation. Moreover, ELISA analysis indicates a reduction in allergen content by 5.98-31.82% with increased ultrasound durations, highlighting the potential of ultrasound treatment in modifying allergenic properties. Therefore, these findings suggest that ultrasound treatment, as a novel approach in food processing, can effectively alter protein structures, reduce fish allergenicity, and further enhance the safety of seafood products and acceptability for individuals with fish allergies. However, further studies need to be conducted involving ultrasound treatments with prolonged durations and increased intensity of exposure.

Conflict of Interest

The authors declare no conflict of interest reported in this paper.

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Contextual transition

Chapter 4 revealed that ultrasound treatment substantially alters the structural and allergenic properties of cod proteins. In the next chapter, we will also evaluate the impact of high-intensity ultrasound (HIU) on the physicochemical properties and *in-vitro* digestibility of Atlantic cod.

Chapter 5

Manuscript IV – Influence of high-intensity ultrasound on physicochemical characteristics and *in-vitro* digestibility of Atlantic cod

Xin Dong * and Vijaya Raghavan

Department of Bioresource Engineering, Faculty of Agricultural and Environmental Sciences,
McGill University, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

*Corresponding Author- Email: xin.dong2@mail.mcgill.ca

ORCID: <https://orcid.org/0000-0002-9052-3563>

5.1 Abstract

This study evaluates the impact of high-intensity ultrasound (HIU) on the physicochemical properties and *in-vitro* digestibility of Atlantic cod (*Gadus morhua*). Various ultrasound durations (0-60 min) were applied to assess changes in color attributes, total antioxidant capacity (TAC), total flavonoid content (TFC), total phenolic content (TPC), total protein content, and *in-vitro* protein digestibility (IVPD). Results indicated HIU maximumly increased TAC, TFC, TPC, and peptide content before digestion by 7.28% (US60), 3.00% (US30), 32.43% (US10), and 18.93% (US60), respectively. While HIU reduced total protein content, it enhanced IVPD by up to 12.24% (US30). Color attributes and electron microscopy reflected structural changes in the cod samples, suggesting the effectiveness of HIU in altering protein structures. These findings highlight HIU's potential as a non-thermal technique for improving the sensory and nutritional quality of Atlantic cod, offering valuable insights for the seafood processing industry and consumers.

Keywords: High-intensity ultrasound; Atlantic Cod; *in-vitro* protein digestibility; physicochemical properties; seafood processing technology

5.2 Introduction

Atlantic cod (*Gadus morhua*), a popular seafood globally, combines high nutritional value with significant culinary versatility (Jafarpour et al., 2020). This species is a rich source of proteins, omega-3 fatty acids, vitamins, and minerals, contributing to its widespread acclaim in human diets (Khalili Tilami & Sampels, 2018). However, the full potential of Atlantic cod is often untapped due to challenges in digestibility and allergenicity, prompting a search for innovative processing methods to enhance its food quality (Zhang et al., 2019).

High-intensity ultrasound (HIU) has emerged as a novel food processing technology, noted for its ability to induce physicochemical changes in food matrices. Utilizing sound waves at frequencies beyond human hearing, HIU offers a non-thermal, environmentally friendly approach, capable of improving the texture, flavor, and shelf life of seafood (Zhou et al., 2023). Specifically, in the context of Atlantic cod, HIU presents an opportunity to modify protein structures, thereby potentially improving the digestibility and reducing the allergenicity (Dong et al., 2020b; Zhang et al., 2018).

In-vitro protein digestibility is a crucial index in assessing the quality of protein-rich foods. The ability of HIU to alter protein conformation, unravelling and exposing new sites, could enhance the digestibility of seafood, such as shrimp (Dong et al., 2020b; Zhang et al., 2018), crab (Yu et al., 2011), and New Zealand Abalone (Bagarinao et al., 2020). Such improvements in protein digestibility are essential for maximizing the nutritional benefits and reducing adverse reactions, particularly in sensitive individuals (Sá et al., 2020). However, few studies to date have investigated the effect of HIU on fish products, despite fish being one of the most common seafood in the human diet.

Therefore, this study aims to comprehensively explore the effects of high-intensity ultrasound on the physicochemical characteristics and *in-vitro* digestibility of Atlantic cod. We investigate how HIU alters protein profiles, impacts texture, and overall quality, and assesses potential enhancements in digestibility. This investigation is poised to contribute significantly to the field of food science, offering new insights into the application of HIU in seafood processing and potentially redefining the consumption standards of Atlantic cod.

5.3 Materials and Methods

5.3.1 Ultrasound-treated Atlantic cod samples preparation, pH and temperature determination

The preparation of cod samples and experimental setup are shown in Figure 5.1. The Atlantic cod meat was obtained from a local seafood market (Montreal, Canada). After cleaning, the cod meat was cut into small pieces. Then, a mincer (GM 200, Retsch) was used to homogenize the cod meat. The homogenized cod was mixed with distilled water and then placed into an ultrasonic cleanser (FS30, Fisher Scientific, USA) for different time durations (0, 5, 10, 20, 30, and 60 mins). Part of the treated samples was used for pH, temperature, color, and microscope observation. A pH meter (accumet AB150, Fisher Scientific, USA) and a thermometer (KT800 AINYPET) were used to immediately determine the pH value and temperature of the samples after treatment time. For each sample, the probe was inserted into the mixture five separate times. The measurements from these five insertions were then averaged, and the resulting mean values were recorded (Table 5.1). The rest of the samples were freeze dried (Freeze dryer, model 7420020, Labconco Corporation, Kansas City, USA) for 48 h and then stored in the fridge at -20 °C for further analysis.

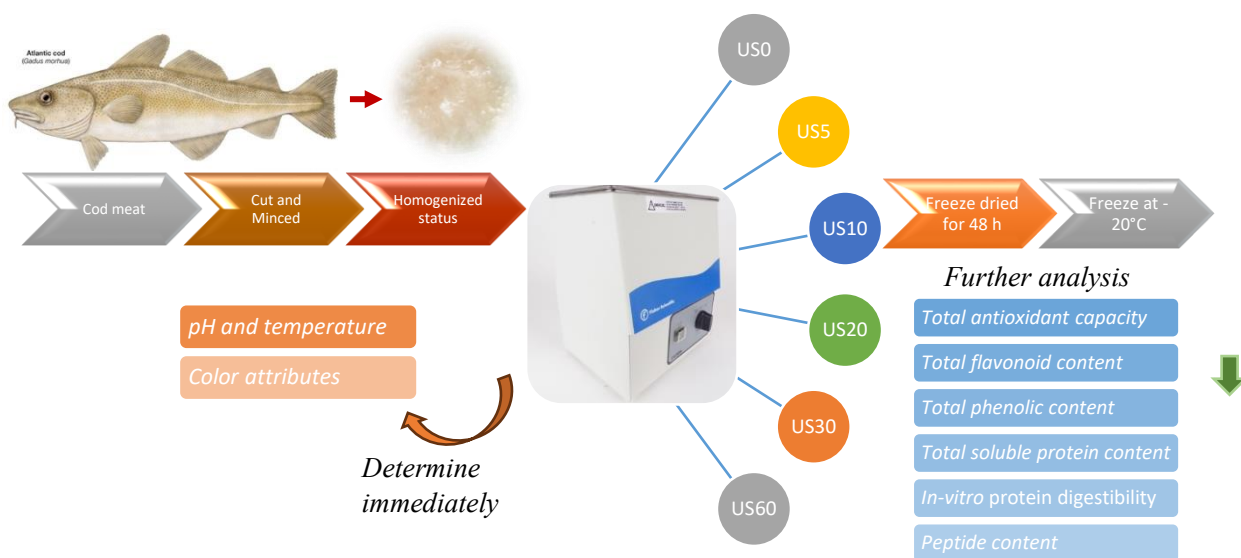


Figure 5.1. Process of sample preparation and experiment overview.

5.3.2 Color attributes

A colorimeter (CR-300 Chroma, Minolta, Japan) with 2° N skylight as the light source was utilized to determine the color attributes of cod samples. The calibration procedure was conducted by placing the hand shank on a specific white tile ($Y = 93.35$; $x = 0.3152$; $y = 0.3212$) until the value on the screen was the same as that on the tile. Three parameters were recorded: they are L^* (light /dark), a^* (red/green) and b^* (yellow/blue) as per the Commission Internationale de l'Eclairage (CIE) Lab color parameters, whose range were from 0 (black) to 100 (white), -60 (green) to $+60$ (red), and -60 (blue) to $+60$ (yellow), respectively (Pathare et al., 2013; Xiao et al., 2010). The total color difference (TCD), hue angle (h), chroma (C), yellow index (YI), and color index (CI), browning index (BI) were evaluated according to the equations noted below (1)-(6) (Chen et al., 2016; Ordóñez-Santos et al., 2017):

$$TCD = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (1)$$

$$h = \tan^{-1} \frac{b^*}{a^*} \quad (2)$$

$$C = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$YI = \frac{142.86b^*}{L^*} \quad (4)$$

$$CI = \frac{180-h}{L^*-C} \quad (5)$$

$$BI = \frac{100(x-0.31)}{0.172} \quad (6)$$

$$\text{where } x = \frac{a^*+1.75L^*}{5.645L^*+a^*-3.012b^*} \quad (7)$$

In above equations, a_0^* , b_0^* and L_0^* represent the color parameters of the sample without ultrasound treatment and were immediately measured after homogenization; a^* , b^* and L^* represent the samples treated by ultrasound treatment and were determined promptly after processing. Each sample was measured eight times to reduce bias to record the mean values.

5.3.3 Total antioxidant capacity (TAC)

The total antioxidant activity of the cod sample was measured by ferric-reducing antioxidant power (FRAP) assay as previously described by Dong et al. (2021b). The absorbance at 593 nm was

measured by a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada). The antioxidant activity was calculated as $\mu\text{mol Fe (II)}/100\text{ mL}$ of cod samples.

5.3.4 Total flavonoid content (TFC)

Total flavonoids content was determined according to the method of Osae et al. (2019). Briefly, 0.5 ml of the extracted solutions were mixed with 2 ml of distilled water, and then 0.15 ml of a 5% (v/v) NaNO_2 solution was added, allowing the reaction to proceed for 6 min. After this initial incubation, AlCl_3 (0.15 mL, 10% v/v) solution was added for a further incubation of 6 min. Subsequently, NaOH (2 mL of a 4% v/v) solution was added, followed by adding distilled water to achieve a final volume of 5 mL. The final mixture was subjected to the absorbance measurement at 510 nm after 15 minutes and it was recorded by a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada). The results were expressed in terms of milligrams of catechin equivalents (CE) per gram of dry weight of the cod sample.

5.3.5 Total phenolic content (TPC)

The total phenolic content was determined by following the methodology (Feng et al., 2021; Osae et al., 2019). Folin–Ciocalteu reagent (1:10 v/v) was diluted by distilled water. The extract solution (1 mL) was mixed with Folin reagent (5 ml), followed by adding Na_2CO_3 (4 ml, 75 g/L). The mixture was vortexed for 10 min and left to incubate for 30 min at 30°C. The absorbance was measured at 760 nm by using a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada). The results obtained were expressed as mg of gallic acid equivalents (GAE) per gram g of dry weight of the cod sample.

5.3.6 Total soluble protein content

Phosphate-buffered saline (PBS, 0.1 M, pH = 7) solution was used to extract freeze-dried cod powder (0.2 g) to assess the total soluble protein content. The mixture was centrifuged at $5000\times g$ for 20 minutes at 4 °C after being incubated for 30 minutes at room temperature. The supernatant was then gathered. By following the instructions, a PierceTM BCA protein assay kit (Thermo Fisher Scientific, Canada) was used to measure the total soluble protein concentration in cod samples.

5.3.7 *In-vitro* protein digestibility

The *in-vitro* digestibility of cod proteins was determined in two stages following the previous

procedure mentioned by Dong et al. (2021b). Freeze-dried cod powder (0.2 g) was mixed with a PBS buffer (0.1 M, 10 mL, pH = 7.4) to a final mixture concentration of 20 mg/mL. The total protein contents before and after each digestion stage were determined by the BCA method. The *in-vitro* protein digestibility (IVPD%) for two stages was calculated using the following equation:

$$\text{IVPD}\% = \frac{\text{protein content before digestion} - \text{protein content after digestion}}{\text{protein content before digestion}} \times 100$$

5.3.8 Peptide content

The peptide content was determined using the o-Phthaldialdehyde (OPA) Reagent. The OPA reagent was prepared by adding 25 mL of 100 mM sodium tetraborate in water, 2.5 mL of 20% (w/v) sodium dodecyl sulfate, 40 mg of OPA dissolved in 1 mL of methanol, 100 µL of β-mercaptoethanol, and ddH₂O to a final volume of 50 mL. A 10 µL of digestion mixture was incubated for 2 min with a 190 µL OPA reagent solution. The absorbances were read at 340 nm using a plate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada). Leucine-glycine was used to obtain the standard curve.

5.3.9 Optical microscope

As described by Dong et al. (2020b), diluted cod samples (15 µL) were stained using 0.1% of toluidine blue (Sigma-Aldrich, Quebec, Canada) solution on the glass slide for 2 min. An optical microscope (Leica DM500, Leica Microsystems Inc., Canada) and imaging software (Leica LAS EZ, Leica Microsystems Inc., Canada) were used to observe the microstructural changes and image capture magnification at 4 times was done.

5.3.10 Statistical analysis

The analysis of variance (ANOVA) feature of the SPSS program (IBM SPSS Statistic, Ver. 29.0.0.0) was used to examine the experimental data. The means were separated using the Duncan, multiple range test, and significance was established at $p \leq 0.05$.

5.4 Results and Discussion

5.4.1 Color attributes

The color attribute is an important index of food visual appeal. The color profile may affect consumer acceptance and preference for food products. As shown in Table 5.2, the initial decrease in L from US0 to US5 was followed by a subsequent increase and finally a decrease at US60 (68 ± 1.1). This indicates more loss of brightness with prolonged treatment. Similarly, J. Wang, J. Wang, et al. (2019) found a slight increase in L^* value up to 12 min and a decrease at 16 min in strawberry juice after ultrasound application (4-16min). This could be attributed to cellular disruptions caused by the cavitation effect of ultrasound (Greenly & Tester, 2015).

The value raised with longer ultrasound treatment time, indicating a shift towards the green coordinate in the cod samples. This, in conjunction with the observed drop in the b from US10 to US20, indicates an initial shift from yellow to blue-green hues. The mechanistic reasons behind such shifts could be related to the breakdown of specific pigments or oxidative reactions induced by ultrasound (Du et al., 2022). Interestingly, the TCD peaked at US10, implying that the most significant color deviation from the reference occurs within the initial 10-min ultrasound treatment. Past this peak, the differences seem to stabilize, suggesting an equilibrium in the color changes. The observed decline in C underscores a potential dulling of color intensity. Such a phenomenon could be linked to the degradation of chromophores or other color-imparting compounds in the cod samples (Pandiselvam et al., 2023). Concurrently, the decrease in the BI suggests reduced browning, a desirable attribute, especially considering the negative connotations associated with browning in food products. The transition in h values from negative to positive territories is indicative of a complex interplay of factors, potentially involving enzymatic activities, pigment interactions, and other physicochemical processes (Li et al., 2021).

The findings illuminate the significant impact of ultrasound treatment on the color attributes of cod samples. Meurer et al. (2020) reported color of foams increased in Aquafaba after the 30-min ultrasound treatment (10-30 min). Kutlu et al. (2022) also concluded the effects of ultrasonication applications on the colors of foods are quite positive. The proper determination of the ultrasonication conditions used is very effective in preserving the color of the food.

Table 5.1. Determination of physicochemical properties (pH, temperature, TAC, TFC, TPC and total protein content) in cod samples during ultrasonic durations.

Treatments	pH	Temperature (°C)	TAC (n/umol/100mg)	TFC (mg/g)	TPC (mg/g)	Total protein content (mg/g)
US0	6.73±0.01 ^b	21.07±0.06 ^d	171.67±1.26 ^c	2.34±0.46 ^a	0.37±0.06 ^c	93.21±1.12 ^a
US5	6.76±0.01 ^a	23.03±0.85 ^c	199.33±3.31 ^a	2.28±0.39 ^a	0.45±0.08 ^b	67.42±0.89 ^c
US10	6.71±0.01 ^c	26.97±0.15 ^b	160.83±1.4 ^d	1.99±0.07 ^b	0.49±0.04 ^a	71.88±0.56 ^b
US20	6.77±0.01 ^a	28.3±0.1 ^b	155.33±1.51 ^e	1.87±0.08 ^b	0.39±0.03 ^d	53.21±0.57 ^f
US30	6.73±0.01 ^b	30.44±0.48 ^a	152.17±2.32 ^f	2.41±0.68 ^a	0.41±0.04 ^c	56.56±0.01 ^d
US60	6.76±0.01 ^a	31.72±0.07 ^a	184.17±1.97 ^b	2.39±0.42 ^a	0.4±0.01 ^{cd}	56.11±0.34 ^e

Table 5.2. Changes in the color attributes of ultrasound-treated cod samples.

Treatment	L	a	b	TCD	C	h	CI	YI	BI
US0	72.32±4.09 ^b	1.12±0.25 ^e	5.84±0.82 ^b	-	5.95±0.8 ^c	1.38±0.05 ^a	2.74±0.15 ^c	11.53±0.32 ^c	7±1.11 ^b
US5	66.9±3.4 ^c	1.92±0.06 ^{cd}	5.93±2.21 ^b	6.23±5.71 ^d	6.26±2.08 ^b	1.23±0.14 ^{ab}	3±0.2 ^b	12.67±0.84 ^b	6.89±3.49 ^b
US10	73.99±15.31 ^{ab}	1.81±0.62 ^d	4.04±2.02 ^c	10.45±3.27 ^a	4.44±2.09 ^d	1.13±0.07 ^b	2.68±0.59 ^d	7.58±2.76 ^d	3.53±1.67 ^d
US20	75.05±1.44 ^a	2.39±0.19 ^b	1.96±0.63 ^d	9.5±1.08 ^b	3.1±0.51 ^e	0.67±0.14 ^c	2.49±0.07 ^e	-3.74±1.25 ^f	4.73±1.01 ^c
US30	75.75±1.12 ^a	2.78±0.22 ^a	0.78±0.9 ^e	7.3±2.64 ^c	2.96±0.43 ^e	0.25±0.29 ^d	2.48±0.06 ^e	1.49±1.72 ^e	1.62±0.91 ^e
US60	68±1.1 ^c	2.08±0.17 ^c	8.11±0.36 ^a	5.33±2.35 ^e	8.38±0.3 ^a	1.32±0.03 ^a	3.04±0.07 ^a	17.05±1.01 ^a	10±0.91 ^a

5.4.2 Total antioxidant capacity (TAC)

The antioxidant capacity (TAC) in cod samples during ultrasound treatments was evaluated. In Table 5.1, the initial antioxidant capacity in US0 was 171.67 ± 18.26 n/umol/100mg. Notably, fluctuations in antioxidant capacity were evident as the ultrasound treatment time increased. In US5, a notable increase in antioxidant capacity was observed, reaching 199.33 ± 36.31 n/umol/100mg. This suggests that a short duration of ultrasound treatment might enhance the antioxidant potential of cod samples. A continued decline in antioxidant capacity was observed at US10 (160.83 ± 10.39 n/umol/100mg), followed by US20 (155.33 ± 17.50 n/umol/100mg) and US30 (152.16 ± 20.32 n/umol/100mg). These findings indicate that there might be a threshold after which the beneficial effects of ultrasound diminish or even revert, leading to a decrease in antioxidant potential (Dzah et al., 2020). However, the highest antioxidant capacity was observed at US60 (184.17 ± 15.97 n/umol/100mg). This suggests that prolonged ultrasound time might have a rejuvenating effect on the antioxidant capacity in cod samples, in spite of not reaching the peak observed in US5.

Similarly, F.-F. Liu et al. (2022) found the antioxidant activities in Mung bean protein hydrolysate which increased with ultrasound power (114 W, 222 W, 330 W, 438 W and 546 W) for 20 min. The superior antioxidant activities were also observed in the treatment with the highest power (546 W). Zou et al. (2019) reported a significant increase in antioxidant properties occurred in chicken plasma protein after ultrasound treatment (20 kHz, 5–30 min). The antioxidant abilities of proteins depend on specific amino acid residues and sequences. Active amino acid residues are transformed and exposed through ultrasound treatment, which contributes to reactions with oxidants (Kong & Xiong, 2006). The variability in results with different durations underscores the complex interactions between ultrasound waves and the biochemical constituents of cod. It is plausible that ultrasound waves might initially disrupt cellular structures, releasing antioxidant compounds (Barba et al., 2015). However, prolonged exposure might degrade these compounds, explaining the dip observed with intermediate durations (Jiang et al., 2006). Ultrasonic treatment would be a promising method to improve the antioxidant capacity of cod, which would broaden the application scope for producing bioactive components in the food industry.

5.4.3 Total flavonoid content (TFC)

The results of total flavonoid content (TFC) in cod samples subjected to ultrasound treatment for various durations are summarized in Table 5.1. The control group (US0) as an essential benchmark displayed a TFC mean of 2.34 ± 0.46 mg/g. After ultrasound durations of 5-20 min, the TFC decreased with different levels. A slight decline in TFC was observed at US5 with 2.28 ± 0.39 mg/g, followed by the continued decrease at US10 (1.99 ± 0.07 mg/g) and US20 (1.86 ± 0.08 mg/g). After ultrasound durations of 30-60 min, an increase was observed at US30 (2.41 ± 0.68 mg/g) and at US60 (2.39 ± 0.41 mg/g) compared to the TFC in control. Prolonged exposure to ultrasound might lead to the disruption of more resilient cellular structures or matrices, which were initially resistant to shorter ultrasound durations. Yusoff et al. (2022) found that ultrasound was effective in extracting bioactive compounds, during which more flavonoids were generated. This can explain the observed increase at treatment of US30 through US60 due to the newly released flavonoids. Nadeem et al. (2018) also found that sonication (20 kHz, 525 W power, 5 min at 15 °C) significantly enhanced the flavonoid content of carrot-grape juice blend. Ultrasound may enhance the release of bioactive compounds through the collapse via cavitation in the surroundings of colloidal particles (Tan et al., 2022). Since there is a correlation between bioactive compounds and antioxidant activity, the total antioxidant activity meanwhile increased as discussed above (Siriamornpun & Kaewseejan, 2017).

5.4.4 Total phenolics content (TPC)

The total phenolic content (TPC) of ultrasound treatment on cod samples is presented in Table 5.1. The total phenolic content varied with varying ultrasound treatment times. As the treatment time increased, the total phenolic content fluctuated. The initial stages of the ultrasound treatment (from US0 to US10) led to an increase in the total phenolic content, reaching its peak at US10 with an average value of 0.49 ± 0.03 mg/g. However, as the treatment time was extended to US20, there was a decrease in phenolic content (0.3927 ± 0.03 mg/g), and this decreasing trend persisted until US30. These results suggest that the efficiency of ultrasound treatment in extracting phenolic compounds might be optimal at shorter durations, specifically around 10 min (Savic Gajic et al., 2019). Prolonged exposure could potentially degrade the phenolic compounds or alter the extractability (Durmus & Kilic-Akyilmaz, 2023). Interestingly, a slight increase was observed again at US60 (0.40 ± 0.01 mg/g), suggesting that longer ultrasound durations might again be

favoring the extraction of phenolics, albeit to a lesser extent than the initial stages. Therefore, ultrasound treatment has a significant influence on the phenolic content extraction from cod samples. Shorter treatment durations, particularly around 10 min, seem to be optimal for maximizing the extraction of phenolic compounds. Milićević et al. (2021) found that the total phenolics from oat and wheat bran fluctuated after the treatment of ultrasound (20–45 kHz). Interestingly, the maximum phenolic was obtained also in the shorter time of extraction (10 min instead of 24 h), which meanwhile could be beneficial in terms of energy efficiency. Notably, both very short and prolonged treatment times do not yield the maximum possible phenolic content. However, J. Wang, S. K. Vanga, et al. (2019) reported that the high-intensity ultrasound (400 W, 25 kHz for 4–16 min) improved the total phenolic in kiwifruit juice, especially at US16 with a significant increase of 108.65%. Thus, other properties should be evaluated to look into the optimal parameters of ultrasound processing for treating cod.

