Study on Potential Allergenicity of *TERASI*, Indonesian Shrimp Seasoning, and its Reduction by Processing Technology

(インドネシア産エビ調味料テラシのアレルギー誘発性と加工技術による低減化に関する研究)

Division of Marine Life Science
Graduate School of Fisheries Sciences
Hokkaido University
北海道大学大学院水産科学院
海洋応用生命科学専攻

Ulfah Amalia
ウルファ アマリア

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主論文の要約

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学位論文題目

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The objective of this study is to investigate the potential allergenicity of *Terasi,* Indonesian fermented shrimp seasoning and discussing the processing technology for the reduction of the IgE-binding ability of *Terasi* final products. The PhD thesis consists of four chapters including one chapter overall explaining this study and three chapters presenting results and discussion of the research.

Chapter 1. (Introduction). *Terasi* is a popular seasoning of Indonesian traditional fermented shrimp paste with unique flavor and excellent nutritional value, which made by mixing dried small shrimp with salt (10–15% w/w). The mixture is grinded and spread out on the ground to dry with sunlight. The salted shrimp meat is solid and subjected to ferment in anaerobic condition at ambient temperature (25–28 ℃) for at least 3 days or longer until the unique aroma has fully established. It is reasonable to regard *Terasi* as a significant component of human diet and nutrition worldwide. However, shrimp, the raw material of *Terasi* contains allergenic protein, tropomyosin (TM), which is a common allergen of invertebrates, such as crab, squid, octopus, and shellfish. Several studies have been reported that proteolysis due to food fermentation reduced food allergenicity, but no investigation about the potential allergenicity of Terasi.

Chapter 2. The study evaluated the safety of *Terasi* (Indonesian fermented shrimp paste), the product characteristics and allergenicity of 20 types of Indonesian commercial Terasi (CT) that meet the Indonesian National Standard were evaluated with a focus on the major shrimp allergen TM. Marked protein hydrolysis of shrimp muscle occurred in all CT samples, and no protein fragments or specific reaction of anti-TM IgG were observed in SDS-PAGE and immunoblot assays. In a competitive enzyme-linked immunosorbent assay using shrimp allergenic patient sera, it was observed a markedly diminished specific IgE reaction of CT compared with that of shrimp muscle, whereas the IgE-binding ability remained in all CT samples. No clear correlation was found between the degree of protein hydrolysis and IgE reactivity. These results indicate that CT could be defined as a low–allergenic processed seafood but has the possibility to be a causative food for shrimp allergy. Direct immunological evaluation is required establishing the food safety of CT, because assessments of protein

profiles and hydrolysis are not useful for determining the safety of *Terasi*. Moreover, among CTs, raw materials and protocol used are less informed; therefore, the following chapters discuss their potential.

Chapter 3. This study examined the effect of the *Terasi* manufacturing process on the loss of the allergen TM and its IgG/IgE-binding ability. *Terasi* was produced from three shrimps, Akiami (*Acetes japonicus*), Okiami (*Euphausia pacifica*), and Isazaami (*Neomysis awatchensis*). Protein degradation and TM IgE-binding activity were examined by immunoblotting using anti-TM rabbit IgG and competitive enzyme-linked immunosorbent assays using shrimp-allergic patients' sera. TM in the materials was degraded during the manufacturing process, and the IgG-specific response in Akiami meat disappeared at the second fermentation step but remained in both Okiami and Isazaami *Terasi*. In contrast, TM IgE-binding ability of TM in all shrimp meats decreased gradually with the progress of the manufacturing process and nearly disappeared in Akiami *Terasi* (AT), indicating Terasi can be recognized as a low allergenic seafood. However, in order to ensure food safety of *Terasi*, an effective mean for decreasing IgE-binding ability of the final product should be introduced to the *Terasi* manufacture, because progress of the IgE-binding loss varied depending on raw materials.

