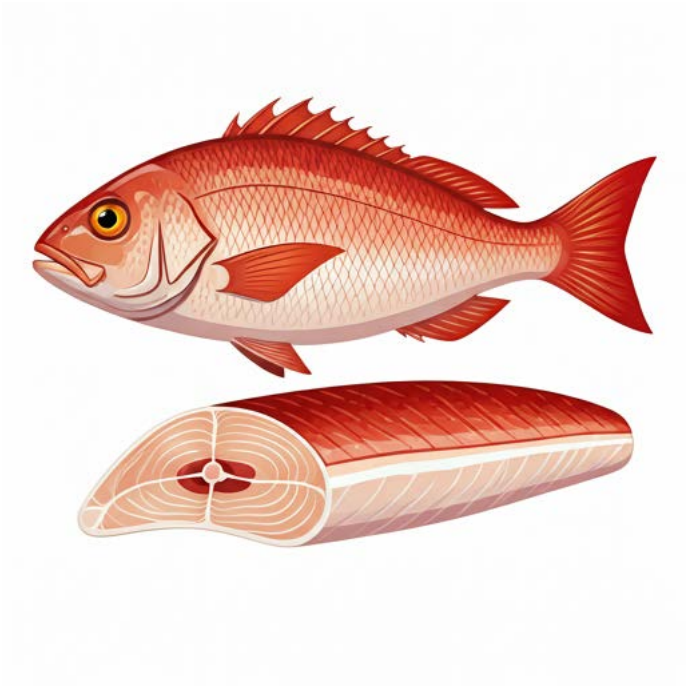
Understanding protein differences to make healthier and safer seafood



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Abstract

Fish allergy represents a major public health challenge, characterised by potentially life-threatening anaphylactic reactions that pose substantial risks to adults and children with fish allergy. Its diagnosis and management are challenging due to species diversity, complexity of fish protein compositions, and under-investigated intra-species variability. To date, only 11 known allergenic proteins have been identified across up to 23 fish species in the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Nomenclature database. Protein profiles, as explored in Chapter 2, demonstrate substantial variability across muscle tissue types, underscoring the critical need for optimised protein extraction protocols, which were fully established for subsequent analyses. Meticulous extraction methodologies are important for achieving high-quality protein recovery and ensuring reproducible proteomic analyses, which are fundamental to characterising the comprehensive allergen repertoire. Analyses presented in Chapter 3 reveal a significant correlation between fish size and protein/allergen abundances by employing proteomic approaches, including SDS-PAGE, immunoblotting, and mass spectrometry analysis. Size-dependent intra-species variability was demonstrated, which adds considerable complexity to fish allergy management. Notably, smaller fish specimens contained higher concentrations of the major fish allergen parvalbumin, while larger fish exhibited a greater abundance of heat-labile allergens. These findings highlighted the critical importance of fish size in allergenomics, proteomics, allergy management, and food safety. Allergen abundance analysis revealed differential protein profiles across distinct red snapper muscle regions, with belly region demonstrating pronounced parvalbumin concentration compared to other muscle regions. The observed variations in allergen abundances across different fish sizes and muscle regions provide a foundational framework for future investigations into fish species from diverse geographical origins. Preliminary studies indicated no statistically significant differences in allergen abundances between Malabar red snapper from the wild and three different farms. This research contributes pivotal insights into the molecular complexity of fish allergens, offering promising implications for improved diagnostic strategies, comprehensive risk assessment, and management of fish allergies.

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Statement contribution of others

Nature of assistance	Contributions	Name	Affiliations
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Statement on the use of generative AI

I hereby declare that during the preparation of this thesis, I utilised DeepL and Claude AI software strictly for language-related purposes. These artificial intelligence tools were employed solely for grammatical refinement, rephrasing, and linguistic optimisation of existing text. It is important to emphasise that the AI software was not used to generate original research ideas, develop content, or conduct any form of data analysis or interpretation. All research concepts, analytical approaches, figures, and data presented in this thesis are entirely my original work, derived from my independent research efforts and intellectual contributions. The AI tools served only as supplementary language-editing resources and played no substantive role in the intellectual or scientific development of this research.

Chapter 1

Exploring fish proteins and allergens for safer and healthier seafood consumption

Abstract

Seafood allergies are a global health challenge, and the prevalence of these allergies is increasing as consumption of both aquaculture and wild-caught fish increases. Despite the global importance of this issue, current research is limited, with only 11 fish allergens identified and registered in the global database. This study comprehensively explores the complex landscape of fish allergenicity, systematically investigating various factors influencing allergen abundances in fish species. Protein profiles in fish are influenced by multiple complex factors including body parts, muscle types, origins (wild versus farmed), and geographical locations, with variations observed in protein composition due to genetic, environmental, and life history traits. These differences may have implications for muscle protein compositions and allergenicity of fish species, which have not been well studied. Furthermore, the significant differences in fish consumption patterns and associated allergy prevalence between different countries highlight the need for comprehensive, region-specific allergy assessments and diagnostic strategies. This study addresses important knowledge gaps and provides a fundamental framework for the development of more accurate diagnostic tools and risk assessment strategies. The results highlight the need for continued research to understand and address this emerging public health issue, with the potential to improve global seafood allergy diagnosis and management strategies.

Keywords: fish allergy, fish allergenicity, allergen abundance, protein profiles, intra-specific, region-specific, species diversity, origins

1. Overview

Seafood, including fish and shellfish, contributes to addressing global food security and provides essential nutritional benefits for human health. Fish is a vital source of essential nutrients, healthy fats, and fat-soluble vitamins, and accounts for a significant portion of the world's food consumption. The diagnosis and management of fish allergy present complex challenges, primarily due to diverse fish species and intra-species variability. Current understanding of fish allergenicity is limited, especially when considering the differences between wild-caught and farmed fish sources. The fisheries industry has been accommodating the demand for fish consumption. According to the State of World Fisheries and Aquaculture, 87 per cent of the estimated 157 million tonnes of aquatic production are directly for human consumption and the remaining is used for non-food purposes such as fishmeal and fish oil (FAO, 2022). The rate of consumption has increased by 3 per cent annually from 1961 to 2019, but the rate dropped in 2020 due to the pandemic and the trend slowly increased in 2022 (FAO, 2022).

Overfishing has become a problem as the demand for seafood has increased, which has led to difficulties in replenishing fish populations. This causes a shortage of fish protein sources to supply the growing world’s population. Aquaculture has emerged as a critical solution to global food security, contributing 67 per cent to the world’s fish production in 2015 (Statistics, 2017). The industry has advanced methods to grow specific fish species across various infrastructural settings, ranging from extensive to intensive farming systems and fish feed development (Addis, 2012). This development not only addresses the growing global demand for protein but also represents a strategic approach to combating hunger and improving nutritional access for an expanding global population. This means that people are more exposed to and reliant on farmed fish as an alternative to wild fish. The increasing reliance on farmed fish has significant implications for allergy research and diagnosis. As people become more exposed to aquaculture-produced fish, the potential for allergenic variations becomes a crucial area of scientific investigation. The differences or alterations in protein structure between wild and farmed fish (Verdegem et al., 2023) may have profound implications for individuals with fish allergies, yet this knowledge remains inadequately explored in current literature.

Critically, the research landscape reveals substantial gaps in understanding the allergenic characteristics of fish from different origins. The complexity of fish allergies extends beyond simple species identification. Scientists and medical professionals are challenged by the lack of comprehensive studies that map allergenic proteins across various fish species and farming environments. This knowledge deficit complicates the development of precise diagnostic tools and management strategies for fish allergies.

The risk associated with fish allergies extends however worldwide, potentially triggering anaphylaxis whether the fish is sourced from wild catches or aquaculture. Multiple variables hinder the comprehensive identification of fish allergens, leaving gaps in our current understanding. This literature review will address the knowledge gaps and limitations of allergen identifications that are influenced by various factors. These gaps are important for improving seafood allergy diagnosis and management.

2. Classification of seafood

Fish and shellfish are often summarised as seafood.

2.1 Shellfish

Shellfish are animal groups that dwell in water and have a shell or shell-like exterior. Shellfish are invertebrates and can be separated into two groups: molluscs and crustaceans (Pascal et al., 2018). The most consumed among the molluscs are bivalve (clams, oysters and mussels), cephalopod (squid and octopus) and gastropods (snails). The most commonly consumed crustacea include Penaidae (prawns and shrimps), Brachyura (crabs) and Achelata (lobster). Since crustaceans fall under the phylum Arthropoda, they share a close phylogenetic relationship with arachnids and insect species. However, it is important to note that the allergens identified in shellfish are distinct from those identified in fish, and will therefore not be discussed further.

2.2 Fish

Fish are vertebrates and can be divided into two main categories: cartilaginous fish (Chondrichthyes: sharks and rays) and bony fish (Osteichthyes) (Betancur-R et al., 2017). The Actinopterygii subphylum of bony fish, finned fish, is a popular group of fish among consumers due to its diversity of flavour, texture and nutritional values. Fish consumption is

an important part of diets in many regions, fish provides the essential fatty acids and vitamin D.

2.2.1 Fish consumption around the world

Fish consumption varies in different regions as the availability of fish stock depends on the geographical location. The most traditionally consumed fish species in Europe include tuna, salmon, cod, Alaska pollock and hake. European countries display significant variation in adult fish consumption recommendations, ranging from 0.1 kg/capita/week in Bosnia and Herzegovina and the Netherlands to 0.5 kg/capita/week in Spain, with most nations suggesting two fish portions weekly (0.2 – 0.3 kg/capita/week) and several countries like Greece, Iceland, and Norway recommending 0.4 g/capita/week (Lofstedt et al., 2021). The different fish dietary intake in Europe is due to the health history studies to improve European cardiovascular health.

Meanwhile, in Asia, annual fish consumption is about 27 kg per capita, this varied across the regions; Japan registered the highest (65 kg), lowest in India (6 kg), Malaysia (45 kg), and Thailand (33 kg) (Dey et al., 2008). Singapore consumes an average of 22 kg of seafood per capita (SFA, 2021). This statistic demonstrates high fish consumption in the Asian region. Fish species commonly consumed in Asia are seabass, catfish, carp, tilapia and tuna. This means that people around the world are exposed to the risk of fish allergies but to very different species.

3. Pathogenesis of food allergy

3.1 Patients' sensitisation to food allergy

An allergic reaction is defined as an adverse immune response by the body to a foreign substance. Allergens can come from the natural environment (pollens, house dust mites, pet dander), foods (nuts, eggs, milk, shellfish), medications and insect bites or stings. In food allergies, adverse reactions are caused by an immune response to antigens after exposure to food. Food allergies can be categorised into two types: Immunoglobulin E (IgE)-mediated and non-IgE-mediated allergy. IgE-mediated food allergy reactions occur rapidly within minutes upon ingestion while it will take hours or days for non-IgE-mediated allergy to react (Chehade, 2007). The allergic reactions of IgE-mediated food allergies result in a multi-organ system including anaphylaxis and non-IgE mediated primarily affecting the gastrointestinal tract (S. Zhang et al., 2021).

In most cases, food allergies are mediated by IgE antibodies, which are produced when the body first encounters an allergen that can affect various organs commonly skin, airways, and the gut. A food allergen can enter the bloodstream through the gastrointestinal tract, leading to symptoms directly at the sites of contact (mouth, oesophageal, and/or intestinal), or in other parts of the body and the allergic reaction occurs immediately upon IgE cross-linking to activate the mast cells and basophils (Valenta et al., 2015). As shown in Figure 1, the introduced allergen binds to the B-cell, a white blood cell type responsible for producing antibodies. B-cell proliferation is stimulated by TH2 lymphocytes and associated cytokines, which promote IgE recombination, and plasma cells differentiate into antibody-producing cells (Sampson et al., 2018). The IgE molecules then bind to the receptors on the surface of the mast cells and basophils (J. Zhang et al., 2021). First exposure to an allergen tends to be asymptomatic; however, the body becomes sensitised to the specific allergen (Galli et al., 2008).

When the body is re-exposed to the same allergen, this allows the antigen to bind to two adjacent allergen-specific IgE molecules of identical specificity and bind the receptors together (Fadal, 1985). This triggers a signalling cascade that causes the release of granules that contain inflammatory mediators such as histamine and other inflammatory chemicals. Histamine produces inflammation by vasodilation, and increased vascular permeability causes mucus secretion, stimulation of sensory nerves, gastric secretion, cardiovascular effects and spasms of smooth muscles (Benly, 2015). Allergic reactions can be mild or severe, depending on the histamine level in the body. Furthermore, the amount and stability of the allergen ingested during digestion, and the permeability of the epithelial barrier are factors that contribute to the type and severity of allergic reactions (Valenta et al., 2015). Those with mild allergies may experience symptoms such as sneezing, runny nose, and watery eyes. Those with severe allergies may experience hives, swelling, difficulty breathing anaphylactic shock or other life-threatening reactions. It is critical to be aware of potential allergic reactions and take the necessary precautions to avoid them.

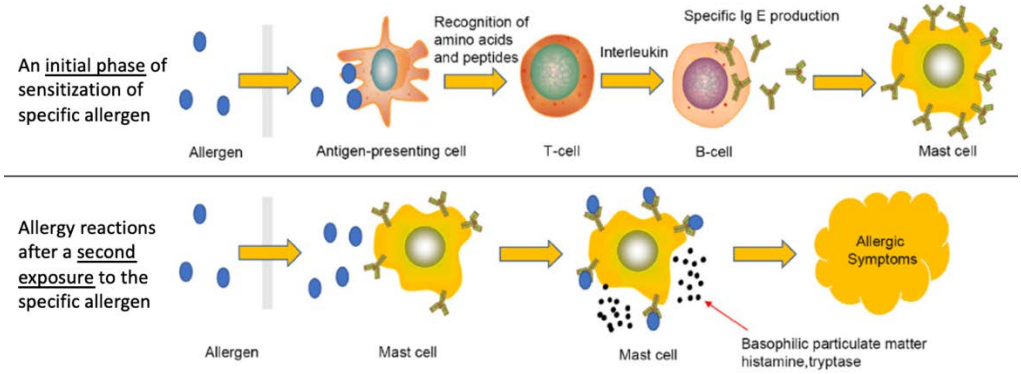

Figure 1: Mechanisms of Sensitisation and subsequent exposure to food allergy (J. Zhang et al., 2021).

3.2 Allergy cross-reactivity

Individuals sensitised to specific allergens may also react to other substances that can contain similar antigens known as cross-reactivity (Kamath et al., 2023). In other words, the antibody recognises a specific epitope (protein patch) on the antigen. Epitopes can be divided into linear and conformational epitopes as binding sites for the antibody (Deng et al., 2018). A linear epitope is a continuous stretch of amino acids that binds to an antibody and does not require tertiary structure to function; contrasted with conformational epitopes, which are discontinuous amino acid sequences that are brought together during protein folding to form an antibody binding site (Najar et al., 2017). It is assumed that an antibody reacts with the epitope if it reacts with a small peptide derived from the linear sequence (made from 8-15 amino acids) or if it reacts when the allergen is observed on the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or immunoblot after the allergen is unfolded, but the crystallographic studies suggest that protein epitopes are conformational: a general picture emerges that does not support the dichotomy between linear and conformational epitopes (Broide, 2001).

In addition, protein structural characteristics determine cross-reactivity, and IgE cross-reactions are initiated by shared features at the primary and tertiary structural levels of proteins, and sequence identity over 70% is required for the cross-reactivity (Ferreira et al., 2004). Cross-reactivity events are highly complicated and can take place either at the B-cell level, T-cell level, mast cell level or even at the structure of the protein level. Antibody-binding is most affected by the surface structure, particularly the epitope, which is part of the surface that interacts with antibodies and cross-reactivity is largely determined by structural factors: two

proteins are cross-reactive if they share structural characteristics (Aalberse, 2000). Unfortunately, it is not possible to derive common rules for allergen-antibody binding sites because the amino acid composition and their physicochemical properties including flexibility, solvent accessibility, lipophilicity, and electrostatic potential are very diverse (Dall'Antonia et al., 2014). The cross-reactivity between allergens is commonly examined in immunological studies. The cross-reactivity towards fish allergens is no exception and will be discussed below.

4. Diagnosis of fish allergy

4.1 Prevalence and distribution of fish allergy

In general, food allergies are common health issues throughout the world, but fish allergies are on the rise. The prevalence of fish allergy varies regionally, reflecting differences in the consumption of specific fish species between countries and the dietary requirements of the global population. In addition, the prevalence of fish allergy varies among adults and children. In Europe, the self-proclaimed fish allergy prevalence was 2.2%, while skin-prick test positivity was 0.6%, specific IgE positivity was 0.7%, and food challenge positivity was 0.1%; lifetime self-reported prevalence and specific IgE positivity were higher in younger age groups (Moonesinghe et al., 2016). Based on the survey, the responses were 2.29% in the Philippines, 0.26% in Singapore and 0.29% in Thailand from a cohort of 22,842 students aged 14 to 16 years old. In Vietnam, about 1.6% of adults have been diagnosed with fish allergy (Le et al., 2020). As allergies are complex and not only manifest in children but can also react to allergens in adulthood. A comprehensive study revealed that seafood allergies affect 5.9% of households, with 2.3% of individuals experiencing any seafood allergy, specifically 2% for shellfish, 0.4% for fish, and 0.2% for both types, demonstrating a significantly higher prevalence among adults (2.8%) compared to children (0.6%) and in women (3.6%) versus men (2%) (Sicherer et al., 2004).

4.2 Adverse IgE-mediated reactions to fish allergy

Allergy reactions to fish can be serious and life-threatening depending on the immunology of allergy-sensitised patients. The pathophysiology of food allergy relies on immune responses triggered by epitopes, which are small amino-acid sequences that are bound by antibodies, and some food allergens possess certain physicochemical properties that enhance their allergenicity (Dupont, 2011). Upon oral exposure to fish proteins, biologically active fish allergens were

detectable in serum samples as early as 10 minutes (Lopata & Lehrer, 2009). Sensitised patients experience IgE-mediated fish allergies, manifesting as acute urticaria, angioedema, respiratory symptoms, gastrointestinal symptoms, and anaphylactic reactions (Pascal et al., 2018).

Individuals with severe allergies to fish and shellfish may suffer health effects when seafood products are cross-contaminated during the processing process and these are known as occupational seafood allergens. In South Africa, fish processing is a common economic activity. Occupational allergic rhinoconjunctivitis and asthma symptom symptoms have been associated with fish among 7% of workers sensitised to fish; 2.6% had occupational allergic rhinoconjunctivitis and 1.8% had occupational asthma as a result of fish exposure in the processing factory (Jeebhay et al., 2008). Sensitised individuals will have the same reactions upon exposure to the allergen during processing or cooking via skin contact or inhalation. In a reviewed study by Lopata et al, occupationally sensitised workers recognise high molecular weight allergens, including glyceraldehyde-3-phosphate dehydrogenase, collagen and vitellogenin (Lopata & Jeebhay, 2013). These allergy symptoms are examined by conventional diagnostic methods including skin prick test, blood testing of the specific IgE antibodies, and oral food challenges.

4.2 Fish allergy diagnostic and immunotherapy

Fish allergy patients can be classified into three groups based on their immunological response patterns towards parvalbumin; 1) poly-sensitised patients who exhibit allergic reactions to multiple fish species, 2) mono-sensitised patients who react exclusively to a single fish species, and 3) oligo-sensitised patients who respond to several fish species due to sensitisation to enolase and aldolase but no IgE reactivity to parvalbumin (Dijkema et al., 2022). Current diagnostic approaches for fish allergy primarily rely on clinical history, skin prick test and specific IgE measurements. Currently, component-resolved diagnostic has emerged as a more precise tool, however, its clinical utility is limited by cross-reactivity among different fish species and it particularly focuses on the major fish allergen parvalbumin from European carp and cod (Calamelli et al., 2019). Regarding treatments, strict avoidance remains the primary management strategy. Apart from avoidance, oral immunotherapy trials for fish allergy have promising results by reducing the IgE reactivity (Ugajin et al., 2021). However, it has considerable challenges from practical issues like taste and odour to immunological complexities such as unpredictable cross-reactivity patterns and protein modifications during cooking, highlighting the critical need for protocol standardisation in this therapeutic approach

(Mack et al., 2022). Studies of the proteomic of a wide range of fish species are crucial to understanding the potential allergen presence and its abundance in the fish muscle tissue to expand the knowledge to improve the diagnostic and immunotherapy strategies.

4.3 Common fish allergens and characteristics of the allergenic proteins

Despite the global consumption of over 1,000 fish species, current allergenic research has identified only 11 allergens across a limited number of 23 fish species, with the most documented in the World Health Organisation/International Union of Immunological Societies (WHO/IUIS) Nomenclature database (www.allergen.org). Notably, striped catfish demonstrate the highest allergen abundance. Furthermore, the AllergenOnline database (www.allergenonline.org) lists 34 fish species as having similar potential allergenic proteins, highlighting the substantial gap in comprehensive allergen mapping across the vast diversity of consumed fish species.

4.3.1 Parvalbumin

In Table 1, the reported allergenic proteins of fish are listed along with their molecular weight. Parvalbumin is the major allergen (Mukherjee et al., 2023), which is commonly found in muscle tissue of most finfish species including cod, salmon, mackerel and tuna. Parvalbumin is responsible for regulating the intracellular calcium ion exchange in fast-twitch muscle with low molecular weight between 10-12 kDa and an acidic isoelectric point. It can be divided into two isoform lineages; alpha-parvalbumin, which is less acidic with an isoelectric point of pI 5.00 or higher, and beta-parvalbumin consisting of more acid parvalbumins with an isoelectric points of pI 4.50 or lower (Goodman & Pechère, 1977). Parvalbumin is characterised as a heat-stable protein. Pilchard parvalbumin was observed to increase the Th2 cytokines and specific IgE responses upon undergoing heat treatment (Dasanayaka et al., 2022).