5.4.5 Total protein content

The changes in the total protein content of cod samples subjected to varying durations of ultrasound treatment were evaluated (Table 5.1). As the ultrasound treatment time increased, a general decline in protein content was noted. At US5 and US10, the protein content lowered by 27.88%–27.67% compared with that at US0. The lowest protein content reached the bottom by 42.91% at US20. This suggests that prolonged exposure to ultrasound might lead to a degradation or structural alteration of proteins, which could be attributed to the mechanical effects of ultrasound, such as cavitation (Deng et al., 2021). However, when the treatment time increased to 30 min and 60 min, the total protein content slightly fluctuated by a decrease of 39.31%–39.80%. Such fluctuations may be indicative of the complex interplay between ultrasound-induced structural changes and other biochemical processes within the cod samples (Tsikrika et al., 2022). Similarly, the total protein content in shrimp also declined after the ultrasound treatment for 5–20 min (Dong et al., 2020b). J. Wang, S. K. Vanga, et al. (2019) also reported that the total protein content in kiwifruit significantly decreased ($p < 0.05$) after the same ultrasound treatment for 4–16 min. Therefore, ultrasound can effectively influence the functional properties of proteins.

5.4.6 *In-vitro* digestibility (IVPD)

5.4.6.1 Protein content after two-stage digestion

Ultrasound treatment has been previously noted for its potential to disrupt cellular structures and enhance the extraction efficiency of various bioactive compounds. Ultrasound also has a significant impact on the protein content after the first stage (pepsin) digestion. In Figure 5.2a, the changes in the protein content before and after digestion are shown. The results manifest a notable decrease in protein content as the ultrasound treatment time increases, with the most pronounced reduction observed at US5, showing a decrease of approximately 49.53% compared to US0. Interestingly, US10 showed a lesser decrease of around 26.99%. This could be due to a transient stabilization of protein structures or other time-dependent factors that need further investigation. Subsequent treatment times (US20, US30, and US60) showed a relatively consistent decrease, hovering around 46-49%.

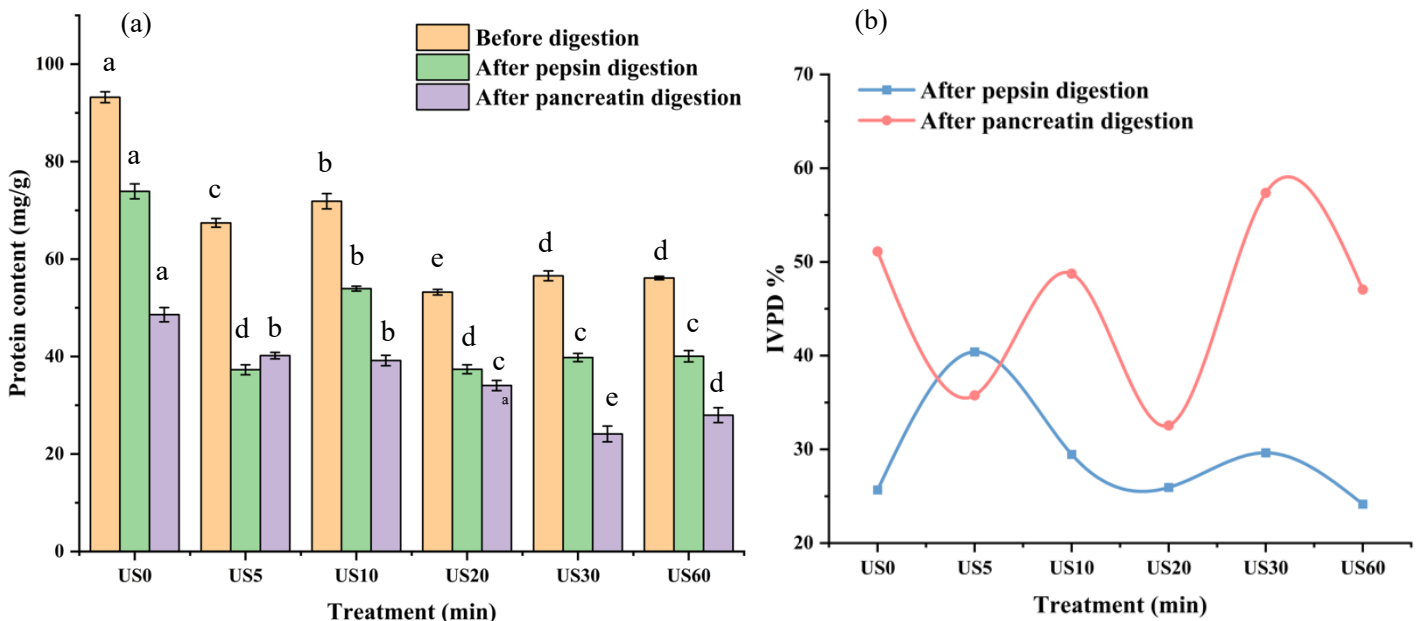


Figure 5.2. Protein content (a) and IVPD values (b) of ultrasound-treated cod samples after two-stage digestion.

Ultrasound treatment significantly influences the protein content of cod samples after pancreatin digestion. The protein content at US5 decreased by approximately 17.29% compared to the untreated sample. As the treatment time progressed, the protein content further decreased, reaching its maximum reduction at US30 with a notable 50.38% decrease. Interestingly, a further increase of 42.44% at US60 showed a slight recovery in protein content, indicating a possible stabilization or change in the protein structures. These findings underscore the intricate relationship between ultrasound treatment duration and the resultant protein content after pancreatin digestion. Understanding these dynamics can be pivotal for tailoring ultrasound treatments in food and biotechnological applications, ensuring optimal protein digestion and extraction.

5.4.6.2 IVPD values

Figure 5.2b demonstrates the changes in the IVPD value of cod samples during two-stage digestion. In the initial pepsin digestion stage, the US5 remarkably decreased protein content by about 57.39% compared to the untreated sample (US0). This pronounced reduction at a mere 5 min of ultrasound exposure underscores the disruptive potential of ultrasound on protein structures. As the ultrasound treatment time progressed, a varying degree of protein content change was observed, with US30 and US60 showing decreases of 15.48% and increases of 5.88%, respectively. Such fluctuations prompt questions about the possible transient effects of ultrasound at different durations. In the subsequent pancreatin digestion stage, most ultrasound treatments (except US30) resulted in a decrease in protein content, with US20 showcasing the most significant reduction of about 36.33%. Interestingly, US30 defied this trend, indicating a 12.24% increase in protein content. This anomaly demands further scrutiny, possibly focusing on the differential effects of ultrasound on protein structures that become more pronounced during pancreatin digestion.

The observed variations across both digestion stages reiterate the intricate interplay between ultrasound treatment and protein digestion. Luo et al. (2021) reported that ultrasound (20 kHz, 200-600 W, 15-45 min) had no obvious impact on the digestibility of infant meat puree in the gastric phase, but it increased the digestibility with the highest IVPD value (80.85%) in the intestinal phase after 600 W for 15 min. This can be explained that ultrasound processing results in protein denaturation and conformational changes, and further benefits in improving the protein digestibility (Salazar-Villanea et al., 2016). However, ultrasound (400 W) is shown to have reduced the digestibility of lotus seed starch-glycerin monostearin complexes in starch. This was because

of the structural changes which led to the hindered hydrolysis of enzymes (Zhuang et al., 2023). Therefore, further investigations are warranted to resolving the exact mechanisms at play, ensuring optimal utilization of ultrasound treatments for enhanced protein digestion in varied applications.

5.4.7 Peptide content

The changes in peptide content in cod samples across different stages of digestion were evaluated in Figure 5.3. Before any digestion, an overall decreasing trend was observed in peptide content for US5 to US30, especially at US5 (30.58%) and US20 (36.29%). Notably, the US60 led to an increased peptide concentration by 18.93%, suggesting potential ultrasound-induced disruptions that may have released peptides from larger protein structures. During pepsin digestion, all ultrasound treatments resulted in a decline in peptide content, with the US30 treatment showing the most pronounced reduction at 46.19% and the least at US60 by 5.75%. This suggests that the action of pepsin, combined with prior ultrasound exposure, could lead to further breakdown of proteins, thereby reducing the detectable peptides. Pancreatin digestion, simulating the conditions of the small intestine, revealed an overall reduction in peptide content for all ultrasound treatments. The US5 treatment led to the most significant decrease at 51.67% whereas US60 at 16.93%. The decrease could be attributed to the combined effects of ultrasound treatment and the specific pH conditions during pancreatin digestion, potentially altering protein structures and making them more susceptible to enzymatic breakdown.

Similarly, Sullivan et al. (2018) found that the peptide content in sorghum was significantly greater by 11.2-17.7% for all ultrasound treatments (5-10 min, amplitude of 20-40%). Thus, while ultrasound treatments undeniably influence protein digestion across different pH stages, the exact mechanisms and interplay between ultrasound exposure duration and pH remain intricate. Diving deeper into these interactions can help in optimizing ultrasound treatments for various applications, ensuring maximum protein digestion efficiency.

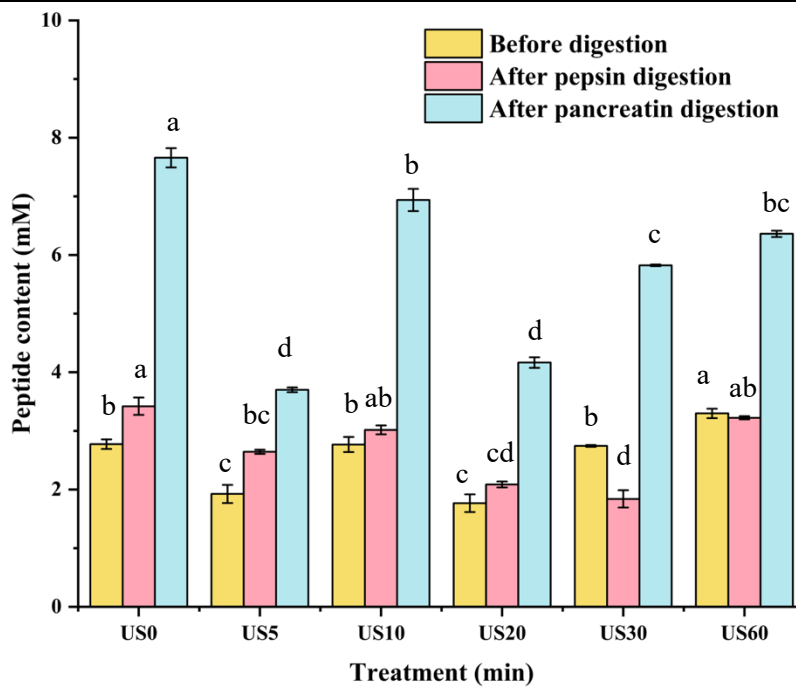


Figure 5.3. Peptide content of ultrasound-treated cod samples before and after two-stage digestion.

5.4.8 Optical microscope observations

To further investigate the morphological changes in cod samples after ultrasound processing, the optical microscope was used to obtain distinct images as shown in Figure 5.4. Micrographs of untreated cod sample (US0) show that they retained the complete shape with smooth membrane and clear color. After ultrasound durations, structure destructions in cod samples were observed at different levels. A rough and deformed membrane was generated with stains at US5. After 10-min ultrasound, the yellow color was presented in cod samples, especially at US 30 and US60. This probably because of the protein denaturation caused conformation alterations, which can affect its optical properties and color (Wang et al., 2017). Additionally, denatured proteins may undergo Maillard reactions with other components in cod sample, leading to the formation of colored compounds (Amaya-Farfan & Rodriguez-Amaya, 2021). Similar results were found in tomato pulp after ultrasound treatment for 15-60 min (Anese et al., 2013) and kiwifruit juice after ultrasound processing for 4-16 min (J. Wang, S. K. Vanga, et al., 2019). Additionally, Jambrak et al. (2014) observed that the formation of partial aggregation in whey protein isolates occurred after a ultrasonic processing (30 min, 40 kHz). These changes are characterized by a more fragmented and less cohesive tissue structure compared to the control samples, indicating that the ultrasound

processing leads to disintegration of muscle fibers and a modification of the protein matrix in cod (Kang et al., 2021). This suggests a potential impact on both the texture and the nutritional quality of the cod, which could have important implications for its culinary applications and consumer acceptability.

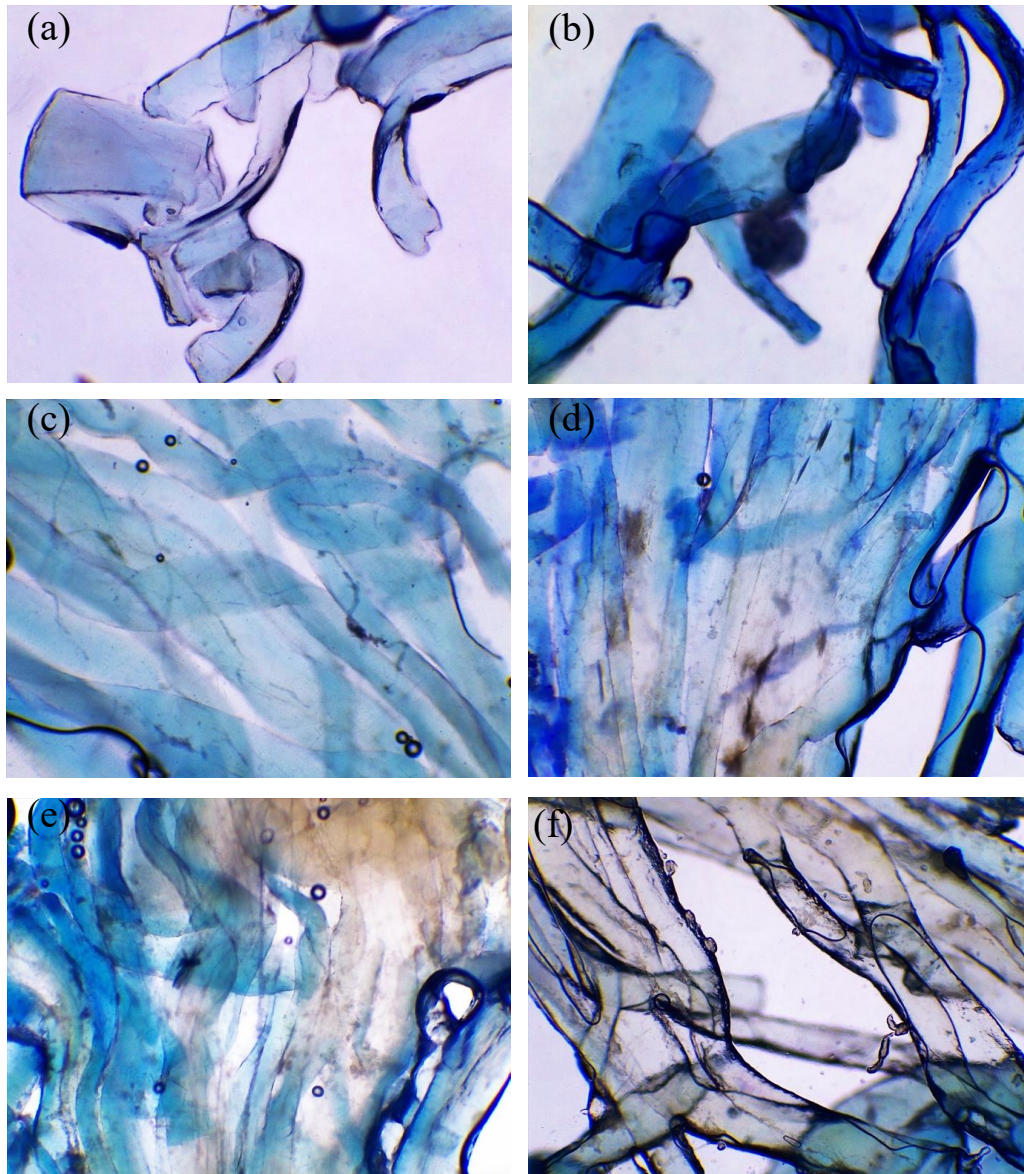


Figure 5.4. Microstructure of cod samples determined by microscope with the magnification of 4 times. US0 (a); US5 (b); US10 (c); US20 (d); US30 (e); US60 (f).

5.5 Conclusion

This investigation into the application of high-intensity ultrasound (HIU) on Atlantic cod (*Gadus morhua*) elucidates significant advancements in seafood processing technology. The study systematically demonstrates that HIU effectively alters the physicochemical properties and *in-vitro* digestibility of cod. Our results reveal a marked influence on color attributes, demonstrating a complex interplay between ultrasound exposure and visual quality of the fish. In the realm of nutritional enhancement, the observed fluctuations in total antioxidant capacity, flavonoid, and phenolic content underscore the nuanced impact of HIU on bioactive compounds. Particularly compelling is the decrease in total protein content contrasted with an increase in protein digestibility, highlighting the potential of HIU in enhancing the nutritional utility of cod for diverse consumer groups, especially those with dietary sensitivities. Electron microscopy provides a deeper understanding of the structural transformations induced by HIU, lending credence to its ability to modify protein configurations. The findings contribute to the broader understanding of ultrasound applications in food processing, paving the way for innovative approaches to enhance the nutritional value and consumer appeal of seafood products. Future research should focus on optimizing ultrasound parameters for specific applications and exploring the scalability of this technology in the seafood industry.

Conflict of Interest

The authors declare no conflict of interest reported in this paper.

Acknowledgment

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Contextual transition

Chapter 5 demonstrated that HIU effectively alters the physicochemical properties and *in-vitro* digestibility of cod. Chapter 6 will investigate another non-thermal processing technique, atmospheric cold plasma treatment. In this chapter, the physicochemical, structural, and allergenic characteristics of Atlantic cod will be evaluated.

Chapter 6

Manuscript V – Cold plasma treatment of Atlantic cod: Insights into the physicochemical, structural, and allergenic characteristics

Xin Dong * and Vijaya Raghavan

Department of Bioresource Engineering, Faculty of Agricultural and Environmental Sciences,
McGill University, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

*Corresponding Author- Email: xin.dong2@mail.mcgill.ca

ORCID: <https://orcid.org/0000-0002-9052-3563>

6.1 Abstract

This study comprehensively explores the impact of atmospheric cold plasma treatment on nutritional, physicochemical, structural, and allergenic transformations in Atlantic cod. The findings revealed that cold plasma notably reduced cod allergenicity by 9.14% at CP10, accompanied by considerable changes in protein secondary structure and microstructure. Additionally, TAC, TFC, and TPC saw their maximum increases by 21.56% (CP10), 68.48% (CP10), and 14.00% (CP30), respectively. Protein content observed before and after a two-stage digestion process could be maximally enhanced by 10.76% and 30.20% at CP20. Meanwhile, the peptide content before and after this digestion process could see its maximum boost by 61.18% and 26.27% at CP30. The digestibility of most samples treated with cold plasma remained almost stable. These results highlight the significant impact of cold plasma on enhancing the nutritional quality and reducing allergenicity in cod, thereby underscoring its promising potential for food allergy management.

Keywords: Cold Plasma; Atlantic Cod; protein structure; allergenicity; physicochemical properties

6.2 Introduction

Atlantic cod (*Gadus morhua*) is a widely consumed fish species known for its nutritional value and economic importance. However, it is also a common allergenic food to induce fish allergy among children and adults (Dong & Raghavan, 2022a). Parvalbumin, the major fish allergen, is a small calcium-binding protein with molecular weights between 10 and 12.5 kDa (Mukherjee et al., 2023). Thus, fish allergenicity can be successfully controlled when the allergenic protein structures are altered (Dasanayaka et al., 2022).

In recent years, the exploration of novel food processing (thermal and non-thermal) technologies has garnered significant attention to enhance food quality and safety. Among these, cold plasma is an emerging nonthermal technology mostly applied to microbial decontamination and food sterilization (Perinban, Orsat, Lyew, et al., 2022). Through the ionization of inducer gas with electrical discharges, cold plasma consisting of electrons, ions, neutral particles, and free radicals can be obtained (Q. Zhang et al., 2021). A few reports also demonstrated a hypoallergenic effect of cold plasma on several common food allergens or allergenic protein fractions, such as prawn, shrimp, and soy (Ekezie et al., 2019; Shriver, 2011; Venkataratnam et al., 2019). However, few studies applied cold plasma to fish, which is one of the most prevalent seafood products. Currently, as the application of cold plasma in the food industry is enhanced, the interactions between plasma and food matrices need to be understood.

Therefore, this study was designed to evaluate the structural, physicochemical, and allergenic characteristics of Atlantic cod following cold plasma treatment. Hopefully, the cod allergenicity can be reduced to further ensure human health, especially the fish-allergic individuals. The optimized processing technique paves the way for future innovations aimed at enhancing food quality and safety. The implications of this study extend beyond the specific context of Atlantic cod, offering broader insights into the cold plasma application in the food industry.

6.3 Materials and Methods

6.3.1 Cold plasma system

Indirect cold plasma treatment of generating plasma-activated water by dielectric barrier discharge plasma at atmospheric pressure was applied in this study (Figure 6.1). The cold plasma system mainly consists of two copper electrodes placed over a quartz tube. The quartz tube acts as the dielectric barrier to carry water during plasma activation. The discharge gap was kept at 4 mm. The copper electrodes are connected to a high-voltage source (PVM 500, Information Unlimited, USA) with 10-kV (peak-peak) voltage and 20-kHz frequency. Premixed argon and oxygen gas mixture in 98%:2% proportion was used to produce NTP within the discharge gap. The gas flow rate was maintained at 4 slm (standard liter per minute) and the water flow rate was 500 mL/ min. Plasma-activated water was generated by activating 400 mL of distilled water for 20 min based on the optimized time (Perinban, Orsat, Garipey, et al., 2022).

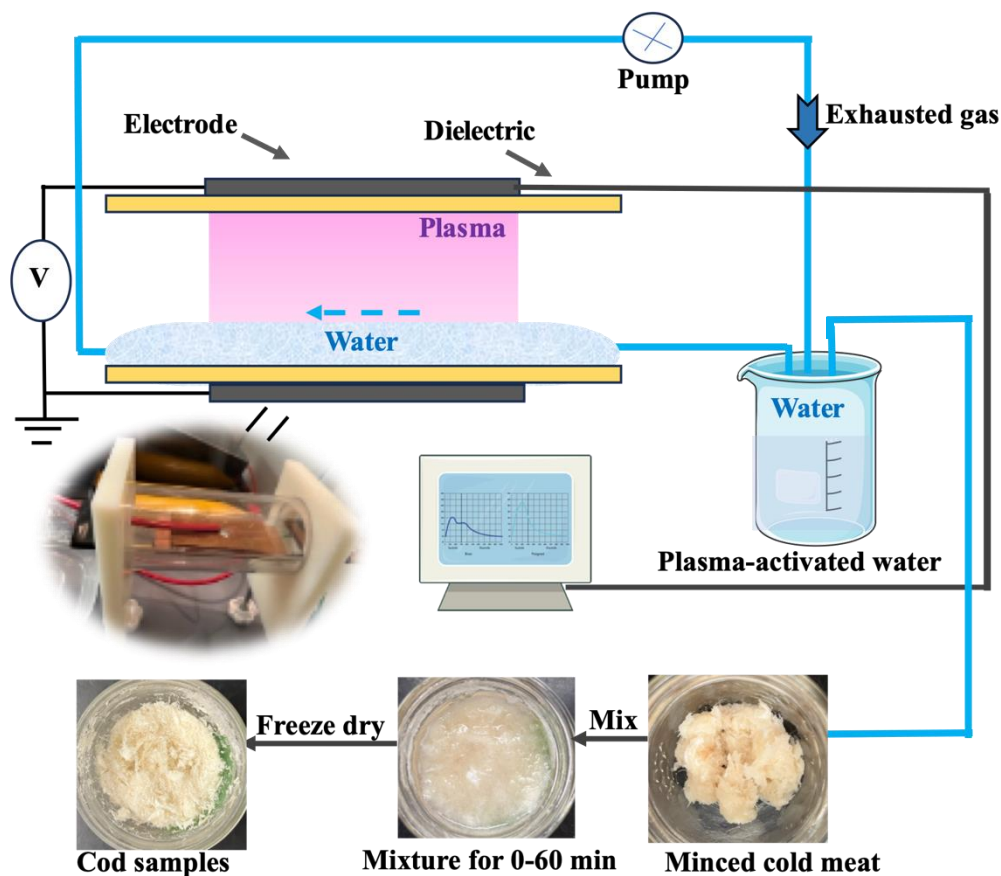


Figure 6.1. Schematic diagram of cold plasma system and sample preparation.

6.3.2 Sample preparation

Atlantic cod meat was purchased from a local seafood market (L'artisan de la mer, Montreal, Canada) and stored at -20 °C until the experiment. Before the treatment, cod fillets were cut into pieces and thawed to room temperature. The fillets were ground and homogenized by a knife mill GRINDOMIX GM 200 (4000 RPM for 90 s, 1000W, Retsch, USA) into minced cod. The minced cod was treated by mixing with plasma-activated water by a ratio of 1:1 (per gram/per milliliter). The treatment time durations were 0 min (CP0), 5 min (CP5), 10 min (CP10), 20 min (CP20), 30 min (CP30), and 60 min (CP60), respectively. The equivalent amount of distilled water was used to treat the control group (CP0). After the treatments, bits of samples were immediately analyzed for color, temperature, pH, and microstructure. The leftover processed samples were subjected to a freeze dryer (7420020, Labconco Corporation, Kansas City, USA) for 48 h. The cod powder was stored at -20 °C until further analysis. All treatments and analyses were performed in triplicates.