Chapter 4. As countermeasures against the technical issues raised in Chapter 3, the backslopping method was applied to the *Terasi* manufacture, and its contribution to reducing the potential allergenicity of *Terasi* was examined. That is, three kinds of starters, the low allergenic commercial Terasi as CT, AT (produced in Chapter 3) and HAT (heat treated AT) were added to manufacturing process of the Isazaami *Terasi* which highly remained IgE-binding ability. This chapter demonstrated that backslopping method using *Terasi* products is an effective manner to produce low allergenic *Terasi* by inducing reduction of IgE-binding ability of TM. Addition of the starter accelerated the fermentation of the raw material, effectively promoting the degradation of the shrimp protein and the reduction of the IgE-binding ability of TM. However, the backslopping effect was dependent on the type of *Terasi* used as a starter, and the commercial *Terasi* used in this study did not contribute to the allergenicity reduction of the final product. Interestingly, *Terasi* added as a starter would have acted primarily as a nutrient source to promote microbial fermentation rather than as a source of fermenting microorganisms and endogenous proteases.

Conclusively, *Terasi* manufacture is an effective manufacturing process to reduce the IgE-binding ability of TM and to ensure the low allergenicity potential. *Terasi* can be recognized as a low allergenic seafood when produced under an appropriate manufacturing condition. It is probable that the measurement of the whole protein hydrolysis is effective for allergenicity evaluation of *Terasi* under certain conditions, and it could be used as a screening index. However, analysing IgE-binding activity of the final products is the most important manner to estimate *Terasi* as a low allergenic seafood. It should be noted that the backslopping method using *Terasi* final products could contribute to improving food safety of *Terasi* by reducing the IgE-binding ability of the final products. I believe that these research results will definitely help ensuring food safety of *Terasi*.

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The journey that one has to embark on, to attain a Doctoral degree, is an arduous one. The foremost challenge in this journey is that of the uncertainty of things that may occur in the foreseeable future and the risk that one is willing to take to overcome them. But then, ***innovation by its very nature involves risk***, and innovation is the very essence of the research leading to a higher degree. The success achieved at the end of this path is because of a collective effort and hard work of the many; and I take this opportunity to express my gratitude to everyone who supported me throughout my journey.

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LIST OF ABBREVIATION

AT = Akiami *Terasi*

C-ELISA = Competitive enzyme-linked immunosorbent assay

CT = Indonesian commercial *Terasi*

HAT = Heated-Akiami *Terasi*

IB = Immunoblotting

IgG = Immunoglobulin G

IgE = Immunoglobulin E

INS = Indonesian National Standardization

IT = Isazaami *Terasi*

ITAT = Isazaami *Terasi* added with AT as starter

ITCT = Isazaami *Terasi* added with CT as a starter

ITHAT = Isazaami *Terasi* added with HAT as a starter

ME = Mercaptoethanol

OT = Okiami *Terasi*

P1–P6 = Shrimp allergy patients' sera number 1–6

PPB =Potassium-phosphate buffer

TBS-T = Tris Buffered Saline-Tween

TM = Tropomyosin

SDS-PAGE = Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

CHAPTER I

INTRODUCTION

1.1 Significance of this study

The generalization of this study would make a significant contribution to the base of knowledge explored the traditionally Indonesian fermented shrimp paste, *Terasi*, from the perspective of food allergy. This study would provide the adequate information about the insight and allergology's overview of Indonesian commercial *Terasi*, most especially for the community of shrimp allergy sufferers that have no idea of its benefits and advantages. Since *Terasi* has a function as a food ingredient, the findings of this study can use of developing their food products, not only in the *Terasi* manufacture. Moreover, developing a new brand awareness will gratify to more demands and loyalty in the future. Future scholars working in the fields of food allergology and marine food science and technology will use this study as a reference when discussing food safety of *Terasi* and developing novel added values in it.

1.2 Aims

This study aimed to evaluate the allergenicity change of shrimp tropomyosin (TM) contained in *Terasi,* Indonesian fermented shrimp paste. I focused on three research subjects:

1. Evaluation of safety of 20 Indonesian commercial *Terasi* from the viewpoint of food allergy, mainly paying the attention to their protein profile and their IgE reactivity.
2. Investigation of specific IgE changes of TM in the manufacturing process of *Terasi* made from different kinds of shrimp.
3. Introducing *Backslopping* method using *Terasi* products as a starter in order to attempt to reduce allergenicity of *Terasi* product.