Predominantly, beta-parvalbumins are found in finfish with reported cross-reactivity between parvalbumins from different fish and other vertebrate species such as frogs (Hilger et al., 2004). Similarly, in a recent study, fish-allergic individuals who have been exposed to fish parvalbumin may experience an allergic reaction if they consume crocodile meat since 70% of fish parvalbumin-sensitised subjects have demonstrated a binding pattern of IgE to heat-stable crocodile parvalbumin (Ruethers et al., 2022). The parvalbumin protein expression varies in different fish muscle tissues. It was observed that parvalbumin content is a) high in the rostral

muscle and decreases in the middle and caudal part of the fish, b) higher in the dorsal compared to the ventral part, and c) higher in the white muscle tissues compared to the dark muscle tissues regardless on species (Y. Kobayashi et al., 2016).

4.3.2 Beta-enolase and Aldolase A

Enolase and aldolase are enzymes in glycolysis processes, which are highly conserved among organisms. They were identified as important fish allergens in cod, salmon and tuna (Kuehn et al., 2013). The molecular weight of beta-enolase is between 47-50 kDa and aldolase A is 40 kDa. It was observed that 56% of the patients had specific IgE antibodies to cod enolase, 24% to salmon enolase and 19% to tuna enolase, meanwhile 37% had specific IgE antibodies to cod aldolase, 16% to salmon aldolase and 13% to an aldolase (Kuehn et al., 2013). However, cross-reactivity of enolase and aldolase between fish and chicken was reported. Fish allergens effectively inhibited IgE binding to chicken homologues in fish allergy patients, and the same was observed in chicken enolase and aldolase-sensitised patients; this was confirmed by skin prick testing (Kuehn et al., 2016).

4.3.3 Tropomyosin

Tropomyosin is a highly conserved protein in muscle and non-muscle tissue of all animals. This protein is responsible for muscle contraction having interaction with actin and troponin in the calcium regulatory system (Smillie, 1979). Tropomyosin produced by invertebrates can cause allergic reactions, including crustaceans (shrimp, lobster, crab, crawfish), arachnids (house dust mites), insects (cockroaches), and molluscs (shellfish), while tropomyosin in vertebrate have been generally considered as non-allergenic. The molecular weight of tropomyosin is between 33-39 kDa. Even though it is commonly found as shellfish and insect allergens, tropomyosin has been detected as an allergen in tilapia. This was confirmed by sera from 10 patients, IgE binding to the pure tropomyosin protein was observed in immunoblotting (Liu et al., 2013). In a study by Fernández et al, patients who complained of gastrointestinal symptoms were positive for IgE binding to the tropomyosin of cod, tuna and swordfish species by immunoblotting, but the skin prick test was negative (González-Fernández et al., 2018). In 2021, heat-stable tropomyosin was reported as an allergen in catfish and salmon species (Ruethers et al., 2021).

4.3.4 Collagen

Since collagen has a high biocompatibility, good bioactivity, and weak antigenicity, it has been studied extensively in the pharmaceutical, biomedical, cosmetic, leather, and film industries (Felician et al., 2018). The presence of collagen-specific IgE was observed in 21% of patients, with a greater proportion of adults (28%) than children (18%) suffering from fish collagen sensitisation (Kalic et al., 2020). Collagen alpha was registered as an allergen in Asian seabass and Atlantic salmon [41].

4.3.5 Other allergens

The group of enzymes responsible for the glycolysis process in the fish species include triosephosphate isomerase (27 kDa), pyruvate kinase (59 kDa), lactate dehydrogenase (36 kDa), glucose-6-phosphate isomerase (62 kDa), glyceraldehyde-3-phosphate dehydrogenase (37 kDa) (Kalic et al., 2021). Based on the WHO/IUIS allergen nomenclature database, these four enzymes are registered as minor fish allergens in striped catfish (*Pangasianodon hypophthalmus*) [41] and showed IgE binding for 6-13 % of fish allergic patients (Ruethers et al., 2021).

Creatine kinase is a key enzyme of cellular energy metabolism, primarily found in vertebrates' muscle and brain tissue. With a molecular weight of 43 kDa, salmon and catfish creatine kinase demonstrated IgE binding capacity [42].

Vitellogenin is present in the vertebrate egg yolk and has been reported as an allergen in salmon roe (Shimizu et al., 2009). Salmon roe allergies were reported in two patient cohorts, one with IgE reactivity to beta components and the other with IgE reactivity to lipovitellin; 45% (9/20) of the patients' sera reacted to lipovitellin from chum salmon and other salmonid lipovitellin, but every patient's serum had an IgE reacted with the beta-component of chum salmon, as well as IgE reactivity to beta-components from other salmonids (Shimizu et al., 2009). The results showed beta component of vitellogenin is the major allergen found in caviar. However, the current research will not include vitellogenin in the data analysis. This research will focus on the protein composition of muscle tissues.

Table 1: Fish allergens, species and allergen names are listed, derived from the WHO/IUIS Allergen Nomenclature database (www.allergen.org)

Fish Allergens	Molecular Weight (kDa)	Fish Species	IUIS Allergen Nomenclature	Characteristic
Parvalbumin	9 - 12	Striped catfish	Pan h 1	Regulating the intracellular calcium ion exchange in fast-twitch muscle
		Grass crap	Cten I 1	
		Common carp	Cyp c 1	
		Atlantic herring	Clu h 1	
		Baltic cod	Gad c 1	
		Atlantic cod	Gad m 1	
		Asian seabass	Lat c 1	
		Rainbow trout	Onc m 1	
		Indian mackerel	Ras k 1	
		Atlantic salmon	Sal s 1	
		Pacific pilchard	Sar sa 1	
		Atlantic mackerel	Sco s 1	
		Ocean perch	Seb m 1	
		Sole	Sole s 1	
		Yellowfin tuna	Thu a 1	
		Swordfish	Xip g 1	

Enolase	47 - 50	Striped catfish	Pan h 2	Enzyme in glycolysis
		Common carp	Cyp c 2	
		Atlantic cod	Gad m 2	
		Atlantic salmon	Sal s 2	
		Yellowfin tuna	Thu a 2	
Aldolase	40	Striped catfish	Pan h 3	Enzyme in glycolysis
		Atlantic cod	Gad m 3	
		Atlantic salmon	Sal s 3	
Tropomyosin	33 - 37	Striped catfish	Pan h 4	Major muscle protein
		Atlantic salmon	Ore m 4	
		Atlantic salmon	Sal s 4	
Collagen Alpha	130 - 140	Asian seabass	Lat c 6	Abundant structural protein in the extracellular matrix of connective tissues
		Atlantic salmon	Sal s 6	
Creatine Kinase	43	Striped catfish	Pan h 7	Enzyme in metabolism
		Atlantic salmon	Sal s 7	
Triosephosphate Isomerase	25	Striped catfish	Pan h 8	Enzyme in glycolysis
		Atlantic salmon	Sal s 8	
Pyruvate Kinase PKM-like	65	Striped catfish	Pan h 9	Enzyme in glycolysis

Glucose 6-Phosphate Isomerase	60	Striped catfish	Pan h 11	Enzyme in glycolysis
Glyceraldehyde-3-phosphate dehydrogenase	36	Striped catfish	Pan h 13	Enzyme in glycolysis
Lactate dehydrogenase	34	Striped catfish	Pan h 10	Enzyme in glycolysis
Vitellogenin (not fish muscle protein)	18	Chum salmon	Onc k 5	Fish yolk protein

5. Factors influencing protein and allergen profile

5.1 Protein differences in body parts and sizes

Fish muscle proteins are categorised into three major protein classes; 1) myofibrillar proteins known as structural proteins (myosin and actin) and regulatory proteins (tropomyosin, troponin and actinin); 2) sarcoplasmic proteins consisting of low molecular weight compounds such as albumins, myoglobin, haemoglobin and enzymes; 3) myoglobin and haemoglobin are responsible for the colour of fish muscles and concentrated in the red muscle (Venugopal & Shahidi, 1996).

The body size of the species influences the proteome profiling analysis. Genetic variation, environmental factors, and life history traits all play a role in the proteomic differences between fish species. A review examined the muscle composition of various fish species, including snappers and seabass, from marine, brackish, and freshwater habitats. The study examined variations in fish body composition by comparing different fish species from diverse marine and freshwater environments, taking into account multiple influential factors such as geographical location, food availability, growth and maturation rates, reproductive characteristics, and survival mechanisms (Ahmed et al., 2022). These comparisons were conducted across distinct species rather than within a single species from different origins and

allergens were not investigated. In addition, the parvalbumin content in white muscle tissues was observed to be higher in the dorsal muscle than ventral muscle, and the rostral muscle higher than the caudal muscle of 12 marine fish species (Y. Kobayashi et al., 2016). Dark muscle tissues had low allergenicity compared to white muscle tissues of horse mackerel and pacific mackerel (A. Kobayashi et al., 2006). However, among fish allergens, only parvalbumin has been investigated in fish muscle tissues of different body parts or specimens of different sizes.

5.2 Protein differences between wild and aquaculture populations

The increasing demand for fish causes wild stocks to be depleted. Overfishing has remained an issue for decades. Farm-raised species serve an alternative supply however wild stock remains a viable food source. The environmental conditions vary as the wild stock is caught in its natural habitat with vast food availability. The diets of farm-raised species are controlled due to the fact they grow in captivity in a controlled environment either in their native region or in an introduced region. Additional innovations such as new feed formulations, feed supplements and selective breeding might influence the protein diversity and abundances.

Wild sea bream expressed higher levels of structural proteins including actin, tropomyosin and myosin light chain while farmed specimens expressed higher levels of glycolytic enzymes such as enolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglucomutase 1, pyruvate kinase and phosphoglycerate (Addis et al., 2010). According to the textural profile, wild sea bass had higher collagen levels than farmed species (Rincón et al., 2016). However, there are no studies published comparing the abundance and immunoreactivity of allergens proteins between wild and farmed fish.

5.3 Protein differences based on geographical locations

Fish species are incredibly diverse, with a wide variety of specimens found in different water bodies all over the world. Water bodies can differ in various ways including physical, chemical and biological characteristics. The key differences among the water bodies are temperature, salinity, pH and oxygen levels as well as different diets that can impact the composition of fish muscle proteins. Sea bass reared in seawater expressed 362 proteins more than those reared in freshwater, according to a study of the effect of salinity on their proteome (Ky et al., 2007). In another scenario, proteome variation was observed in European whitefish between brackish and freshwater conditions (Papakostas et al., 2014). The temperature of the water conditions is

widely affected by the climate, seasons, depth and location. Proteins associated with cytoskeletons, carbohydrate metabolism, proteolysis, amino acid conversion and catabolism, cellular defence stress responses, and protein synthesis differed in fish adapted to 8°C versus 22°C (Ibarz et al., 2010). The key variations of water bodies influence the fish protein compositions that play a role in the species' texture, flavour and nutrient value. Furthermore, each species of fish population has its unique behaviour, diet and habitat. It is therefore important to understand the impact of environmental factors on the protein and allergen composition in fish.

6. Conclusion

Seafood allergies are becoming an increasingly global health concern. The complexity of the human immune system's response to various allergens and the incomplete knowledge of the fish proteome continues to present considerable challenges in the diagnosis and management of fish allergies. This literature review provides an insight into the current understanding of factors that influence protein expression and the allergenicity of fish. Diversity of fish species can have unique characteristics due to environmental factors including variations in water salinity, temperature, food availability and behaviour. Furthermore, fish specimens from different origins, such as wild-caught and farm-raised, seem to have distinct proteome profiles. In addition, species from different geographic locations should be analysed to determine the factors responsible for differences in proteome profiles. The geographical locations can influence the allergenicity of the potential proteins which could lead to a better understanding of how allergenicity is affected by environmental conditions.

There are no published research studies on the proteomic composition of fish species with a specific focus on food allergens. Although various species are observed in established allergen databases, it is crucial to understand the proteomic level within the species. This could identify the potential allergens that might be missed if only studying a single representative specimen and lead to a more comprehensive understanding of fish protein diversity and its impact on allergenicity.

7. Research plans

Advanced and standardised proteomic approaches are required to enable detailed comparisons between specimens. Hence, this thesis addresses two critical research topics: Firstly, different

mechanical protein extractions were explored and highly standardised protocols were developed to ensure reproducibility of proteomic analysis. Secondly, intra-species variability in proteomic composition and allergenicity was evaluated.

Current research lacks standardized fish protein extraction techniques, hindering comprehensive comparisons of allergen profiles and abundance across fish species. There are different ways of extracting the proteins from fish muscle tissues. Methodological variability in fish protein and allergen research is important due to the diverse ways fish allergic patients are exposed. The allergenicity of fish can change depending on various factors. This is addressed in Chapter 2. A robust protein extraction technique must account for these diverse exposure scenarios to provide meaningful insights for allergic patients and researchers. Protein recovery can also differ from multiple extraction methods and buffers used to extract the proteins. Hence, **Chapter 2** develops and optimises a reliable protein extraction method specifically for fish muscle tissue. This chapter aims to achieve high-quality protein recovery for subsequent protein analysis by investigating the diversity of protein profiles of each fish tissue representative and its allergen contents. The complexities of fish muscle proteins underscore the importance of developing comprehensive extraction methods that can capture the full range of potential allergenic variations.

Chapter 3 research study focuses on the protein and allergen composition influenced by the fish sizes from one farmed fish species (*Lutjanus malabaricus*, Malabar red snapper). Proteins were extracted using the protein extraction protocol optimised in Chapter 2. The investigation focused on understanding how protein profiles vary across different sizes and muscle locations. Fish of varying sizes may exhibit differences in protein expression due to the growth stages and developmental needs. This could relate to the different body parts as they may have different proteins tailored to their specific functions. The primary focus is on identifying and quantifying the 11 fish allergen abundances within these variations. By examining specimens across multiple sizes and from various muscle locations, the research seeks to map the complex proteins and allergens within a fish species. This approach allows us to characterise how allergenic protein changes during different developmental stages and how it varies across different muscle tissues.

Future studies building upon Chapter 3 investigate the protein variability in specimens of two commercially important fish species in Southeast Asia: the Malabar red snapper (*Lutjanus malabaricus*) and the Asian seabass (barramundi, *Lates calcarifer*). The overarching

hypothesis is that the allergenicity of fish specimens of the same species differs depending on origin. This research study aims to investigate the influence of aquaculture practices on protein variability by comparing wild-caught fish with those raised in aquaculture facilities. This variability could stem from several factors, including variations in fish feed, environmental factors and genetic variations between farmed stock. By analysing protein profiles and abundance across farms, it seeks to determine if these aquaculture practices significantly alter protein composition in these fish species, especially the major allergens and their allergenicity. While it is important to focus on protein diversity within species and species sourced from various farms, **future studies** can broaden the scope to investigate the impact of geographical locations on protein/allergen profiles in different species. Fish inhabiting different geographical regions encounter diverse environmental conditions such as water temperature, salinity and dietary adaptations that could potentially influence protein expression. Recent studies on shrimp species observed to have origin-dependent protein composition which has an impact on the allergenicity of the shrimp (Dorney et al., 2024). However, there are no studies on the allergenicity of fish species comparing different origins including geographical locations, wild capture and aquaculture capture.

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Chapters overview

Chapter 1: Introduction to understanding the differences in fish proteins to make healthy and safer seafood.

• address the health concerns of seafood allergy around the world which causes life-threatening anaphylaxis.

• This chapter compiles a comprehensive list of registered fish allergens and their functional roles within the muscle tissues.

• Identify the factors that could influence the protein variability which impacts the allergenicity of the protein.

• Address the knowledge gaps in understanding the protein differences in fish species.

Chapter 2: Optimisation of protein extractions for proteomics analysis of fish tissue

• Exploring the different extraction methods and establishing the standardised protein extraction

method that is most suitable for muscle tissues.

• Address the challenges of extracting proteins from fish muscle tissues.

• Prioritising the optimised extraction method to achieve high-quality protein recovery and abundance.

• Explore the diversity of protein profiles of various fish muscle tissues and the impact on the allergen profile.

Chapter 3: A comparative analysis of protein profiles across *Lutjanus malabaricus* (Malabar

red snapper) muscle tissues.

• Investigate the protein profiling of different red snapper tissues to understand the aspects that influence the differences in protein.

• Comparing the protein diversity in various muscle tissues and body locations of the red snappers.

• Explore the protein differences between fish due to developmental stage, environmental adaptations and genetic variations.

• Understanding the protein's abundance with a focus on parvalbumins and other known and putative allergens.

Future studies

1. Analysis of protein variations of the closely related genus of red snapper species. This is to understand the impact of species differences on the abundance and distribution of allergens.

2. Comparison of protein variations between farmed species depending on various locations and

aquaculture practices.

3. Investigate the protein variability that is influenced by environmental conditions by analysis of species from various geographical regions.

Chapter 2

Optimisation of protein extraction from fish muscle tissues for standardised proteomics

Abstract

Fish is packed with essential nutrients, healthy fats and fat-soluble vitamins, and contributes to a significant portion of global food consumption. However, the majority of consumers with fish allergy exhibit a reactivity to bony fish species and parvalbumin is a major fish allergen. The muscle tissues of commercially important fish such as Malabar red snapper (*Lutjanus malabaricus)* and Asian seabass (barramundi, *Lates calcarifer)* are composed of distinct layers of white meat, red meat and a skin layer. Individual muscle tissues are distinctive, reflecting their specialised functions, and biological sample analysis is inadequate for a comprehensive study of protein variation. This study aims to further optimise fish protein extractions to foster comprehensive analyses of complex protein compositions in muscle tissues. Fish proteins were extracted by various mechanical extraction including mincing and blending into powder after snap-freeze and freeze-drying of muscle tissues. Mechanical extractions and individual tissue protein extraction allowed for a deeper understanding of protein abundance and potential allergens presence in both fish species. White muscle tissues of red snapper yielded the highest protein content and the highest abundance of parvalbumin, the major fish allergen. Two parvalbumin isoforms were found in white meat and skin whereas red meat contains only one isoform in red snapper. The observation of protein profiles of each muscle tissue allows for the optimisation of protein extraction methods. This study established a highly standardised fish extraction procedure to ensure the reproducibility of proteomic analysis.

Keywords: Fish allergy, pelagic fish, muscle tissues, mechanical protein extractions, protein profiles, protein abundance, allergens distribution, parvalbumin

1. Introduction

Seafood allergies are a major global health concern(Davis et al., 2020). Allergic reactions to fish occurred on first exposure in 171 (65%) Filipino, 7 (41%) Singaporean, and 4 (67%) Thai children after their first year of life (Connett et al., 2012). In Singapore, a study diagnosed fish allergy in 108 children, primarily affecting 62% of infants and young children, with threadfin, salmon, and cod being the most common allergens, often leading to skin reactions and, less frequently, anaphylaxis (Tan et al., 2023). Eleven allergens were identified in fish muscle from up to 23 fish species with parvalbumin often referred to as the major allergen (Ruethers et al., 2018). Parvalbumin was found to be more abundant in white muscle tissues compared to red muscle and more abundant in sedentary fish compared to migratory species (Kobayashi, Yang, et al., 2016). The protein levels differ in each muscle tissue due to the differences in function of various types of muscle tissues. This possibly includes the allergens distribution correlated to the red and white meat of various fish species

Fish rely on white muscle for rapid bursts of movement, making it ideal for escaping predators or capturing prey (Omidvar et al., 2024). White muscle fibres are thick and powerful, but they contain fewer blood vessels compared to other muscle types (Alam, 2007). This means they receive less oxygen. However, white muscle utilizes a special energy production process that doesn't require a lot of oxygen, allowing for those short bursts of speed. Red muscle tissue is underneath the skin layer located along the lateral line, responsible for sustained and slow-continues movement (Boland et al., 2018). The dark pigment originates from the high concentration of myoglobin that contains haemoglobin within the myofibrillar proteins (Alam, 2007). Lastly, the intermediate pink muscle is located in between the red and white muscle tissues and serves an intermediate functionality of both muscle tissues, allowing the fish to swim continuously at high speed (Omidvar et al., 2024). Moreover, the diversity of proteins in fish species provides insight into the identification of potential allergens and their distribution among different muscle tissues.

The preparation and consumption of fish exhibit variability across different cultural and regional contexts. For instance, the consumption of raw fish as sashimi is particularly prevalent in certain geographical regions. Furthermore, a wide range of fish processing and cooking techniques, including the preparation of fish paste, drying, roasting, grilling, and poaching, can significantly alter the structure and properties of fish proteins (Bhat et al., 2021). Fish allergy

patients can be exposed to allergenic proteins through various fish preparations. Therefore, protein extraction methods for proteomic analysis should mimic the breakdown of proteins during digestion.

A standard protein extraction method is crucial to obtain high-quality and reproducible protein recovery. In most studies of fish allergens, the fish proteins were extracted by mincing and homogenising the muscle tissues (Campion et al., 2012; Kobayashi, Yang, et al., 2016; Ruethers et al., 2021). However, the methodology used is not sufficient due to the complexity of the protein variation in each muscle tissue. Hence, the inclusion of technical replicates plays an essential role in showcasing the rigorous standardisation of the methods employed for fish protein extraction. Technical replicates refer to the repeated analysis of the same sample using the same method. The purpose is: firstly, to validate the consistency of the extraction procedure, and secondly to ensure the reproducibility of results. The technical replicates aim to establish that the methods utilised should represent a consistent and replicable approach to extracting fish proteins. These technical replicates serve as a quality control measure to access the standardisation of the extraction process. This is crucial in scientific research, particularly in proteomic analysis, as minor error variations significantly impact the outcome.

This study aims to evaluate the efficacy of different mechanical protein extraction methods to further optimize the extraction process and to characterize protein profiles in a range of muscle tissues, thus facilitating the development of a reliable analytical approach. Optimising these extraction protocols is crucial as it will establish a foundation for future protein profiling research.