6.3.3 Color attributes

A colorimeter (CR-300 Chroma, Minolta, Japan) with 2° N skylight as the light source was utilized to determine the color attributes of cod samples. The calibration procedure was conducted by placing the hand shank on a specific white tile ($Y = 93.35$; $x = 0.3152$; $y = 0.3212$) until the value on the screen was the same as that on the tile. Three parameters would be recorded: L^* (light /dark), a^* (red/green) and b^* (yellow/blue) in terms of the Commission Internationale de l'Eclairage (CIE) Lab color parameters, whose range were from 0 (black) to 100 (white), - 60 (green) to + 60 (red), and - 60 (blue) to + 60 (yellow), respectively (Pathare et al., 2013; Xiao et al., 2010). The total color difference (TCD), hue angle (h), chroma (C), yellow index (YI), and color index (CI), browning index (BI) were evaluated according to the equations below (1)-(6) (Chen et al., 2016; Ordóñez-Santos et al., 2017):

$$TCD = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (1)$$

$$h = \tan^{-1} \frac{b^*}{a^*} \quad (2)$$

$$C = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$YI = \frac{142.86b^*}{L^*} \quad (4)$$

$$CI = \frac{180-h}{L^*-C} \quad (5)$$

$$BI = \frac{100(x-0.31)}{0.172} \quad (6)$$

where

$$x = \frac{a^*+1.75L^*}{5.645L^*+a^*-3.012b^*} \quad (7)$$

In these equations, a_0^* , b_0^* and L_0^* represent the color parameters of the sample without ultrasound treatment and were immediately measured after homogenization; a^* , b^* and L^* represent the samples treated by ultrasound treatment and were determined promptly after every processing treatment. Each sample was measured eight times to reduce bias.

6.3.4 Total antioxidant capacity (TAC)

The total antioxidant activity of the cod sample was measured by ferric-reducing antioxidant power (FRAP) assay as previously described by Dong et al. (2021b). The absorbance at 593 nm was measured by a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada). The antioxidant activity was calculated as $\mu\text{mol Fe (II)}/100 \text{ mL}$ of cod samples.

6.3.5 Total flavonoid content (TFC)

Total flavonoids content was determined according to the method of Osae et al. (2019). Briefly, 0.5 ml of the extracted solutions were mixed with 2 ml of distilled water, and then 0.15 ml of a 5% (v/v) NaNO_2 solution was added, allowing the reaction to proceed for 6 min. After this initial incubation, AlCl_3 (0.15 mL, 10% v/v) solution was added for a further incubation of 6 min. Subsequently, NaOH (2 mL of a 4% v/v) solution was added, followed by adding distilled water to achieve a final volume of 5 mL. The final mixture was stood for 15 min and then the absorbance at 510 nm was recorded by a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada). The results were expressed in terms of milligrams of catechin equivalents (CE) per gram of dry weight of the cod sample.

6.3.6 Total phenolic content (TPC)

The total phenolic content was determined by following the methodology (Feng et al., 2021; Osae et al., 2019). Folin–Ciocalteu reagent (1:10 v/v) was diluted by distilled water. The extract solution (1 mL) was mixed with Folin reagent (5 ml), followed by adding Na_2CO_3 (4 ml, 75 g/L). The mixture was vortexed for 10 min and left to incubate for 30 min at 30°C . The absorbance was measured at 760 nm by using a microplate reader (Synergy HTX Multi-Mode Reader, BioTek

Instruments, Canada). The results obtained were expressed as mg of gallic acid equivalents (GAE) per gram g of dry weight of the cod sample.

6.3.7 *In-vitro* protein digestibility

The *in-vitro* digestibility of cod proteins was determined in two stages following the previous procedure motioned by Dong et al. (2021b). Freeze-dried cod powder (0.2 g) was mixed with a PBS buffer (0.1 M, 10 mL, pH = 7.4) to a final mixture concentration of 20 mg/mL. The total protein contents before and after each digestion stage were determined by the BCA method. The *in-vitro* protein digestibility (IVPD%) for two stages was calculated using the following equation:

$$\text{IVPD}\% = \frac{\text{protein content before digestion} - \text{protein content after digestion}}{\text{protein content before digestion}} \times 100$$

6.3.8 Peptide content

The peptide content was determined using the o-Phthaldialdehyde (OPA) Reagent. The OPA reagent was prepared by adding 25 mL of 100 mM sodium tetraborate in water, 2.5 mL of 20% (w/v) sodium dodecyl sulfate, 40 mg of OPA dissolved in 1 mL of methanol, 100 μL of β -mercaptoethanol, and ddH₂O to a final volume of 50 mL. A 10 μL of digestion mixture was incubated for 2 min with a 190 μL OPA reagent solution. The absorbances were read at 340 nm using a plate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada). Leucine-glycine was used to obtain the standard curve.

6.3.9 FT-IR analysis

Fourier transform infrared (Nicolet Magna 158,750 FTIR, Nicolet Instrument Corp., Madison, WI) spectroscopy was used to investigate secondary structures in cod protein. The absorbance was scanned in the spectra region (700–4000 cm^{-1}) with a resolution of 4 cm^{-1} by OMNIC software (Version 8, Thermo Nicolet Co., Madison, WI). The software OriginPro 2022 was used for data analysis.

6.3.10 CD spectral analysis

Circular dichroism (CD) spectroscopy was used to examine the secondary structures of cod protein. Circular dichroic measurements were determined by Chirascan (Applied Photophysics, USA) with the thermostat set at 23.4 °C. Each sample was prepared with PBS to a concentration of 6 mg/mL and filtered through a 0.22 μM membrane. All Samples were scanned from 190 to 260 nm with a

bandwidth of 1 nm, time per point used was 0.5 s and the scanning time for once was 65 s. A cuvette with a path length of 1 mm was filled by 185 μ L of sample for each sample measurement. The final spectrogram was averaged by five accumulated spectra. The DichroWeb (<http://dichroweb.cryst.bbk.ac.uk>) was used for the analysis of protein secondary structures.

6.3.11 SDS-PAGE analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the following protocol. The protein extract (10 μ L) was mixed with β -mercaptoethanol and 2x Laemmli sample buffer and then heated at 95 °C for 5 min. Running buffer was prepared by 10 $\times$ Tris/Glycine/SDS mixed with distilled water. Samples were loaded for 10 μ L per lane, and a molecular weight marker (10–250 kDa) (Bio-Rad, Philadelphia, PA, USA) was loaded for 5 μ L. Electrophoresis was performed in a vertical unit (Mini-PROTEAN® Tetra System, BIO-RAD, Philadelphia, PA, USA) at 200 V. After electrophoresis, gels were washed in water for 5 min. After removing water, the gel was stained by Bio-Safe™ Coomassie G-250 Stain (Bio-Rad, USA) for 1 hr and then was rinsed with water for 30 min. The image was captured using a digital camera (Canon, EOS Rebel SL2 DSLR Camera, America).

6.3.12 ELISA analysis

A sandwich ELISA (Fish Parvalbumin ELISA Kit, Arigo Biolaboratories) was utilized to quantify the parvalbumin content in the cod samples according to protocol. After incubation and washing following microplate procedures several times, the solution color changed from blue to yellow. The absorbances were read at 450 nm by a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada).

6.3.13 SEM observation

Microstructural changes of cod samples were observed by a Scanning Electron Microscope (SEM) (TM3000, Hitachi High-Technologies Corporation., Tokyo, Japan) was applied to observe microstructural changes in cod samples. A piece of freeze-dried cod sample was transferred to the measuring platform for observation. For microstructural visualization, the images were captured when they were magnified 100 times.

6.3.14 Optical microscope

As described by Dong et al. (2020b), diluted cod samples (15 μ L) were stained using 0.1% of toluidine blue (Sigma-Aldrich, Quebec, Canada) solution on the glass slide for 2 min. An optical microscope (Leica DM500, Leica Microsystems Inc., Canada) and imaging software (Leica LAS EZ, Leica Microsystems Inc., Canada) were used to observe the microstructural changes and image capture (magnification at 4 times).

6.3.15 Statistical analysis

The analysis of variance (ANOVA) feature of the SPSS program (IBM SPSS Statistic, Ver. 29.0.0.0) was used to examine the experimental data. The means were separated using the Duncan, multiple range test, and significance was established at $p \leq 0.05$.

6.4 Results and Discussion

6.4.1 Color, pH and temperature

Table 6.1 presents the effects of CP treatment on cod fillets, as evidenced by changes in color parameters, pH levels, and temperature over different treatment durations. The L^* values, indicative of lightness, varied significantly across treatments, with CP20 showing the highest lightness (77.85 ± 0.30) and CP5 the lowest (59.71 ± 2.20). This suggests that CP treatment can cause noticeable changes in the lightness of cod fillets, potentially due to protein denaturation or surface dehydration (Huang et al., 2019). The a^* values, representing the green-red chromatic scale, remained statistically consistent across treatments, indicating that redness/greenness was not significantly affected by cold plasma. The b^* values, representing the blue-yellow chromatic scale, showed some variability, with CP10 having the lowest b^* value, indicating less yellowness. The TCD and Chroma (C) values highlight the overall color changes due to CP treatment. CP5 showed the most significant color change (TCD: 10.03 ± 0.70), which might be attributable to surface oxidation (Ekielski et al., 2013). The consistency in Chroma across most treatments suggests that the intensity of color remained relatively constant, despite variations in lightness and yellowness. The Hue (h) and Yellow Index (YI) further support these observations. The Hue varied slightly, indicating minor changes in the color tone, while the Yellow Index showed notable differences, particularly at CP30, which had the highest YI value (13.40 ± 0.12), suggesting increased yellowness.

The pH levels demonstrated a downward trend with increased treatment duration, with CP5 showing the lowest pH (6.07 ± 0.03). This could be attributed to chemical changes in the fish tissue, possibly due to acidic compounds generated during plasma treatment. The temperature of the cod also varied with treatment duration, but not in a consistent pattern. CP5 showed the highest temperature (25.20 ± 0.10 °C), which might influence biochemical reactions in the fish tissue.

6.4.2 Total antioxidant capacity (TAC), total flavonoid content (TFC) and total phenolic content (TPC)

The treatment of cod with cold plasma for varying durations (0–60 min) yielded notable changes in the total antioxidant capacity (TAC), total flavonoid content (TFC), and total phenolic content (TPC) (Table 6.1). The TAC values exhibited a significant increase, with the highest value observed in the CP10 sample (215.17 ± 2.95 n/umol/100mg), indicating a peak in antioxidant activity with a maximum increase of 21.56%. The findings demonstrate that cold plasma treatment can affect the bioactive compound profiles in cod. Similarly, Farias et al. (2020) revealed that plasma (200 Hz) significantly increased the antioxidant capacity of apple juice. The increase of TAC at CP10 could be attributed to the plasma-induced formation of antioxidant compounds or the release of bound phenolic compounds (Yu & Beta, 2015).

The TFC fluctuated with a maximum value recorded at CP10 (2.78 ± 0.73 mg/g) with an increase of 68.48%, followed by a slight decrease with longer treatment durations. This pattern indicates a time-sensitive response of flavonoids to plasma exposure. The initial increase in flavonoid content could result from the breakdown of complex compounds into simpler flavonoid structures. However, prolonged exposure seems to lead to a gradual degradation or loss of these compounds (Hemmati et al., 2021).

The TPC results demonstrated a slight fluctuation but were almost stable across different treatment times, with the highest value observed at CP30 with an increase of 14.00%. A similar increase of TPC was observed in the blueberry (Ji et al., 2020) and avocado (Batista et al., 2021), while a similarly slight decrease was observed in the acerola juice (Fernandes et al., 2019). Liao et al. (2018) revealed that the decreased phenolic values were probably due to the destroyed structures of the aromatic ring because the phenolics are vulnerable to ozone assaults. Based on the TAC, TFC, and TPC results, the optimal treatment time appears to be CP10, balancing the enhancement of antioxidant capacity and flavonoid content while maintaining the phenolic content.

Table 6.1. Changes in the physicochemical properties (color, pH, temperature TAC, TFC, and TPC) of cod samples treated by cold plasma.

		CP0	CP5	CP10	CP20	CP30	CP60
Color attributes	L	70.08±1.79 ^b	59.71±2.20 ^d	69.35±2.83 ^b	77.85±0.30 ^a	64.46±3.28 ^c	58.37±1.95 ^d
	a	-1.12±0.25 ^a	-1.08±0.14 ^a	-1.16±0.02 ^a	-1.44±0.16 ^a	-1.59±0.16 ^a	-0.71±0.10 ^a
	b	5.84±0.82 ^a	4.92±0.71 ^{ab}	4.19±0.44 ^b	5.83±0.61 ^a	6.41±0.64 ^a	2.10±0.95 ^c
	TCD	-	10.03±0.70 ^b	6.58±0.62 ^d	7.60±0.48 ^c	4.16±0.44 ^c	11.83±0.17 ^a
	C	5.95±0.80 ^a	3.67±0.86 ^b	6.59±0.89 ^a	6.01±0.63 ^a	6.60±0.66 ^a	2.22±0.92 ^b
	h	-1.38±0.05 ^b	-0.61±0.25 ^a	-0.83±0.78 ^{ab}	-1.33±0.01 ^b	-1.23±0.01 ^{ab}	-1.21±0.12 ^{ab}
	CI	2.74±0.15 ^{ab}	2.23±0.01 ^c	2.48±0.04 ^{bc}	2.63±0.17 ^{abc}	2.96±0.30 ^{ab}	3.10±0.48 ^a
	YI	11.53±1.32 ^b	11.29±1.67 ^b	9.07±1.35 ^c	11.07±0.89 ^b	13.40±0.12 ^a	5.07±0.85 ^d
pH		6.73±0.01 ^a	6.07±0.03 ^e	6.29±0.01 ^d	6.44±0.02 ^c	6.60±0.01 ^b	6.29±0.00 ^d
Temperature (°C)		24.23±0.06 ^b	25.20±0.10 ^a	24.03±0.06 ^{bc}	24.10±0.10 ^{bc}	23.93±0.32 ^c	24.17±0.15 ^{bc}
TAC (n/umol/100mg)		177±2.61 ^c	183.5±4.29 ^{bc}	215.17±4.95 ^a	186.17±3.79 ^b	191.5±5.77 ^b	147.67±6.01 ^d
TFC (mg/g)		1.65±0.16 ^b	2.55±0.53 ^a	2.78±0.73 ^a	2.11±0.06 ^{ab}	2.33±0.48 ^{ab}	1.85±0.1 ^{ab}
TPC (mg/g)		0.5±0.08 ^{ab}	0.43±0.07 ^{ab}	0.46±0.11 ^{ab}	0.5±0.04 ^{ab}	0.57±0.08 ^a	0.4±0.01 ^b

Table 6.2. Determination of protein and peptide content (before/ after pepsin/ after pancreatin digestion) and IVPD values.

Treatment (min)	Protein content (mg/g)			Peptide content (mM)			IVPD (%)	
	Before digestion	After pepsin digestion	After pancreatin digestion	Before digestion	After pepsin digestion	After pancreatin digestion	After the first stage	After the second stage
CP0	90.34±1.60 ^b	63.90±2.45 ^b	33.48±2.62 ^c	3.22±0.41 ^b	2.81±0.13 ^b	6.32±1.17 ^b	29.27	58.02
CP5	47.91±0.48 ^c	29.93±2.25 ^c	23.52±0.70 ^d	1.85±0.06 ^c	1.61±0.22 ^c	3.67±0.46 ^{cd}	37.52	50.91
CP10	50.77±1.74 ^c	39.28±3.77 ^d	32.84±0.87 ^c	1.72±0.14 ^c	1.62±0.13 ^c	3.04±0.72 ^d	22.63	35.32
CP20	100.06±4.09 ^a	78.34±1.66 ^a	43.59±3.82 ^a	3.75±0.51 ^b	3.46±0.49 ^a	4.55±0.68 ^c	21.71	56.44
CP30	85.59±3.84 ^c	65.21±2.24 ^b	39.86±4.09 ^{ab}	5.19±0.32 ^a	3.57±0.37 ^a	7.98±0.48 ^a	23.81	53.42
CP60	80.28±2.19 ^d	52.71±5.80 ^c	34.92±5.76 ^{bc}	3.29±0.5 ^b	3.55±0.48 ^a	7.58±0.39 ^a	34.34	56.50

6.4.3 *In-vitro* protein digestibility and peptides

The effects of cold plasma treatment on the fluctuation of total soluble protein content and peptide generation were investigated (Table 6.2). Before the digestion, compared with that of CP0, protein content showed a decreasing trend at CP5 (46.97%), CP10 (43.80%), CP30 (5.26%), and CP60 (11.13%). However, protein content observed before and after a two-stage digestion process could be maximally enhanced by 10.76% and 30.20% at CP20. The peptides exhibited a decrease in both CP5 (42.57%) and CP10 (46.55%). However, peptide concentrations after 20 min showed an increase at CP20 (16.29%), CP30 (61.18%), and CP60 (2.12%). The peptide content before and after this digestion process could see its maximum boost by 61.18% and 26.27% at CP30. The two-stage digestion was *in vitro* simulated gastric and intestinal digestion, corresponding to enzymes of pepsin and pancreatin, respectively. The IVPD values after pepsin digestion fluctuated from 21.71–32.52%. After pancreatin digestion, IVPD values showed an overall decrease in comparison to CP0 (58.02%). The lowest IVPD was found at CP10 (35.32%), followed by CP5 (50.91%), CP30 (53.42%), CP20 (56.43%), and CP60 (56.50%). Kheto et al. (2023) found that the IVPD of guar seed flour increased maximum of 2.9 % after being treated by a plasma reactor (5–20 min) at different power levels (10 and 20 kV). This might be because cold plasma inhibited enzymatic digestibility by inducing the development of complex protein structures (Kheto et al., 2023). Jian Wang et al. (2023) revealed that the IVPD rose by 11.93% in chickpea protein treated by atmospheric pressure plasma jets. The treatment altered the secondary and tertiary structures of chickpea protein, which caused the side chains of amino acids to unfold. This structural change made a large number of cleavage sites visible, improved protease binding and ultimately increased IVPD (Mollakhalili-Meybodi et al., 2021). Moreover, Dabade et al. (2023) reported that cold atmospheric pressure non-thermal needle electroplating plasma treatment decreased the trypsin inhibitor activity of soybeans, therefore increasing IVPD. However, long-term atmospheric pressure plasma jets can also trigger a decreased IVPD. This can be explained as the disulfide bond formation causing protein aggregation and the absence of proteinase binding sites (Jian Wang et al., 2023).

3.4 FTIR spectroscopy and CD spectroscopy

The FTIR data revealed the molecular changes, involving fatty acids, proteins, polysaccharides, and other compounds (Dziuba et al., 2007). As shown in Figure 6.2a, in the fatty acid region ($3100\text{--}2800\text{ cm}^{-1}$), notable differences in peak intensities were observed across samples. The CP10 and CP20 samples showed an increased absorption, indicating possible alterations in the fatty acid composition or structure due to plasma treatment. The CP30 and CP60 samples, however, exhibited less pronounced changes, suggesting a threshold beyond which plasma treatment does not significantly alter fatty acids (Veettil et al., 2023). In the amide region ($1800\text{--}1500\text{ cm}^{-1}$), crucial for protein analysis, it revealed distinct variations in the amide I and II bands. The CP5 and CP10 samples exhibited shifts in peak positions, possibly indicating changes in protein secondary structures, such as alterations from α -helix to β -sheet. The minimal changes at CP0, CP20, CP30, and CP60 suggest that plasma treatment might have a non-linear effect on protein structure. In the mixed region ($1500\text{--}1200\text{ cm}^{-1}$), it showed complex patterns due to overlapping signals from proteins, fatty acids, and phosphates (Ma'alifah et al., 2022). A gradual shift in peak intensity and position was evident in samples treated for longer durations (CP20, CP30, and CP60), hinting at cumulative molecular alterations due to prolonged plasma exposure. In the polysaccharide region ($1200\text{--}900\text{ cm}^{-1}$), it displayed significant changes, especially in CP10 and CP20 samples. These changes might be attributed to plasma-induced modifications in carbohydrate structures or interactions between carbohydrates and other macromolecules (Szymanska-Chargot et al., 2015). In the “fingerprint region” ($900\text{--}700\text{ cm}^{-1}$), known for its unique absorption patterns, it indicated specific molecular changes in the samples. The CP10 sample showed the most distinct pattern, suggesting that this treatment duration led to unique molecular rearrangements or formations (Kozisek et al., 2023).

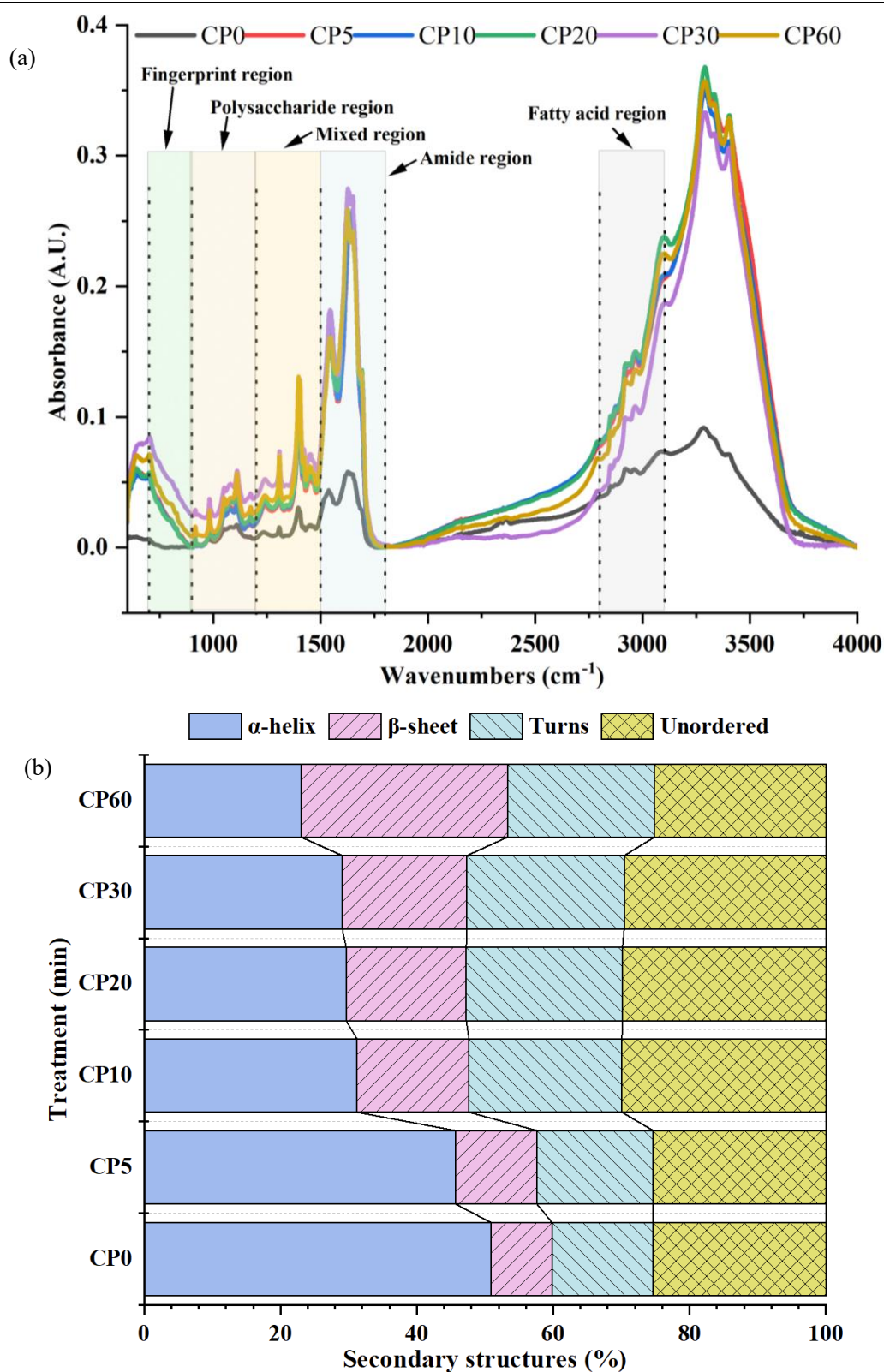


Figure 6.2. Molecular changes determined by FTIR spectra (a) and the percentage changes of protein secondary structure by CD spectra (b).