1.3 Food allergy

1.3.1 Overview of pathogenic mechanism of food allergy

In the world today, food allergy is becoming a bigger health concern, and not just in Westernized countries, but also in developing countries (Loh and Tang, 2018). Food allergy is described as an immunological hypersensitivity (allergy symptom) brought on by ingesting a specific food. It is also known as" an occurrence in which symptoms that are harmful to the living body are induced by the antigen-specific immunological mechanism after oral route, dermal exposure or acute exposure of the causative food" (Japanese Society of Pediatric Allergy and Clinical Immunology, 2020) and also defined as "an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" (Boyce *et al.*, 2010).

Across every country, food allergy is mostly triggered by milk, egg, and wheat. Moreover, the "Big 8"–a term used to describe the top eight food allergens–include five kinds of foods (fish, crustacean, shellfish, peanuts, and soybeans). Fish, crustacean, and shellfish are classified as causative foods in most all countries (Saeki, 2018). Shrimp, cow's milk, fish, eggs, and ant eggs are the top five foods in Northern Thailand that induce food allergy in children, with shrimp being the most frequent dietary trigger (Lao-araya and Trakultivakorn, 2012). In Hong Kong, Taiwan, Singapore, and Thailand, shellfish is the major source of anaphylaxis in adults and older children (Smit *et al.*, 2005; Thong *et al.*, 2005; Techapornroong *et al.*, 2010; Hsin *et al.*, 2011).

Normally, food components are not recognized as foreign materials when consumed and digested as nutrients in the intestinal tract. On the other hand, food allergy arises when our living body incorrectly perceives food components as foreign materials due to a biological dysregulation system (oral immune tolerance). Food allergy is classified into immunoglobulin E (IgE) mediated and cell-mediated hypersensitivities, and almost all food allergic symptoms

are accompanied by IgE involvement (Kumar *et al.*, 2012). Specific antibodies (IgE) can interact at specific locations on allergens (allergenic proteins) called epitopes to cause an immune system reaction. Hypersensitivity begins as a result of the interaction between allergens and IgEs via epitopes.

The mechanism of IgE-mediated food causing allergic reaction is as follows (Bischoff, 2007; Singh and Balla, 2008; Kumar *et al.*, 2012; Saeki, 2018):

(1) When digested foods containing allergen are perceived as harmful substance, specific antibodies (IgE) to food allergen are produced in our body. (2) A food allergen's specific IgE binds to the surface of mast cells (establishment of sensitization). (3) When the sensitized allergen in food is ingested again, it is bound to specific IgE at the surface of the mast cells. (4) The allergen-IgE reaction triggers release of chemical substances, such as histamine and leukotriene from the mast cells. (5) As shortly as the irritating substances come into contact with different regions of the body, they begin to cause vasodilation, increased vascular permeability, smooth muscle contraction of the bronchi, and peripheral vascular irritation. Adverse symptoms subsequently emerged as a result (urticaria, erythema, itching, conjunctivitis, vomiting, and diarrhea). Food allergy systemic symptoms manifest quickly, and severe shock symptoms (anaphylaxis), such as a rapid decrease in blood pressure and loss of consciousness coupled with organ dysfunction, may result in fatalities.

1.3.2 Seafood Allergy

The feature of seafood allergy is existence of IgE cross-reactivity among allergens in various kinds of seafood, reflecting marine bioresources, which include allergen with high structural similarity. There are distinctive allergens in different types of seafood, including fish, crustaceans, shellfish, and cephalopods. Understanding of the IgE-binding epitopes in each allergen is helpful to comprehend the overview of IgE cross-reactivity among seafood (Saeki,

2018). TM displays IgE cross-reactivity in marine invertebrates, such shrimp, crab, squid, octopus, and shellfish (Elsayed and Bennich, 1975; Reese *et al.*, 1999). IgE cross-reactivity makes dealing with seafood allergies more challenging in countries where a steady food distribution network makes a variety of seafood widely available. Therefore, ensuring the food safety of marine bioresources requires awareness of IgE cross-reactivity among seafood (Saeki, 2018).