2. Materials and methods

2.1 Muscle sample collection

Specimens of two fish species, Malabar red snapper (*Lutjanus malabaricus*) and Asian seabass (barramundi, *Lates calcarifer*), were collected from a farm in Singapore and were kept chilled upon delivery.

The fish were descaled and cleaned. The weight and length were recorded for data collection purposes. The fish was then filleted to collect the dorsal muscle in the specific location shown in Figure 1 (shaded region). A straight cut was made along the line starting at the dorsal fin (point A) and anal fin (point C). The section of point B was half the length of the distance

between points A and C. An additional straight cut was made along the vertebrae (point D). Finally, the dorsal muscle tissues were isolated by filleting the shaded region.

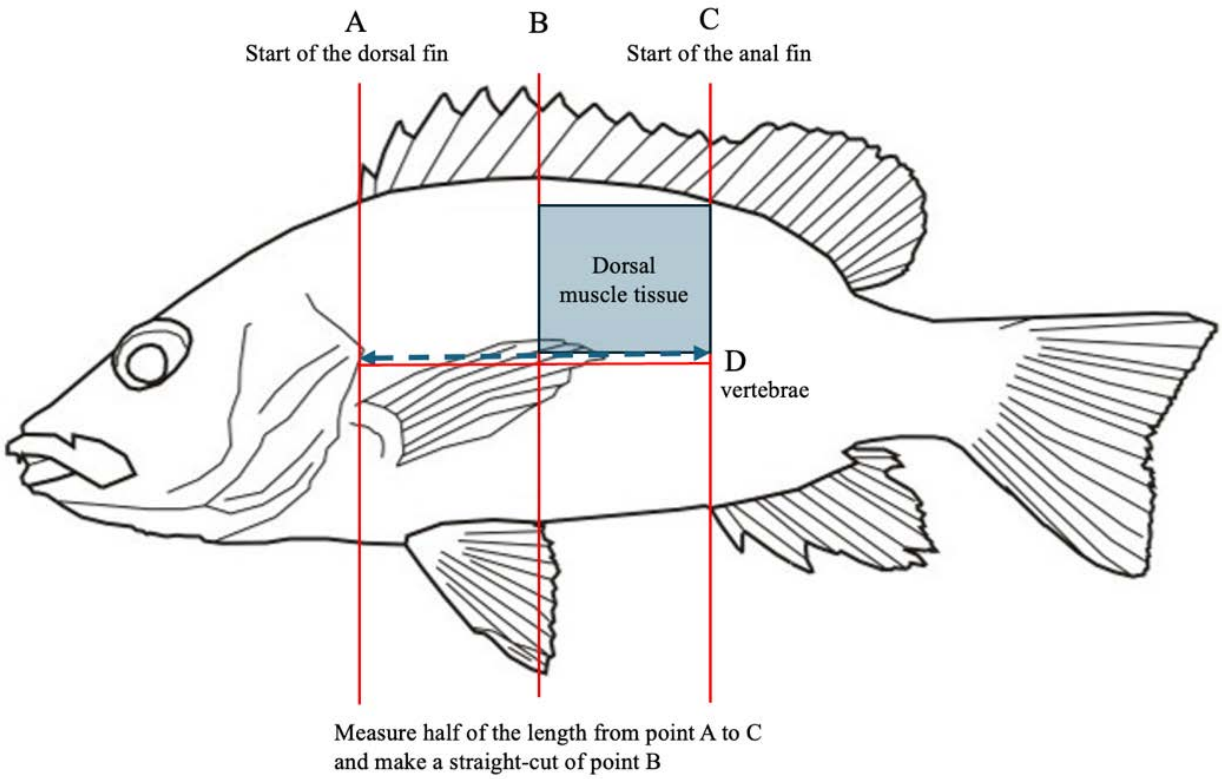

Figure 1: Illustration of standard sample collection of the dorsal muscle tissue (shaded region) based on the measurement of two distinct fins; dorsal fin and anal fin.

2.2 Mechanical protein extractions from fish tissues

2.2.1 Raw and heat extraction

The protein extractions were divided into two groups (Figure 2): raw and heat-treated. For raw extraction, muscle tissues without skin of seabass were prepared by mincing, blending into a powder with liquid nitrogen, and freeze-drying. Tissue from only one specimen for each extraction was utilised. For further optimisation snapper was the species of focus. Individual muscle tissues (white meat, red meat, and skin) from one specimen were collected and processed using liquid nitrogen methods to obtain powder. Whole red snapper powder muscle tissues (with skin) were compared with homogenised and non-homogenised techniques. Technical replicates of whole red snapper powder were incorporated for final optimisations of protein extraction. In addition, technical replicates of minced snapper muscle tissues were compared with optimised red snapper powder extracts.

For heat extractions, the fish fillets were first heated at 95°C for 20 minutes. This was done before incubation, after incubation, and after centrifugation. Tissue from only one specimen for each extraction was used. All samples were incubated under agitation at 4°C for 19 hours and then centrifuged at 12,108 x *g*.

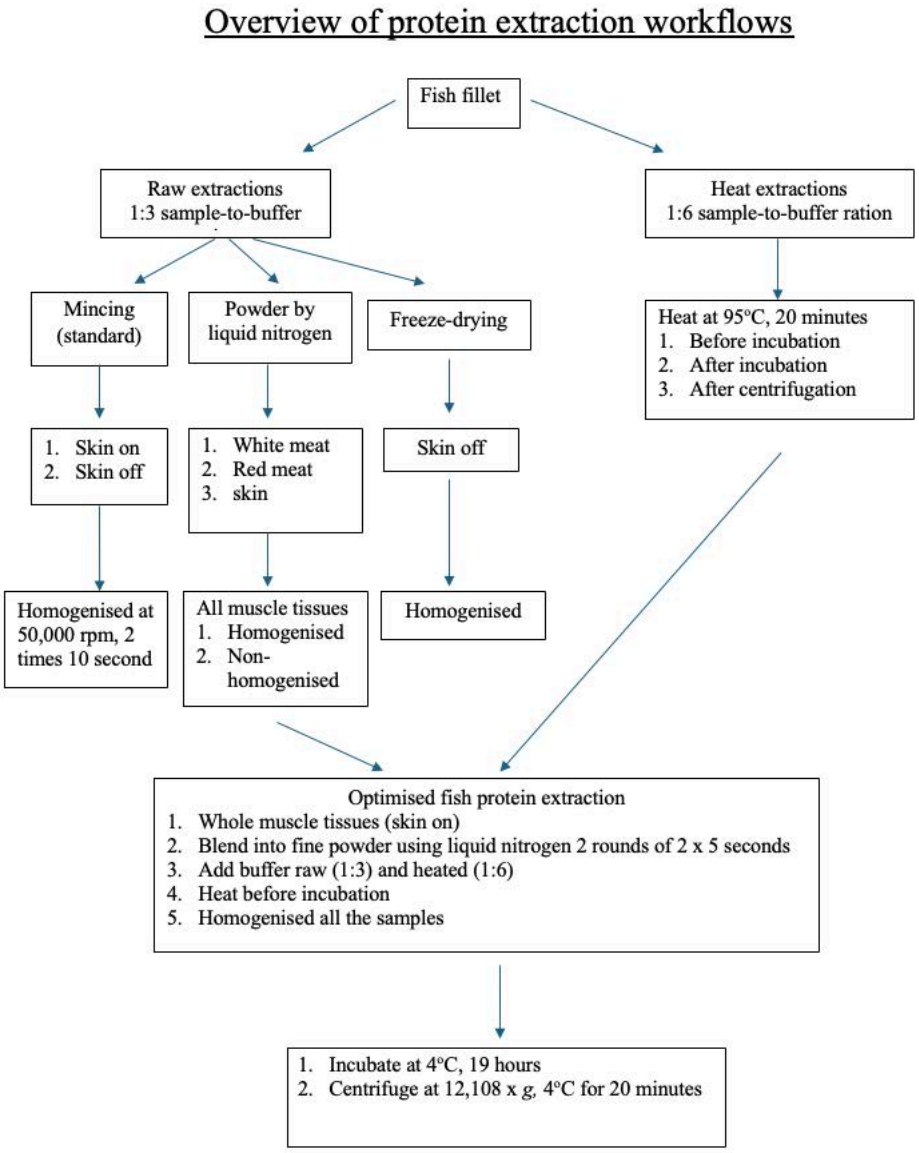

Figure 2: An overview of protein extraction workflows that were divided into raw and heated fish extracts involving various mechanical extraction techniques, different specimens, homogenisation and heat treatments.

2.2.1.1 Sample-to-buffer ratio

Phosphate buffer saline (PBS) is a common buffer used in seafood protein extraction (Nugraha et al., 2021; Ruethers et al., 2019; Taki et al., 2023). The samples were extracted in chilled PBS

(Bio-Rad Laboratories), pH 7.4. The ratio of sample to buffer for the raw extraction was 1:6 (1 g of tissue sample: 6 mL of PBS), while the heated extraction ratio was 1:3. The heated samples were heated to 95°C for 20 minutes and then cooled down on ice for 3 minutes.

2.2.1.2 Skin on vs skin off

The muscle fillets were thawed for approximately 5 minutes. The fillet including the skin layer was diced into 1 cm x 1 cm cubes treated as samples with skin on. For skin-off samples, the skin was removed, and the muscle tissue was diced into cubes.

2.2.1.3 Extraction with liquid nitrogen (LN2)

The diced samples were snap-frozen using liquid nitrogen (LN2). They were then blended three times for five seconds each using the A11 basic analytical grinder (IKA) with a beater. The powder was mixed thoroughly and blended again following the same steps after pouring more LN2, until it became a fine powder. Finally, the powder was weighed to achieve the desired raw sample-to-buffer ratio.

2.2.1.4 Homogenise vs non-homogenise

The samples were homogenized on ice at 50,000 rpm two times for about 10 seconds with a 10-second break in between intervals using the homogeniser with dispersing tool HT1010 (DAIHAN). After each sample was homogenized, the homogenizer probe was cleaned with ultrapure water and a 70% ethanol solution and dried before being used for the next sample. This was done to avoid the risk of protein contamination between samples. Samples that were not homogenized, were gently mixed before incubation

2.2.1.5 Heat Treatments

The samples were heated at 3 different stages after adding the buffer. Firstly, the sample was heated at 95°C for 20 minutes on a heating block and cooled down on ice for 3 minutes followed by homogenising. Secondly, the samples were first homogenised and incubated followed by heating. Thirdly, the sample was heated after completing the centrifugation steps.

2.2.1.6 Incubation

The samples were incubated on a digital orbital shaker at 120 rpm at 4 °C for 19 hours. The samples were centrifuged at 12,108 x *g* at 4 °C for 20 minutes, to separate the supernatant and

pellet. The supernatant was transferred to a 15 mL Falcon tube and aliquots of 1 mL. The samples were stored at -80 ºC until further use.

2.2.3 Freeze-dried extraction

A sample preparation protocol was performed where one gram of diced muscle tissue underwent overnight lyophilisation using a FreezeZone freeze dryer (Labconco). The moisture content loss was determined by weighing the dehydrated tissue. The dried sample was then suspended in 3mL of PBS and homogenised at 50,000 rpm for 10 seconds. They were then incubated on an orbital shaker at 120 rpm, at 4°C for 19 hours. The samples were then centrifuged at 12,108 x g for 40 minutes at 4°C to collect the supernatant.

2.2.4 Optimised standard fish protein extraction

Muscle tissue was thawed for approximately 3 minutes and diced into small pieces (~1 cm3 cubes), including the skin layer. The samples were then further optimised by snap-freezing the muscle tissues. The muscle tissue was blended in 2 rounds of 3 x 5 seconds each to obtain a fine powder. Chilled PBS buffer was added to the samples with a ratio of 1 gram of powder to 3 mL of PBS for raw samples and 1 gram of powder to 6 mL of PBS for heated samples. For the heated samples: The samples were heated at 95°C for 20 minutes and cooled down on ice for 3 minutes before being homogenised. Both the raw and heated samples were homogenised at 2 x 50,000 rpm for approximately 10 seconds on ice. Once processed, the samples were incubated on an orbital shaker at 120 rpm, at 4°C for 19 hours. Then, the samples were centrifuged at 12,108 x *g*, at 4°C for 20 minutes. The supernatant was quickly transferred to 15mL tubes to avoid the fat layers, and 1mL aliquots of the supernatant were taken for further analysis. Finally, the supernatants were stored at -80°C.

2.3 Total protein quantification - Bicinchonic acid (BCA) assay

2.3.1 Pierce BCA protein assay kit

The supernatant was thawed at 4°C, vortexed briefly, and then centrifuged at 21,000 x g at 4°C. The supernatant was immediately transferred to a separate microcentrifuge tube, taking care to avoid contamination from the pellet.

Total protein concentration was determined using the Bicinchoninic Acid (BCA) colourimetric assay based on copper reduction. Samples were prepared in triplicate by pipetting 20 µL into a 96-well plate, with raw samples diluted 5-fold and heated samples diluted 2-fold with PBS

buffer. These dilution factors were selected to ensure protein concentration fell within the optimal assay range (0.5 - 1.0 mg/mL) for accurate measurement. PBS buffer served as both diluent and blank standard in the quantification assay.

The protein quantification was performed using Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific). The standard curve was prepared using a pre-diluted standard from 0.125 mg/mL to 2.0 mg/mL. The assay was conducted by combining samples with working reagents in a 1:8 ratio, with 200 µL of working reagents added per well. Following gentle shaking at 300 rpm for 30 seconds and then incubated at 37°C for 30 minutes. Total protein concentration was determined by measuring absorbance 562 nm on a plate reader (FLUOstar® Omega, BMG Labtech). Based on the results, samples were diluted to achieve a final protein concentration of 2.5 mg/mL and 1.25 mg/mL for raw and heated extracts, respectively.

2.3.2 Pierce dilution-free rapid gold BCA protein assay kit

Another protein quantification was performed using Dilution-free Rapid Gold BCA Protein Assay kits (Thermo Fisher Scientific), which measured protein concentrations up to 10 mg/mL, following the manufacturer's protocol, further optimisation was done to ensure the absorbance was below 1. The samples and standards were prepared in triplicate by combining 5 µL of each with 100 µL of working reagents. The mixture was shaken at 300 rpm for 30 seconds and was incubated at room temperature for 5 minutes. The absorbance was measured at 480 nm. Importantly, the assay was measured within 10 minutes. Similarly, the sample was normalised to a final concentration of 2.5 mg/mL.

2.4 Total Protein Profiles - Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Hand-cast SDS-PAGE gels were cast using the double-wide multi-caster kit with 1.0mm, 16 cm height (CBS Scientific). The gels comprised 6% stacking and 12% resolving acrylamide/bis solution, which were degassed before casting. After preparation, gels were stored overnight at 4 ^{o}C in 0.1 M Tris buffer (diluted with milliQ water).

Samples were prepared using a reducing 5X loading dye containing 0.5M of dithiothreitol. After brief mixing, samples were heated at 95 ^{o}C for 5 minutes, followed by cooling at room temperature for 3 minutes and centrifuged at 21,000 x *g* for 5 minutes at 4^{o}C.

A double-wide mini vertical with 20 cm width and 16 cm height (CBS Scientific) and casted gels were assembled and a chilled 1X SDS running buffer was added to the brim before the removal of the combs. Protein loading volumes were optimised to 8 µg of proteins and 4 µL of both pre-stained and unstained molecular weight markers (Bio-Rad Laboratories)for SDS-PAGE. The gels were run at 60V for 15 minutes and 180V for 3 hours (depending on the gel length). Chilled 1X running buffer was added every 1 hour of gel running to prevent the gels from drying up and the system from overheating.

The gel was stained overnight in a Coomassie solution with a final composition of 0.1% CBB R-250 (Bio-Rad Laboratories), 40% methanol, and 7% acetic acid. The gels were destained twice for 20 minutes each using a destaining solution containing 40% methanol and 7% acetic acid and then stored in a 5% acetic acid solution. The gel images were scanned at a resolution of 50 nm with 700 channels using the Odyssey M imaging system (Li-Cor). Densitometric analysis was performed using GelAnalyser software to calculate the molecular weights of the protein bands and the intensity of the peak.

2.5 Parvalbumin detection - Immunoblotting

The SDS-PAGE protocol described above was repeated using 4 µL protein and 2 µL markers. Before transfer, 0.84mm filter paper and 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories) were equilibrated in tris-glycine transfer buffer with 20% methanol for 15 minutes. Once the gel was ready, the gel was equilibrated for 1 minute in the transfer buffer. The proteins were transferred to the membrane in between the assembled gel sandwich using the Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories) at 23V for 20 minutes. The membrane was dried at room temperature and then heat-treated at 60 ^{o}C for 20 minutes to ensure the proteins were fixed on the membrane. The membranes were carefully stored until further incubation with antibodies. After transfer, the gels were stained in the Coomassie solution to evaluate the transfer efficiency.

The membranes were rehydrated in PBS and then blocked using 1x casein for 45 minutes on a 3D Rocker (Benchwaver™) at 23 rpm. Subsequently, the membranes were incubated with 10 mL of a commercial primary monoclonal anti-parvalbumin antibody (Parv-19, Sigma-Aldrich) diluted in a 1:3000 mixture of 0.2X casein (Sigma-Aldrich) and PBS-T (0.05% Tween-20) for 45 minutes. Parv-19 was previously used in the identification of fish parvalbumin (Saptarshi et al., 2014). Following incubation, the membranes were rinsed quickly and washed three times for 5 minutes each with PBS-T.

Membranes were then incubated with Goat anti-Mouse IgG (H+L) Secondary Antibody, DyLight™ 800 4X PEG (Invitrogen, Thermo Fisher Scientific), diluted 1:10,000 in a mixture of 0.5X casein and PBS-T, for 45 minutes in the dark.

After incubation, the membranes were rinsed quickly and washed three times for 5 minutes each, followed by a single 10-minute wash with PBS-T and another 10-minute wash with PBS. Finally, the membranes were dried in dark conditions and scanned at a 50 nm resolution using the 700 and 800 channels of the Odyssey M imaging system.

3. Results

3.1 Comparison of protein profiles from different extractions of seabass tissue

Multiple protein extraction methods were conducted to evaluate each method using seabass muscle tissue without the skin layer. The methods included mincing, snap-freeze with liquid nitrogen and freeze-drying. All three extraction methods resulted in similar protein profiles (Figure 3). However, sample powder from snap-frozen and freeze-dried methods enhanced the intensity of protein bands at 15 – 37 kDa. Raw samples of minced seabass did not have the protein band at 27 kDa, which was observed in powder from snap-frozen and freeze-dried samples. The impact of heat treatment varied between heat-treated extraction methods. Based on the Dilution-free BCA assay, the heat-treated samples of minced tissues showed 56% protein denaturation. The result enabled the identification of heat-stable proteins on SDS-PAGE gel. In contrast, freeze-dried samples demonstrated heat stability (20% reduction), maintaining protein profiles similar to their raw state (non-heat-treated sample), with only minor changes observed in the 15 - 20 kDa. Visually in Figure 3B, the two parvalbumin isoforms at 11 and 12 kDa remained consistent across all extraction methods (both raw and heated samples), as confirmed by immunoblotting with monoclonal anti-parvalbumin antibody.

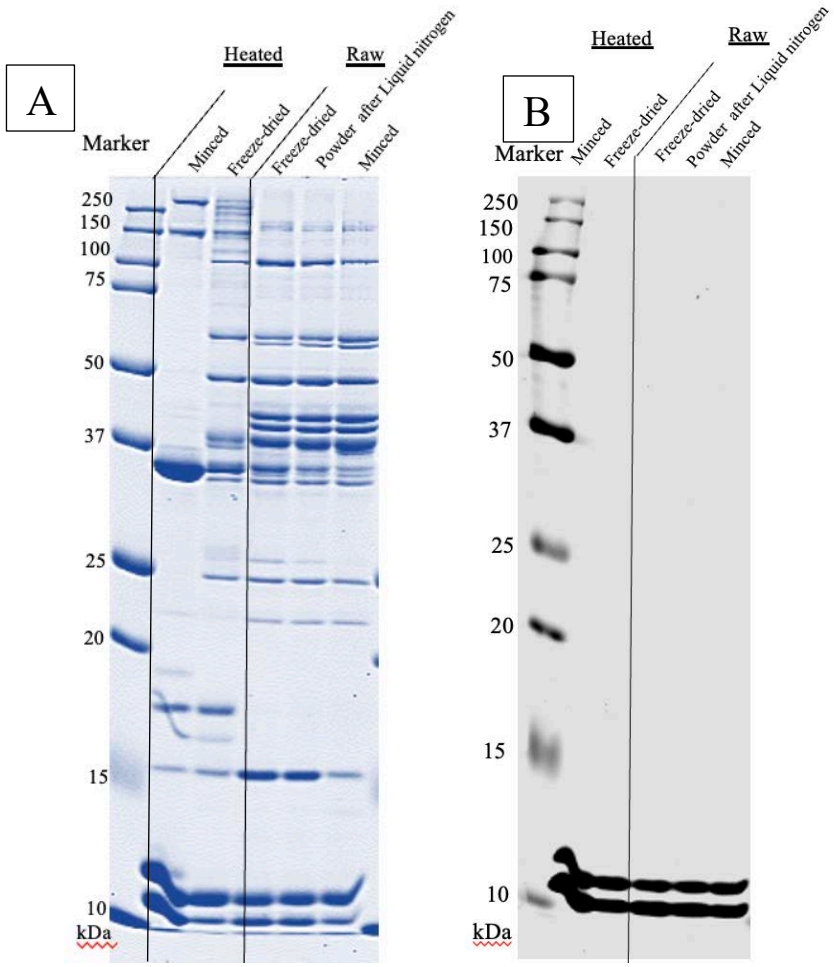

Figure 3: SDS-PAGE gel and immunoblot results of seabass proteins extracted with different mechanical methods. **A)** SDS-PAGE of raw seabass muscle proteins extracted by mincing, using liquid nitrogen and freeze-drying was observed to have similar protein profiles, however, there was high protein recovery at 15 – 37 kDa. The protein content of the heated freeze-dried extract exhibited a notable reduction in comparison to the non-heated extract. Conversely, the heating process resulted in a considerable degradation of proteins from the minced tissue. **B)** Immunoblots of allergen-specific antibody of parvalbumin which showed parvalbumin binding are not different between each of the extraction methods.