The FTIR results reveal that cold plasma treatment can induce significant molecular modifications in cod, with varying impacts based on treatment duration. The distinct changes at the CP10 sample across multiple spectral regions suggest a threshold at which plasma treatment induces maximum molecular alterations. This could be due to an optimal combination of factors such as reactive species generation, electromagnetic effects, and thermal changes at this specific plasma exposure duration. In contrast, the less pronounced changes at the CP0, CP5, CP20, CP30, and CP60 samples indicate that the effects of plasma treatment are not directly proportional to the duration of exposure. This non-linear response highlights the complexity of plasma-protein interactions and underscores the importance of optimizing treatment conditions to achieve desired molecular modifications without compromising the nutritional and structural integrity of seafood products.

The CD analysis provided insightful information about the secondary structural changes of cod protein. The variations in the percentages of α -helix, β -sheet, turns, and unordered structures are shown in Figure 6.2b. With the increased cold plasma duration, α -helix content decreased while β -sheet and turns increased with relatively stable unordered structures. These results suggested plasma can induce conformational changes in the cod protein. Decreased α -helix content could be attributed to possibly oxidative modifications or the hydrogen bond breakage that stabilizes the α -helix structure (Z. Zhang et al., 2020). The increase in β -sheet and turns might be indicative of protein aggregation or unfolding processes, common in stress responses. Protein aggregation often involves a transition from α -helix to β -sheet structures (Housmans et al., 2023). The relatively stable proportion of unordered structures is due to a limited impact of cold plasma on the unordered or random coil regions of the cod protein.

3.5 Allergenicity

ELISA analysis was used to determine the allergenicity of cod parvalbumin treated with cold plasma (Figure 6.3a). The concentration of parvalbumin at CP0 (1.493 ug/mg) is the highest. After cold plasma treatment, CP10 reached the lowest allergen content with a decrease of 9.14%, followed by CP60 (8.64%), CP20 (6.07%), CP5 (5.44%), and CP30 (4.38%). Compared with CP0, a slight decrease at CP5 and a more pronounced decrease at CP10 suggest that cold plasma treatment can reduce allergenic protein content. However, the subsequent

increases at CP20 and CP30 indicate that the relationship between plasma treatment time and parvalbumin content is not linear and may be influenced by other factors such as protein denaturation, aggregation, or conformational changes that expose or hide epitopes recognized by the ELISA. Followed by another decrease at CP60, the allergenicity showed a significant fluctuation in the treated cod samples. The prolonged treatment seems to further diminish the detectability of allergenic proteins. This reduction is probably due to extensive protein degradation, which could lead to the loss of epitopes or the creation of aggregates that are not readily detectable by ELISA.

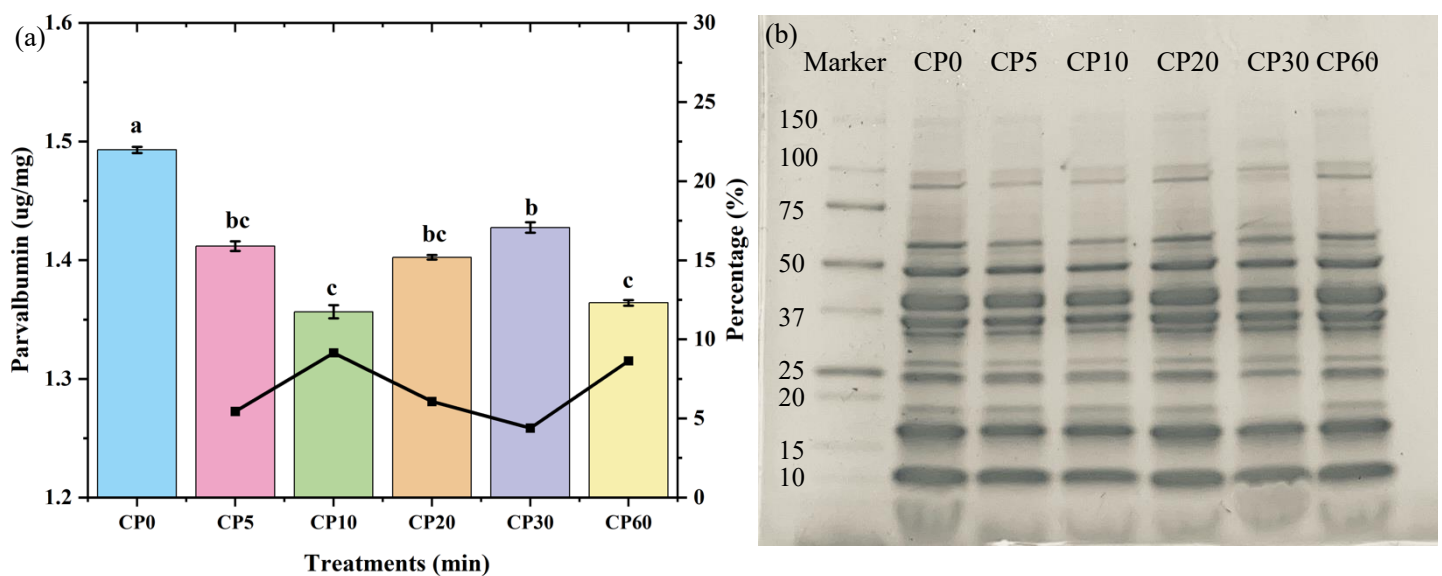


Figure 6.3. ELISA analysis of parvalbumin content (a) and SDS-PAGE result (b) cod samples treated by cold plasma.

Venkataratnam et al. (2020) reported that cold plasma (52 kHz, 32 kV, and 118 s) reduced the antigenicity of Ara h 1 by up to 65% and Ara h 2 by up to 66% from dry, whole peanut and defatted peanut flour by using competitive ELISA. Ng et al. (2021) revealed that cold plasma (5-8kV, 25kHz, and 0-30 min) decreased the antigenicity of casein and α -lactalbumin, whereas increasing the antigenicity of β -lactoglobulin in bovine milk. The decreased allergenicity may be owing to the cold plasma treatments causing the conformational structure to have changed and released hydrophobic or hydrophilic residues that allow antigenic epitopes to be destroyed or reoriented. The increased allergenicity might be due to new epitopes generated after cold plasma treatment, which further increased IgE binding potential (Sathe et al., 2016).

Moreover, SDS-PAGE profiles of cod proteins treated by cold plasma treatment are shown in Figure 6.3b. The image suggests that the lower band intensity was observed in cold plasma-treated samples compared with the CP0, which corresponded to the ELISA results. This probably correlates with decreased allergenic protein content due to cold plasma treatment. The band located approximately at 10 kDa is cod parvalbumin, with the thinnest band in CP10. However, the band intensity between the treated cod samples was not visibly changed. Similarly, Ng et al. (2021) found that cold plasma (30 min) did not change the band intensity of β -lactoglobulin in milk. Zhou et al. (2016) also reported that the band intensity of bovine serum albumin remained unchanged after cold plasma treatment. This suggests that plasma exposure could not have a significant effect on the cod allergenicity and slight total protein degradation occurred (Venkataratnam et al., 2019).

3.6 Microstructure

The impact of cold plasma on the morphology obtained by the optical microscope and SEM of cod samples is illustrated in Figures 6.4 & 6.5. The optical microscope images showed a comparison among cod samples with and without cold plasma treatment. In Figure 6.4, the CP0 had a smooth shape and a clearer blue color, whereas the treated cod samples were somewhat damaged with a deformed shape and brown color. These results are consistent with the SEM results. In Figure 6.5, the surface matrix of the CP0 was compact and tightly packed. After the cold plasma treatment, most samples showed a slightly rough surface, especially in CP10. The CP10 had increased surface flaws and crinkled irregular forms, all of which pointed to the deterioration of the polymeric chains (Sadeghi et al., 2021). This could be explained by protein molecules being broken up and the sample surface being looser due to electrons, ions, and other high-energy particles during the discharge process (Yu et al., 2020). Meanwhile, the marked structural destruction observed in CP10 suggests a critical threshold in cold plasma duration at which protein structures undergo significant alteration. Cold plasma treatment either below or above a 10-min duration does not significantly alter protein structures in cod. In the CP10 sample, the extent of structural unfolding and subsequent exposure of hydrophobic regions may reach a critical level. This can lead to the aggregation of unfolded proteins, as hydrophobic regions from different molecules interact and bind together. Such aggregation is often

irreversible and results in the formation of large protein complexes (Khanikar et al., 2022). In contrast, shorter or longer treatment times (as in CP0, CP5, CP20, CP30, and CP60) might not induce sufficient unfolding or might lead to other types of modifications that do not favor aggregation to the same extent.

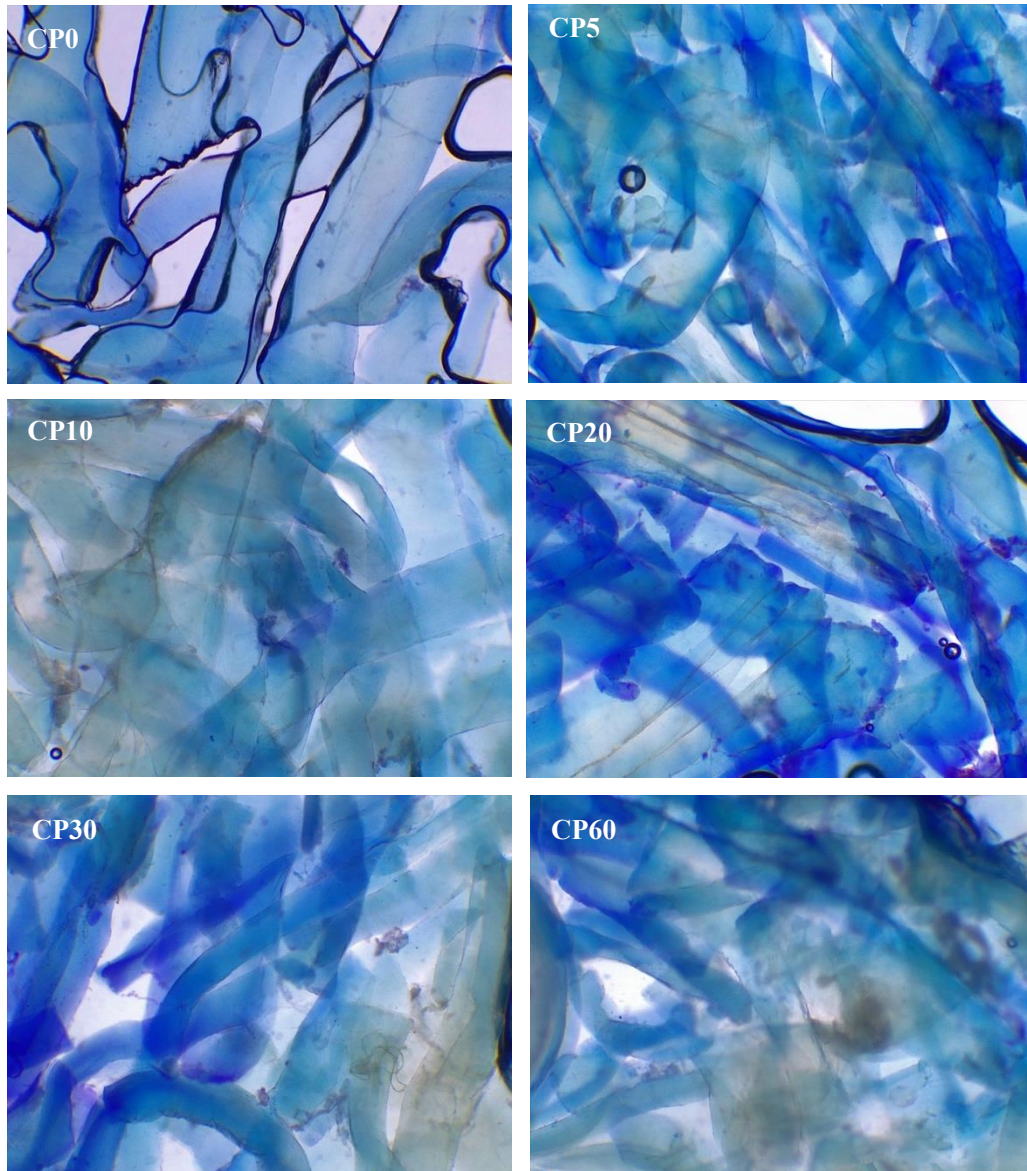


Figure 6.4. Optical microscope images of cod samples treated by cold plasma (magnification of 4 times).

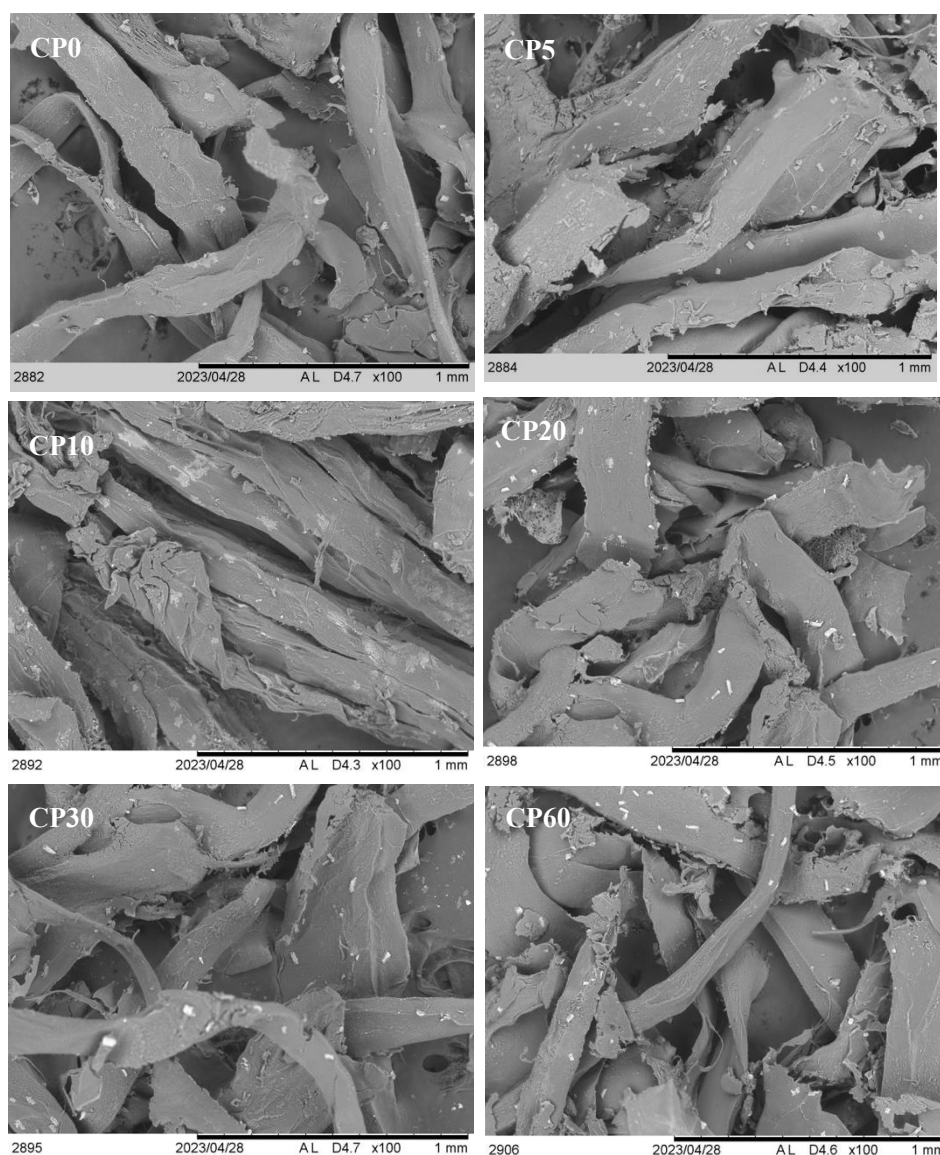


Figure 6.5. SEM images of cod samples treated by cold plasma (magnification of 100 times).

6.5 Conclusion

This investigation illustrates the profound effects of atmospheric cold plasma treatment, as a revolutionary method, for enhancing the nutritional attributes and reducing the allergenic properties of Atlantic cod. The findings reveal a significant reduction in cod allergenicity by 9.14%, alongside noteworthy alterations in the protein and peptide compositions, which were observed to increase notably after a two-stage digestion process. These modifications are critical for the advancement of dietary management practices and for addressing the growing concerns surrounding food allergies. The research also demonstrates an impressive stability in the digestibility of the treated samples, indicating the robustness and reliability of cold plasma treatment. The increased levels of TAC, TFC, and TPC further emphasize the potential of cold

plasma to substantially enhance the quality of food products. Ultimately, the results of this study not only augment our understanding of the impact of cold plasma on food but also underscore its vast potential as a tool for improving food safety, nutrition, and allergenicity profiles. This work sets a precedent for future research in food science, offering a promising direction for the development of innovative strategies in the management of food allergies.

Conflict of Interest

The authors declare no conflict of interest reported in this paper.

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Contextual transition

In the last chapter, cold plasma treatment induced significant modifications in protein composition and structure of cod. The previous chapters mainly involved various thermal and non-thermal treatments. In Chapter 7, thermal glycation, which is a combined (hybrid) treatment, will be applied to investigate the structural change and allergenic properties of Atlantic cod.

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Chapter 7

Manuscript VI – Modifications of the Structure and Allergenic Characteristics of Atlantic Cod Induced by Thermal-Glycation Treatment

Xin Dong * and Vijaya Raghavan

Department of Bioresource Engineering, Faculty of Agricultural and Environmental Sciences,
McGill University, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

*Corresponding Author- Email: xin.dong2@mail.mcgill.ca

ORCID: <https://orcid.org/0000-0002-9052-3563>

7.1 Abstract

This study investigates the effects of thermal glycation on the structural and allergenic properties of Atlantic cod proteins. Techniques like FTIR spectroscopy, CD spectra, SDS-PAGE, ELISA analysis, and SEM are employed. The research findings highlight significant modifications in the protein secondary structures and microstructures treated by thermal glycation. The combination of microwave (150 °C) and glucose could significantly decrease the cod allergen content by up to 16.16% and significantly increase IVPD to 69.05%. Glucose showed better performance than lactose when combined with microwave heating. In comparison to 100 °C, the treatments at 150 °C induced higher IVPD and peptides in cod. This study offers a novel perspective on the relationship between protein processing techniques and allergenicity, providing valuable information for both food industry professionals and consumers concerned with food allergies.

Keywords: Thermal Glycation; Atlantic Cod; Protein Structure; Allergenicity; Food Processing Techniques.

7.2 Introduction

Fish has become an increasingly important source of protein in human diets worldwide. However, the allergenicity of fish has become a growing concern, with reports of adverse reactions to fish consumption increase. In particular, Atlantic cod has been identified as a common cause of food allergies (Kalic et al., 2021). Parvalbumin is the major fish allergen commonly found in fish muscle tissue, which belongs to the calcium-binding protein family with stable, heat-resistant and cross-reactivity attributes (Mukherjee et al., 2023; Tsai et al., 2023). It is noteworthy to acknowledge that the implementation of food processing techniques may lead to a reduction in the allergenicity of parvalbumin by altering its structure.

In comparison to conventional thermal techniques, microwave heating shows many advantages, such as a high heating rate, more uniform heating effect, less energy consumption, ease of operation, and environment-friendly. Microwaves can alter the native structure of proteins, and therefore might potentially affect the ability of certain proteins to be recognized by IgE of sensitized subjects. Also, microwave heating has a less negative influence on the flavor and nutritional compounds of food sources during processing (Salazar-González et al., 2012).

Thermal glycation is a chemical reaction between reducing sugars and proteins, leading to the formation of advanced glycation end-products (AGEs) (Zhang et al., 2022). AGEs have been associated with a range of physiological consequences, including the exacerbation of allergic reactions. Wu et al. (2020a) reported that thermal glycation may result in changes to the structure of proteins and decreases the nutritional value of food products. Zhao et al. (2017) reported that thermal-glycation treatment (glucose at 60 °C for 72 h) led to a reduction of IgE binding in recombinant silver carp parvalbumin detected by dot blot. However, the structural changes and allergenic properties of Atlantic cod upon exposure to thermal-glycation are still not well understood. In this study, Atlantic cod was subjected to thermal-glycation processing to investigate the changes in allergenicity, protein structures, and digestibility.

7.3 Materials and Methods

7.3.1 Sample preparation

Atlantic cod meat was purchased from a local seafood market (L'artisan de la mer, Montreal, Canada) and stored at -20 °C until the experiment. Before the treatment, cod fillets were cut into pieces and thawed to room temperature. The fillets were ground and homogenized by a knife mill GRINDOMIX GM 200 (4000 RPM for 90 s, 1000W, Retsch, USA) into minced cod. Glucose (D-Glucose, Anhydrous, Granular Powder/Certified ACS, Fisher Scientific) and lactose (α -D-Lactose monohydrate, ACS, Fisher Scientific) solutions were prepared for 2 mg/mL by using distilled water. The minced cod was treated by mixing with glucose and lactose solutions by a ratio of 1:1 (per gram/per milliliter) in the glass tube. Mini WAVE Digestion Module (SCP Science, 115 V, 60 Hz, 15 A, 1000 W, Canada) was used at 100 and 150 °C for 10 min. The thermal-glycation treatments were set as (1) Control, (2) MW100, (3) MW150, (4) MW-LAC100, (5) MW-LAC150, (6) MW-GLU100, (7) MW-GLU150, respectively (Table 7.1). The processed samples were subjected to a freeze dryer (7420020, Labconco Corporation, Kansas City, USA) for 48 h. The cod powder was stored at -20 °C until further analysis. All treatments and analyses were performed in triplicates.

Table 7.1. Experimental setup of thermal glycation treatments for cod samples.

Treatments	Thermal treatment	Temperature (°C)	Sugars	Duration (min)
Control	–	RT	–	10
MW100	Microwave	100	–	10
MW150	Microwave	150	–	10
MW-LAC100	Microwave	100	Lactose	10
MW-LAC150	Microwave	150	Lactose	10
MW-GLU100	Microwave	100	Glucose	10
MW-GLU150	Microwave	150	Glucose	10

7.3.2 Total soluble protein content

Total soluble protein content was determined by extracting freeze-dried cod powder (0.2 g) with phosphate-buffered saline (PBS) solution (0.1 M, pH = 7). Following 30-min incubation at room temperature, the mixture was centrifuged at 5,000×g, 4 °C for 20 min. The supernatant

was collected for subsequent analysis. The total soluble protein content in cod samples was determined using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Canada) following the provided protocol.

7.3.3 Peptide content

The peptide content was determined using the o-Phthaldialdehyde (OPA) Reagent. The OPA reagent was prepared by adding 25 mL of 100 mM sodium tetraborate in water, 2.5 mL of 20% (w/v) sodium dodecyl sulfate, 40 mg of OPA dissolved in 1 mL of methanol, 100 µL of β-mercaptoethanol, and ddH₂O to a final volume of 50 mL. A 10 µL of digestion mixture was incubated for 2 min with a 190 µL OPA reagent solution. The absorbances were read at 340 nm using a plate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada). Leucine-glycine was used to obtain the standard curve.

7.3.4 *In-vitro* protein digestibility

The *in-vitro* digestibility of cod proteins was determined in two stages following the previous procedure motioned by Dong et al. (2021b). Freeze-dried cod powder (0.2 g) was mixed with a PBS buffer (0.1 M, 10 mL, pH = 7.4) to a final mixture concentration of 20 mg/mL. The total protein contents before and after each digestion stage were determined by the BCA method. The *in-vitro* protein digestibility (IVPD%) for two stages was calculated using the following equation:

$$\text{IVPD}\% = \frac{\text{protein content before digestion} - \text{protein content after digestion}}{\text{protein content before digestion}} \times 100$$

7.3.5 FTIR analysis

Fourier transform infrared (Nicolet Magna 158,750 FTIR, Nicolet Instrument Corp., Madison, WI) spectroscopy was used to investigate secondary structures in cod protein. The absorbance was scanned in the spectra region (1200–1800 cm⁻¹) with a resolution of 4 cm⁻¹ by OMNIC software (Version 8, Thermo Nicolet Co., Madison, WI). The software OriginPro 2022 was used to fit secondary structural peaks in the amide I band (1700-1600 cm⁻¹). During peak-fitting progress, the second derivative spectrum was used to identify the component bands. Following the appropriate wavelength and peak areas obtained, the proportion of each secondary structure in cod proteins can be estimated.

7.3.6 CD spectra analysis

Circular dichroism (CD) spectroscopy was used to examine the secondary structures of cod protein. Circular dichroic measurements were determined by Chirascan (Applied Photophysics, USA) with the thermostat set at 23.4 °C. Each sample was prepared with PBS to a concentration of 6 mg/mL and filtered through a 0.22 µm membrane. All Samples were scanned from 190 to 260 nm with a bandwidth of 1 nm, time per point used was 0.5 s and the scanning time for once was 65 s. A cuvette with a path length of 1 mm was filled by 185 µL of sample for each sample measurement. Five accumulated spectra averaged the final spectrogram. The DichroWeb (<http://dichroweb.cryst.bbk.ac.uk>) was used to analyze protein secondary structures.