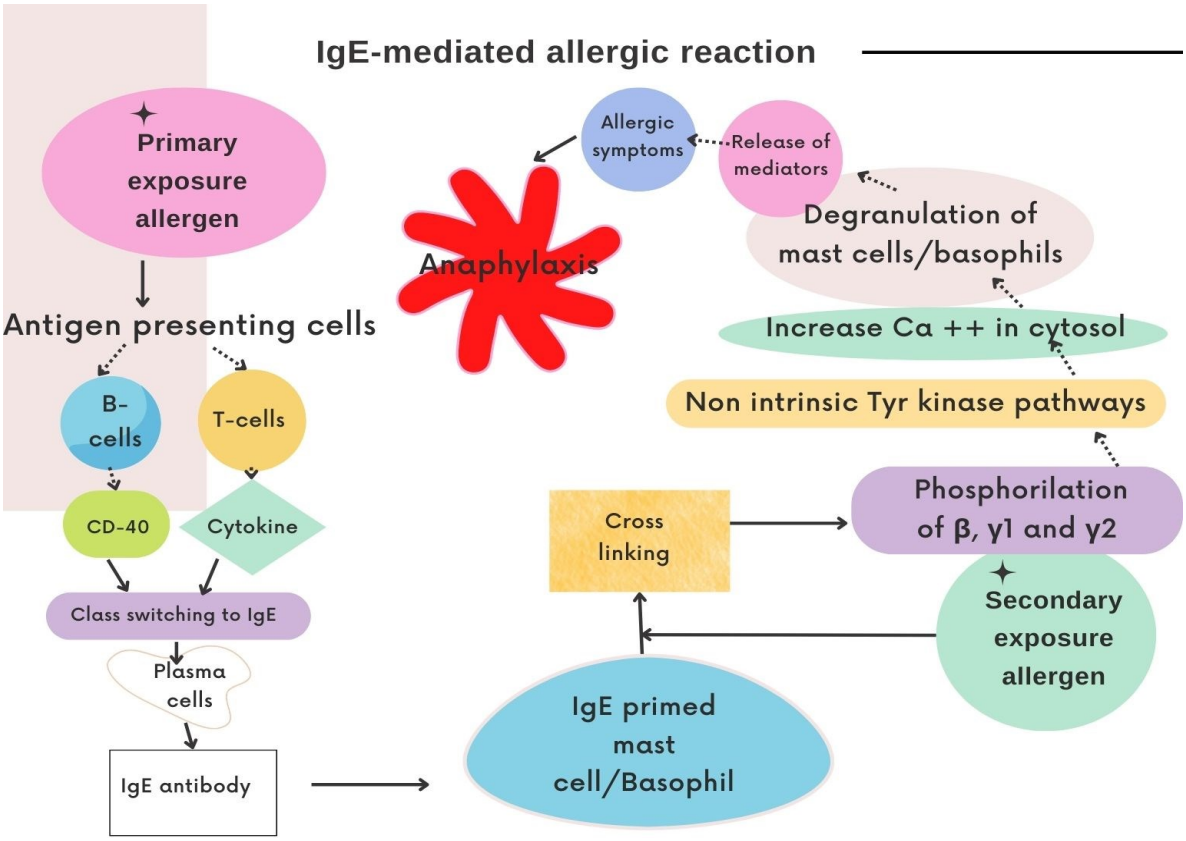

Figure 1.1. IgE-mediated allergic reaction mechanism (Source: Kumar *et al.*, 2012). (This figure was received the license from the publisher to reuse and redrawn by the author without changing the meaning of the original pictures).