3.2 The protein profiles diversity of different types of red snapper tissues

3.2.1 Protein variability in each muscle tissue

A comparative protein analysis was conducted using three distinct snapper tissues: white meat, red meat and skin. Quantitative analysis revealed significant variations in protein concentration across tissues where white meat exhibited the highest concentration at 6.5mg/mL, followed by red meat at 2.9 mg/mL and skin tissue at 1.1 mg/mL. After SDS-PAGE, each muscle tissue

exhibited unique protein profiles and most importantly parvalbumin content was high in the white muscle tissues compared to the skin and red muscle tissues (Figure 4A). White meat and skin contained two parvalbumin isoforms, however in contrast red muscle tissue had only one parvalbumin isoform. The immunoblot further confirmed the parvalbumin isoforms on the muscle tissues as it showed intense binding on white meat compared to skin tissue, and only the higher molecular weight isoform in red meat (Figure 4B).

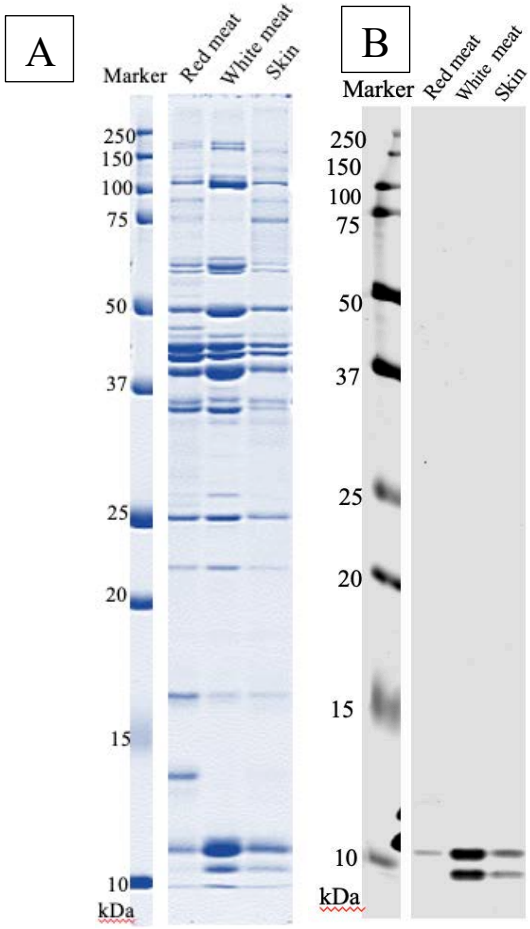

Figure 4: The diversity of protein profiles of different red snapper muscle tissue powder extract using liquid nitrogen **A)** Raw extracts of red meat, white meat and skin had unique total protein diversity on SDS-PAGE gel. White meat had the most protein abundance followed by red meat and skin tissue. **B)** Allergen-specific antibody parvalbumin binding to bands at 11 and 12 kDa. The binding intensity was reflected in the Coomassie stain gel, which demonstrated that white meat and skin tissue exhibited two parvalbumin isoforms, while red meat displayed a single isoform.

3.2.2 Homogenise vs non-homogenise

The protein levels of homogenised and non-homogenised samples were compared from extracted red snapper powder. It was observed that the non-homogenised powder exhibited a tendency to clump together and was not effectively mixed after 19 hours of incubation. In Figure 5, the protein profiles of the two mechanical extractions were found to be identical. Furthermore, no notable differences were observed in protein concentration, with the homogenized sample exhibiting a concentration of 5.3 mg/mL and the non-homogenized sample displaying a concentration of 5.2 mg/mL. The homogenisation process did not have a considerable impact on the protein profile and parvalbumin bands at approximately 10 kDa.

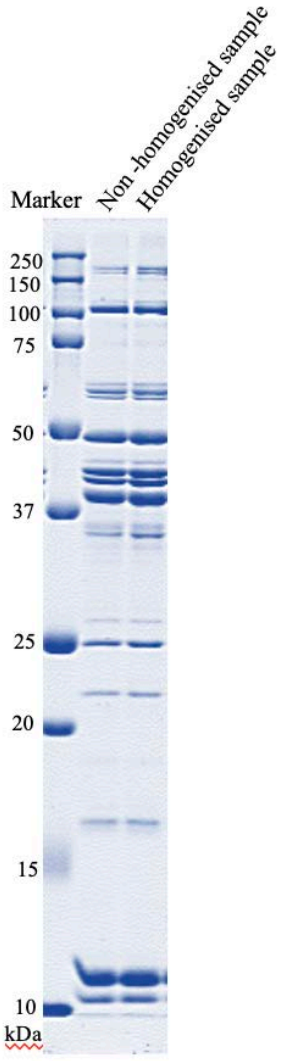

Figure 5: Analysis of protein yield and quality using homogenisation and non-homogenisation during mechanical protein disruption. There were no considerable differences in the densitometric analysis.

3.2.3 Comparison between the heat treatments

Heat was applied at three different stages: 1) before incubation, 2) after incubation and 3) after centrifugation steps. Analysis of heat treatment timing on protein extraction revealed distinct trends and variations in protein profiles (Figure 6A). The sample heated before incubation yielded lower protein content compared to the sample heated after incubation, as it observed differences at a higher molecular weight of 37 – 75 kDa. The sample heat-treated after centrifugation yielded two highly heat-stable proteins at 11, 12 and 36 kDa. These two proteins were identified as tropomyosin and two parvalbumin isoforms (Ruethers et al., 2021). The immunoblot further confirmed the presence of parvalbumin isoforms in all heated extracts (Figure 6B).

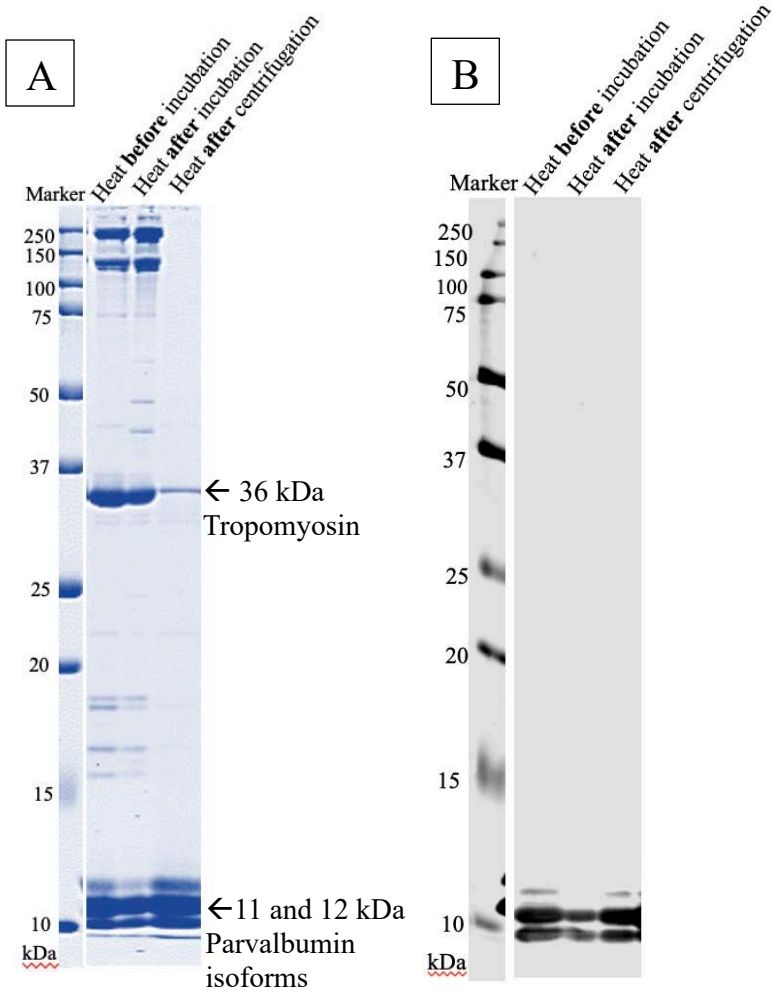

Figure 6: The diversity of heat-stable proteins in heated extracts. **A)** SDS-PAGE of three stages of heat treatments where the samples were heated before the incubation, heated after incubation and heated after centrifugation. Parvalbumin and tropomyosin were annotated based on

previous research studies (Ruethers et al., 2021) **B)** Binding of allergen-specific antibody to parvalbumin of heated extracts on immunoblot.

3.3 Technical Replicates of Standard vs Optimised Extraction

Protein profiles of the technical replicates of the standard raw without skin extract showed that there is a lack of consistency in the protein bands at 11, 15 and 20 kDa (Figure 7A). Based on the result, further optimisation of the protein extraction method was required. The optimised method of blending the fish muscle into powder using liquid nitrogen showed that the profiles were identical. The allergens were annotated based on the molecular weight of salmon, catfish and seabass from previous research studies (Kalic et al., 2020; Ruethers et al., 2021). A similar trend was observed in the immunoblot, detecting parvalbumin isoforms, - consistent signal for technical replicates of the optimised snap-frozen with LN2 extraction (Figure 7B). The lack of reproducibility in protein band representation at 11 and 12 kDa in minced muscle tissue (without skin) has shown differences in parvalbumin band detection in immunoblot.

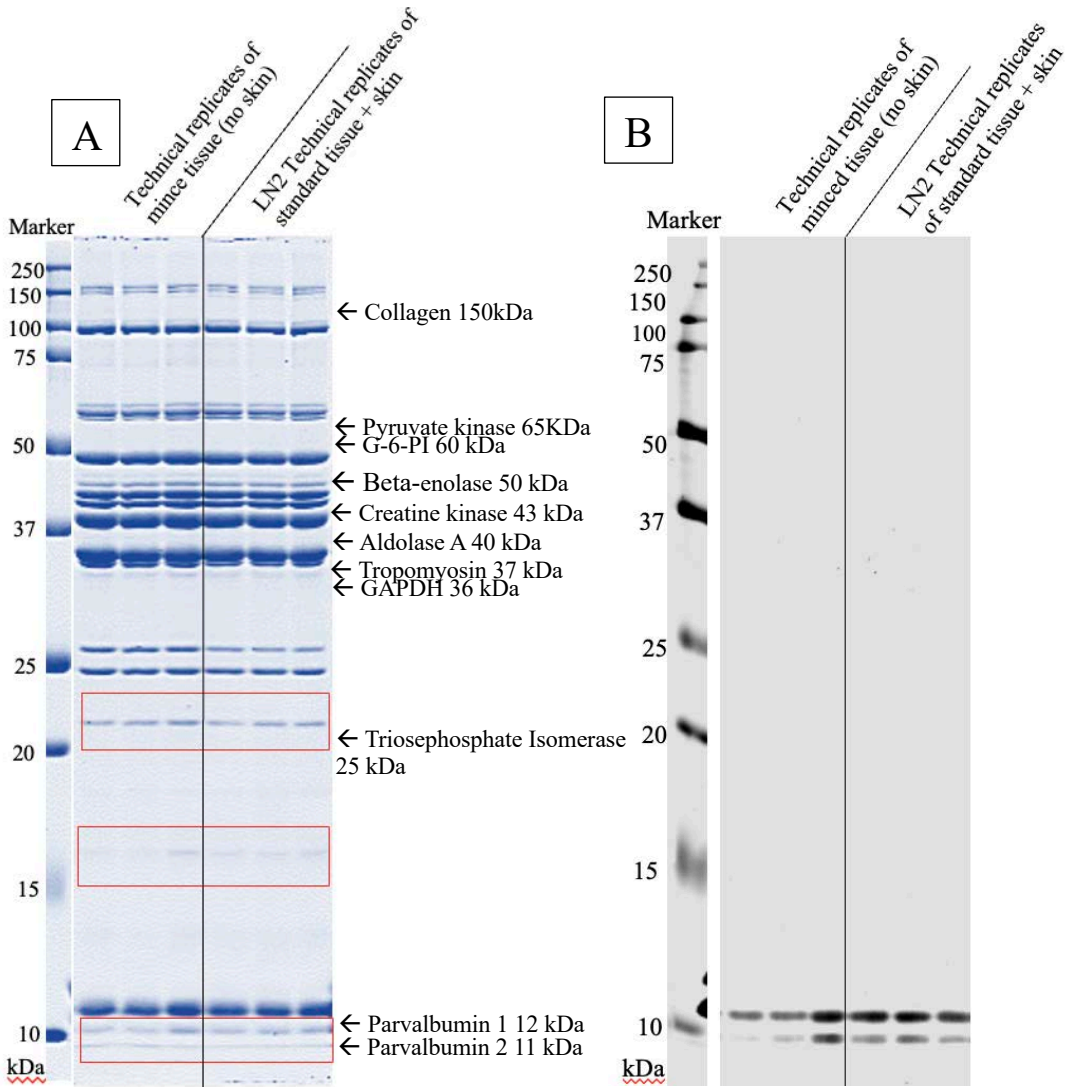

Figure 7: Comparison of technical replicates protein profiles of two types of extraction methods**. A)** Total protein of technical replicates of minced muscle tissue without skin and whole muscle tissue including the skin blended into powder using liquid nitrogen. The proteins are annotated at the molecular weight based on previous research studies (Kalic et al., 2020; Ruethers et al., 2021) **B)** Immunoblot of allergen-specific binding to parvalbumin.

4. Discussion

Patients with fish allergy are exposed to fish in a variety of ways, including consumption and preparation, and this study provides insight into the effects of protein extraction at the molecular level. The diversity of proteins in each muscle tissue allows the optimisation of fish protein extraction to have high-quality reproducible protein analysis. In the field of proteomics, fish proteins are extracted in different extraction buffers depending on the objective of downstream analysis. The extraction buffer has an impact on determining the protein recovery in a sample. The different composition of buffer plays a part in the pH levels, ionic strength and detergent properties that affect the solubility of the proteins (Ugwu & Apte, 2004). Selecting the ionic strength buffers able to extract the targeted proteins. Common non-denaturing phosphate buffers can yield high molecular weight proteins and denaturing buffers are used to target smaller proteins such as troponin, tropomyosin and actin (Malva et al., 2018). High pH and salt buffers are known to improve the recovery and enrich specific proteins however further analysis observed that neutral buffers can extract more potential allergens (Nugraha et al., 2021). Hence, PBS buffer is selected to extract water-soluble proteins of red snapper muscle tissues.

In this chapter, a standardised protein extraction method was established. Raw protein extracts demonstrated considerable protein profile differences between white meat, red meat and skin off/on. Heated protein extracts enable to identification of thermostable proteins by denaturing the heat-labile or heat-sensitive proteins. Raw and heated extraction methods are designed to mimic consumer preferences in terms of the consumption and preparation of fish in the kitchen. The preliminary extraction method of mincing and freeze-drying fish muscle tissues does not show differences in protein levels. Freeze-drying the muscle tissues removes 98% of moisture by sublimation at low temperatures and pressure below the triple point of water capable of preserving the muscle tissues that are unstable in solution (Nguyen et al., 2014). Freeze-dried fish are rarely consumed daily but are popular for pets' food. Hence, the minced samples of raw and heat treatment are the standard methods of extracting the proteins from fish muscle tissue. However, proceeding with protein extractions by mincing the muscle tissues' proteomics analysis is not ideal. The protein profiles of technical replicates of the minced method showed a lack of consistency, suggesting that mincing muscle tissues as a standard fish protein extraction has its limitations. The challenge of standard protein extraction is to ensure that the tissue sample is standardised in all extracts. There could be bias in selecting the correct tissues

and introduce contamination of other tissues during the extraction. This suggests that there could be tissues that are not equally distributed, and contamination of other tissues causes dilution of the target proteins. Another issue of mincing the muscle tissues reduces the integrity of the samples. Prolonged thawing and processing of the samples at room temperature induces protein degradation resulting in variations in total proteins. In thawed muscle tissue, the sarcoplasmic reticulum is disrupted and swollen which causes an increase in the cavities (holes) allowing water to absorb and alter the muscle contractile filaments (Ayala et al., 2005).

The optimised extraction adds a new element to the standard raw extraction by snap-freezing the muscles and blending them into powder. It can maintain a cool temperature and ensure that the protein is well preserved to obtain high-quality protein recovery. From the allergy preceptive, the optimised extraction method can give a consistent and reproducible profile by preserving the heat-sensitive proteins (e.g., triosephosphate isomerase at 25 kDa and glyceraldehyde-3-phosphate dehydrogenase at 36 kDa). This is crucial to eliminate the possible assumptions of protein variability from the mechanical extraction. Muscle tissues processed into powder allow equal distribution of tissues presentative including the skin layer. In addition, homogenisation plays a part in disrupting the muscle tissues as well as thoroughly mixing the samples to maintain consistency. In theory, the powder sample would slowly dissolve, and the protein would disintegrate in the extraction buffer during the incubation. Nevertheless, this was not the case for the non-homogenised powder sample. The powder was observed to clump and not disperse adequately in the sample tube during incubation due to the insolubility of the powdered muscle tissue in PBS buffer. The tendency of the sample to clump during the incubation is not ideal causing potential error of non-consistency of protein recovery. However, homogenisation and non-homogenisation techniques do not have a considerable impact on the protein concentration.

The analysis of technical replicates of minced fish muscle highlighted a lack of reproducibility and this research analysis tackles the issues of standard protein extraction by understanding the components of fish muscle tissues. In teleost fish, skeletal muscle is segmented with myotomes and each myotome is separated by a thin layer of connective tissues known as myocommata (Boland et al., 2018). In addition, fish muscle tissue is divided into 3 main types of muscle: white muscle, outermost red muscle and intermediate pink. The muscle is enveloped by a skin layer. All organisms have anatomical structures to carry out biological functions related to the tasks to perform to survive and reproduce. This applies at the cellular level where muscle cells

contain contractile fibres for movement and signal cells are responsible for signal transmission. The proportion of each muscle tissue depends on the fish species. As an example, red muscle tissues are the main tissues in tuna and mackerel species, however, the white muscle tissues are major muscle tissues in most pelagic fish species. It is established that white muscle tissue contains a high glycogen content, which enables the fish to perform sudden and rapid swimming movements whereas red muscle tissue contains a high lipid content, allowing for the sustained, continuous movement necessary for slow swimming (Omidvar et al., 2024).

The movement of the fish involved muscle contraction activity which requires energy in the form of ATP. Actomyosin, a protein composed of actin and myosin role important role in muscle contraction and ATPase activity was found to be three times higher in the white muscle compared to the red muscle (Nag, 1972), these were observed in SDS-PAGE results of white and red muscle proteins. Both white and red muscle tissues are important in the cellular activity of muscle contraction that is used in movements. Muscle contraction requires energy forms of adenosine triphosphate (ATP). Two major pathways of ATP regeneration are used in muscle oxidative and glycolytic pathways. Oxidative pathways occur in the red muscle fibre which is rich with myoglobin and allows the pyruvate (product of glucose) oxidised in mitochondria to generate ATP thus white muscle fibre pyruvate is converted into glycolytic pathway (Listrat et al., 2016). Parvalbumin was observed to have two isoforms in white meat and skin layer raw extract however, red meat only contained one isoform. Parvalbumin is a calcium-binding protein, primarily found in fast-twitch muscles responsible for rapid bursts of movement (Arif, 2009). Calcium ions are stored in cortisol and the sarcoplasmic reticulum during muscle relaxation, and it is released from the sarcoplasmic reticulum to bind to troponin triggering the muscle contraction process (Berchtold et al., 2000). Parvalbumin transports calcium ions back to the sarcoplasmic reticulum to continue the cycle (Berchtold et al., 2000). These parvalbumin isoforms are related to physiological function in muscle adaptation and development with high abundance in the white muscle tissues rather than red muscle tissue.

Lastly, heat treatment methods mimic household fish cooking methods such as boiling and poaching. In the typical style of fish consumption, it is common to cook fish first. In the comparison of SDS-PAGE profiles, most of the proteins at ~37 to 75 kDa such as pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, enolase, aldolase, creatine kinase, GAPDH and triosephosphate isomerase were denatured during the heating process. Hence, the heat treatment of muscle tissue is ideal for analysis to identify the heat-stable proteins of fish

that cause allergic reactions after food processing. Parvalbumin is present after various cooking methods including steaming, boiling, frying, baking, microwave and air-frying of salmon and grass carp species (Leung et al., 2024). The total proteins denatured most of the protein's presence in raw extract. The heat causes protein coagulation and disintegration of myofibrils resulting in liquid occupying the cavities within the muscle fibres (Ayala et al., 2005). Interestingly, two major fish allergens, parvalbumin and tropomyosin are still present in the heat-treated samples after centrifugation known as broth in cooking terminology. Food processing may reduce the allergenicity of heated fish extract (Jiang & Rao, 2021), however, there are heat-stable allergens remaining in the extracts. Research studies of canned salmon and sardine showed that heat-stable allergens parvalbumin, tropomyosin and collagen still demonstrated IgE binding (Taki et al., 2023). Three collagen isoforms in fish skin were observed in Pacific mackerel after heating at a high temperature of 120 ^{o}C (Kobayashi, Kuriyama, et al., 2016).