7.3.7 SEM observation

A Scanning Electron Microscope (SEM) (TM3000, Hitachi High-Technologies Corporation., Tokyo, Japan) was applied to observe microstructural changes in cod samples. A piece of freeze-dried cod sample was transferred to the measuring platform for observation. For microstructural visualization, the images were captured when they were magnified 100 times.

7.3.8 SDS-PAGE analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the following protocol. The protein extract (10 µL) was mixed with β-mercaptoethanol and 2x Laemmli sample buffer and then heated at 95 °C for 5 min. Running buffer was prepared by 10× Tris/Glycine/SDS mixed with distilled water. Samples were loaded for 10 µL per lane, and a molecular weight marker (10–250 kDa) (Bio-Rad, Philadelphia, PA, USA) was loaded for 5 µL. Electrophoresis was performed in a vertical unit (Mini-PROTEAN® Tetra System, BIO-RAD, Philadelphia, PA, USA) at 200 V. After electrophoresis, gels were washed in water for 5 min. After removing water, the gel was stained by Bio-Safe™ Coomassie G-250 Stain (Bio-Rad, USA) for 1 hr and then was rinsed with water for 30 min. The image was captured using a digital camera (Canon, EOS Rebel SL2 DSLR Camera, America).

7.3.9 ELISA analysis

A sandwich ELISA (Fish Parvalbumin ELISA Kit, Arigo Biolaboratories) was utilized to quantify the parvalbumin content in the cod samples according to protocol. After incubation and washing in microplate procedures several times, the solution color changed from blue to yellow. The absorbances were read at 450 nm by a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Canada).

7.3.10 Statistical analysis

The analysis of variance (ANOVA) feature of the SPSS program (IBM SPSS Statistic, Ver. 29.0.0.0) was used to examine the experimental data. The means were separated using the Duncan, multiple range test, and significance was established at $p \leq 0.05$.

7.4 Results and Discussion

7.4.1 Protein and peptide content

The protein content of cod samples was determined (Figure 7.1a). Compared to the control (86.84 mg/g), the protein content of all samples treated by thermal glycation could be significantly increased. The highest protein content before digestion was observed in the MW150 (158.04 mg/g) and MW-GLU150 (123.42 mg/g), while the lowest was in the MW-LAC100 (30.17 mg/g) and MW100 (31.96 mg/g). After pepsin digestion, an increase in protein content was observed in all treated samples compared to the control (11.28 mg/g). The highest protein content was still found in the MW-GLU150 (55.88 mg/g) and MW150 (54.13 mg/g) with a significant difference, while other samples showed an increase without a significant difference. The pancreatin digestion led to different results compared to the pepsin digestion, with some treatments (MW100, MW150 and control) showing a higher protein content after pancreatin than after pepsin digestion. Following pancreatin digestion, the highest protein content was again in the MW150 sample (71.66 mg/g) with a significant difference, and the lowest was in the MW-LAC100 sample (16.03 mg/g). The results indicated that thermal glycation treatments, especially using microwaves at higher temperatures, significantly impact the protein content in cod. The MW treatment at 150°C consistently resulted in the highest protein content before or after digestion, which suggests that this treatment might help in retaining or making more proteins soluble. There's a noticeable difference in protein content

post-pepsin and post-pancreatin digestions. This suggests that different digestive enzymes interact uniquely with the protein structure altered by thermal treatments, impacting the solubility and breakdown of proteins. The inclusion of glucose and lactose in the treatment (especially at 100°C) generally resulted in lower protein content compared to the highest results (MW at 150°C). This could be due to the Maillard reaction between sugars and proteins, affecting protein structure and solubility (Ashaolu et al., 2023). The higher solubility of proteins post certain treatments at higher temperatures (like MW at 150°C) could be beneficial for dietary purposes and in the food industry.

For the peptide content before the digestion, compared with the control (3.09 mM), two treated samples including MW150 (4.94 mM) and MW-GLU150 (3.84 mM) showed a significant increase whereas others significantly decreased (Figure 7.1b). The lowest peptide content was found in the MW-LAC100 sample (1.03 mM). In comparison to the pre-digestion levels, pepsin digestion appeared to reduce peptide content significantly in most samples except for the MW100 and MW-LAC150 samples which increased slightly. After pepsin digestion, the highest peptide content was found in the MW-GLU150 sample (3.64 mM), and the lowest in the MW-LAC100 sample (0.97 mM). Afterwards, the pancreatin digestion resulted in a general increase in peptide content compared to both pre-digestion and post-pepsin digestion levels for all samples. Following pancreatin digestion, the highest peptide content was in the MW150 sample (8.54 mM) and MW-GLU150 sample (7.13 mM) with a significant difference, and the lowest was in the MW-LAC100 and MW100 samples (around 2.80 mM). The MW treatment at 150°C consistently resulted in the highest peptide content, particularly after pancreatin digestion. This suggests that microwave treatment at higher temperatures might be more effective in preserving or enhancing peptide solubility and availability. The different impacts of pepsin and pancreatin digestions on peptide content indicate that these enzymes have distinct modes of action on the protein-peptide matrix, especially after thermal treatments (Ashaolu et al., 2023). The addition of sugars in the treatment process (especially lactose at 100°C) generally resulted in lower peptide content compared to the highest results. This could imply that sugars may interact with protein structures in a way that affects peptide release or solubility

(Dwek, 1996). The increased peptides after digestion in the high-temperature microwaved treatment could improve the bioavailability of peptides and potential health benefits.

7.4.2 *In-vitro* protein digestibility

The IVPD results of cod samples with thermal glycation treatments are shown in Figure 7.1c. After the first stage (pepsin) digestion, the treated samples showed an overall decreased IVPD compared with that of the control (87.05%). The highest and the lowest IVPD percentages were in the MW150 sample (61.45%) and the MW-GLU100 sample (39.37%), respectively. After pancreatin digestion, the IVPD values of most treated samples showed a decreasing trend except for the MW-GLU150 sample. The final IVPD percentage was highest for the MW-GLU150 sample (69.05%) with an increase of 19.26% in comparison to the control (57.90%). The MW100 treatment resulted in the lowest final IVPD percentage (36.45%). These results indicated a significant impact of thermal glycation treatments on the digestibility of proteins in cod. The MW treatment at 150°C and the addition of glucose at the same temperature generally maintained or improved digestibility, while the MW treatment at 100°C significantly reduced it. Moreover, there is a notable difference in IVPD percentages between pepsin and pancreatin digestions. This suggests that these enzymes have different efficiencies in digesting the modified protein structures resulting from the various thermal treatments. The addition of sugars, particularly glucose at 150°C, seems to enhance the digestibility of proteins post-treatment, which could be attributed to alterations in protein structure that make them more susceptible to enzymatic breakdown (Joye, 2019). Therefore, thermal glycation treatments by using microwaves at higher temperatures and glucose significantly influence the digestibility of cod proteins. This provided suggestions for designing processing methods to optimize the nutritional value of cod. Conversely, the IVPD values of whey protein α -lactalbumin decreased from 25.91% to 10.93% with the increasing temperature (60–100°C) (Wu et al., 2021). Because protein digestion and absorption are important for human health, an increase in protein digestibility results in an enhancement of protein nutrition and could be beneficial to the human body.

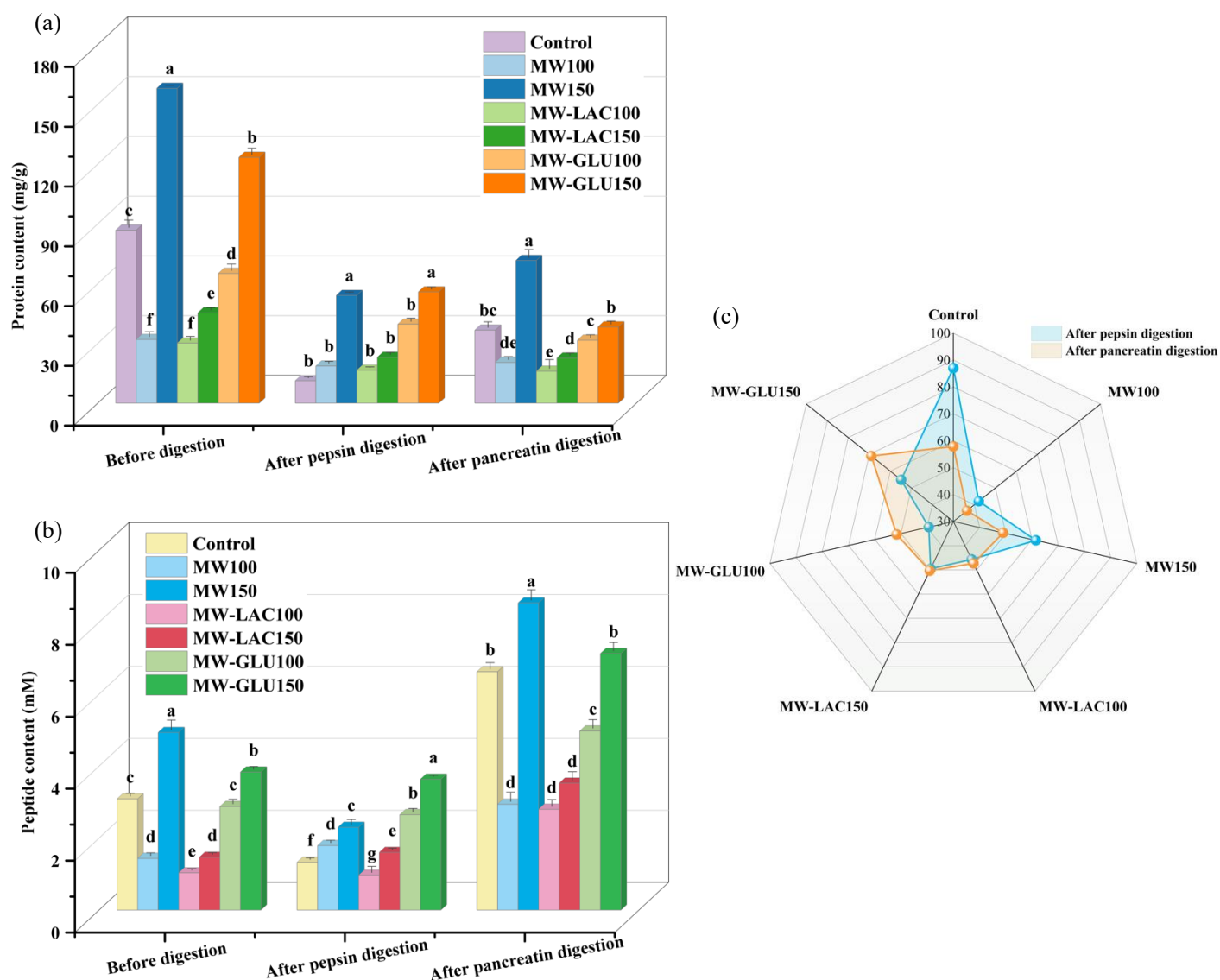


Figure 7.1. The protein content (a) and peptide content (b) of cod samples before and after each stage of digestion; the IVPD percentage (c) after two-stage digestion.

7.4.3 FTIR and CD spectra analysis

The impact of different thermal glycation treatments on the protein secondary structure of cod was evaluated by FTIR and CD spectra. In Figure 7.2a, the absorbance values vary significantly across the different treatments. The MW-GLU150 treatment showed a notable difference in absorbance, which might indicate significant protein structural changes. Further, the FTIR analysis also revealed significant alterations in the percentage of protein secondary structure. Compared with the control, MW100 showed a notable increase in β -sheet (35.38%) and α -helix

(26.54%) contents. In MW150, there was a similar β -sheet content to the control but a decrease in α -helix and unordered structures. MW-LAC and MW-GLU Treatments showed a remarkable increase in β -sheet content, especially in MW-GLU100 (39.20%), indicating a profound impact of glucose in promoting β -sheet formation. The FTIR results demonstrate that thermal glycation treatments can significantly alter the secondary structure of proteins in cod. These changes could be due to the Maillard reaction between proteins and sugars or heat-induced denaturation (Cardoso et al., 2019). The alterations in the amide I and II bands suggest changes from α -helices to β -sheets, which are often associated with protein aggregation or denaturation. Alterations in protein structure could also influence the digestibility and allergenicity of the proteins (Rahaman et al., 2016).

The CD spectroscopy results provide insights into the secondary structure of cod protein. The data shows variations in CD spectra across treatments, indicating alterations in the protein's secondary structure due to the thermal glycation process (Figure 7.2b). For the percentage changes, in comparison to the control, MW100 demonstrated a slight decrease in β -sheets (13.3%) and an increase in α -helices (37.3%), whereas MW150 showed an increase in β -sheets (22.2%) and a decrease in α -helices (14.4%). In MW-LAC100 & MW-LAC150, a marked increase in α -helices (67.5% and 70.4%, respectively) with reduced β -sheet content was observed. Moderate changes with increased β -sheet content in MW-GLU100 (17.7%) and balanced β -sheets (12.0%) and α -helices (33.4%) in MW-GLU150. Higher temperatures and adding sugars could lead to more pronounced structural changes, possibly due to enhanced Maillard reactions and heat-induced denaturation. This is evident from the distinct CD profiles of samples treated with MW-GLU and MW-LAC at 150°C compared to the control. Similarly, Ma et al. (2013) found that increasing amounts of the α -helix and β -sheet in egg white ovalbumin were observed after 30-min glycation by glucose. Wu et al. (2020b) revealed that the secondary structure of glycated β -conglycinin gradually changed from α -helix and β -sheet structures to the random coil with increasing thermal processing degree (100–180°C).

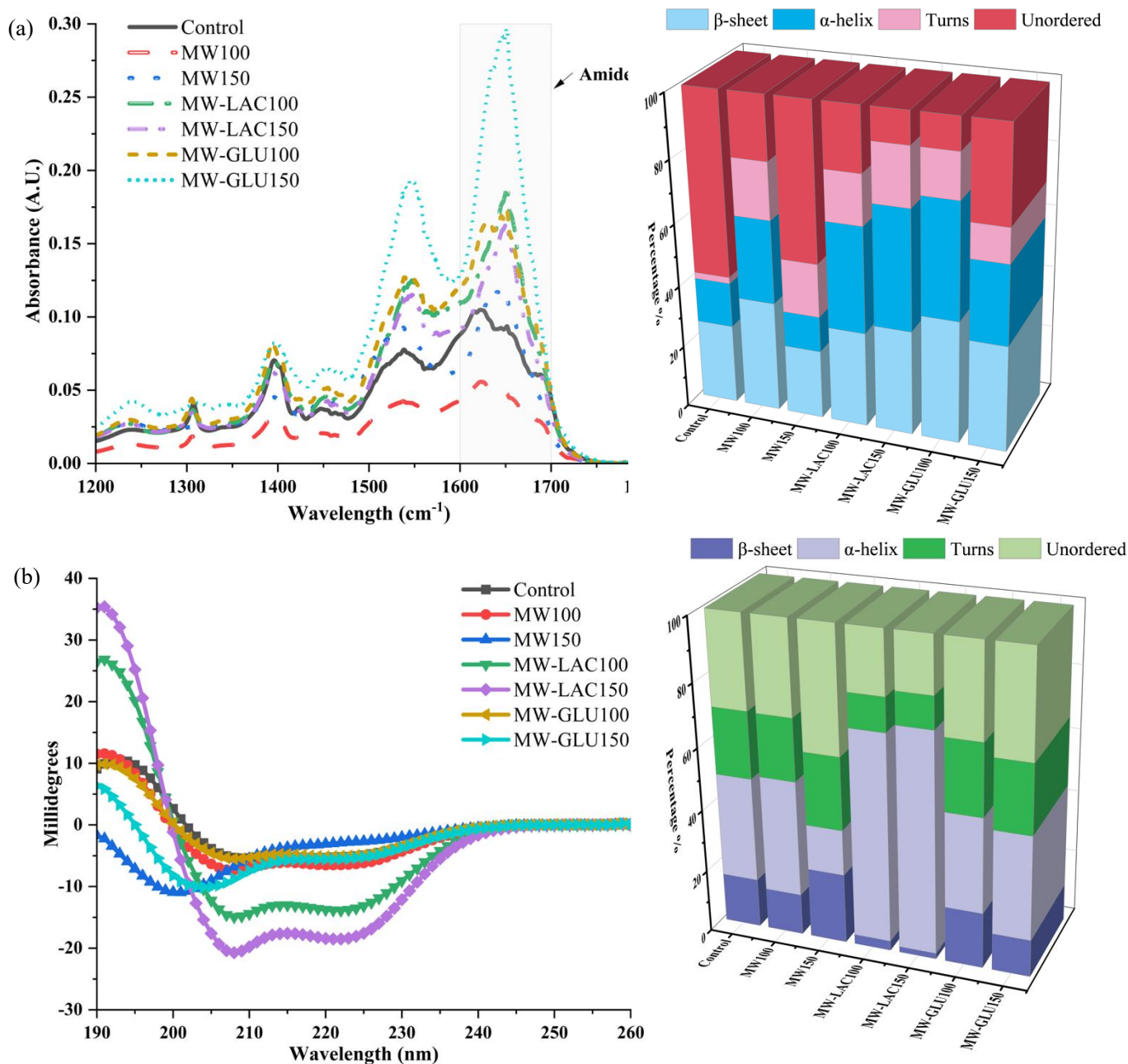


Figure 7.2. FTIR spectra and the percentage changes of protein secondary structure by FTIR spectra (a); CD spectra and the percentage changes of protein secondary structure by CD spectra (b).

FTIR spectroscopy and CD spectroscopy provided different estimates of protein secondary structures due to their distinct measurement techniques and sensitivities. FTIR spectroscopy has been recognized for its sensitivity to the secondary structure of proteins, particularly its ability to detect β -sheet structures due to the distinctive absorption patterns in the amide I region (1600–1700 cm^{-1}) (Baird et al., 2020). As for CD spectroscopy, it is generally more sensitive to α -helical content because α -helices produce a strong dichroic signal in the far-UV region (200–250 nm) (Wang et al., 2017). Additionally, CD spectroscopy typically analyzes liquid samples, while FTIR spectroscopy analyzes solid samples. Environmental factors such as pH, ionic strength, and solvent composition can also influence the results (Zhu et al., 2018b).

7.4.4 ELISA analysis and SDS-PAGE

ELISA analysis was used to quantify the major cod allergen parvalbumin following various thermal glycation treatments (Figure 7.3a). The parvalbumin content showed an overall increase at 100 °C whereas a decrease at 150 °C. The parvalbumin in MW100 showed a significant increase (9.60%). In MW-LAC100 and MW-GLU100, the parvalbumin concentration increased slightly with no significant difference compared with the control. MW150 and MW-LAC150 showed a slightly lower parvalbumin concentration compared to the control but no significant change in allergenicity percentage. MW-GLU150 showed the lowest parvalbumin content with a significant reduction in allergenicity percentage (16.16%). The ELISA results indicate that thermal glycation treatments can modulate the allergenicity of cod. The reduced concentration of parvalbumin treated at 150°C suggests that higher temperatures may denature the allergen, thereby potentially reducing its allergenicity. The presence of glucose at 150°C (MW-GLU150) could further decrease the allergenic potential. This could be attributed to the formation of advanced glycation end-products (AGEs) that may affect the immunoreactivity of parvalbumin (Huang et al., 2023). However, lactose and glucose at 100°C could slightly increase cod allergenicity without significant change. Similarly, Gruber et al. (2005) found that heating (100°C for 90 min) with sugars (glucose, maltose, or ribose) led to a slightly increased allergenicity in peanuts. Ma et al. (2013) revealed that heating and glucose together could significantly reduce the allergenicity of egg white ovalbumin, especially with a longer heating time (30–60 min). The allergenicity changes occurred during the Maillard

reaction. The protection of the epitopes by the covalent attachment of reducing sugar chains may be the mechanism behind this. Additionally, Maillard reactions could change the allergen structure by exposing hidden interior epitopes to bind IgE antibodies or destroying conformational epitopes (Ma et al., 2013).

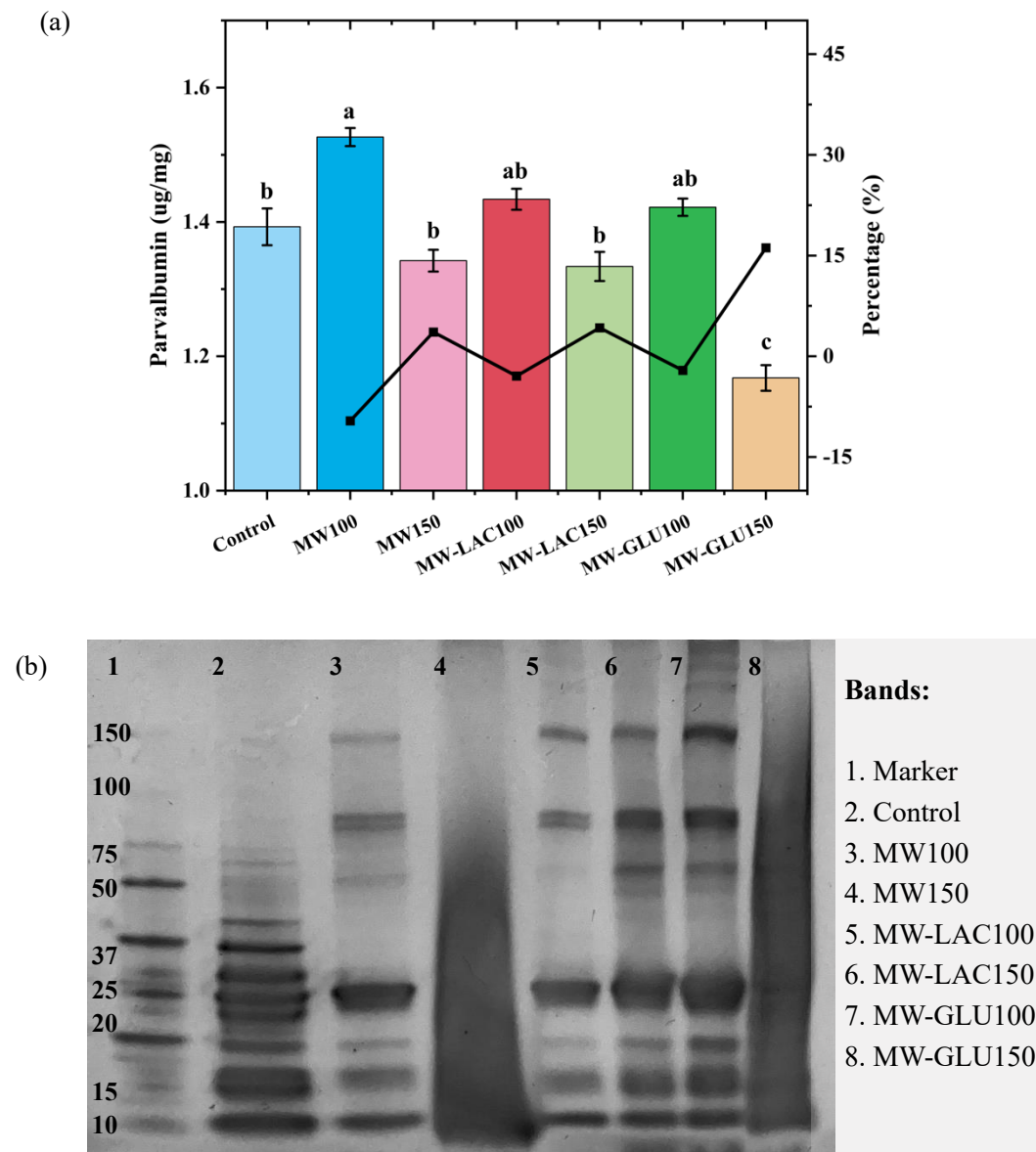


Figure 7.3. ELISA analysis of parvalbumin content (a) and SDS-PAGE result (b) cod samples before and after thermal-glycation treatment.

The SDS-PAGE analysis was conducted to determine the protein profiles of cod samples with various thermal glycation treatments (Figure 7.3b). Lane 2 (Control) displayed a distinct banding pattern characteristic of untreated cod proteins. Lane 3 (MW100) and Lane 5 (MW-LAC100) showed a slightly altered banding pattern from the control, indicating changes in protein structure or degradation. Lane 4 (MW150) and Lane 8 (MW-GLU150) exhibited diffuse banding patterns with clear smearing. They were the most destroyed bands with further alterations in the protein profile, suggesting more pronounced thermal effects at higher temperature (150°C) leading to the structural degradation (Kamble et al., 2020). Lane 6 (MW-LAC150) and Lane 7 (MW-GLU100) showed similar band intensity and the possible presence of new bands, possibly due to Maillard reaction products and sugars added (Ashaolu et al., 2023). The SDS-PAGE results revealed that microwave heating (especially at 150°C) led to protein modifications including unfolding, aggregation, or fragmentation. These changes are more noticeable at higher temperatures, as evidenced by the diminished band intensities in Lane 4 (MW150) and Lane 8 (MW-GLU150). Thus, thermal glycation treatments at higher temperatures may reduce the allergenicity of cod by decreasing parvalbumin.