1.3.3 Tropomyosin as a major allergen in shrimp

Shrimp is the most common of food that causes allergic responses in people, ranging in severity from moderate to life-threatening; and it has emerged as a global problem for people's health (Dong and Raghavan, 2022). Shrimp allergy typically results in symptoms including widespread pruritus, urticarial itching, angioedema, gastrointestinal, pulmonary, and systemic anaphylaxis (Sicherer *et al.*, 2004). It is triggered on by allergens found in different varieties of shrimp include North Sea shrimp (*Crangon crangon*), Brown shrimp (*Penaeus aztecus*), Greasy back shrimp (*Metapenaeus ensis*), Sakura shrimp (*Sergia lucens*), Kuruma shrimp (*Marsupenaeus japonica*), Juvenile white shrimp (*Litopenaeus vannamei*), Pacific white shrimp (*Litopenaeus vannamei*), Indian prawn shrimp (*Fenneropenaeus indicus*), Speckled shrimp (*Metapenaeus monoceros*), Tiger shrimp (*Penaeus monodon*), etc (**Table 1.1**) (Faisal *et al.*, 2019; Dong and Raghavan, 2022).

The major allergen in shrimp has been confirmed as the muscle protein TM. This substance is belonging to a family of highly conserved proteins, that mostly found in the muscle of all species of vertebrates and invertebrates (Reese *et al.*, 1999; Khanaruksombat *et al.*, 2014). However, there has been no report for other minor allergens until now (Dong and Raghavan, 2022). TM only acts as a food allergen in invertebrates, such as shrimp, crab, squid, octopus, and shellfish (Reese *et al.*, 1999). On the other hand, vertebrate TM do not act as a food allergen; only tilapia TM has been reported as an allergen (Liu *et al.*, 2013).

TM was initially identified as a squid allergen (Miyazawa *et al.*, 1996), and subsequently recognized as a common allergen of marine invertebrates (between crustacea and mollusca) (Leung *et al.*, 1996). TM is a constituent protein of muscle fiber binding to actin filament in muscle (Wai *et al.*, 2017), which is composed of two α-helical chains (each molecular mass is 34–38 kDa) taking a coiled structure (Khora, 2016; Saeki, 2018). Other study reported TM has a secondary structure of coils that is 36 kDa (Leung *et al.*, 2014). Shanti *et al.* (1993) observed

that the heat-stable fraction, which dominated the allergenicity of the extracts, contained TM as a soluble allergen. TM makes up 6% of all shrimp proteins and contains epitopes, which are distinct allergenic areas that interact with Immunoglobin E (IgE) antibodies to produce allergic reactions (Silke, 2017; Ahmed *et al.*, 2018). There are up to eight IgE-binding sites in shrimp muscle (Pen a 1) and non-muscle cells; and allergic invertebrate TM have the highly conserved of the amino acid sequence homology (89.1–98.6% in shrimps and crabs (10 species of crustacean); and 91.2–99.6% in squid and octopus (four species)). The amino acid sequence homology between crustaceans and mollusks is not particularly high; it ranged from 61.3–64.4% for crustaceans (12 species) and cephalopods (4 species), and from 54.6–63.7% for crustaceans and shellfish (19 species) (Emoto *et al.*, 2009); consequently, IgE cross-reactivity has been observed among these aquatic bioresources (Saeki, 2018). With a slightly acidic isoelectric point and minimal glycan changes, TM is water-soluble (Lopata *et al.*, 2010).

Table 1.1. Main crustacean allergen

Biomedical name	Molecular mass (kDa)	Allergen	Main species
Tropomyosin	34–38	Pen i 1	Indian white shrimp
		Pen a 1	Northern brown shrimp
		Cra c 1	North Sea shrimp
		Cra g 1	Pacific oyster
		Lit v 1	White leg shrimp
		Exo m 1	White legged freshwater
		Mel l 1	Shrimp king prawn
		Met e 1	Greasy backed shrimp
		Scy p 1	Mud crab
		Pen m 1	Black tiger shrimp
Arginine kinase	40–45	Lit v 2	White leg shrimp
		Pen m 2	Black tiger shrimp
		Scy p 3	Mud crab
		Cra c 2	Nort Sea shrimp
Myosin light chain	17.5–23	Cra c 5	North Sea shrimp
		Hom a 3	American lobster
		Lit v 3	White shrimp
		Pen m 3	Black tiger shrimp
		Scy p 3	Mud crab
		Art fr 5	Brine shrimp
Sarcoplasmic calcium-binding protein	20–25	Cra c 4	North Sea shrimp
		Cra a 4	Pacific oyster
		Lit v 4	White shrimp
		Scy p 4	Mud crab
		Pen m 4	Black tiger shrimp

Source: Guo *et al.* (2021); Dong and Raghavan (2022).