In addition, freeze-dried (lyophilisation) samples were able to preserve the proteins. The removal of 90% of water maintained the native quaternary structure of the proteins resulting in improved recovery of enzyme activities during rehydration (Anchordoquy et al., 2001). Hence, protein can stay intact in the matrix and heat treatment would not be effective in removing the heat-labile protein. These concluded extreme temperatures in processing canned fish (Taki et al., 2023) and conventional cooking methods are not able to denature the allergens. Among various heating methods tested, ohmic heating technology in food processing demonstrated superior efficacy in reducing the allergenicity of Japanese eel parvalbumin, achieving approximately 65% decrease in IgE/IgG binding capacity compared to other thermal processing techniques (Li et al., 2024). It was observed that the application of microwave treatment at 150°C in the presence of glucose resulted in a notable reduction in the allergenicity of Atlantic cod, with a 16% decrease recorded. (Dong & Raghavan, 2024). Still, fish-allergic patients are advised to avoid exposure or consumption of any fish products.

This research study established the standardised protein extraction method as fish muscles have unique distinct muscle tissues. This is to ensure high-quality fish protein recovery and credible downstream proteomic analysis of subsequent research studies.

Conclusion

The various protein extraction methods simulated diverse allergen exposure scenarios and fish processing techniques. This study revealed a remarkable diversity within the red snapper's muscle protein profiling. Distinct protein profiles were observed across different muscle types, likely reflecting their specialised functions. Red snapper, being a pelagic fish known for strong swimming, likely relies heavily on proteins optimised for sustained movement. The protein profiles visualised using SDS-PAGE gel electrophoresis appear tailored to the specific functions of each muscle tissue. Furthermore, individual analysis of protein profiles from each muscle tissue provided deeper insights into protein abundance and allergen distribution. Recognising the variation in parvalbumin isoforms across tissues offered valuable information. Parvalbumin is a common fish allergen, and understanding its distribution can be crucial for allergy research. This study highlighted the impact of mechanical extraction methods on protein recovery. Optimising these methods is crucial for future research. A combination of techniques like snap-freezing (processing tissues into powder), and homogenisation can help preserve protein quality and integrity. This optimisation is reflected in identical technical replicates, which serve as a quality control measure for the entire proteomic analysis. The present research study demonstrates a highly standardised extraction technique that enables the reproducibility of subsequent research analyses. This leads to a more robust and credible body of scientific knowledge, advancing the field.

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Size-dependent fish allergen profiles in red snapper (*Lutjanus malabaricus*)

Highlights (3-5 bullet points of max 85 characters incl. space)

- Proteomic analyses revealed the impact of fish size on protein/allergen abundance
- Size-dependent intraspecies variability adds complexity to fish allergy management
- Small fish contain more of major fish allergen parvalbumin compared to large fish
- In large fish, most heat-labile fish allergens are more abundant (e.g., enolase)
- Fish size matters for proteomics, allergenomics, allergy management and food safety

Abstract (150 words)

Fish allergies are a major public health concern, posing complex food safety challenges due to high species diversity, species-specific allergenicity and under-investigated intraspecies variability. This study investigated the variability of allergen profiles in commonly farmed Malabar red snapper (*Lutjanus malabaricus*) with a focus on fish size and body regions. Proteins were extracted from 24 collectively farmed snappers (212 to 413 mm) and their composition was examined with a highly standardised proteomics approach. The total protein profiles differed across the various fish sizes and muscle locations. Quantitative immunoblotting and mass spectrometry analysis demonstrated that smaller fish exhibited a higher abundance of the major fish allergen parvalbumin compared to larger fish. However, the larger fish displayed an increased abundance of most heat-labile allergens. Size-dependent allergen abundances were discovered in snapper, which should be considered for proteomic and allergenomic analyses for all fish as well as in allergy management and food safety approaches.

Keywords: fish allergy, intra-species variability, food safety and allergy management, parvalbumin, allergenicity, fish sizes, muscle regions

1. Introduction

Fish can trigger life-threatening allergic reactions upon ingestion and inhalation of fish proteins (allergens), which are recognised by Immunoglobin E (IgE) antibodies in affected, sensitised consumers. The rising fish consumption has heightened the risk of fish allergy globally, with regional prevalences of up to 3% reported in general populations (Moonesinghe et al., 2016).

Parvalbumin, a low molecular weight (10 – 13 kDa) calcium-binding protein that plays a critical role in muscle contraction, has been identified as the major fish allergen (Ruethers, et al., 2018). The two distinct phylogenetic lineages of parvalbumin are alpha- and beta-parvalbumin which have differences in amino acid sequence and isoelectric point (Arif, 2009). Beta-parvalbumin is predominantly expressed in bony fishes and most fish-allergic individuals are sensitised to it whereas cartilaginous fish (sharks and rays) predominantly contain alpha-parvalbumin with lower IgE binding capacity (Ruethers et al., 2018; Stephen et al., 2017). Allergic reactions to fish are most frequently caused by IgE-binding of highly cross-reactive bony fish beta-parvalbumin (Kalic et al., 2021). Fish-allergic patients showed IgE reactivity towards four tropical fish species such as threadfin, Indian anchovy, pomfret and tengirri (mackerel) as well as Baltic cod, however, patients from Europe also showed binding towards these tropical fish species regardless of prior exposure to only European species (Lim et al., 2008).

Species- and tissue-specific allergenicity has been investigated in previous studies with a focus on parvalbumin levels. White muscle tissue contains more parvalbumin, which is therefore considered of higher allergenicity compared to the red muscle tissue (A. Kobayashi et al., 2006; Y. Kobayashi et al., 2016). The proportion of red and white muscle tissues varies among fish species. Tuna and mackerel are often considered less allergenic species as they have a higher portion of red muscle (A. Kobayashi et al., 2006). It was also observed that the anterior region of fish is more allergenic compared to the posterior region due to higher parvalbumin content (Lee et al., 2012). Apart from parvalbumin, a few studies have reported IgE binding to other proteins from specific fish species, which are therefore listed as fish allergens in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature database (www.allergen.org): beta-enolase, aldolase A, tropomyosin, collagen, creatine kinase, triosephosphate isomerase, pyruvate kinase, lactate dehydrogenase

(DH), glucose-6-phosphate isomerase (G-6-PI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(Kalic et al., 2021).

The impact of fish sizes on parvalbumin content in the muscle tissues has not been thoroughly investigated. Fish of different sizes may exhibit differences in protein expression because of their different growth stages and developmental requirements. Previous studies observed that the parvalbumin content in the white muscle tissues of barbel increased as the length reached from 1 cm to 5 cm (larva stage) and slowly decreased as the sizes increased to 60 cm (adult) (Huriaux et al., 1997). The tails of small fish seem to contain more parvalbumin due to increased tail beat frequency and accelerated rate of muscle relaxation, allowing smaller fish to burst into movement in escape from predators (Rodnick & Sidell, 1995). Proteomic analysis of different sizes of wild snakehead (*Channa striata*) caught at different seasons, concluded that smaller fish expressed more proteins compared to larger fish (Gam et al., 2006). However, protein profiles were considered similar with no detailed comparisons of protein abundances and a lack of standardisation with many variabilities among wild-caught specimens from different seasons.

This study investigates and compares the protein composition and allergen abundance in Malabar red snapper (*Lutjanus malabaricus*) muscle tissues in different body regions of specimens across a wide range of sizes. Red snapper is an economically important fish species, which is commonly consumed globally and a key aquaculture species in the Asia-Pacific region. This is the first study comparing protein profiles and allergen abundances in fish of different sizes with all other parameters controlled in an aquaculture environment.

2. Materials and methods

2.1 Sample collection

Fingerlings were sourced from Malaysia and reared under identical conditions in one floating cage along Singapore's Johor Strait for nine months. More than 24 snappers were harvested on the same day with fish sizes ranging from 212 mm to 413 mm in size (detailed in Supplementary Table S1) - any fish showing signs of illness or morphological abnormalities were excluded from further analyses. All specimens were maintained on ice during transport and processing. Dorsal muscle tissues were used to analyse protein variations in fish sizes. Following standard protocols, the fish were descaled and cleaned with tap water. Distinct muscle tissues were collected from four anatomical regions (head, dorsal, tail and belly; illustrated in Supplementary Figure S1). Each muscle section was carefully removed using a clean filleting knife, individually bagged, and immediately stored at -80°C.

2.2 Protein extraction

Protein extraction procedures were optimised and standardised. Muscle tissues were briefly thawed (<5 minutes at room temperature) and cut into 8 cm^3 cubes. Following safety protocols, the tissues were snap-frozen in liquid nitrogen (LN2) and pulverised using an analytical mill with a beater motion (A11 Basic; IKA). A cool temperature was maintained by the introduction of LN2 into the mixer at both ends of the round. Proteins were extracted in phosphate buffer saline (PBS, pH 7.4) with 1:6 (w:v) sample-to-buffer ratio. The powder was homogenised in the buffer on ice at 50,000 rpm for 10 seconds using a homogeniser (dispersing tool HT1010; DAIHAN®). The dispersing tool was cleaned using ddH_2O and 70% ethanol after processing each sample to avoid cross-contaminations. Homogenised samples underwent overnight incubation at 4°C on a digital orbital shaker at 120 rpm for 19 hours followed by centrifugation at 12,108 x *g* at 4°C for 20 minutes. The supernatant was transferred and stored at -80°C until use.

2.3 Total protein quantification - Dilution-free rapid gold BCA assay kit

Samples were thawed at 4°C, briefly vortexed and centrifuged at 21,000 x *g* at 4°C for 10 minutes. The supernatant was used for protein quantification using the Pierce™ Dilution-Free™ Rapid Gold BCA Protein Assay kit (Thermo Fisher Scientific) following the manufacturer's protocol with modifications to achieve absorbance readings below 1. In brief,

5 µL samples and pre-diluted bovine serum albumin standards were prepared in triplicate. Next, 100 µL of working reagents were added to the samples and standards. The mixture was shaken at 300 rpm for 30 seconds and incubated at room temperature for 5 minutes. The absorbance was measured at 480 nm within 10 minutes. All samples were normalized with PBS to a standardised concentration of 2.5 mg/mL, aliquoted in 20 µL volumes and stored at -80ºC until single use.

2.4 Total protein profiling

Protein profiles were resolved by Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The 5X reducing loading buffer was formulated using 0.5 M dithiothreitol, 10% SDS, 50% glycerol, 0.5% bromophenol blue, and 0.25 M Tris at pH 6.8. A total volume of 20 µL of 2.5 mg/mL protein sample was combined with 5 µL of loading buffer, followed by vortexing and centrifugation. The samples were then heated at 95°C for 5 minutes and centrifuged at 21,000 x *g* prior to loading on the gel.

Gels were hand casted using a double wide multi-caster kit (CBS Scientific, San Diego, California, USA), comprised of 6% acrylamide/bis-acrylamide stacking gels and 12% resolving gels. Protein separation was performed using a mini vertical double-wide 20cm x 16cm system (CBS Scientific), with 8 µg of protein loaded per well. Electrophoresis was conducted in three sequential stages: 60V for 15 minutes, 120V for 30 minutes and followed by 200V for 3 hours. Protein bands were visualised by Coomassie Brilliant Blue R250 (Bio-Rad Laboratories) staining and scanned with an Odyssey M® imaging system (LI-COR Biosciences, Lincoln, Nebraska, USA). Densitometric analysis was performed using GelAnalyser software. The molecular weight of each band was calculated by applying the Rf value to log molecular weight formula (-0.475 x LN(Rf) + 1.0433).

2.5 Detection of the major fish allergen parvalbumin

Of each sample, 4 µg of protein was separated by SDS-PAGE followed by semi-dry transfer with tris/glycine buffer (20% methanol) onto a 0.2 µm nitrocellulose membrane using a Trans-blot semi-dry transfer cell (Bio-Rad Laboratories) at 23 V for 20 minutes. The dried membrane was blocked with casein (Sigma-Aldrich) in PBS for 45 minutes. Primary monoclonal anti-parvalbumin antibody Parv-19 (Sigma-Aldrich) was diluted 1:3,000 in PBS-T containing 0.2X casein and incubated with the membrane for 45 minutes. The membrane underwent washing steps with PBS-T before subsequent incubation with the immunofluorescence secondary

antibody DyLight 800 goat anti-mouse (H+L) 4X PEG (Invitrogen), diluted 1;10,000 with PBS-T containing 0.5X casein, for 45 minutes in the dark. After washing, antibody-binding was visualised using the above-mentioned Odyssey M system with subsequent densitometric analyses using GelAnalyser software.

2.6 Mass spectrometry analysis

The protein composition of all extracts was analysed by mass spectrometry after tryptic digestion, based on previously described methods (Ruethers et al., 2019; Taki et al., 2023). In brief, 28.6 µg of whole raw protein was processed utilising the S-Trap™ micro spin column digestion protocol (ProtiFi, Fairport, New York, USA). The extracts were chemically processed through three critical steps: reduction of sulfate bonds with tris (2-carboxyethyl) phosphine (TCEP), alkylation of disulfate bonds with iodoacetamide and enzymatic digestion using trypsin, following the manufacturer's recommendation protocol.

The recovered peptides were subsequently analysed by nanoESI-LC-MS/MS using an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) equipped with a nanoflow HPLC (Ultimate 3000 RSLC, Dionex). The LC system was equipped with an Acclaim Pepmap nano trap column (Dinoex-C18, 100 Å, 75 µm x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dinoex-C18, 100 Å, 75 µm x 50 cm). Tryptic peptides (1 µL) were injected to the enrichment column at an isocratic flow of 5 µL/min of 2% v/v CH3CN containing 0.1% v/v formic acid for 5 min applied before the enrichment column was switched in-line with the analytical column. The eluents were 5% DMSO in 0.1% v/v aqueous formic acid (solvent A) and 5% DMSO in CH3CN with 0.1% v/v formic acid (solvent B) and the gradient timetable was as follows [time (min), %B]: [0, 3], [6, 3], [7,4], [82, 25], [86, 40], [87, 80], [90, 80], [90.1, 3], [95, 3]. The solvent flow rate was 300 nL/min and the column compartment was maintained at 50°C throughout the analysis. Compounds were ionised using nano-ESI operated in positive ion mode with a spray voltage of 1.9 kV, ion transfer tube temperature of 275 °C and RF of 50%. MS1 spectra were acquired over the range *m/z* 350-1400 at a resolution of 120,000 with a maximum injection time of 50 ms and normalised AGC target of 250%. Peptide ion fragmentation was carried out using data-independent acquisition with 50 x 14.7 *m/z* windows from *m/z* 360.5 – 1046.5 with a 1 *m/z* overlap between adjacent windows. Ions were activated using HCD with a normalised collision energy of 30%, a maximum injection time of 55 ms and normalised AGC target of 2000%. MS2 spectra were acquired at a resolution of 30,000 over the *m/z* range 200-2000.

Raw data were processed with Spectronaut (v19.1.240806.62635; Biognosys) using Direct Data-Independent Acquisition (DIA) with default parameters against a red snapper protein database in FASTA format based on in-house genome assembly and annotation for Malabar red snapper (*Lutjanus malabaricus*). Oxidised methionine and protein N-terminal acetylation were set as variable modifications and carbamidomethylation of cysteine was set as a fixed modification. Peptide-spectral matches, peptide and protein false-discovery rates were set to 1%. Peptide ions were quantified at the MS2 level. Relative protein abundances were expressed by the intensity-based absolute quantification (iBAQ) by dividing the total peptide intensity by the number of peptides observed for a given protein (Fabre et al., 2014). Proteins were grouped and the relative abundance of all fish allergens was calculated as iBAQ%.

3. Results

3.1 Size-dependent total protein profiles

The fish length was used as the primary, most standardised measurement to categorise fish size in this study. There was a positive correlation between fish length and body weight with a p-value of <0.0001 (supplementary Figure S1). Differences were observed in the total protein profiles after separation by molecular weights (Figure 1). The protein profiles revealed 20 most prominent protein bands which exhibited distinct patterns when comparing fish samples of different sizes. Technical replicates (samples 21, 22, and 23) displayed consistent protein patterns, validating the reproducibility of the mechanical extraction method. Notable differences in protein bands were observed at molecular weights of 36, 35, 27 and 15 kDa. The abundance of the minor parvalbumin isoform (11 kDa) showed reduction with higher levels in smaller fish (212 mm) compared to larger fish (413 mm). A similar trend was observed for a band at 15 kDa. In contrast, proteins at approximately 35 and 25 kDa demonstrated a positive correlation with fish size, showing increased abundance in larger specimens.

3.2 Densitometric analysis from the smallest and largest fish

Densitometric analysis was performed to analyse the intensities of 20 protein bands with their respective molecular weights (Figure 2A). The analysis revealed that 12 out of 20 protein bands exhibited up- or down-regulated abundance, with high variation (coefficient of variation, >10%) between the smallest and largest fish. A trend of increasing abundance was observed for higher molecular weight protein (194, 61, 58, 40, 30, 23 and 22 kDa), proportionally with increasing fish size. In contrast, the abundance decreased for low molecular weight proteins (11, 15 and 20 kDa). The 11 kDa parvalbumin band was reduced by 75% from fish 1 to fish 26 while it was only 5% variation for the 12 kDa parvalbumin band.

3.3 Detection of the major fish allergen parvalbumin

Immunoblotting was conducted to confirm the presence of the major fish allergen parvalbumin at 12 and 11 kDa (Figure 2B). Antibody-binding intensity was observed to be higher in the smallest fish compared to the largest fish. In small fish, both 11 and 12 kDa parvalbumin bands were equally abundant while the 12 kDa band was much more abundant compared to the 11 kDa band in larger fish based on SDS-PAGE profiles. However, the signal intensity on the

immunoblot to both parvalbumin bands was comparable in both small and large fish, indicating a differential composition of parvalbumin isoforms.

3.4 Relative abundance of parvalbumin isoforms

The relative abundance of parvalbumin isoforms was expressed in intensity-based absolute quantification (iBAQ) percentage in the protein extract by mass spectrometry analysis. In-solution tryptic digestion identified three predominant parvalbumin isoforms in 26 fish samples (Figure 3). Each parvalbumin isoform were plotted with increasing in fish size. Parvalbumin-3 (12 kDa) was the most abundant isoform, followed by two parvalbumin-beta-like isoforms (11 kDa). The relative abundance of the 12 kDa parvalbumin-3 isoform decreased from 15% to 6% as the fish length increased. Similarly, the average abundance of the parvalbumin beta-like isoforms reduced from 5% to 2% with increasing fish size. The reduction trend of 3 parvalbumin isoforms were significant (p-value <0.001).

3.5 Abundance of fish allergens in small and large fish

The trends of relative abundance of parvalbumin isoforms had decreased as fish size increased (Figure 3), which allowed further analysis to be conducted by grouping the fish into the smallest and largest fish groups to examine other fish allergens listed in the WHO/IUIS database. Differential of 11 known fish allergens was compared across different fish sizes (Figure 4). This analysis was conducted by compiling data for five specimens each from the small fish group (248.80 ± 36.75 mm) and the large fish group (402.20 ± 7.7 mm). Allergen abundance varied between small and large fish groups: creatine kinase was the most abundant allergen (23% and 21%) followed by aldolase (8% and 12%), parvalbumin (12% and 7%), GAPDH (7% and 11%), enolase (4% and 7%), triosephosphate isomerase, TPI (3% and 4%), pyruvate kinase (2% and 4%), lactate DH (2% and 3%), G-6-PI (0.6%), tropomyosin (0.5 and 0.6%), and collagen (0.1%), respectively. Significant differences between the small and large fish were noted for seven allergens. The relative abundance of parvalbumin was significantly higher ($p < 0.0001$) in small as compared to large fish. Conversely, the glycolysis enzymes enolase, aldolase, TPI, pyruvate kinase, lactate DH and GAPDH were significantly higher ($p < 0.01$) in abundance in large fish. Differences in the abundance of tropomyosin, collagen, creatine kinase and G-6-PI were not statistically significant.

3.6 Comparison of SDS-PAGE and parvalbumin detection in different body regions

Total protein profiles and antibody-binding patterns were analysed to compare different muscle regions (head, tail, belly, and dorsal) in five large 391 mm – 413 mm fish (Figure 5). The protein concentration was highest in extracts from the head region (7.4 mg/mL) followed by dorsal region (7.2 mg/mL), tail region (6.3 mg/mL) and belly region (4.9 mg/mL). SDS-PAGE protein profiles were distinct for tissues from each of the different muscle regions (Figure 5A). Two prominent protein bands were observed at 15 kDa across all muscle regions, with the highest abundance observed in the head, followed by the belly, dorsal, and tail regions respectively. A similar pattern was noted for parvalbumin bands (11 and 12 kDa), where the head region showed notably higher parvalbumin levels compared to other muscle tissues. The tail region displayed faint protein bands at 13 and 17 kDa. Immunoblot analysis revealed that the head (rostral) region contained higher parvalbumin amounts compared to the tail (caudal) region (Figure 5B). No differences in parvalbumin content were observed between the dorsal (upper) and belly (ventral) regions. Tail muscle tissue showed lower levels of parvalbumin compared to all other body regions.

3.7 Mass spectrometry analysis of relative abundance in muscle locations

Protein analysis was conducted on one specimen of fish ID 21 (399 mm) representing the largest fish group to compare protein profiles across different muscle regions (Figure 5). In-solution mass spectrometry was employed to quantify the relative abundance of 11 known fish allergens across different muscle tissue regions (head, dorsal, tail, and belly) of red snapper (Figure 6). Parvalbumin was most abundant in the belly region (10%) followed by head (8%), dorsal (7%) and tail (4%) muscle regions. Aldolase exhibited higher expression (14%) in the dorsal muscle tissues compared to the other regions. Creatine kinase had elevated levels in both the dorsal and tail muscle tissues, 14% and 19% respectively. TPI showed higher relative abundance specifically in the tail muscle region (6%) of the fish. Several other allergens like enolase, aldolase, tropomyosin, pyruvate kinase, lactate DH, G-6-PI and GAPDH also exhibited differential abundance patterns across the tissue locations.

4. Discussion

This study aimed to evaluate the possible correlation between allergen abundances and fish size within a single fish species, Malabar red snapper. Previous studies on protein profiling in different specimens of the same fish species did not compare abundances of individual proteins and focused on non-standardised wild barbel fish (Huriaux et al., 1997) and snakehead fish (Gam et al., 2006). We have analysed farmed specimens that were all growing in the same floating cage to ensure identical feed availability and environmental conditions, with fish length as only variable.