7.4.5 SEM observation

The microstructural changes of the samples were observed by SEM at 100 magnifications (Figure 7.4). After treatments, the apparent structure of the proteins was altered from block to broken and the protein surfaces turned from smooth to rough. Additionally, protein aggregations and protein folding were noted, which were probably due to the modifications of the second and tertiary structures of proteins (Luo et al., 2013). Thus, microstructural changes were related to structural changes and may further affect allergenicity. Similar results were also observed in other research. Pi et al. (2022) reported that the microstructure changed with the holes and rough surfaces generated in soybeans after boiling (100°C) and autoclaving (121 °C) for 20 min.

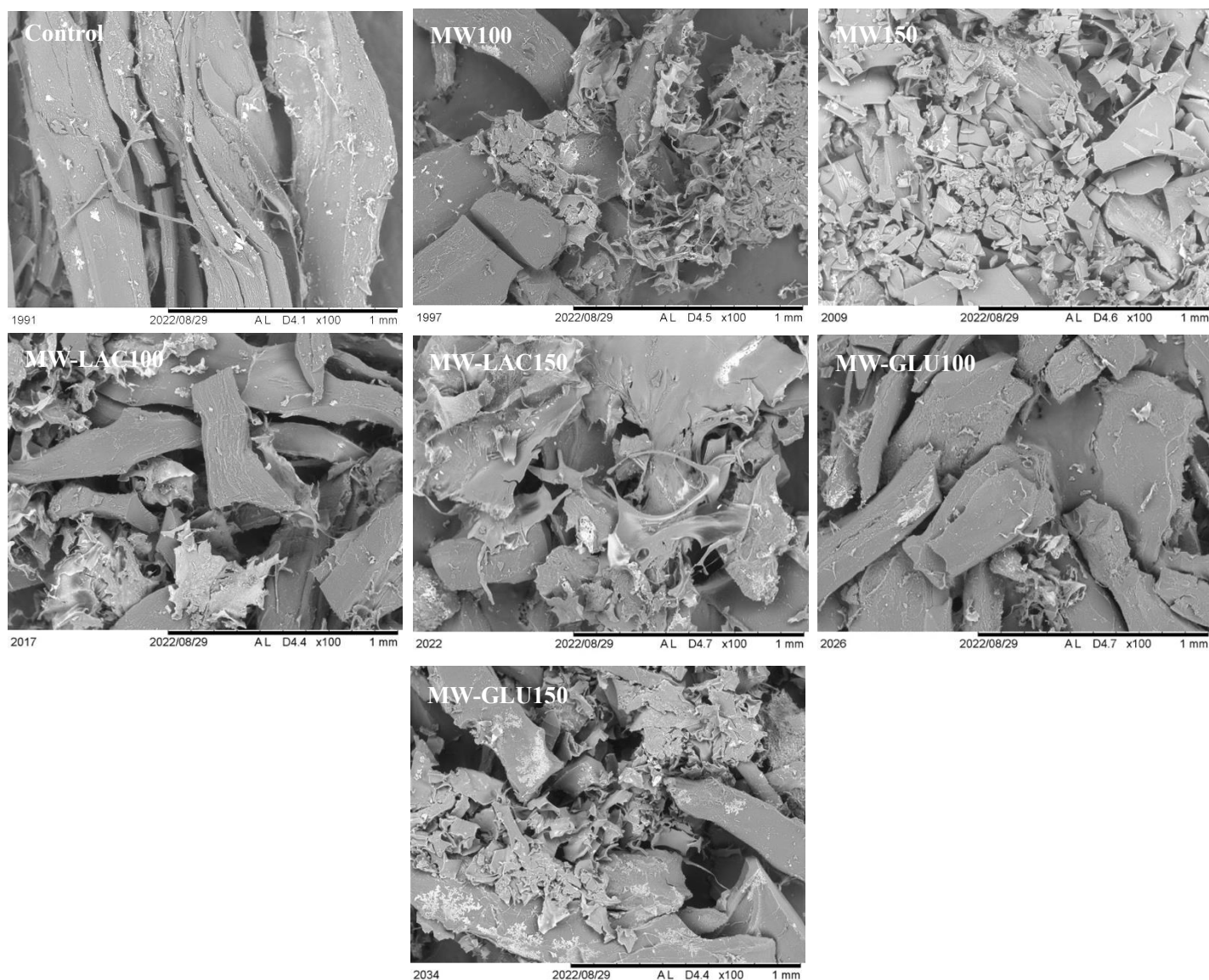


Figure 7.4. SEM images of cod samples treated by thermal glycation (magnification of 100 times).

7.5 Conclusion

In conclusion, the study reveals significant insights into the impact of thermal glycation on fish proteins. The total soluble protein content, as analyzed in the study, indicates marked changes in the protein composition of Atlantic cod following thermal glycation treatments. Furthermore, the peptide content showed substantial variations, suggesting alterations at the molecular level due to thermal processing. The IVPD percentage illustrated the digestibility changes in the protein structure as a consequence of the glycation process. The IVPD was calculated as the percentage difference between protein content before and after digestion. This data is pivotal in understanding how thermal glycation affects protein bioavailability and allergenicity.

Moreover, ELISA analysis provides essential insights into the allergenic properties of the glycated proteins. These results are crucial for comprehending how thermal processing alters the allergenic potential of fish proteins, which has significant implications for individuals with seafood allergies and for the food processing industry. Overall, this study underscores the profound impact of thermal glycation on the structural and allergenic properties of Atlantic cod proteins. These findings not only contribute to the scientific understanding of protein chemistry in the context of food processing but also have practical implications in developing processing methods that could potentially reduce allergenicity in seafood, thereby making it safer for consumption by a broader population.

Conflict of Interest

The authors declare no conflict of interest reported in this paper.

Acknowledgment

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Contextual transition

In previous chapters, we treated Atlantic cod by using thermal, non-thermal, and combined processing techniques. Utilizing a comprehensive analysis, the research revealed the effect of processing on physicochemical, structural, and allergenic transformations in cod samples. In Chapter 8, we will apply molecular dynamics (MD) simulations to cod major allergen parvalbumin. This may provide more insights at the molecular level.

Chapter 8

Manuscript VII – Molecular modeling simulations of thermal and pressure processing in Atlantic cod major allergen parvalbumin Gad m 1

Xin Dong * and Vijaya Raghavan

Department of Bioresource Engineering, Faculty of Agricultural and Environmental Sciences,
McGill University, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

*Corresponding Author- Email: xin.dong2@mail.mcgill.ca

ORCID: <https://orcid.org/0000-0002-9052-3563>

8.1 Abstract

Atlantic cod is a highly nutritious seafood product. Parvalbumin Gad m 1, one of its main allergens, has been identified as a potent inducer of fish allergies. In this study, molecular dynamics (MD) simulations were employed to investigate the impact of external thermal force fields at different temperatures (ranging from 300 to 375 K) and pressures (1 and 3 kbar) on the secondary structures and functional properties of Gad m 1. The results revealed significant structural changes in Gad m 1, including reversible molecule transformations. Additionally, the properties of the molecule's surface were influenced by various factors, such as the solvent-accessible surface area (SASA) and snapshots representing compactness within the protein structure. Notably, higher pressure (3 kbar) resulted in decreased values for the root mean square deviation (RMSD) and radius of gyration (Rg). This study provides valuable insights into the molecular-level characteristics of allergenic structures and offers suggestions for optimizing food processing parameters in the management of food allergies.

Keywords: Fish allergy; GROMACS; molecular modeling; parvalbumin; secondary structures

8.2 Introduction

Fish constitutes a variety of valuable nutrients, such as protein, omega-3 fatty acids, vitamins, and minerals (N. Li et al., 2020). Fish products are beneficial to human health such as reducing the risk of cardiovascular diseases and various cancers (Ashfaq et al., 2020; Krittanawong et al., 2021). The worldwide production of fish is 154 million tons per year, and its consumption shows an increasing trend with around 18.5 kg per capita per year (Can et al., 2015). In various geographical regions, the consumption of fish in terms of species is varied and different. For example, cod, salmon, and tuna are the most popular species in Europe, while catfish, tilapia, and perch are consumed more in Asia-Pacific regions (www.fao.org). Among them, Atlantic cod ranks as the top consumed fish in Portugal with a consumption of approximately 60,000 tons per year, which is popularly exported all over the world (Piccirillo et al., 2013). However, fish allergy is regarded as one of the “big nine” food allergies. It is reported that approximately 0.1–0.4% of the population worldwide suffers from fish allergy, and the prevalence in the pediatric population is up to 7% (Buyuktiryaki et al., 2021; Sharp & Lopata, 2014). Clinical symptoms triggered may range from mild allergic reactions to severe anaphylaxis, and even lead to death (Dong et al., 2020a). At present, patients with an allergy to one or more fish species are suggested to avoid consuming most or all fish products (Dijkema et al., 2020).

Common fish allergens identified include parvalbumin, enolase, tropomyosin, and aldolase. Among them, parvalbumin ranks the top allergen in many fish species, such as Atlantic cod (Gad m 1), cod fish (Gad c 1), salmon (Sal s 1), carp (Cyp c 1), and mackerel (Sko j 1) (Kubota et al., 2016; Mohammadi et al., 2017). It is a calcium-binding protein with a molecular weight of 9–14 kDa, and it is a digestion-resistant and heat-stable protein (Swoboda et al., 2002). Ketnawa and Liceaga (2017) reported that microwave treatment (90 °C for 5 min) decreased the allergenicity of trout frame protein hydrolysates by 55-93% due to the effect on protein conformation. Similarly, Dong et al. (2021b) found that microwave treatment (75-125 °C for 5-15 min) reduced the allergenicity of shrimp tropomyosin by up to 75%, which was strongly associated with the alteration of the secondary structure of shrimp proteins. Vanga et al. (2019) investigated a simulation study about the influence of temperature (300 K-373 K) and pressure (1 bar-6 kbar) deviations on soy allergen Gly m 4. They found significant structural changes in

the molecule in terms of solvent-accessible surface area and radius of gyration, leading to the compaction at increased pressures, molecule swelling at lower temperatures and higher pressures (300 K and 6 kbar) (Vanga et al., 2019). For the influence of temperatures and pressures on parvalbumin Gad m 1, Somkuti et al. (2012a) reported that Gad m 1 partially unfolded at 5-kbar pressure, which formed a less ordered and less stable stage at higher pressures due to dissociation of calcium. It is well documented that increased pressure and temperature induced the irreversible unfolding of the protein.

To date, numerous studies applied processing methods to alter the conformation of allergenic proteins and change the functional properties of these proteins. The techniques utilized to assess the conformational information of proteins generally include fourier transformation infrared (FTIR) spectroscopy, circular dichroism (CD) spectroscopy, and nuclear magnetic resonance (NMR) (Dong et al., 2020b; Moraes et al., 2014; Zhu et al., 2018a). However, only limited information can be provided regarding the alterations of protein structures. Recently, it has been found that the gaps between the microscopic levels (regarding time and length) and macroscopic levels (real-time experiments) can be minimized through the application of computational knowledge (Allen, 2004). MD simulation is widely used for many food proteins, such as soy allergen, soy trypsin inhibitor, peanut allergen, egg allergen, kiwifruit allergen, and milk allergen, treated by electric fields at different external stresses and temperatures (Saxena et al., 2019; Vagadia et al., 2016; Vanga et al., 2015; Vanga et al., 2019; J. Wang et al., 2020; Zhu et al., 2021). However, no studies focus on the parvalbumin Gad m 1 for MD simulation. This present study investigated the effect of thermal and pressure treatments on the secondary structure of Gad m 1 where functional properties including the root mean square deviation (RMSD), root mean square fluctuations (RMSF), radius of gyration (Rg), and solvent accessible surface area (SASA) are discussed. Hopefully, this research can be a visual prediction for the conformational changes and functional properties of parvalbumin Gad m1 by food processing technique of thermal stresses.

8.3 Materials and Methods

8.3.1 Molecular dynamic (MD) simulations

In the present study, Groningen Machine for Chemical Simulations (GROMACS) software from Stockholm Center for Biomembrane Research, Stockholm, Sweden was applied to run the MD simulations based on the classical MD algorithm (Hess et al., 2008). Gad m 1, allergenic cod parvalbumin, is one of the major elicitors of IgE-mediated reactions in fish-allergic patients (Moraes et al., 2014). The protein data file of Gad m 1 in PDB format (access code: 2MBX) was downloaded from Protein Data Bank (PDB). According to the GROMACS tutorial, the Amber94 force field and TIP3P water model were opted for the simulation conditions of force field and solvent, respectively. In the MD simulation system, the protein molecule was enclosed in a cubical water box with dimensions of 2.0 nm × 2.0 nm × 2.0 nm containing 5581 water molecules.

Before starting the simulation, energy minimization (EM) should be done to ensure no steric clashes or inappropriate geometry in the simulation system. Two factors of E_{pot} (potential energy) and F_{max} (maximum force) in the EM process are set as negative and not more than 1,000 kJ mol⁻¹ nm⁻¹, respectively. To begin real dynamics, solvent and ions around the protein must be in equilibrium through two steps, including NVT (constant number of particles, volume, and temperature) for stabilizing the system temperature and NPT (constant number of particles, pressure, and volume) for stabilizing the system pressure and the density. The 100-ps equilibrations with the steepest descent for 50 000 steps were used to achieve a well-equilibrated system. To run production MD for data collection, a temperature setting of 300 (control), 325, 350 and 375 K accompanied by a pressure setting of 1 bar and 3 kbar were chosen for the study (Table 8.1). Each temperature was maintained for 2 ns (2000 ps), and temperature sets were in terms of a complete loss of IgE reactivity at 413 K (Kubota et al., 2016). Hence, this study consists of eight molecular dynamic simulations carried out at various temperatures and pressures. Related studies concluded that the electric field could foster protein folding or might have a negative effect on secondary structure in terms of its damage (Dong et al., 2021b; Saxena et al., 2019). To evaluate the effect of the temperatures and pressures on the secondary structure of protein molecule Gad m 1, the functional properties such as RMSD, RMSF, Rg, and SASA were taken into consideration.

Table 8.1. Experiment setup in the MD simulated systems.

Protein	Temperature	Pressure	Equilibration	Time duration
Gad m 1	300 K (control)	1 bar, 3 k bar	100-ps	2 ns (2000 ps)
(Atlantic cod	325 K	1 bar, 3 k bar	equilibrations with	
main allergen,	350 K	1 bar, 3 k bar	steepest descent for	
PDBID: 2MBX)	375 K	1 bar, 3 k bar	50 000 steps	

8.3.2 Secondary structure

The secondary structures of Gad m 1 were analyzed by the performance of the timeline trajectory analysis tool in visual molecular dynamics (VMD) software according to the timeline tutorial. Timeline calculations were performed using a set of built-in analysis methods to generate the 2D box plot (Isralewitz, 2012). The formats of data files used for timeline analysis were converted based on the VMD guideline from SASSIE web (https://sassie-web.chem.utk.edu/training/auc_2015/files/lab_I.pdf).

8.3.3 Root mean square deviation (RMSD)

The RMSD is a primary tool to measure deviations in molecule according to the atomic movement induced by the external stress. It is an important parameter to characterize the conformational changes of proteins. The following equation is used for the calculation of RMSD:

$$RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^N |r_{\text{final}}(i) - r_{\text{initial}}(i)|^2} \quad (1)$$

where N is the number of protein atoms, and $r_{\text{final}}(i)$ and $r_{\text{initial}}(i)$ represent the final and initial coordinates of an atom i , respectively (Vanga et al., 2015).

8.3.4 Radius of gyration (Rg)

The Rg is defined as the distribution of atoms in the protein molecules with respect to its center of mass. It is basically used to evaluate the overall size of a chain molecule (Budi et al., 2005; Singh et al., 2013). Besides, Rg is an index of the compactness of protein molecules. It is accepted that a low Rg value indicates that the number of atoms packed around the center of protein molecule is relatively high (Lobanov et al., 2008). The structural change of a protein during MD simulations can be quantified by Rg with the equation as follows:

$$Rg = \sqrt{\frac{1}{N} \sum_{i=1}^N |r(i) - r_{\text{center}}|^2} \quad (2)$$

where N is the number of protein atoms, and $r(i)$ and r_{center} are the coordinates of an atom i and the center of mass, respectively (Singh et al., 2013).

8.3.5 Root mean square fluctuation (RMSF)

The RMSF is a parameter to evaluate the deviation between the position of atom i and some reference position in a protein molecule. The measurement of RMSF changes in Gad m 1 are computed with the following equation (Pitera, 2014):

$$RMSF_i = \sqrt{\frac{1}{T} \sum_{t_i=1}^T |r_i(t_i) - r_i^{ref}|^2} \quad (3)$$

where r_i^{ref} is the reference position of atom i and T is the time.

8.3.6 Solvent accessible surface area (SASA)

The SASA is used as a tool to evaluate the surface area of a protein molecule that is available to have interactions with other molecules and solvents. It is confirmed that even slight structural deviations of protein molecules can lead to changes in their surface properties, which in turn affect the intermolecular interactions in proteins (Singh et al., 2013; Vagadia et al., 2016). Besides, Lee and Richards (1971) suggested that the accessible surface area is an area to place the center of the solvent molecule without disturbing other atoms nearby. This study used GROMACS software by built-in commands with the following equation to determine SASA changes:

$$SASA = A = \sum (R / \sqrt{R^2 - Z_i^2} \times D \times L_i) \quad (5)$$

where A denotes the surface area, R denotes the radius of the atom, L_i is the length of the arc drawn on a given section i , and Z_i is the perpendicular distance of section i from the center of the sphere.

8.4 Results and Discussion

8.4.1 Secondary structure

Gad m 1 (PDB ID: 2MBX), a prominent allergen in cod belonging to the parvalbumin family, has been extensively analyzed through molecular dynamics simulations. While its crystal structure remains unelucidated, the secondary structural components and Ramachandran plot are depicted in Figure 8.1. Analysis via PDBsum reveals Gad m 1's composition of 109 residues, predominantly α -helices (59 residues, 54.1%) and 3/10 helices (3 residues, 2.8%), with the remainder classified as other structural types. In Figure 8.1c, there are six α -helices and one

3/10 helices. It can be seen that α -helices are present between the residues from 9-18 (ASP-ALA), 27-33 (HIS-LYS), 41-51 (PRO-ILE), 61-71 (GLU-PHE), 80-90 (ASP-GLY), 100-108 (VAL-LYS). The 3/10 helices are present between the residues from 36-38 (LEU-ALA). Fig. 1d indicates the structural domain of Gad m 1, in which the red cylinders represent α -helices with residue numbers.

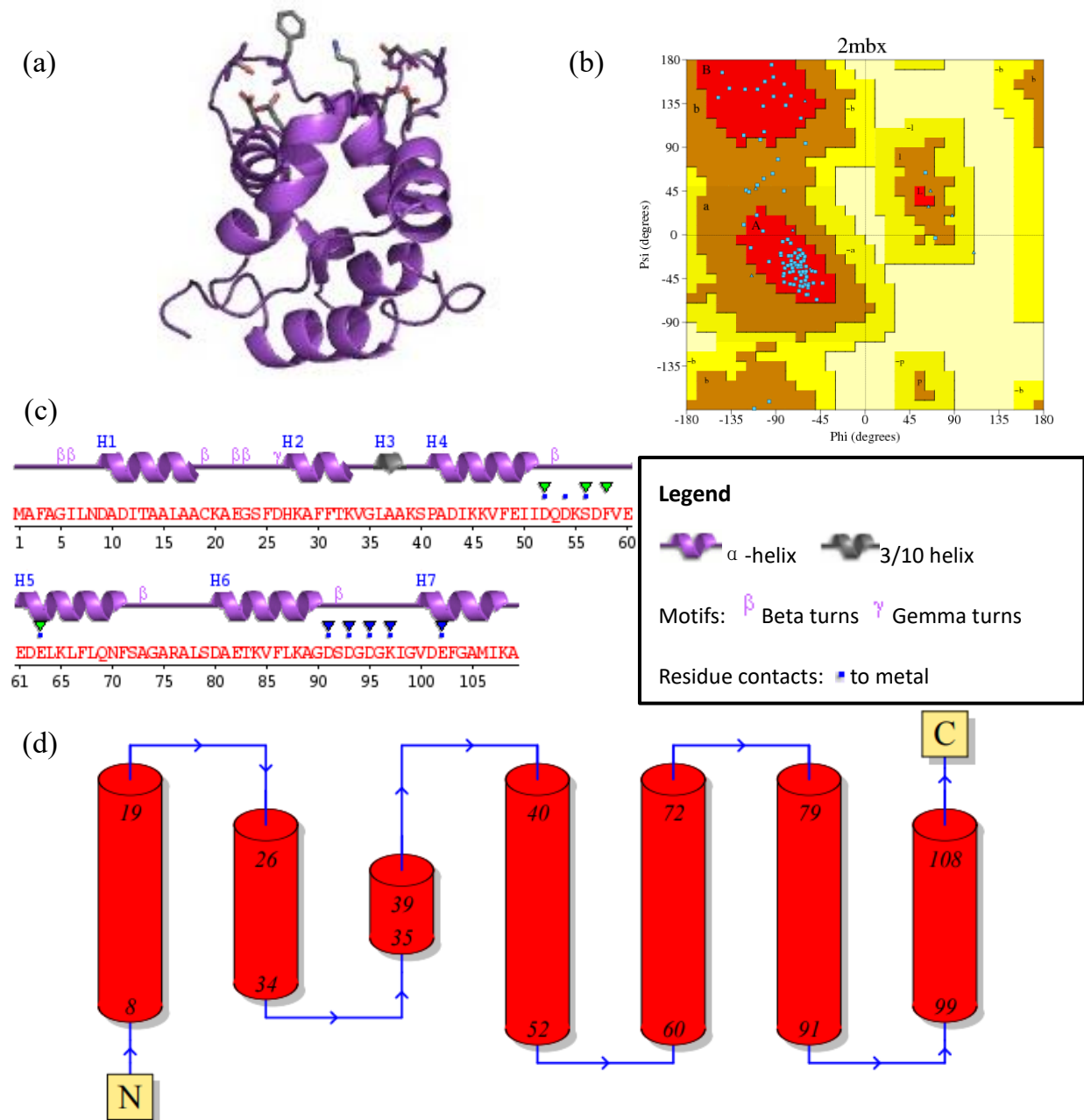


Figure 8.1. Schematic diagrams of cod parvalbumin Gad m 1 (PDBID: 2MBX) adapted from the PDBsum: (a) Secondary structure of protein chain; (b) Ramachandran Plots; (c) Sequence display for secondary structure; (d) Topology diagram of the structural domain (red cylinders:

α -helices; arrows: direction of the protein chain from the N- to the C-terminus; numbers: residue number given in the PDB file).

The timeline analysis in visual molecular dynamics (VMD) is applied to visualize the secondary structural changes in cod parvalbumin Gad m 1. In Figure 8.2, the Y and X-axes represent the protein residues and the time/frame numbers, respectively. It can be observed that changes in protein secondary structure occurred during thermal and pressure treatments with the electric fields. When Gad m 1 was subjected to the external electric fields at 300 K with stress enhancement from 1 bar to 3 kbar (Figures 8.2a & 8.2b), amounts of 3/10 helices converted to α -helices between residues 16-49. 3/10 helices, as intermediate conformations, commonly exist in the early period of protein denaturation (Daggett & Levitt, 1992). Meanwhile, α -helices were replaced by turns between residues 65-71 as the processing time raised, indicating the onset of the protein denaturation. At 300 K (Figures 8.2a & 8.2b), significant losses of coils between residues 58-61 and 97-100 were observed in protein, whereas they were almost stable in later treatments (Figures 8.2c-8.2h). For 3/10 helix and α -helix between residues 16-49, a similar deviation was observed at 325 K compared with 300 K (Figures 8.2c & 8.2d). However, a reverse transformation of increased 3/10 helix and decreased α -helix occurred at 350 K between residues 16-49, with turns produced between residues 34-47 (frame 11-47) (Figures 8.2e & 8.2f). These findings agree with the results obtained from simulation studies of kiwifruit and egg under thermal and pressure treatment in an electric field (J. Wang et al., 2020; Zhu et al., 2021). At 375 K, a large amount of 3/10 helix disappeared to transform to α -helix between residues 16-49, 61-70, and 100-103 (Figures 8.2g & 8.2h). This can be explained that partial transformations of α -helix under longer time periods might be hidden due to the limited time scale of simulations (Müller et al., 2021).