1.3.4 Immunological method for evaluating processed foods

In food allergy research, it is essential to use IgE that reacts with major allergens. In this paper, ELISA and immunoblotting are use, that utilize the reactivity between allergens and specific IgE. Different individuals may exhibit different clinical shrimp allergy symptoms (Khora, 2020). As of now, avoiding the intake of suspected allergens for patients with shrimp allergy is the greatest method to prevent developing food allergy (Dong *et al.*, 2021). The divergent findings in shrimp allergenicity may affected by the degradation of protein and the diverse transformation routes (such as deamination, decarboxylation, reduction of disulfide bonds) of distinct shrimp matrices (Liu *et al.*, 2019). Similar study was also noted the shrimp allergenicity may produce varying results even the same allergen is detected due to variations in the shrimp matrix and detection techniques (Griesmeier *et al.*, 2010).

Most research on shrimp allergen detection techniques mentioned above performed protein-based immunological assays with variety of effectiveness. The most popular technique for measuring allergenic protein in food products is ELISA. It is based on the recognition of one or more allergens in food products by monoclonal or polyclonal antibodies, with a detection limit of 0.15 mg/kg and as parts per million (ppm) (Jeoung *et al.*, 1997; Monaci and Visconti, 2010). Direct, indirect, sandwich and competitive ELISA assays, as depicted in **Figure 1.2**, are the four types of ELISA assays typically employed for allergen identification (Mandal and Paul, 2022).

Following protein extraction, the experimental technique for ELISA includes a number of incubation and wash steps. In order to determine the sample concentration, a standard curved created by a standard allergenic protein that has been diluted is used (Karsonova *et al.*, 2020). Due to its maturity, simplicity, high sensitivity, and specificity, ELISA technique has been widely employed in the routine identification of food allergens (Xu *et al.*, 2022; Dong and Raghavan, 2022). The current research shows that immunoassays, such as Western blot and

indirect ELISA, are without a doubt the primary method for determining how processing affects shrimp allergen levels. The mechanism is made clearer by the fact that adhesion of antibodies to processed allergenic proteins results in altered ability to cause allergic reactions (Verhoeckx *et al.*, 2015). In addition, according to the studies, the protein-based ELISA exhibits a generally sufficient sensitivity in the simultaneous quantification of seafood protein.

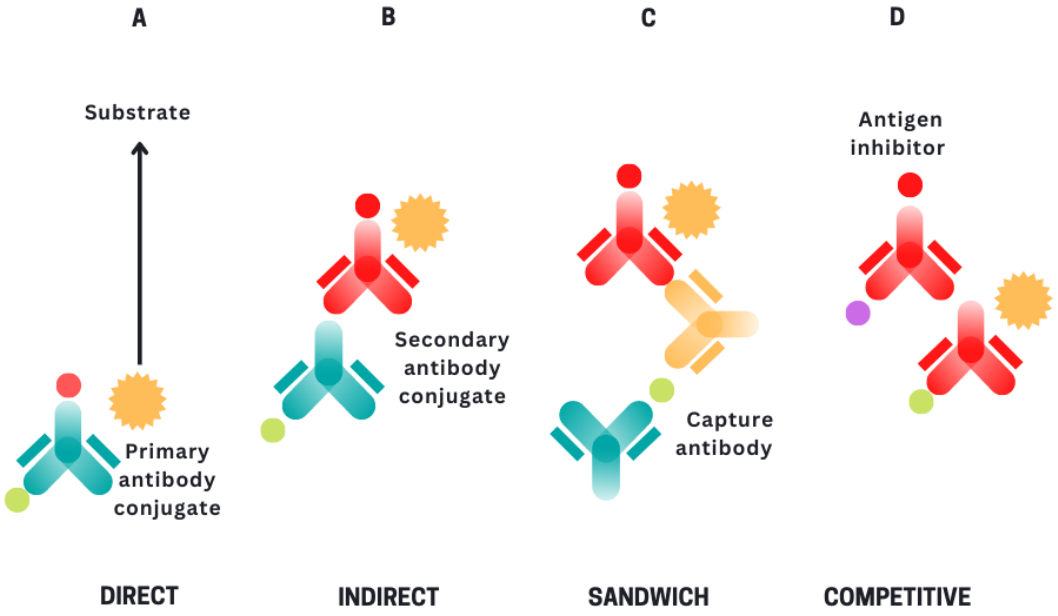

Figure 1.2. The types of ELISA assays (Source: Mandal and Paul, 2022). (This figure was received the license from the publisher to reuse and redrawn by the author without changing the meaning of the original pictures).