Protein profiles demonstrated clear distinctions in the protein abundances between the small and large red snapper fish. This is likely due to the differences in muscle physiology and energy metabolism demands as fish grow and develop. Fish growth is dependent on environmental variability as such desirable temperature, food availability and prey-predator relationship. In teleost, skeletal muscle tissues can optimise muscle performance in adaptation to the environment with indeterminate growth known as muscle plasticity (Joyce, 2023). As fish in the developmental phase or at different life cycle demands of energy, skeletal muscle contains high plasticity of contractile and metabolic rate in myotome components which house muscle fibre, nerve fibre, connective tissue, fibroblasts, skeletal osteocytes (cells in bone), adipocytes (fats storage) and capillary endothelial cells (Nemova et al., 2021).

Smaller red snapper fish exhibited higher levels of parvalbumin, the major fish allergen, compared to larger fish. Red snappers are demersal fish species, which are known as fast-swimming fish. These allow small red snapper to move faster than the larger snapper due to various reasons such as quick escapes and manoeuvres resulting in high parvalbumin content. In vertebrate organisms, muscle contraction process starts with calcium ions released from the sarcoplasmic reticulum and binds to troponin C allowing tropomyosin to interact with myosin and actin while parvalbumin shuttles calcium ions from troponin back to the sarcoplasmic reticulum during muscle relaxation (Arif, 2009). The interaction between parvalbumin and tropomyosin is a crucial factor during the contraction-relaxation process. Therefore, the levels of both proteins show a similar trend, with an increase in fish size corresponding to a decrease in parvalbumin and tropomyosin abundance. This suggests smaller fish may be more allergenic than larger fish for individuals primarily sensitised to parvalbumin.

Larger fish contained significantly more of the other allergens enolase, aldolase, triosephosphate isomerase, pyruvate kinase, L-lactate DH and GAPDH, likely to reflect their increased energy metabolism demands. Hence, larger fish would pose higher allergy risks to individuals primarily sensitised to fish allergens other than parvalbumin and tropomyosin. Previous studies on yellow perch have also identified parvalbumin, aldolase A, creatine kinase, pyruvate kinase, and GAPDH as proteins associated with body mass and body length of different ages (Reddish et al., 2008). Glycolysis is primary pathway for glucose catabolism in fish muscle tissues, leading to the formation of pyruvate and generating a small amount of energy (Kaushik & Schrama, 2022). The enhanced expression of these glycolytic enzymes suggests a higher rate of glucose metabolism and ATP production necessary for sustained growth (Kocmarek et al., 2014).

Creatine kinase (43-49 kDa) was found to be the most abundant allergen in Malabar red snapper regardless of fish size. This enzyme is responsible for cellular energy homeostasis and ATP regeneration (Sahlin & Harris, 2011). Previous IgE binding analysis with serum from 32 – 42 fish-allergic subjects identified major allergens at 51 kDa in both golden snapper (*Lutjanus johnii*) and mangrove red snapper (*Lutjanus argentimaculatus*), along with additional major allergens at 48 kDa in golden snapper and 42 kDa in mangrove red snapper, identified as creatine kinase by mass spectrometric analyses (Rosmilah et al., 2005).

The high parvalbumin content in the head and dorsal regions can be explained by the higher concentration of fast-twitch muscles used to burst muscle movement. This is consistent with previous studies demonstrating a higher abundance of parvalbumin in muscle tissues located near the rostral region compared to the caudal region of different species of bony fishes (Coughlin et al., 2007; Y. Kobayashi et al., 2016; Lee et al., 2012; Thys et al., 2001). The diversity of protein content in different body parts is derived from the distribution variety of proteins to carry out movement functions. Muscles are responsible for initiating movement hence, muscle tissues located near the head contain a high abundance of proteins. High parvalbumin content at the head or rostral part of the fish correlated with a faster rate of muscle contraction and the tail or caudal part tended to have a slower rate. The rostral region requires rapid muscle contraction as fish jaw and gills have a strong movement enabling feeding and respiration (Huby & Parmentier, 2019). The tail region contained less of the 11 kDa parvalbumin compared the head region, likely as it is primarily used for propulsion and

continuous steady swimming movement, which is associated with 12 kDa parvalbumin-3 (Herr & Dennis, 2004).

This study highlights the importance of considering the size of fish specimens when investigating protein and allergen profiles. Understanding the distribution and abundance of key allergens across different fish tissues and sizes can inform more accurate allergy research.

Conclusion

It is well known that the allergenicity of fish muscle tissue depends on the tissue type and body region. However, there is a gap of knowledge regarding the abundance of allergens in fish of different sizes. This study focused on fish allergens in red snapper. Muscle tissues were collected from farmed red snapper of the same age, with the snapper's growth environment well controlled by keeping the fish in the same cage for over nine months, providing similar types of feed, and maintaining consistent environmental conditions. Size variations after harvest were reasonably high, ranging from 212 mm to 413 mm corresponding to 195 g to 1330 g, which could be explained by the genetic variations of snappers due to their growth metabolism. The protein composition in red snapper is highly diverse and serves specific biological functions and adaptations. Understanding size-dependent protein variations can reveal novel insights into fish allergenicity and uncover previously unexplored patterns of protein composition. The abundance of eight fish allergens differed depending on fish sizes. Small red snapper had a higher content of the major fish allergen parvalbumin, a calcium-binding protein that allows rapid bursts of movement and manoeuvres for predators and prey capture. As the fish grow larger, there was an increase in glycolytic enzymes, which support the metabolic demands of sustained growth. Altogether, smaller fish may trigger stronger allergic responses due to their higher parvalbumin content compared to larger fish. However, larger fish may present an increased allergy risk to individuals sensitised to fish allergens other than parvalbumin and tropomyosin. Accordingly, fish size is an important factor to consider when assessing the allergenic potential of different fish samples. This study explored allergen abundances, however, IgE binding capacities of small and large fish are yet to be conducted. The severity of allergic reactions in individuals with fish allergy may depend on the size of the fish, which should be further investigated. In this study, we demonstrate relationships between fish size, body part, protein compositions and allergen abundances to advance our understanding of intra-species variabilities. Size-dependent allergen abundances should be considered for proteomic and allergenomic analyses for all fish as well as in allergy management and food safety approaches.

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Figures

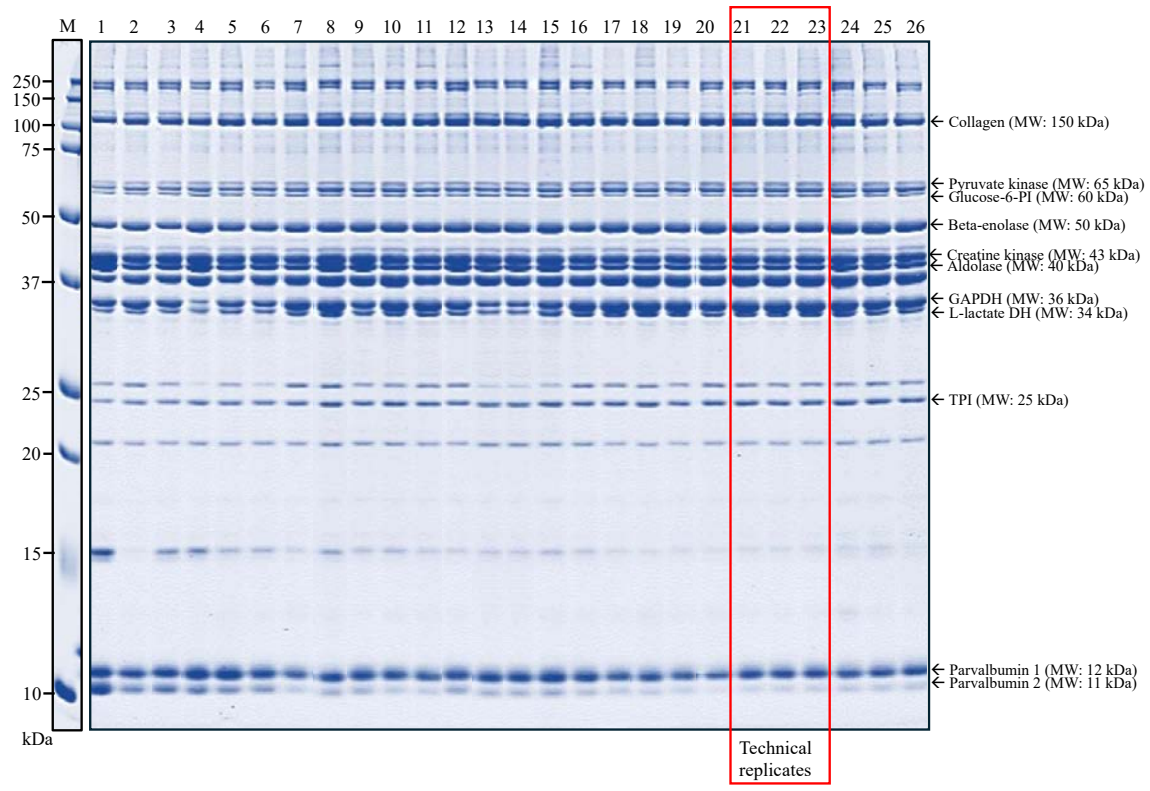

Figure 1: SDS-PAGE protein profiles of raw extracts from muscle tissue collected from red snapper of different sizes, with the smallest fish measuring 212 mm (1) and the largest measuring 413 mm (26) as detailed in Table S1. Samples 21, 22 and 23 were the technical replicates incorporated in the analysis. Prominent differences were observed at 15, 27, 35 and 36 kDa. Bands containing fish allergens have been identified based on their molecular weight and mass spectrometric analyses after in-gel tryptic digestion in previous studies (Kalic et al., 2020; Ruethers et al., 2021) M: molecular weight marker, DH: dehydrogenase, G-6-PI: glucose-6-phosphate-isomerase, GAPDH: glyceraldehyde-3-phosphate-dehydrogenase, TPI: triosephosphate isomerase.

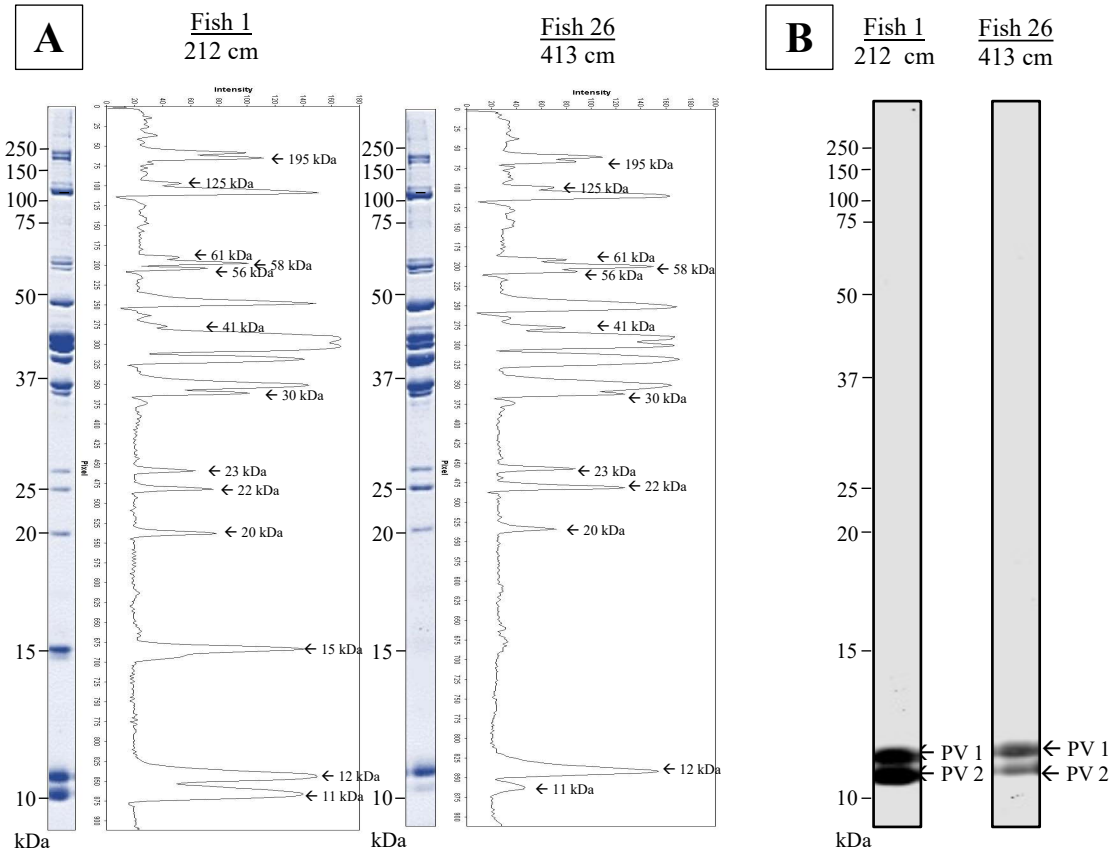

Figure 2: A comparative analysis of the protein profiles of raw extracts from two different size groups of fish is presented. **A)** Densitometric analysis of the intensity of 20 protein bands in extracts from the smallest fish 1 (212 mm) and the largest fish 26 (413 mm). The molecular weight of each band was calculated, and the bands indicated with arrows had differences in the trend over increasing size (refer to supplementary table S2 for detailed analyses of intensity peak differences). The larger fish exhibited increased protein band intensities at 125, 61, 58, 56, 41, 30, 23, and 22 kDa, and decreased protein band intensities at 195, 20, 15, and 11 kDa. **B)** Immunoblot membrane was incubated with a monoclonal anti-parvalbumin antibody (Parv-19). Parvalbumin isoforms were detected at 11 and 12 kDa. The signal intensity was about 50% lower for bands in the extract from the larger as compared to the smaller specimen. M: molecular weight marker, PV: Parvalbumin

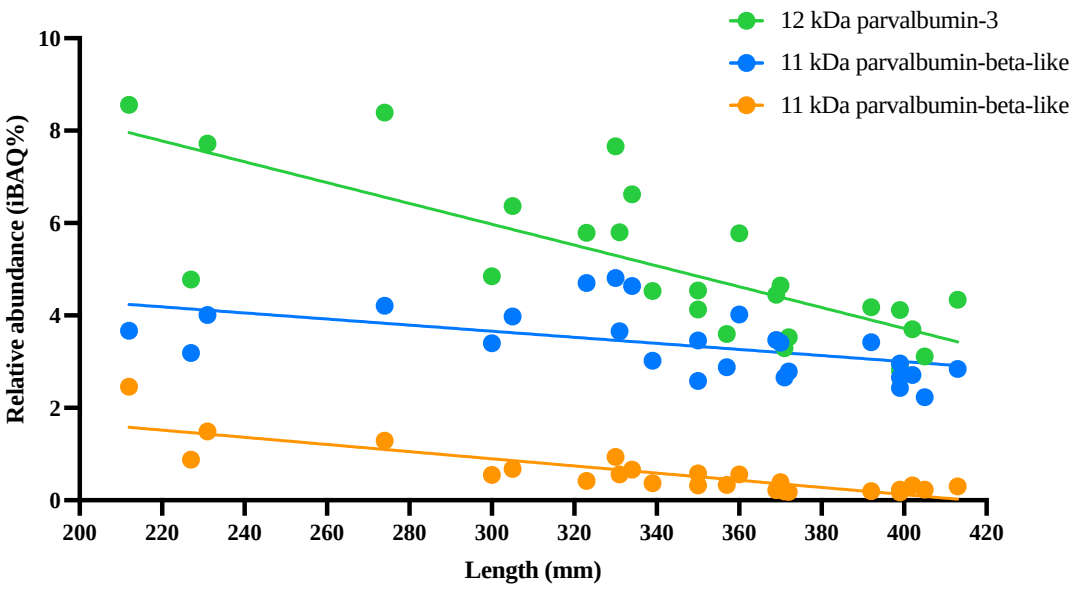

Figure 3: The three most abundant parvalbumin isoforms were quantified in the raw extracts of dorsal muscle tissue from the Malabar red snapper by mass spectrometric analysis after in-solution tryptic digest. The correlation between fish length and abundance of three parvalbumin isoforms showed a statistically significant decrease in the relative abundance of all three parvalbumin isoforms over increasing size of the fish specimens (p-value <0.001).

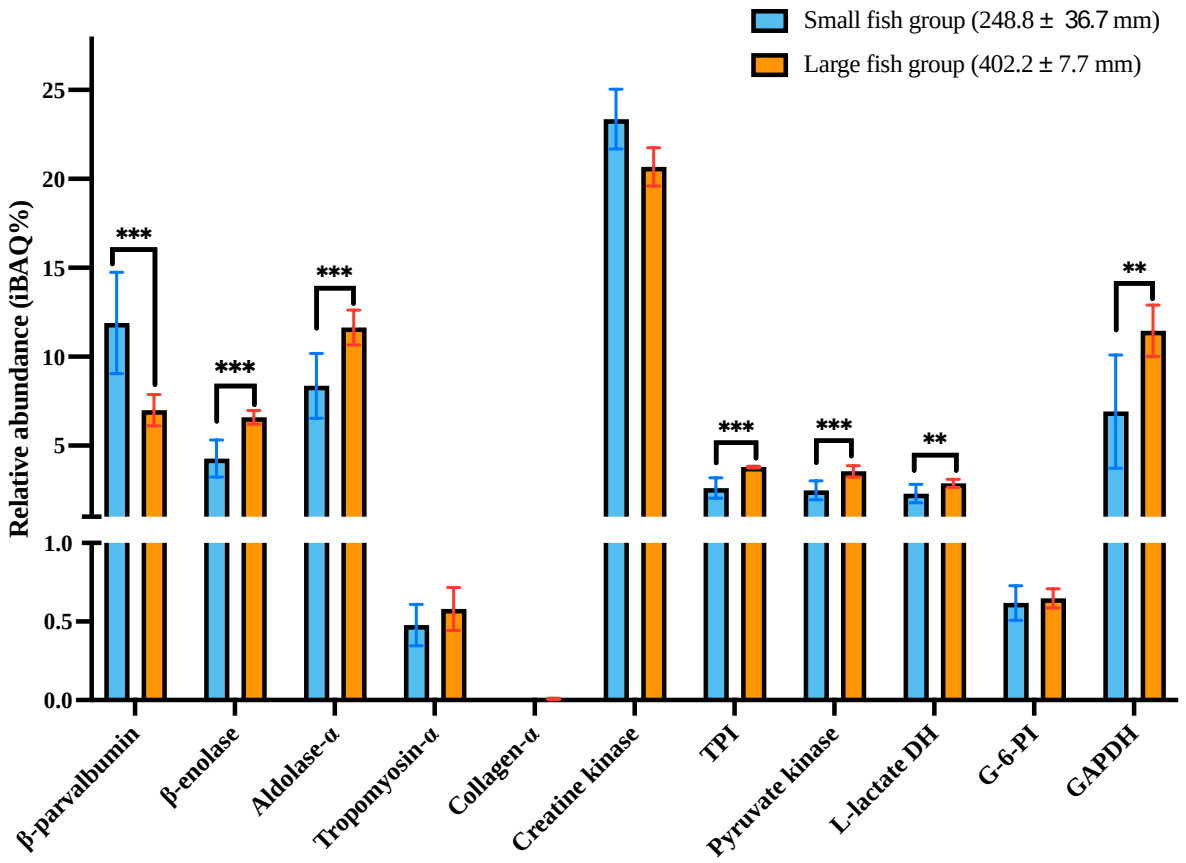

Figure 4: Relative abundance of 11 fish allergens in raw protein extracts from five small (248.80 ± 36.75 mm) and five large specimens (402.20 ± 7.7 mm) of red snapper expressed in iBAQ% (intensity-based absolute quantification percentage). iBAQ% was calculated after tryptic digestion with subsequent mass spectrometric analyses. Fish allergens are defined according to WHO/IUIS Allergen Nomenclature (www.allergen.org) as beta-parvalbumin, beta-enolase, aldolase alpha, tropomyosin alpha, collagen alpha, creatine kinase, triosephosphate isomerase (TPI), pyruvate kinase, L-lactate dehydrogenase (DH), glucose-6-phosphate-isomerase (G-6-PI), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Significant value (P<0.001***, P<0.01**)

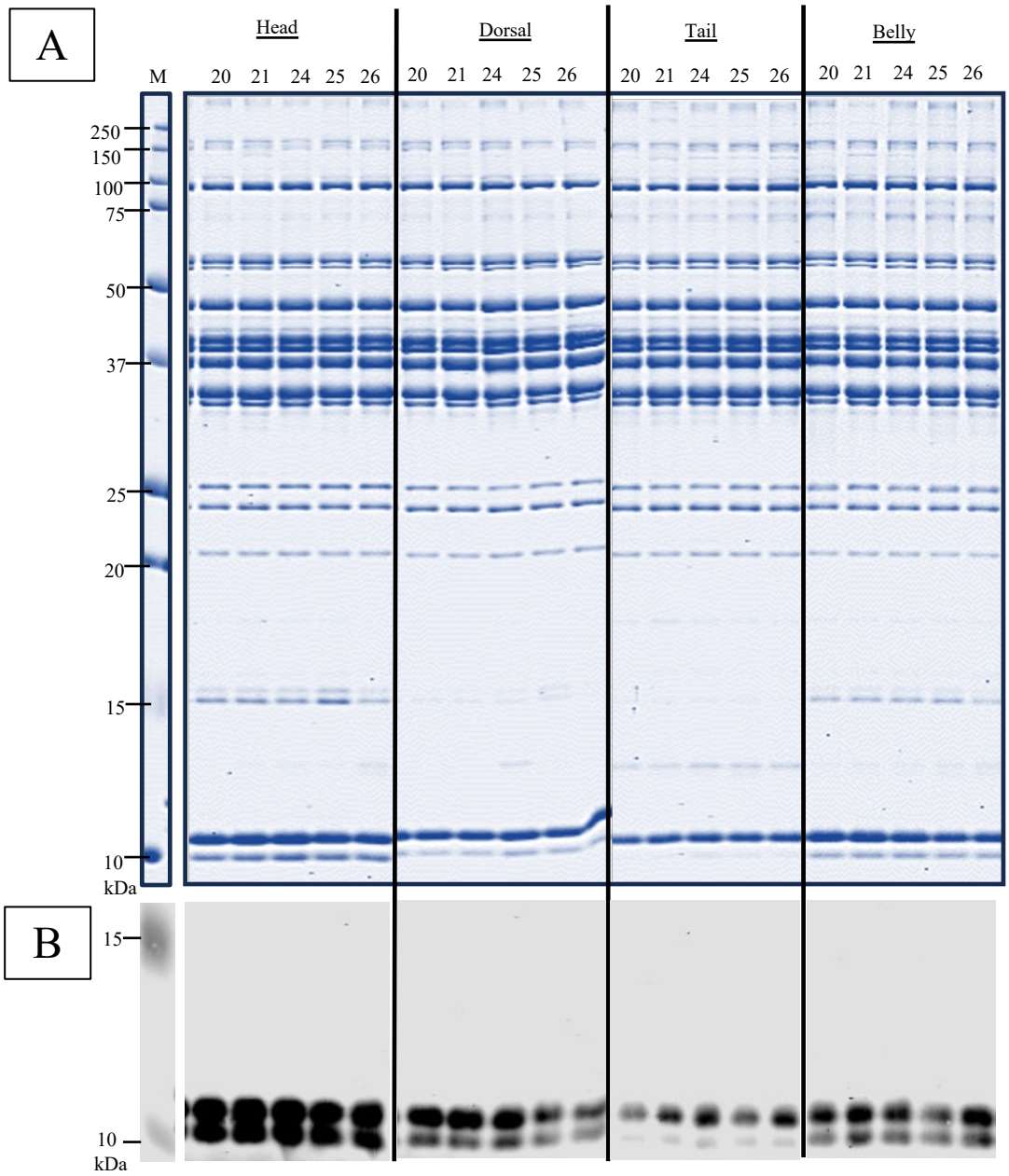

Figure 5 Protein profiles of raw extracts from the head, dorsal, tail, and belly muscle tissues of red snapper (samples 20 – 26 in Table S1; 392 - 413 mm) with subsequent parvalbumin-detection. **A)** All protein profiles demonstrated prominent protein bands at the molecular weight range of parvalbumin, 10-12 kDa. Tail muscle tissues were observed to have one predominant parvalbumin band while head muscle, belly muscle and dorsal muscle contained two. **B)** Detection of parvalbumin bands in head, dorsal, tail, and belly muscle tissues using a monoclonal anti-parvalbumin antibody revealed that parvalbumin abundance was highest in head muscle, followed by dorsal and belly muscle, and lowest in tail muscle. No signals were observed above 15 kDa.