8.4.2 Root mean square deviation (RMSD)

As shown in Figure 8.3, an overall increase in RMSD values of Gad m 1 was observed under thermal stresses during 2 ns. When simulated pressure was set at 1 bar (Figure 8.3a), RMSD values at 350 K remarkably increased with respect to the reference (300 K), while no obvious changes were obtained at 325K and 375 K. J. Wang et al. (2020) observed similar results, especially RMSD values of Act d 2 in kiwifruit which also increased between 300-350 K.

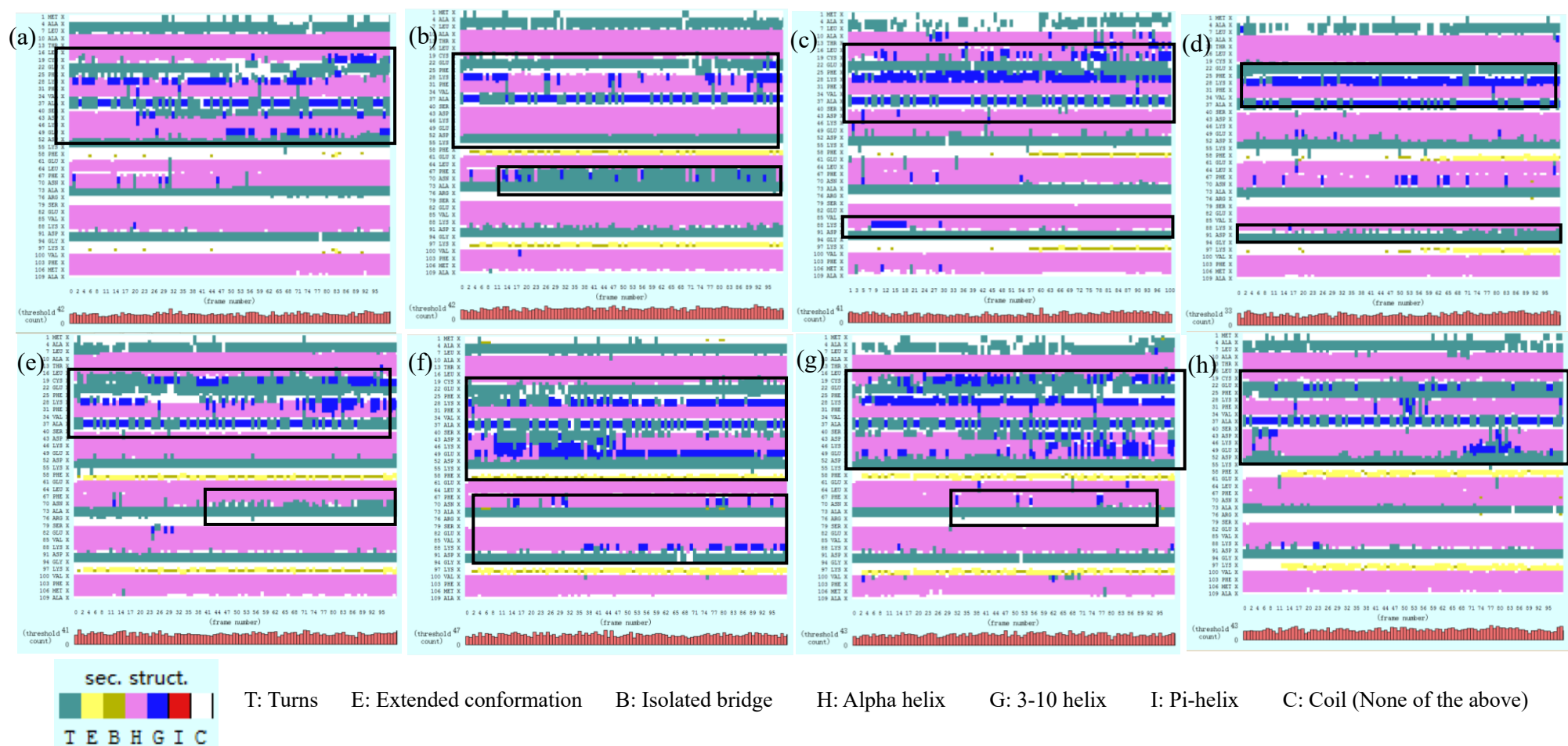


Figure 8.2. Secondary structure changes of cod parvalbumin Gad m 1 under electric field with thermal and pressure treatments: (a) 300 K, 1 bar, (b) 300 K, 3 kbar, (c) 325 K, 1 bar, (d) 325 K, 3 kbar, (e) 350 K, 1 bar, (f) 350 K, 3 kbar, (g) 375 K, 1 bar, (h) 375 K, 3 kbar.

The enhanced RMSD values might be caused by the increased thermal sensitivity of allergenic protein at a specific simulated temperature (J. Wang et al., 2020). At 3 kbar (Figure 8.3b), no significant differences were visualized in thermal simulations between 300-350 K, only small increments were observed at 375 K. This remained constant where RMSD values corresponded to the previous simulation study for soy Gly m 4, which might be attributed to the impact of external stresses on the RMSD reaching its maximum level (Vanga et al., 2019). Compared to 1 bar, overall RMSD values at 3 kbar significantly reduced between 300-350 K. However, RMSD values at 3 kbar were slightly higher than those at 1 bar at 375 K. The observation of lower RMSD values at higher pressures is in agreement with the results obtained from thermal simulation of soy allergen between 1 bar to 6 kbar (Vanga et al., 2019). The fluctuations of RMSD values above agree with the changes in protein secondary structures, which are probably due to the secondary structural losses and protein unfolding process induced by thermal stresses.

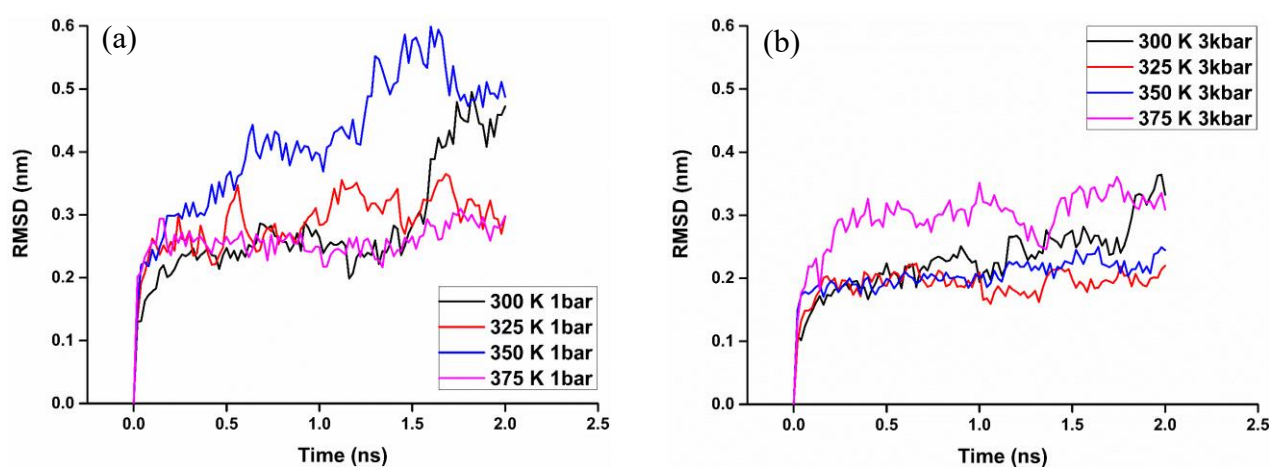


Figure 8.3. Changes of root mean square deviation (RMSD) in cod parvalbumin Gad m 1 simulated by increasing temperatures at 300 K, 325 K, 350 K, and 375 K under different pressures at (a) 1 bar and (b) 3 kbar.

8.4.3 Root mean square fluctuation (RMSF)

The changes of RMSF at various temperatures (300-375 K) and pressures (1 bar and 3 kbar) during the 2-ns simulation are presented in Figure 8.4. There was an overall fluctuation of RMSF values with the increasing atoms at each temperature, although a sharp decrease was observed in the first 100 atoms (Figure 8.4a). Most peaks were found under 325 K thermal simulation around atoms of 300, 800, 1300 and 1400, with one peak obtained at 350 K around atom 1100. However, no obvious interspecific differences in temperature affects average RMSF. At the strengthened pressure of 3 kbar, an overall increase of RMSF values was noticed with increasing applied temperature between 300-375 K thermal simulation (Figure 8.4b). This result agrees with the thermal simulation study on bovine β -lg for allergen epitopes (Saxena et al., 2019). When the higher temperatures were applied, protein epitopes lost their rigidity and further resulted in higher fluctuations. In Figure 8.4b, many sharp peaks are shown in atoms at 375 K, such as atoms around 100, 300, 600, 1200, and 1600. Moreover, peaks of thermal simulations at 325 K and 350 K were also recorded at atoms around 800 and 600, respectively. J. Wang et al. (2020) also observed the fluctuation of atoms in the kiwifruit allergenic protein molecule. These sharp peaks produced might be attributed to the secondary structural changes as previously discussed, such as the transformation of helices and turns. Similarly, Dong et al. (2018) found changes in RMSF values across the sequence under thermal simulation in marine mollusks. RMSF characteristics can reflect the thermal distortion of binding sites and perturbation of the conformational alterations (Dong et al., 2018).

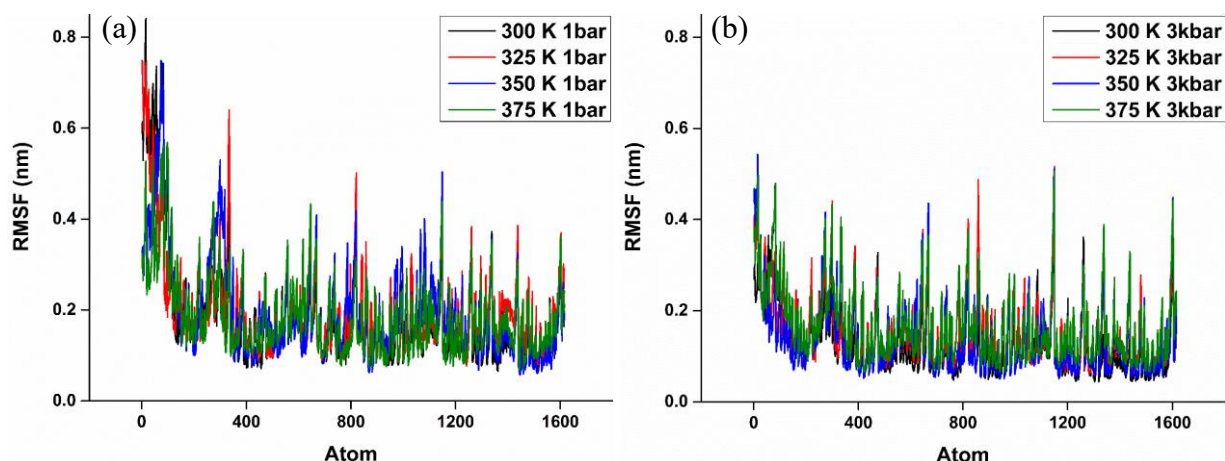


Figure 8.4. Changes of root mean square fluctuation (RMSF) in cod parvalbumin Gad m 1 simulated by increasing temperatures at 300 K, 325 K, 350 K, and 375 K under different pressures at (a) 1bar and (b) 3 kbar.

8.4.4 Radius of gyration (Rg)

Rg is considered as a parameter to imply the degree of compactness and folding stability. In the characterization of proteins, steady Rg values indicate protein compactness and stable folding; conversely, variable Rg values infer weak folding in proteins (Smilgies & Foltá-Stogniew, 2015). Figure 8.5 demonstrates the overall reduction trends of Rg in Gad m 1 allergen with increasing temperatures from 300 K to 375 K and stresses from 1 bar to 3 kbar. In Figure 8.5a, it was seen that the Rg values at 375 K significantly declined compared with those at 300 K. The constant fluctuations accompanying similar tendencies were observed between 325 K and 350 K, but both of their final Rg values at 2 ns were lower than that at 300 K. It was reported that radius of gyration correlates with the compactness of atomic packing (Lobanov et al., 2008). Therefore, the reduction of Rg values recorded at the increasing temperatures probably indicates the protein conformations to be packed when subjected to higher temperatures (Zhu et al., 2021). Compared to Rg values reported at a pressure of 1 bar, Rg values at 3 kbar showed a slight decrease, especially the Rg values at 350 K (Figure 8.5b). Despite the fluctuations observed at each temperature, the Rg values at 2 ns were all reduced to lower values compared to Rg at 300 K. However, based on the limited simulation time,

temperature possibly may not have any effect on the core packing of the molecule due to fluctuated R_g between 325-375 K (Saxena et al., 2019).

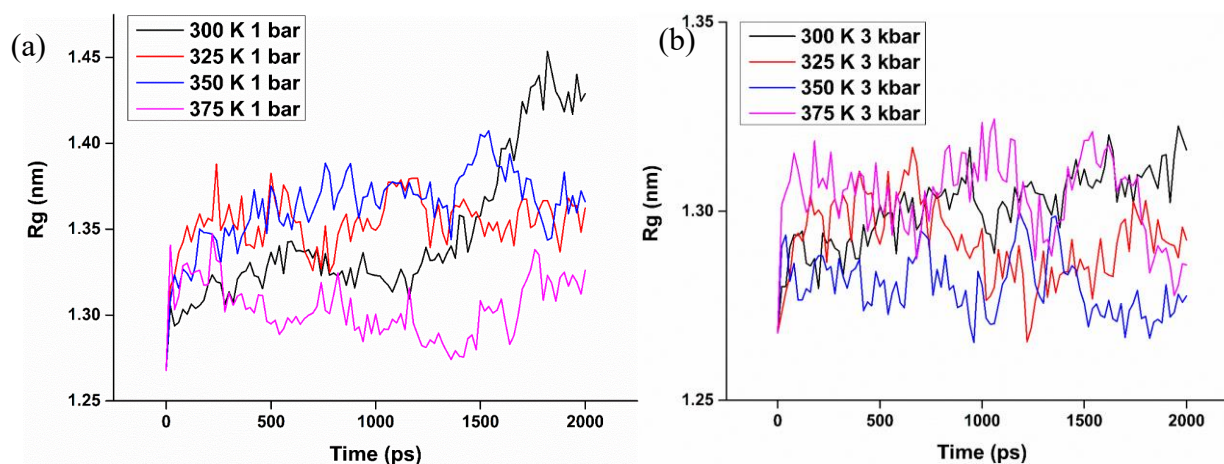


Figure 8.5. Changes of radius of gyration (R_g) in cod parvalbumin Gad m 1 simulated by increasing temperatures at 300 K, 325 K, 350 K, and 375 K under different pressures at (a) 1 bar and (b) 3 kbar.

8.4.5 Solvent accessible surface area (SASA)

SASA parameter is used to infer the interactions between protein and solvent or protein-ligand complexes and solvent (Gogoi et al., 2021). Protein surface characteristics play a significant role in affecting the functional properties of protein. Structural confirmations of the protein can determine the surficial properties. It is reported that even a slight conformational deviation is possible to induce alterations in surface properties and protein intermolecular interactions, which further leads to changes in functional properties (Saxena et al., 2019).

In this study, SASA was analyzed for Gad m 1 simulated by thermal stresses. According to Figure 8.6, the SASA values between 325-375 K overall dropped compared to the control at 300 K after 2 ns simulation when protein was applied with the increased temperatures and stresses. As described in Figures 8.6a-8.6c, the SASA values of control (300 K, 1 bar) were noticed to have an obvious increasing trend, implying new binding sites formed on the surface of the molecule during the simulation (Zhu et al.,

2021). Although the fluctuations between 325-375 K occurred, the SASA values at 375 K constantly reduced. When the stress was enhanced to 3 kbar, the average SASA values were lower than those at 1 bar due to the compression in the molecule. Similar results were also observed by Vanga et al. (2019) in a simulation study for soy allergen with thermal stresses (Vanga et al., 2019). The analysis for SASA results probably involves compactness in the protein structure, and the closed water inlet valves of the internal cavities. The decreased SASA values correspond to the decreased Rg values as discussed previously, which both imply the structural compression of protein in an aqueous medium (Ebrahimi et al., 2021).

Besides, the snapshots of the molecular structures of Gad m 1 simulated by thermal stresses were also obtained by using VMD (Figure 8.7). As the temperature increased during the thermal simulation at 1 bar, almost no obvious changes in the molecule structure were observed between 300 and 350 K (Figures 8.7a-8.7c). It was noticed that the multimeric form started converting into monomeric form at 375 K, which indicates high temperatures can effectively affect the surface properties of the protein molecule (Figure 8.7d). Moreover, basic residues protruded outward initially which began shifting inward in the core of the molecule gradually at 3 kbar (Figures 8.7e-8.7h). Similar changes in molecule surface were also observed in studies of egg avidin (Zhu et al., 2021) and peanut (Vanga et al., 2015). Under the thermal stresses, the unfolding of the molecule was observed with the increased temperatures, which agrees with the results of the alterations of the secondary structure and SASA as per the previous discussion.

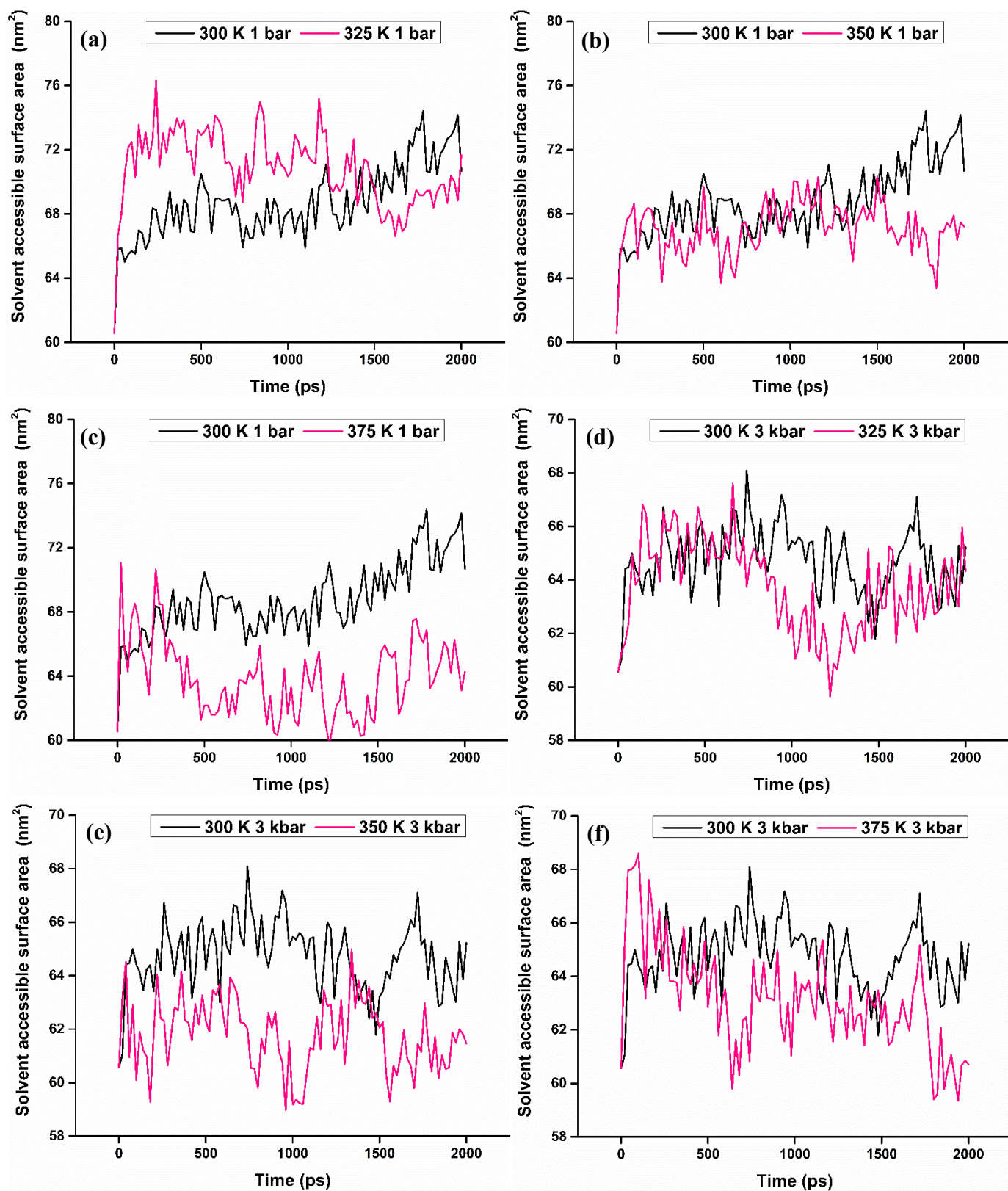


Figure 8.6. Solvent accessible surface area (SASA) variations in cod parvalbumin Gad m 1 simulated by thermal stresses (300-375 K, 1 bar/ 3 kbar).

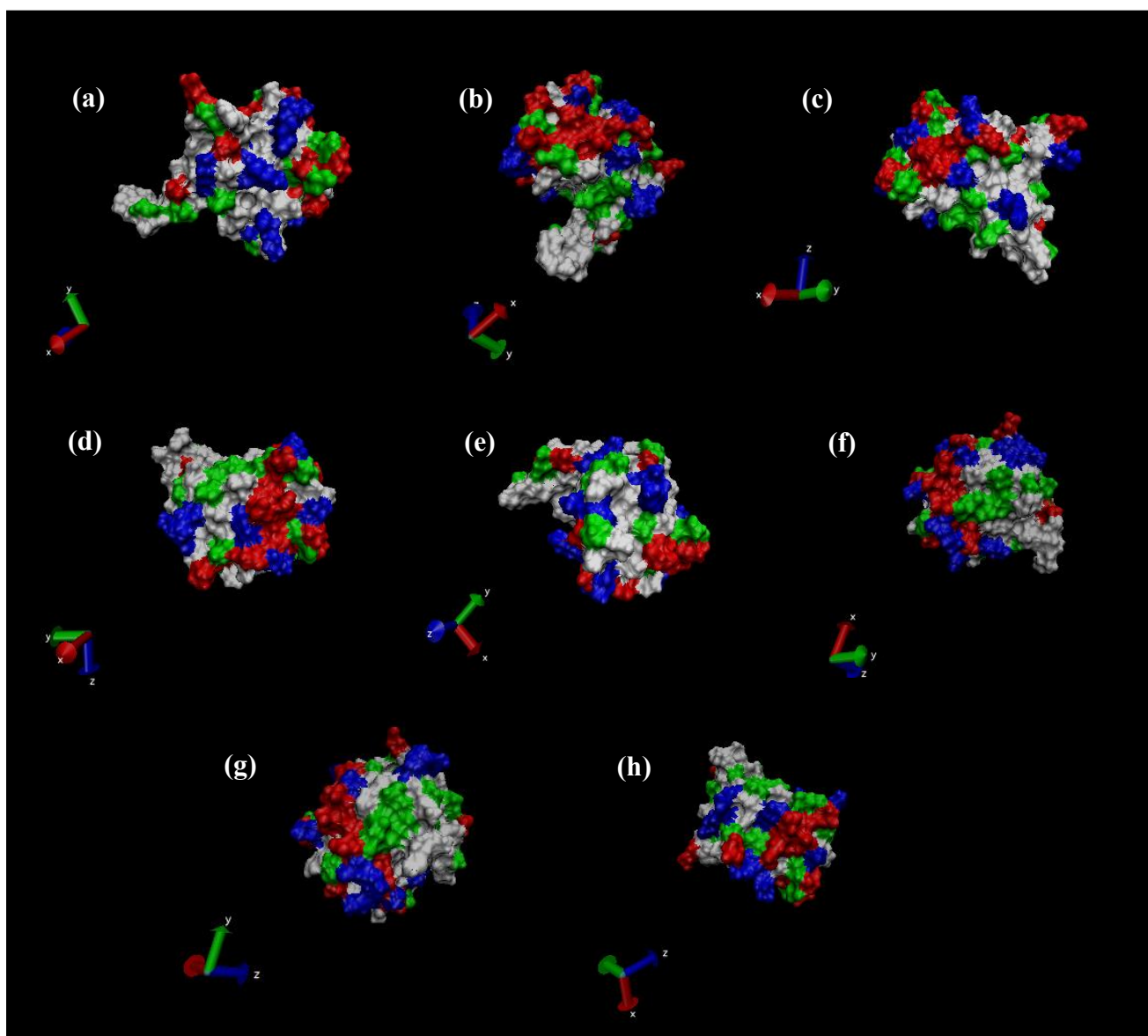


Figure 8.7. Snapshots of the surface properties of parvalbumin Gad m 1 after thermal simulations: (a) 300 K, 1 bar; (b) 325 K, 1 bar; (c) 350 K, 1 bar; (d) 375 K, 1 bar; (e) 300 K, 3 kbar; (f) 325 K, 3 kbar; (g) 350 K, 3 kbar; (h) 375 K, 3 kbar. Note: residue color code: polar residues (green), non-polar residues (white), basic residues (blue), and acidic residues (red).