Large quantities discrepancies in allergenic proteins, as well as false positive or false negative results, may emerge from various processing technique, antibody composition, experimental procedures, reagents, and standard (Asensio *et al.*, 2008; Parker *et al.*, 2015). Another drawback of immunoassays is that, depending on the processing method used, protein solubility may change, which may have an impact on the following detection using protein-based methods (Mattison *et al.*, 2016). A recent sandwich ELISA approach based on poly- and monoclonal antibodies (PcAb and mAb) was created by Zhao *et al.* (2022) to identify shrimp tropomyosin and this innovative technique demonstrated greater detectability and tolerance to matrix in processed foods.

1.4 Factor affecting allergenicity of processed foods

1.4.1 Effect of various food-processing method on shrimp allergenicity

Daily food processing has the ability to change the allergenicity of foods by changing the physico-chemical characteristics of proteins (Rahaman *et al.*, 2016; Cabanillas and Novak, 2019). The extent of such adjustments varies depending on various elements, including the processing circumstances, the type of food being considered, the allergenic content, etc (Cabanillas and Novak, 2019).

An intensified band of purified tropomyosin (Pen j 1) from raw kuruma prawns (*Marsupenaeus japonicus*) after heat treatment (20–80 ℃) was observed by SDS-PAGE and Western blotting (Usui *et al.*, 2013). Similarly, by SDS-PAGE, no changes were identified in the allergenicity of tropomyosin from shrimp (*Penaeus monodon*) and crab (*Scylla paramamosain*) with several heat treatments (steaming, baking, frying, microwave roasting, grilling, and boiling) (Lasekan and Nayak, 2016; Liu *et al.*, 2018). On the other hand, a high-intensity microwave treatment (75–125 ℃ for 5–15 min) might dramatically lower the allergenicity of shrimp (*Litopenaeus vannamei*) by 75% measured by a sandwich ELISA (Dong

et al., 2021). This is due to the possibility that higher temperatures could lessen allergenicity as a result that extremely high thermal processing could lead in irreversible aggregation with covalent and hydrophobic connections (Arámburo-Galvez *et al.*, 2018; Peram *et al.*, 2013). Due to the breakdown of TM molecules under high-intensity ultrasound, Zhang *et al.* (2018) discovered that ultrasound (20 kHz, 100–800 W, 15 min) dramatically reduced the allergenicity of shrimp tropomyosin with the removal of immune response at 800 W as determined by immunoblotting and ELISA.