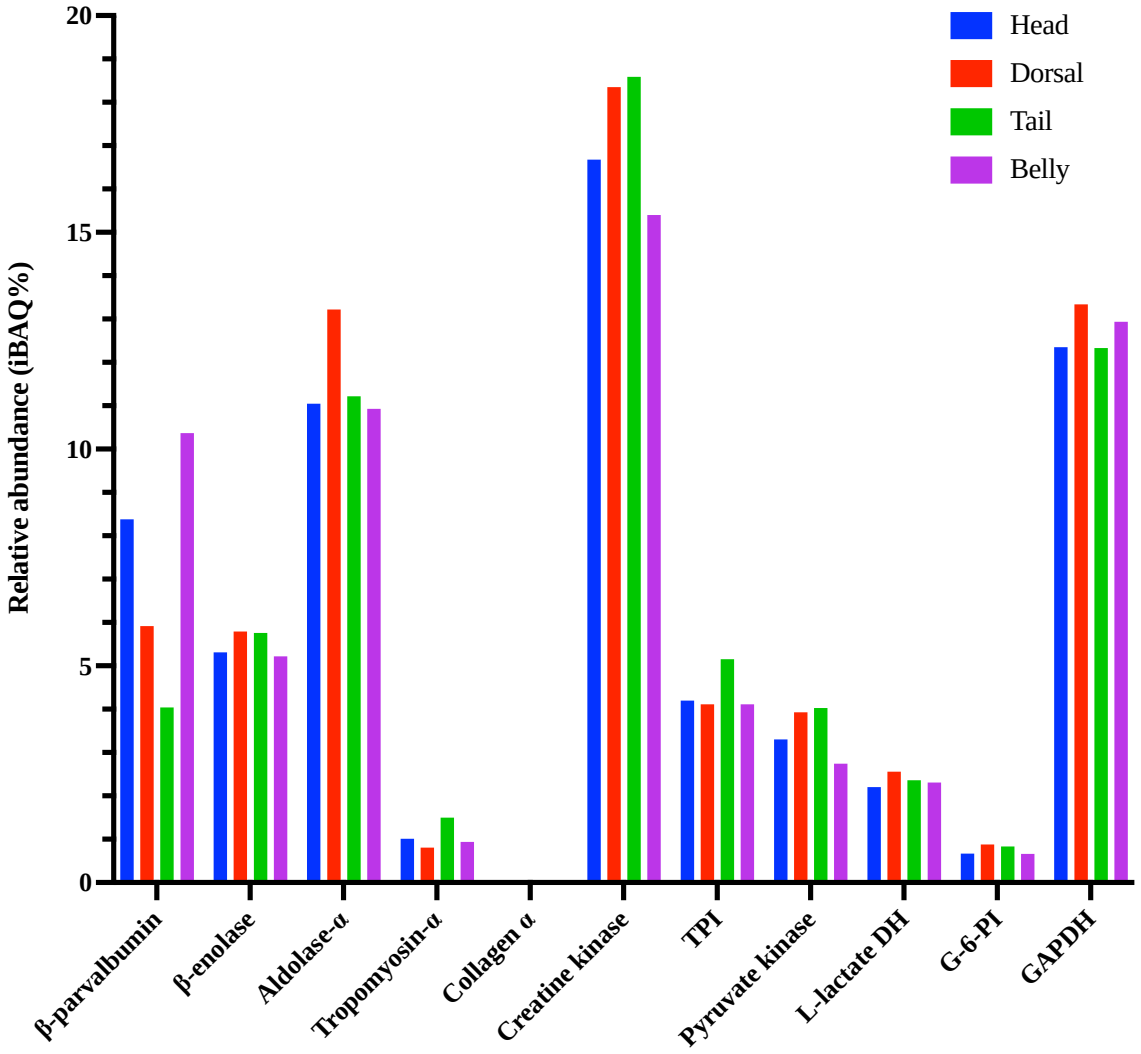

Figure 6: Relative abundance expressed in intensity-based absolute quantification percentage (iBAQ%) of 11 known fish allergens in head, tail, belly and dorsal muscle of red snapper after tryptic digestion with subsequent mass spectrometric analyses. Results for Samples 21, 22 and 23 in Table S1 are shown. The fish allergens are beta-parvalbumin, beta-enolase, aldolase alpha, tropomyosin alpha, collagen alpha, creatine kinase, triosephosphate isomerase (TPI), pyruvate kinase, L-lactate dehydrogenase (DH), glucose-6-phosphate-isomerase (G-6-PI), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) based on WHO/IUIS Allergen Nomenclature (www.allergen.org).

Supplementary figures

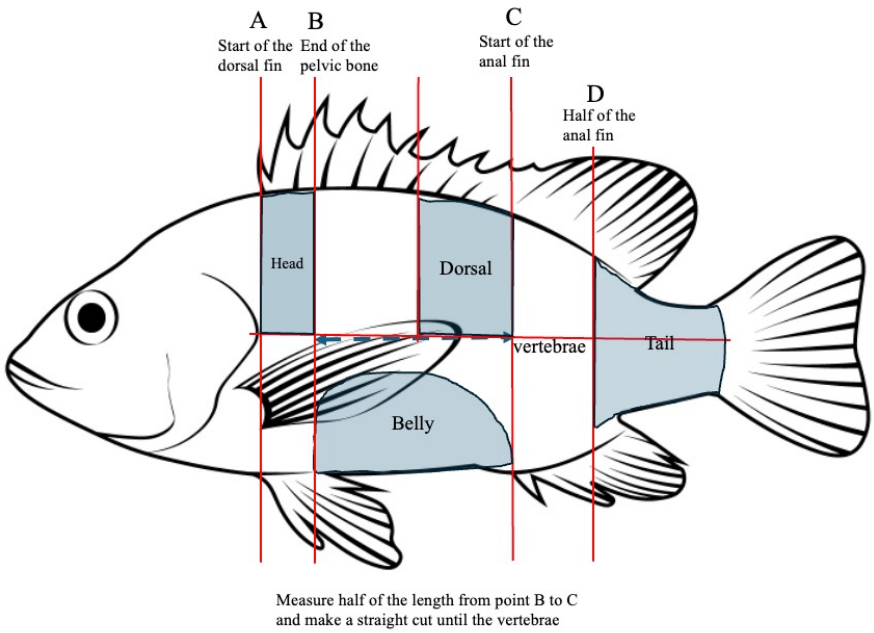

Figure S1: Illustration of analysed muscle tissue sections in red snapper. Head, dorsal, tail and belly muscle tissues were defined using points A (the front end of the dorsal fin), B (the end of the pelvic bone), C (the front end of the anal fin) and D (half of the anal fin).

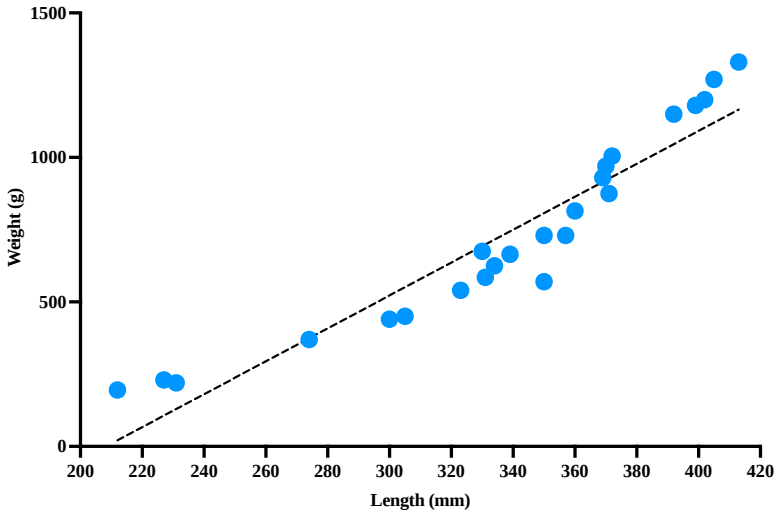

Figure S2: Positive correlation between length and weight (correlation efficient, *r* value = 0.9458). Length was used as the primary variable to investigate protein compositions and allergen abundances in fish of different sizes.

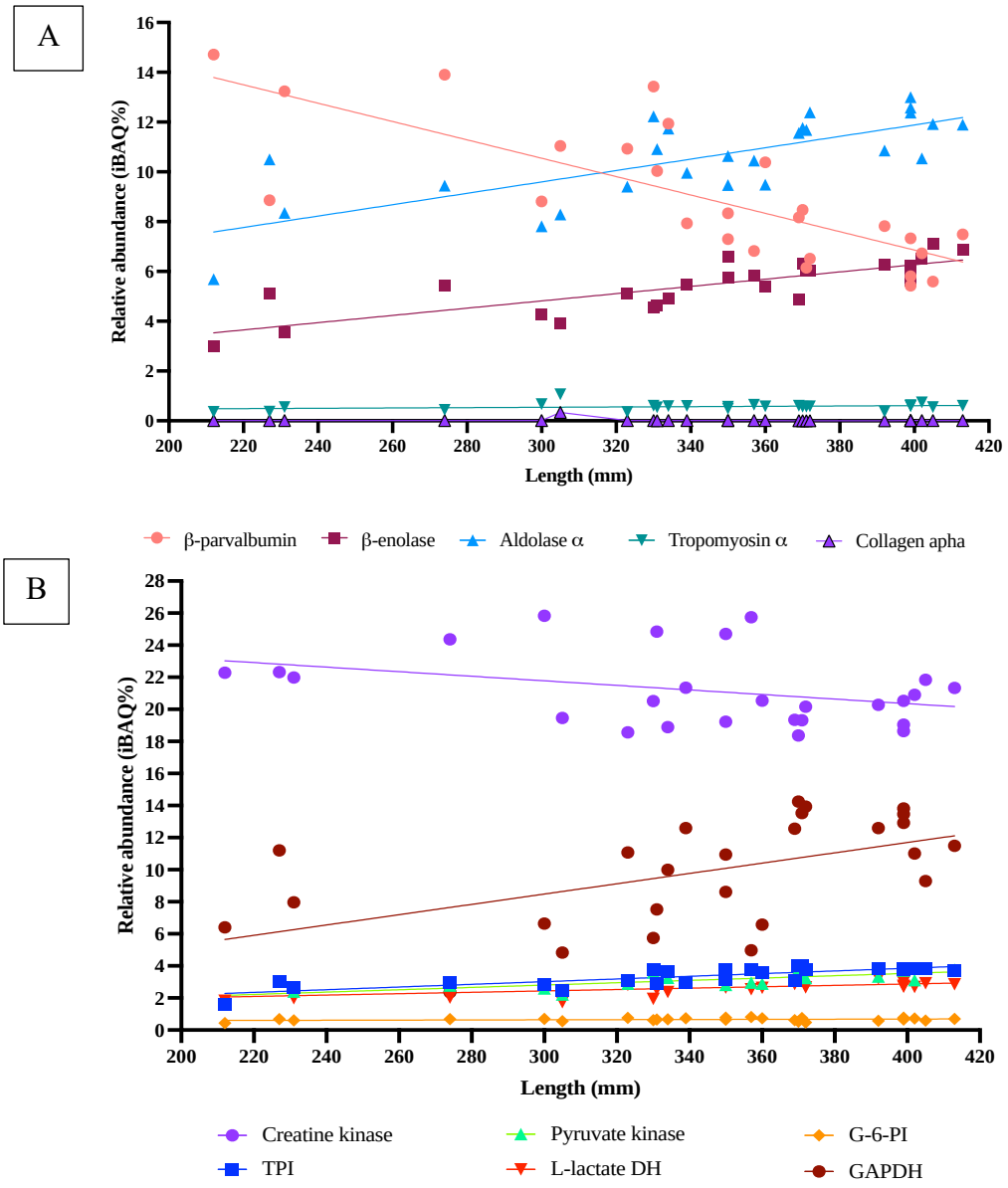

Figure S3: The relative abundance of fish allergens identified in whole raw extracts of red snapper. **A)** Size-dependent abundance of five well-described fish allergens. Beta-parvalbumin abundance significantly decreases with fish size (p-value <0.0001). Conversely, the abundance of beta-enolase and aldolase alpha increased over the sizes (p-value <0.0001). Tropomyosin and collagen abundance showed no significant differences (p-value > 0.1). **B)** Size-dependant abundance of other heat-labile fish allergens: Creatine kinase was the most abundant allergen in red snapper muscle tissues - a non-significant decrease over fish size was observed (p-value > 0.08). In contrast, glycolysis enzymes, including triosephosphate isomerase (TPI), pyruvate kinase, and L-lactate dehydrogenase (DH), exhibited a significant increase in abundance over size. No significant increase with p-value >0.1 was observed for G-6-PI and GAPDH).

Supplementary table

Table S1: Details and photos of 28 farmed Malabar red snapper (*Lutjanus malabaricus*) specimens collected for this study. The fish ID corresponds to the analysed respective protein extracts. IDs 21, 22 and 23 reflect technical replicates from protein extraction onwards. Unhealthy fish were excluded from detailed analyses and have no ID.

Fish ID SDS-PAGE	Length (mm)	Weight (g)	Fish image	Observation
1	212	195		-
2	227	230		-
3	231	220		-
4	274	370		-
	275	335		Morphological unhealthy - Deformed eye
	293	495		Morphological unhealthy - Deformed eye

5	300	440		-
6	305	450		-
7	323	540		-
8	330	675		-
9	331	585		-
10	334	625		-
11	339	665		-
	341	710		Morphological unhealthy – pale in colour
12	350	570		History – grow in a different cage

13	350	730		-
14	357	730		-
15	360	815		-
16	369	930		-
17	370	970		-
18	371	875		-
19	372	1005		-
	386	1085		Morphology unhealthy – big belly and deformed tail

20	392	1150		-
21,22,23	399	1180		-
24	402	1200		-
25	405	1270		-
26	413	1330		-

Table S2: Densitometric analysis of SDS-PAGE protein profiles of Malabar red snapper fish 1 1 and fish 26 based on the gel shown in Figure 2. The coefficient of variation (CV) was calculated for each sample to determine the degree of variability between the peak values of each molecular weight. A CV value <10% indicates a low degree of variability. Protein bands with high CV value (> 10%) are reflected in Figure 2.

Molecular weight (kDa)	Peak value		CV%	up-/down-regulated
	Sample 1 (212 mm)	Sample 26 (413 mm)		
218	121	127	3%	+
195	137	103	20%	-
125	62	81	19%	+
111	177	183	2%	+
61	61	90	27%	+
58	120	172	25%	+
56	85	103	14%	+
46	172	184	5%	+
41	49	90	42%	+
38	185	185	0%	=
37	185	186	0%	=
35	168	184	6%	+
31	170	184	6%	+
30	124	151	14%	+
23	74	99	20%	+
22	90	145	33%	+
20	92	80	10%	-
15	164	34	93%	-
12	169	157	5%	-
11	161	49	75%	-

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Further results and discussion not included in the manuscript

Preliminary investigation of fish allergen profiling across different origins

The analysis of fish size data has been established as an area of study and further research may be conducted on the allergenicity of fish from different origins. This preliminary study aimed to investigate the protein/allergen profiles of Malabar red snapper (*Lutjanus malabaricus*) of different origins. Malabar red snapper is a commercially importance fish species and has high consumption in the Asia-Pacific region. To achieve this, red snapper specimens were sourced from two distinct sea-cage farms in Singapore, as well as from wild-caught populations in Indonesia. All fish were selected within a standardised market size range of 290-350 mm (430-720 g). Standardised dorsal muscle tissue samples were collected from specific muscle sections (detailed in Supplementary Figure 1, Chapter 3) and extracted using a snap-frozen protein extraction method established in Chapter 2. Protein samples (10 µg each) were separated using SDS-PAGE gel electrophoresis.

The SDS-PAGE methodology requires further optimisation. Preliminary comparative analysis of protein profiles between wild and farmed red snapper revealed significant differences in protein abundance at specific molecular weights: 60 kDa, 27 kDa, and 15 kDa (Figure 1). However, no significant variations were observed in the protein profiles between different wild-caught and farmed fish populations. To further investigate these differences, additional farmed fish were sourced from Malaysia, and in-solution tryptic digest mass spectrometry analysis was conducted to quantify allergen abundances from four origins.

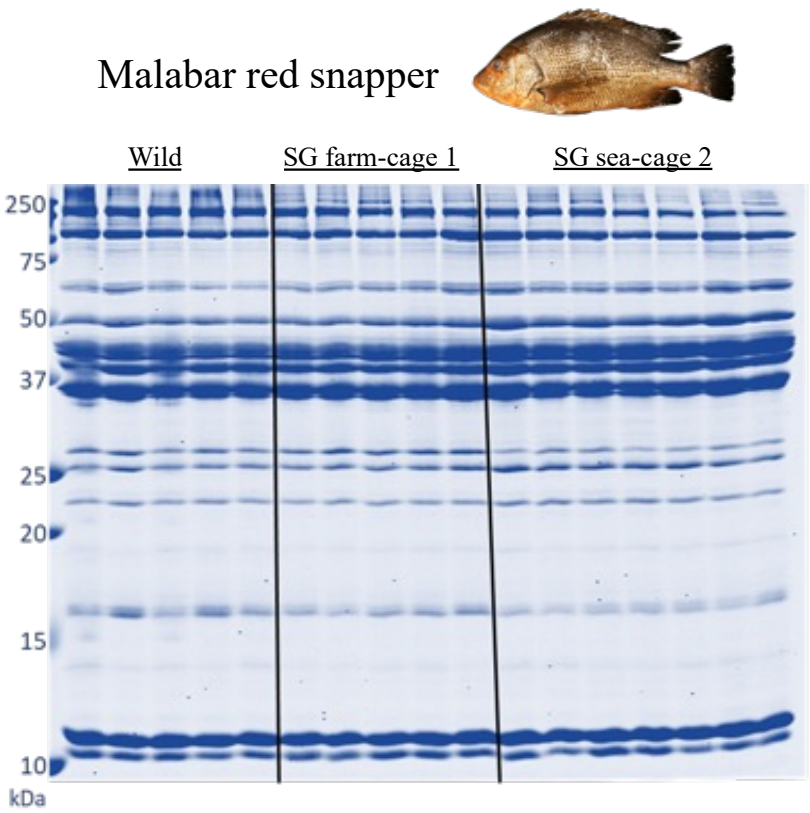

Figure 1: SDS-PAGE gel of Malabar red snapper that was sourced from three locations: wild-caught and two farms in Singapore. It observed the differences in protein bands at 60 kDa, 27 kDa and 15 kDa.