8.5 Conclusion

Parvalbumin is a major fish allergen leading to fish allergy in fish-allergic individuals. Gad m 1 is identified as the main allergen present in Atlantic cod. Molecular dynamic simulation is a vital strategy in evaluating the structural changes in this molecule. In the food industry, thermal and pressure processing treatments are used as popular techniques which are applied to induce alterations in protein structures, further leading to changes in functional properties and allergenicity. In the present study, the effect of temperature and stress deviation under a simulated electric field was investigated in Gad m 1 cod allergen. The secondary structures of Gad m 1 were changed as per the analysis of the VMD timeline. The secondary structures including α -helices, 3/10 helices, turns and coils were converted under thermal stresses in specific residues. Notably, 3/10 helices converted to α -helices at 300 K whereas the reverse transformation occurred at 350 K, after which 3/10 helix transform back to α -helix at 375 K. The RMSD values of Gad m 1 overall increased with the increasing temperatures applied during 2 ns, and the enhanced pressure of 3 kbar caused lower RMSD values compared with those at 1 bar. The RMSF values at 1 bar fluctuated with the increasing atoms with a sharp reduction at first, and temperature effects were not obvious on average RMSF. The RMSF values at 3 kbar similarly fluctuated with the increasing atoms and showed an overall increasing trend with increasing temperatures. The Rg values of Gad m 1 allergen showed the overall reducing trend with increasing temperatures and stresses. Besides, the average Rg values at 3 kbar were lower than those at 1 bar. The SASA values between 325 and 375 K overall dropped in comparison to those at 300 K. The snapshots of the molecular structures of Gad m 1 at 1 bar showed that multimeric form converted to monomeric form at 375 K, with almost no obvious changes between 300 and 350 K. Moreover, basic residues protruded outward initially began shifting inward to the core of molecule gradually at 3 kbar. At present, MD simulation studies can be used as an effectively predictable database for the optimized setting of food processing parameters. More in-depth simulation studies focusing on

thermal stresses on the secondary structure and functional properties of parvalbumin are needed for better management of fish allergy. Furthermore, the use of simulations on purified proteins may not predict the impact of real processing when the proteins are in a food matrix. Further work is needed to validate how well the modeling software produces structural changes by using experimental studies.

Abbreviations

MD	Molecular dynamics
GROMACS	Groningen machine for chemical simulations
PDB	Protein Data Bank
VMD	Visual molecular dynamics
EM	Energy minimization
NPT	Constant pressure, constant temperature
NVT	Constant volume, constant temperature
RMSD	Root mean square deviation
RMSF	Root mean square fluctuations
SASA	Solvent accessible surface area

Declaration of Competing Interest

The authors declared that no conflict of interest to report in this work.

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Contextual transition

In previous chapters, we delved into various processing techniques applied to Atlantic cod, including thermal, non-thermal, and combined treatments, and extended this exploration to include molecular-level modeling of its primary allergen. The next chapter will present a comparative analysis of the allergenicity, digestibility, and bioavailability of Atlantic cod following the treatments used in Chapter 3 to 7. Additionally, we will also state the mechanism and clinical diagnosis of seafood allergy, offering readers a comprehensive understanding and laying the groundwork for potential future collaborative research.

Chapter 9: Comprehensive scholarly discussion

The doctoral research described in this thesis was aimed to comprehensively investigate and optimize the currently popular food processing techniques to improve food safety and quality. The treatments discussed in this research include thermal treatments (oven, steam, microwave, wet heat, and dry heat), non-thermal treatments (ultrasound and cold plasma), and combined treatments (thermal glycation). By evaluating and comparing the nutritional, structural and allergenic properties of treated Atlantic cod, the optimal processing parameters were obtained. Moreover, the processing techniques effectively lowered cod allergenicity to address the safety problems. These provided useful information for both food industry professionals and consumers concerned with food allergies. It will also make producing hypoallergenic food products to be possible in the future, which further improve food safety and food quality.

In Chapter 2 (literature review), we clarified seafood consumption and classification, the prevalence and symptoms of seafood allergy, biochemical characteristics of the major seafood allergens, and highlighted the recent advances in processing techniques (thermal, non-thermal, combined treatments) and main allergen detection methods for seafood products. Seafood is common table food, but seafood allergy is a public concern for human health. There are three categories of adverse responses caused by seafood based on different mechanisms: (1) Immunologic responses, including IgE and non-IgE anaphylaxis; (2) Toxic responses (i.e. marine biotoxins); and (3) Food intolerance (Acker et al., 2017; Bahna, 2016; Dong et al., 2021a). For the immunologic responses, IgE-mediated reaction is typically induced rapidly with fatal anaphylaxis developing within minutes to a few hours after the intake. Non-IgE-mediated reactions are typically chronic. Compared with IgE-mediated response, it is regarded as a more difficult disease to control with food avoidance alone (Anvari et al., 2019). Adverse responses triggered by the latter two categories often show the resemblance of clinical symptoms with seafood anaphylaxis, where the necessary diagnostic analysis of IgE antibody reactivity is needed to distinguish a true seafood allergy and other adverse responses (Davis et al., 2020).

Typically, seafood anaphylaxis is divided into two phases by sequence, the sensitization phase for the first ingestion of allergens, followed by the effector phase for the later intake of the same allergens (Fu et al., 2019; Palomares, 2013). In the sensitization phase, when allergens enter the human body, gut or skin antigen-presenting cells (APC) drive T cell differentiation into Th2 cells. Afterward, B cells are driven to switch and mature into main IgE-producing cells, and seafood allergy is consequently induced (Berin & Sampson, 2013). In the effector phase, when the specific allergens enter, the mast cells and basophils are activated and release potent inflammatory mediators. The mediators finally stimulate target organs to trigger allergic reactions (Fu et al., 2018). To distinguish seafood allergy, the mechanism of seafood tolerance is used where the regulatory T cells developed by the antigen presentation in the Gut-Associated Lymphoid Tissue (GALT) drive regulatory B cells to produce main IgG antibodies to seafood allergens, followed by the potentiality of B cells driving the secretion of IL10 and the production of IgG4 (Berin & Sampson, 2013).

The symptoms caused by IgE-mediated allergic reactions are usually rapid and severe, involving almost all organ systems, such as the skin, respiratory, gastrointestinal, cardiovascular, and neurological system (Anvari et al., 2019). The symptoms induced by non-IgE-mediated allergic responses commonly include seafood protein-induced enterocolitis syndrome (FPIES), allergic proctocolitis (AP), seafood protein-induced enteropathy (FPE), and celiac disease (Connors et al., 2018).

To prevent seafood allergies, effective and accurate diagnosis is essential for clinicians and patients. A double-blind placebo-controlled food challenge (DBPCFC) is regarded as a gold standard despite of the limitations of its utility. Seafood allergy diagnosis usually involves oral food challenges (OFC), skin prick tests (SPT), and allergen-specific immunoglobulin E (ssIgE) (Gupta et al., 2018), with novel strategies such as basophil activation tests (BAT) and component-resolved diagnosis (CRD). Until now, oral food challenge (OFC) is the reference standard for the assessment of seafood allergy and the severity of the clinical reactions (Kansen et al., 2018).

SPT and ssIgE are commonly used as sensitive methods to detect IgE sensitization. Based on the probability of clinical reactivity during oral food challenges, cut-offs serving as an important indicator have been generated to improve the specificity of SPT and ssIgE. Normally, specific IgE to allergens can differentiate species-specific allergens from cross-reactive allergens. This is helpful to distinguish between a true/potentially seafood allergy from the pollen-food syndrome or clinically irrelevant sensitization (Gomes-Belo et al., 2018).

Besides, basophil activation tests (BAT) are promisingly novel methods to be used in clinical applications for diagnosis with high specificity and sensitivity (Gupta et al., 2018). BAT can complement specific IgE to cause a delay of OFC in patients with a positive BAT (Gomes-Belo et al., 2018). In a study targeting the patients of 12- to 45-year-olds with fish and shrimp allergy, BAT results also showed a high correlation with DBPCFC severity scores (Song et al., 2015).

In addition, component-resolved diagnosis (CRD) is an emerging diagnostic approach in recent years. It assesses epitope-specific IgE by using a single allergen to predict individualized sensitization and reaction risks (Tong et al., 2018). Using prawn tropomyosin-sIgE has been confirmed to have a better positive predictive value for clinical reactions in comparison to SPT or ssIgE (Pascal et al., 2015). Despite CRD being promising, a diagnostic OFC is still necessary for an accurate evaluation (Wong et al., 2019).

As mentioned above, diagnosis for seafood allergy involves a thorough history, a series of tests including SPT, ssIgE, BAT, CRD, and OFC for diagnostic confirmation. However, the diagnosis of seafood allergy is still mainly based on the clinical history at present (Gomes-Belo et al., 2018). The management of seafood allergy should be tightly regulated, including different cells, cytokines, and other immune components. To date, avoiding seafood allergens strictly is the only standard way to clinically care for allergic individuals since immunotherapy is almost unavailable (Fu et al., 2019). Under the current scope from the literature allergen-specific immunotherapy is considered as the most promising treatment strategy, whereas for fish or shellfish allergy, there are still no published clinical trials for the evaluation of specific

oral immunotherapy (Ebisawa et al., 2015; Lozano-Ojalvo et al., 2018).

In Chapter 3 to Chapter 7, the effect of processing methods on the physicochemical and allergenic characteristics of Atlantic cod was thoroughly investigated. Thermal treatments including oven, steam, microwave, wet heat, and dry heat were discussed in Chapter 3, non-thermal treatments including ultrasound in Chapters 4 and 5 and cold plasma in Chapters 6, and combined treatments of thermal glycation in Chapters 7. All of these chapters discussed the changes of cod allergenicity, digestibility, and peptide content.

Regarding the changes in cod allergenicity, non-thermal treatment (ultrasound and cold plasma) led to an overall decrease, while thermal and thermal-glycation treatment caused both decrease and increase (Figure. 9.1). Among these treatments, steam maximumly reduced cod allergen content by 46.33%, followed by US60 (31.82%), wet heat (31.64%), microwave (24.71%), US30 (20.83%), and MW-GLU150 (16.16%). Despite cold plasma treatments resulting in the lowest allergen content reduction, notably with CP10 at 9.14%, the overall allergenicity changes in cold plasma-treated samples were modest.

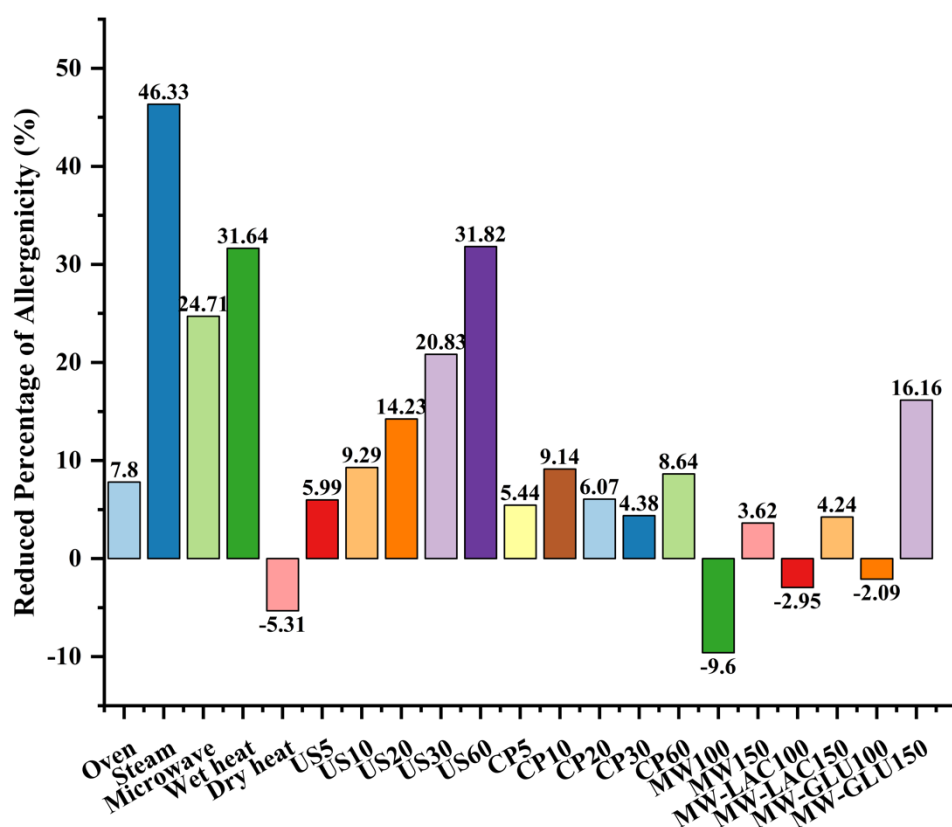


Figure. 9.1. The summary of the percentage changes of cod allergenicity in thermal, ultrasound, cold plasma, and thermal glycation treated samples.

Most treatments reduced IVPD percentage of cod samples, except for US30 and MW-GLU150 showing an increase of 12.24% and 19.26%, respectively (Figure. 9.2). Thermal treatments caused an overall lower IVPD compared to other treatments. This result is in accordance with the research findings stated in the previous work. In comparison to non-thermal processing, thermal processing methods destroy nutritional components present in food sources, despite of significantly reducing food allergenicity as described by Dong et al. (2021a). To satisfy consumer demands for high-quality, convenient, and minimally processed food products, non-thermal food processing is a good substitute for conventional thermal processing. They are made to prevent high processing temperatures and the adverse effects of heat on food. They have an impact on a number of characteristics, both microscopic and macroscopic, including rheology, flavor, process stability, texture, and appearance (Barbhuiya et al., 2021).

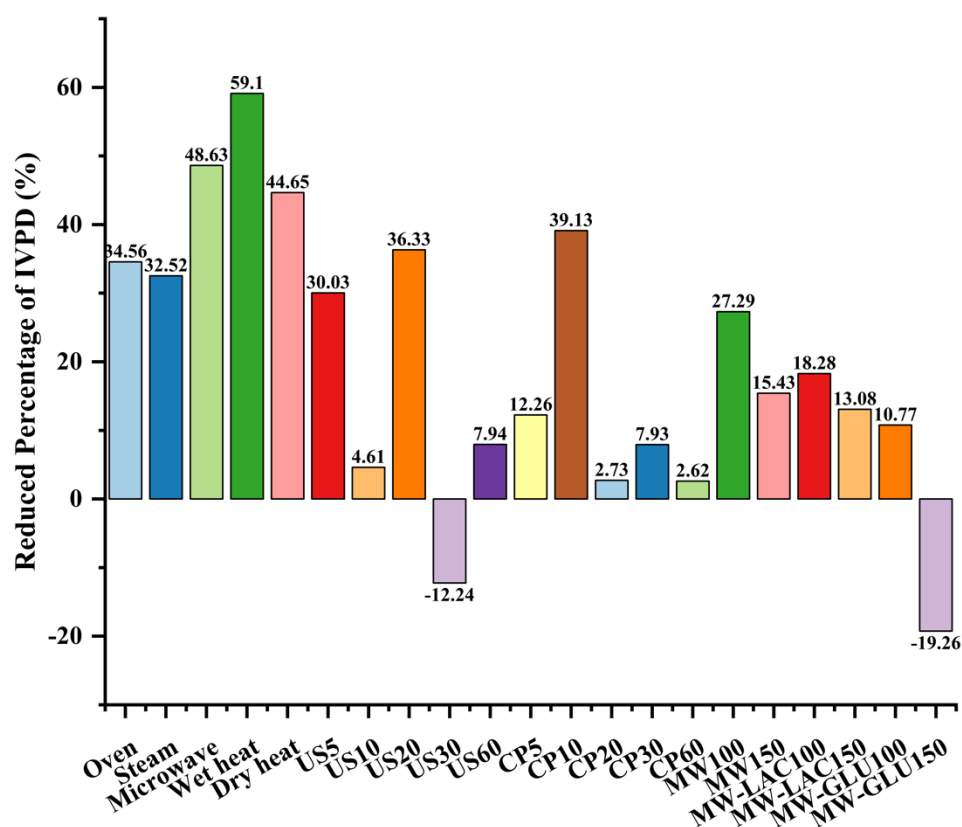


Figure. 9.2. The summary of the percentage changes of IVPD in cod samples treated by thermal, ultrasound, cold plasma, and thermal glycation.

Peptides are important bioactive compounds derived from functional foods for human health. They can be found naturally, formed during processing, or extracted from other sources (Butnariu & Sarac, 2019). Protein sources from seafood products have been utilized to produce bioactive peptides, such as salmon (Neves et al., 2017), chub marckerel (Bashir et al., 2018), stone fish protein (Auwal et al., 2017), and shrimp shell discard (Ambigaipalan & Shahidi, 2017) in other reported studies. In this work, several processing treatments increased the cod peptide content to higher bioactivity (Figure 9.3). MW150 led to the highest increase of peptides by 28.82% compared to the control, followed by CP30 (26.2%), CP60 (19.87%), dry heat (16.78%), and MW-GLU150 (7.59%). The bioactive peptides produced can mitigate oxidative stress and lipid peroxidation caused by free radicals generated during oxidation activities of a human body (Tadesse & Emire, 2020).

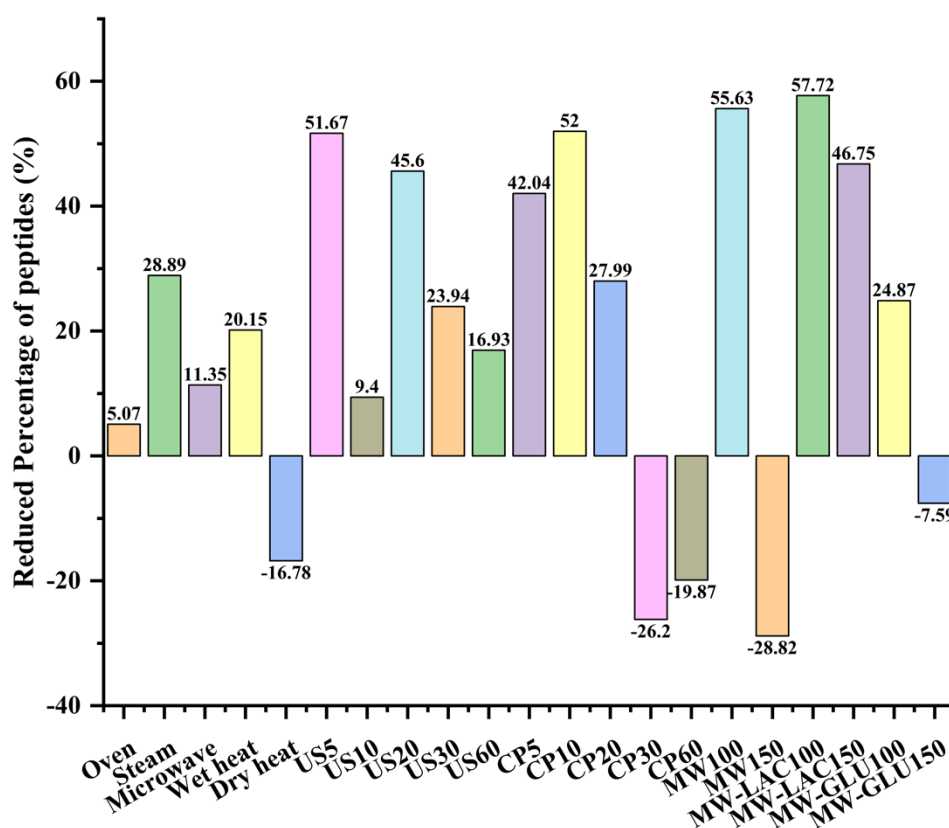


Figure. 9.3. The summary of the percentage changes of peptide in cod samples treated by thermal, ultrasound, cold plasma, and thermal glycation.

Thermal processing has emerged as the most effective method in reducing the allergen content of cod, with steam processing achieving a notable maximum reduction of 46.33%. However, this method also resulted in the lowest digestibility rates, adversely affecting protein absorption in the human body and leading to nutritional loss. In contrast, ultrasound processing not only showed promising results in reducing allergen content but also enhanced digestibility by 12.24%. However, it led to a decline in peptide content and bioavailability. Cold plasma treatment had a minimal impact on allergenicity and a slight decrease in digestibility, but it increased peptide levels especially at longer-time treatments (CP30 and CP60). These observations suggest that non-thermal treatments have a better performance in preserving the original quality and nutritional values of food, as described by Dong et al. (2021a) in previous work. Regarding the thermal-glycation treatment, the MW-GLU150 resulted in a 16.16% reduction in allergen content, with a 19.26% increase in digestibility and a 7.59% rise in peptide

content. This treatment uniquely achieved the dual goal of lowering allergenicity while enhancing digestibility and bioavailability. This outcome aligns with the findings of Dong and Raghavan (2022a), which indicated that hybrid treatments (a combination of thermal and non-thermal processing) outperform singular treatments (thermal or non-thermal processing only).

Chapter 10: Summary and Conclusions

Seafood allergy is a global health problem. Effective processing techniques are powerful in reducing allergenicity by modifying the allergen conformations and improving food nutritional properties. The three categories of food processing techniques are thermal, non-thermal, and combined (hybrid) treatments. Compared to thermal treatments, non-thermal treatments usually show better results in retaining the nutritional value and sensory quality of seafood products. Compared to a single treatment, combined (hybrid) treatments have a better performance with higher efficiency in eliminating seafood allergens. In Chapter 3, thermal treatments effectively reduced cod allergen content by 7.80%–46.33%. Steam demonstrated the best performance leading to the lowest allergen content (46.33%) and the highest *in-vitro* protein digestibility (55.29%) in cod. Changes to the secondary and tertiary structures were noted with higher β -sheets and lower α -helices. More structural destructions with fragments and micro holes were observed with increasing processing durations. In Chapters 4 and 5, high-intensity ultrasound decreased cod allergen content by up to 31.82% at 60 min and enhanced protein digestibility by up to 12.24% at 30 min. A significant reduction in total soluble protein content and a shift in protein secondary structure from α -helices to β -sheets and unordered structures was evidenced by FTIR and CD spectroscopy. UV spectroscopy, SEM, and electron microscopy analyses further confirmed these structural changes, indicating protein denaturation and potential Maillard reactions. SDS-PAGE results showed protein degradation and aggregation, particularly in sample treated for longer durations (60 min). In Chapter 6, the maximum TAC and TFC were observed at CP10, with the protein destruction leading to the lowest allergen content and IVPD by 9.14% and 35.32%, respectively. In Chapter 7, the combination of microwave (150°C) and glucose could significantly decrease the cod allergen content by up to 16.16% and significantly increase IVPD to 69.05%. Glucose showed better performance than the lactose when combined with microwave heating. In comparison to 100°C, the treatments at 150°C induced higher IVPD and peptides in cod. In Chapter 8, the study provides valuable insights into properties of parvalbumin molecule influenced by various

temperatures and pressures, including reversible molecule transformations. Additionally, the properties of the molecule's surface were influenced by various factors, such as the SASA and snapshots representing compactness within the protein structure. Notably, higher pressure (3 kbar) resulted in decreased values for the RMSD and Rg. All work deeply delves into the impact of processing techniques on the physicochemical, structural, and allergenic transformations of cod. The research provides useful suggestions for the optimization of food processing techniques in the food industry, which is essential for future food allergy management, thus contributing to the cause of human health worldwide.

Recommendation for future work

1. While processing techniques have reduced the allergenic potential of cod to some extent, the actual response after intaking fish by the allergy-related patients remains uncertain. Hence, there is a need for further studies focusing on clinical and immunological responses.
2. Future work could contain a broader nutritional assessment, including factors like texture, solubility, and amino acid content. This would provide a more comprehensive understanding of the physicochemical properties and contribute to a more thorough evaluation of food quality.
3. The development of more advanced processing techniques, with higher efficiency and improved outcomes, is essential for enhancing the quality and safety of food products.
4. In this study, only thermal glycation was explored as a combined treatment method. Future research should consider other combined approaches, such as integrating multiple non-thermal methods like ultrasound, cold plasma, and glycation.
5. Molecular dynamics simulation serves as an insightful tool for visualizing protein structural changes under various temperature and pressure conditions. However, the current simulations are limited by short durations due to computational constraints. Future research should aim to extend these simulations to 200 ns or longer, allowing for a better integration with experimental data to verify the modeling results.

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