The research studies on the allergenicity of single fish species of different origins are still limited. This preliminary relative abundance was expressed using intensity-based absolute quantification percentage (iBAQ%) of various allergens found in raw extracts of Malabar red snapper samples from different origins, including wild-caught fish from Indonesia and fish from three different aquaculture farms: SG farm-cage 1 and SG farm-cage 2 are from Singapore farms and MY farm-cage was from Malaysia farm (Figure 2). Quantitative of known fish allergens between wild-caught and three-farmed Malabar red snapper populations using the in-solution tryptic digest mass spectrometric analysis. Mass spectrometry analysis revealed the major allergen parvalbumin shows the highest abundance (8%) in Singapore's farm-cage 1 compared to the other origins and farm-cage from Malaysia had the lowest abundance of these allergen (6%). A metabolism enzyme creatine kinase exhibited a high abundance of allergen in red snapper for all origins where farm-cage 1 from Singapore contained the highest abundance of creatine kinase (24%) followed by the three origins (21%), Even though, there were

significant differences in relative abundance between all allergens (p-value <0.001) however, no allergens exhibited statistical differences regardless of fish origins (p-value = 0.552).

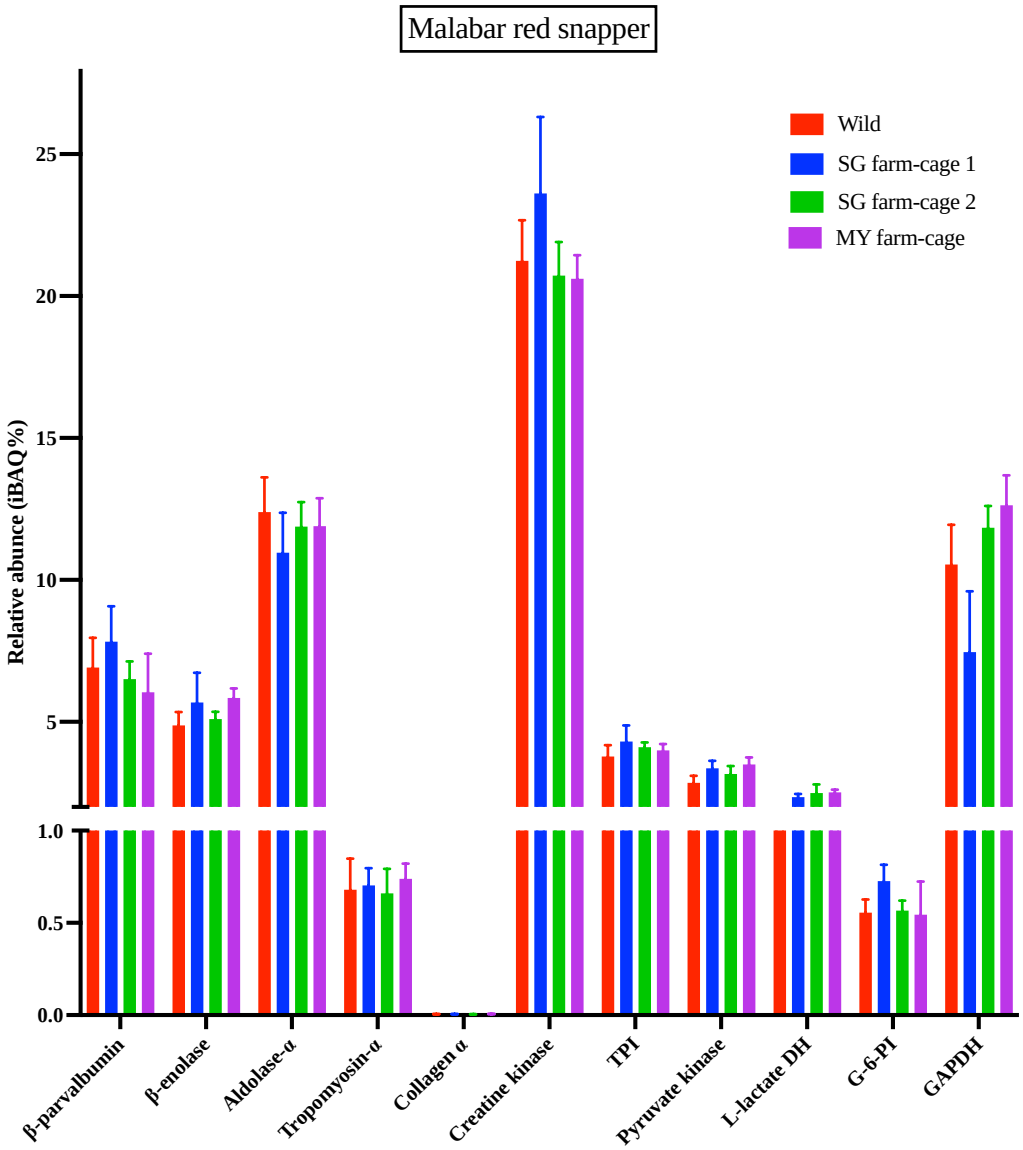

Figure 2: The relative abundance of 11 known fish allergens expressed as iBAQ% by comparing wild-caught and farmed Malabar red snapper using in-solution trypic digest mass spectrometric. ANOVA analysis revealed significant differences (p-value < 0.0001) between all fish allergens, however, no allergen showed statistically significant changes (p-value = 0.552) regardless of fish origins. The following fish allergens were identified: beta-parvalbumin, beta-enolase, aldolase alpha, tropomyosin alpha, collagen alpha, creatine kinase, TPI, pyruvate kinase, L-lactate DH, G-6-PI and GAPDH. TPI: triosephosphate isomerase, DH: dehydrogenase, G-6-PI: glucose-6-phosphate isomerase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, SG: Singapore, MY: Malaysia

The preliminary research presented in this study did not reveal any significant differences in allergen profiles between the wild-caught and farmed Malabar red snapper and Asian seabass samples, two species that are highly relevant in Asia-Pacific. Proteomics data suggests some variation in the abundance of individual allergens, such as parvalbumin and allergenicity between wild-caught and farmed fish appears to be largely comparable based on the information provided. However, it is important to note that the allergenicity of Malabar red snapper from different geographical locations has not yet been fully explored. Further investigations would be necessary to understand how factors such as habitat, environmental conditions, and farming practices may influence the allergen composition of this fish species. To confirm and expand upon these initial findings, additional analyses focused on IgE binding profiles would be valuable. Assessing the specific IgE reactivity of the identified allergens could provide more definitive insights into the potential differences in allergenicity between wild-caught and farmed Malabar red snapper, as well as across geographical regions. Overall, this preliminary research serves as a starting point for a more comprehensive understanding of the allergen characteristics of Malabar red snapper. Further investigation, including IgE-binding studies and a broader geographical survey, would be necessary to draw more conclusive findings and a full understanding of the allergenicity of this commercially important fish species.

The concept of investigating the allergenicity of Malabar red snapper from different origins (Chapter 3) was extended to another economically important species in the Asia-Pacific region: the Asian seabass (barramundi, *Lates calcarifer*). However, due to the decline of wild populations and the prevalence of aquaculture, determining the origin of specific fish has become challenging. This variability limits the scope of preliminary investigations.

Asian seabass were sourced from three different farms in Singapore, with a standardised market size range of 380-450 mm (720-1300 g). Standardised dorsal muscle tissue samples were collected from specific muscle sections (detailed in Supplementary Figure 1, Chapter 3) and extracted using a snap-frozen protein extraction method established in Chapter 2. Protein samples (10 µg each) were separated using SDS-PAGE gel electrophoresis. The protein profile of SDS-PAGE analysis revealed notable differences in protein profiles (Figure 1) among the three Asian seabass farms, particularly in the 15-37 kDa range. The underlying causes of these protein differences remain unclear. Potential factors include genetic variation within the species and variations in the origin or diet of the fish.

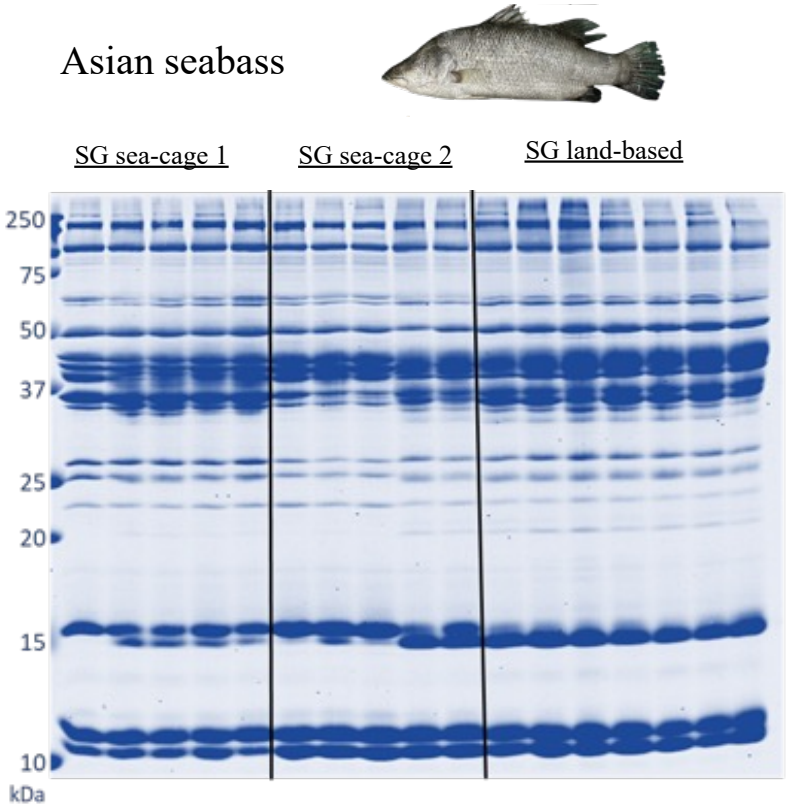

Figure 1: Preliminary SDS-PAGE protein profiling of Asian Seabass from three different farms in Singapore: two of the farms were floating-cage farming and one land-based farming. Asian

seabass protein profiles in both floating-cage farming have two protein bands at 15 kDa while only one protein band is present in land-based farmed seabass. Also, land-based farmed seabass has higher protein abundance at 20 kDa.

Chapter 4

General discussion and future direction:

Understanding protein differences to make healthier and safer seafood

Seafood plays a crucial role in global food security and human nutrition. The global fish consumption rate increased by 3% annually - consumption of aquatic food per capita has increased to 21 kg in 2021 (Sean Sampson, 2024). However, the growing demands have led to challenges such as overfishing, creating difficulties in replenished populations and meeting the nutritional needs of an expanding global population. To address these challenges, aquaculture has emerged as a vital solution. In 2018, finfish aquaculture contributed 88% to the world's fish production (FAO, 2020), potentially improving food security and addressing global hunger. The increase in fish consumption has heightened the risk of IgE-mediated fish allergy.

The current approach to diagnosing fish allergy involves a multifaceted process. Clinicians first gather a detailed clinical history and conduct a thorough assessment of the patient's symptoms and medical background. This is typically followed by an oral food challenge, skin-prick testing and allergen-specific IgE (Sindher et al., 2022). Nevertheless, there are still obstacles to overcome in the clinical diagnosis of fish allergy. Further research is required to develop more precise diagnostic techniques, such as component-resolved diagnostics, to reduce the necessity for oral food challenges, which carry an inherent risk of anaphylaxis (Reginald et al., 2024). The diversity of fish species and the lack of adequate intraspecies studies present a challenge to the diagnosis and management of fish allergy.

As discussed in Chapter 1 of this thesis, the prevalence of fish allergy varies significantly across different regions of the world. This is largely due to the immense diversity of fish species consumed, which span evolutionary distant classes. The wide range of fish species used in different culinary traditions contributes to the disparities in fish allergy prevalence observed globally. Chapter 1 reviewed what is known about fish proteins and allergens and highlighted the significant health concern posed by seafood allergies, which can trigger life-threatening reactions in both adults and children. This chapter discusses the variability in fish protein profiles and their impact on allergenicity, emphasising the need for further research to identify

fish allergens across intra- and inter-species. Fish species are widely spread across geographical origins, but intra-species variability is under-investigated, and there are no studies comparing allergen abundances in fish of different origins.

To achieve that, this thesis addresses two central objectives. Firstly, optimising protein extraction methodologies is a fundamental aspect often overlooked in proteomic research. This research aims to ensure reproducible and reliable sample analysis by developing and refining extraction techniques. Secondly, the thesis examines the protein variations influenced by fish sizes and muscle regions.

Importance of protein extraction optimisation

The preparation and consumption of fish varies considerably depending on the cultural and regional context. Fish may be consumed raw as sashimi, a culinary practice particularly prevalent in specific geographical regions. Additionally, diverse fish processing and cooking techniques encompass the preparation of fish paste, drying, roasting, grilling, and poaching fish which altered the fish proteins (Bhat et al., 2021). In Chapter 2, a selected range of extraction methods was examined to closely mimic the preparation of fish typically in a home kitchen setting. This approach was adopted to gain an understanding of the molecular-level implications of various extraction techniques.

The protein extraction represents a critical methodological component in the research. It has potentially significantly influenced protein recovery quality, which directly impacts the subsequent analytical results and overall reliability. The complexity of fish muscle resulted in the need to further optimise the current protein extraction protocol. Fish muscle proteins are classified into three categories based on their functional and structural characteristics (Ochiai & Ozawa, 2020). The first group includes structural and regulatory myofibrillar proteins, which include myosin, actin, tropomyosin, and troponin. The second group are sarcoplasmic proteins, which consist primarily of glycolytic enzymes, creatine kinase, myoglobin, and parvalbumin. The third group is stroma protein, which consists primarily of extracellular matrix proteins such as collagen. This complex protein composition inherently complicates the extraction process, thus demanding optimisation strategies to achieve high-quality proteomic analysis.

Chapter 2 optimisation protocol has elevated temperatures during the protein extraction facilitated the recovery of high protein levels and a comprehensive protein profile of the fish

samples, as demonstrated by the protein profiles of the lyophilised and snap frozen. These techniques effectively prevented drastic temperature fluctuations and minimised protein degradation during extraction by maintaining fish muscle tissues at cryogenic temperatures using liquid nitrogen. This approach ensured the extracted proteins closely represented their native tissue state (Gokoglu & Yerlikaya, 2015), which is important for studying protein structure and function, particularly in the context of allergenicity. Preserving heat-labile proteins and allergens within fish muscle tissues was a primary consideration. Through meticulous standardisation and optimisation of the extraction protocol, sample-to-sample variability was substantially reduced, resulting in more consistent analytical outcomes. The refined extraction methodology has direct implications for enhancing the reproducibility and reliability of molecular-level sample analysis. Successfully optimising the protein extraction protocol ultimately facilitated subsequent investigations into protein and allergen composition variations influenced by fish size in Malabar red snapper.

Fish size matters

Proteomic analyses of Malabar red snapper muscle tissues, body parts and fish sizes provided insights into the sources and abundance of fish allergens. The abundance of a major fish allergen, parvalbumin varies between different muscle types and muscle regions of fish which are well-studied (A. Kobayashi et al., 2006; Y. Kobayashi et al., 2016; Omidvar et al., 2024). White muscle tissues, which constitute most of the fish's muscle mass, contained higher levels of parvalbumin. In contrast, a thin layer of red muscle tissues along the vertebrae line and beneath the skin demonstrates lower parvalbumin. In addition, the anterior region of the Malabar Red Snapper was shown to have increased allergenicity compared to the posterior region, a finding that is supported by previous research (Y. Kobayashi et al., 2016; Lee et al., 2012).

Chapter 3 study's primary objective was to explore the complex relationship between fish size, age, and protein composition. This study's comprehensive approach, examining 24 collectively farmed Malabar red snapper (*Lutjanus malabaricus*) ranging from 212 to 413 mm, demonstrates the intricate nature of fish allergen profiles. By utilising a highly standardised

proteomics methodology, including quantitative immunoblotting and mass spectrometry, the research offers valuable insights into the dynamic world of fish allergenicity and the potential implications for individuals with fish allergies.

As fish grow and mature, their muscle protein composition undergoes substantial transformations, revealing an intricate interplay between size, age, and biochemical components (Jobling, 2002; Mommsen & Moon, 2001). Proteomic analysis revealed a fascinating size-dependent allergen profile: smaller fish demonstrated a higher abundance of the major fish allergen parvalbumin, while larger fish showed increased levels of seven other known fish allergens including enolase and aldolase. These findings have significant implications for fish allergy management and food safety. The research suggests that the severity of allergic reactions may be influenced by the size of the fish consumed. Smaller fish, with their higher parvalbumin levels, potentially pose a greater risk of severe allergic reactions. Conversely, larger fish, while containing lower amounts of the primary allergen, may still trigger intense allergic responses due to the increased presence of other allergens.

The study highlighted the complexity of fish allergenicity, emphasising the critical role of intraspecies variability. By demonstrating size-dependent variations in allergen profiles, this research contributes to a more nuanced understanding of fish allergies. The findings underscore the importance of comprehensive proteomic analyses in developing targeted approaches to allergy management and food safety.

Future fish directions

1. Allergenicity of fish in different sizes and muscle regions

An important limitation in Chapter 3 and current research is that the frequency of IgE binding to small and large fish has not been studied. This presents a compelling opportunity for future research to explore whether the observed size-related variations in parvalbumin content and other allergens, such as tropomyosin, collagen, creatine kinase, enolase, aldolase, pyruvate kinase, triosephosphate isomerase, glucose-6-phosphate isomerase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase, translate into differences in allergic reactions. The allergenicity of muscle regions (head, dorsal, tail and belly muscle tissues) has not fully investigated. Further investigation of this issue can provide researchers with invaluable insights into the potential allergenic risks associated with the consumption of fish of different sizes and help to develop more effective strategies for allergen management and consumer safety.

2. Allergen distribution of fish from different origins

It is well-established that the allergenicity of fish can vary depending on several factors, including the type of muscle tissue (white versus red), the specific muscle region, and the fish species (Y. Kobayashi et al., 2016; Lee et al., 2012; Lim et al., 2008; Ruethers et al., 2018; Van Do et al., 2005). Much of the existing research has primarily focused on the major fish allergen, parvalbumin. Yet, fish allergens abundant in fish species from diverse geographical locations (the origin of fish was caught or farmed), as well as aquaculture practices (e.g., fish feed and water conditions), have not been fully investigated. There are prior studies investigating the allergenicity of various fish species, muscle tissues and body regions. High allergenicity of white muscle tissues compared to dark muscle tissues (A. Kobayashi et al., 2006; Lee et al., 2012). Head (anterior) regions of the fish contained high parvalbumin levels and lesser in the tail (posterior) region (Y. Kobayashi et al., 2016; Lee et al., 2012). Large migratory fish such as swordfish and tuna are less allergenic due to the low parvalbumin level due to decreasing muscle contraction-relaxation activity which is demonstrated in the swimming muscles of rainbow trout (Coughlin et al., 2007) and striped bass (Rodnick & Sidell, 1995).

Furthermore, most research studies focus on the muscle protein composition of wild and farmed fish species to compare their nutritional factor, and muscle and flesh quality traits. A research study (Addis. *et al,* 2010) investigated the proteomic changes of gilthead sea bream from different farmed locations and wild stock to enable the production of higher-quality fish (Addis et al., 2010). There were notable differences between farmed and wild sea bass in terms of proximate composition (protein, lipid and moisture content), colour and texture, fatty acid profiles, and free amino acid profiles (Fuentes et al., 2010).

The risk of fish allergies extends worldwide, with the potential for severe reactions like anaphylaxis across both wild-caught and aquaculture fish. There are possibilities of variations in protein composition and allergen distribution between wild-caught and farmed fish are influenced by a multitude of factors, including genetic background, environmental conditions, farming practices, and geographical origins. Aquaculture practices may introduce variability that can substantially impact fish protein composition. The introduction of a variety of fish feeds is an effective method of managing fish allergy, as an EDTA-enriched diet has been shown to reduce IgE antibody reactivity (De Magalhães et al., 2020). The variety of farming infrastructures, including sea cage and land-based farming, results in different environmental conditions that have the potential to influence protein expression and allergen concentrations.

Farmed fish are subjected to controlled breeding and environmental conditions that can alternatively modify protein expressions. Wild-caught fish exhibit protein variations influenced by natural habitat diversity, migration patterns and ecological interactions. This research highlights the need for comprehensive proteomic mapping to understand how these different origins impact protein structure, allergens distribution and potential health risks.

Building a Foundation for Safer Seafood

The risk of severe fish allergies extends worldwide, with the potential for severe reactions like anaphylaxis across both wild-caught and aquaculture fish. However, the current state of research remains insufficient to comprehensively identify and understand the full spectrum of allergenic proteins in fish. This knowledge gap is particularly concerning regarding the variability of allergenic fish proteins across different geographical origins and production methods. As the global population becomes increasingly reliant on aquatic food sources, unravelling these protein composition variations is not just a scientific endeavour, but a critical public health imperative. This research provides a foundational framework for future investigations mapping fish protein allergens across diverse origins, including wild-caught and farmed fish.

A key finding of this study is that fish size significantly impacts allergen abundance and profiles which was not fully investigated from published journals. Smaller fish contain considerably higher levels of the major allergen parvalbumin compared to larger fish. However, larger fish exhibit elevated levels of other known fish allergens, including enolase, aldolase, pyruvate kinase, triosephosphate isomerase, glucose-6-phosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase. Furthermore, the distribution of these allergens varies across different muscle regions within the fish. Parvalbumin was most abundant in the belly region, followed by the head, dorsal, and tail muscle tissues. Aldolase exhibited higher expression in the dorsal muscle, while creatine kinase had elevated levels in both the dorsal and tail regions. Triosephosphate isomerase showed a specific increase in the tail muscle.

The goal of this research extends beyond scientific curiosity – it aims to ensure safer and more transparent seafood consumption. By unravelling the molecular complexities of fish protein composition, researchers can contribute to developing more sophisticated allergy screening methods and advances in food safety protocols. Comprehensive research examining the allergenicity of diverse fish species, as well as their geographical distribution and consumption patterns, is crucial for improving the accuracy and reliability of fish allergy diagnosis. By gaining a deeper understanding of the regional variations in fish allergy prevalence, healthcare professionals can better tailor their diagnostic approaches and provide more effective management strategies for individuals with fish allergies.

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