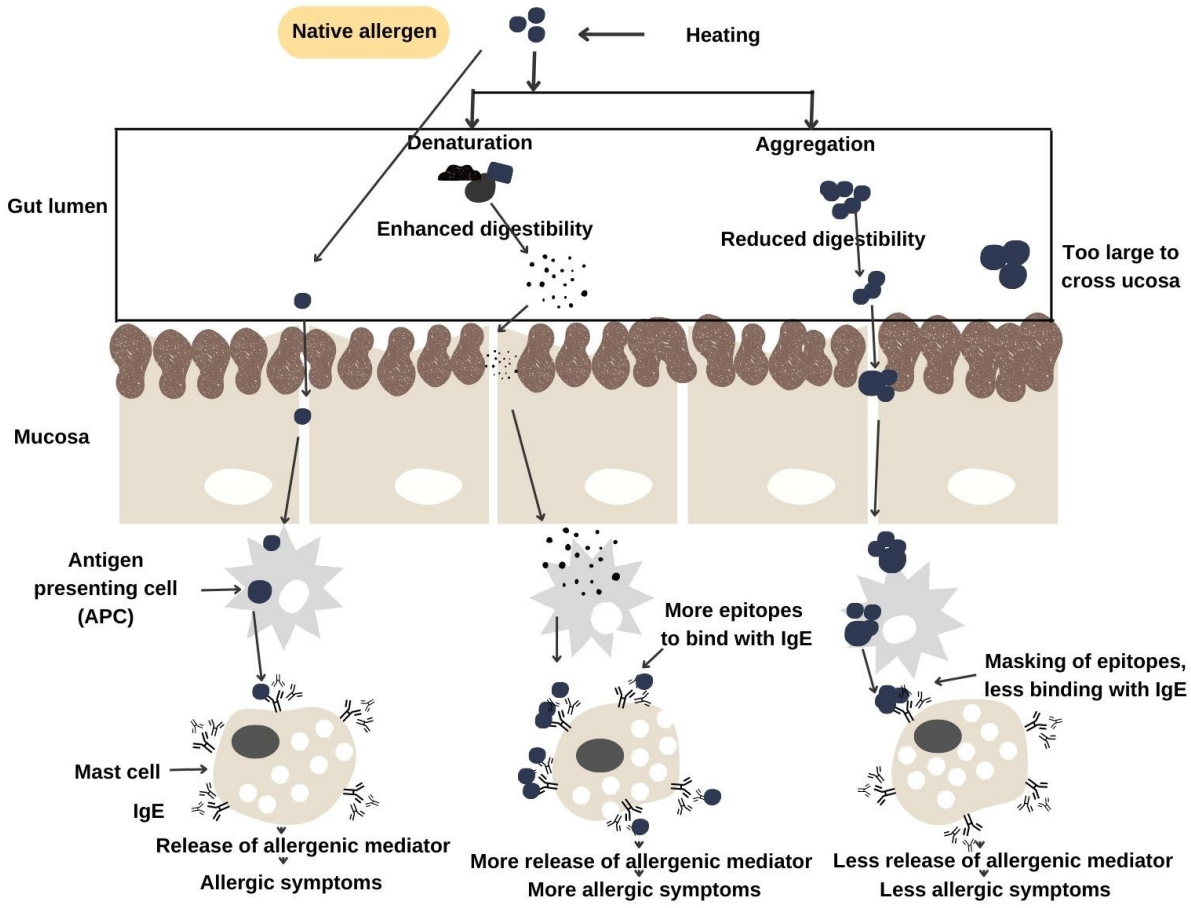

Figure 1.3. Heating effect on immunoreactivity of allergen (Source: Rahaman *et al*., 2016). (This figure was received the license from the publisher to reuse and redrawn by the author without changing the meaning of the original pictures).

1.4.2 Fermentation impact on food allergenic protein

Non-thermal processing has the advantage of occasionally making food more nutrient-dense than food processed by thermal processing (Rahaman *et al.*, 2016). Its impact on food allergenicity has been studied. Interestingly, fermentation is one of the most among non-thermal techniques (high pressure, ultrasound, gamma radiation, and enzymatic hydrolysis) that are being recommended to reduce the food allergenicity. Compared to other processing techniques, fermentation can significantly decrease food allergenicity without being limited by safety reason (e.g., irradiation) and high cost (e.g., high pressure) (Pi *et al.*, 2021).

Several studies reported the degradation of allergenicity of soybean major allergen in *Natto* (Japanese traditional fermented soybean) (Yamanishi *et al.*, 1995); fermented soybean, soymilk, and soy sauce (Seo and Cho, 2016; Biscola *et al.*, 2017; Yang *et al.*, 2018; Xia *et al.*, 2019; Cao *et al.*, 2019 and Pi *et al.*, 2021). Other studies revealed that fermentation was also reduces the beta lactoglobulin's allergenicity in cow's milk (Ehn *et al.*, 2005; Chen *et al.*, 2012; Bu *et al.*, 2013; Yao *et al.*, 2015) and the allergenicity of wheat allergen (El-Mecherfi *et al.*, 2022).

In the food industry, fermentation is essential for enhancing food quality and microbiological stability. When bacteria interact with food substrates like sugars, alcohol is produced, carbon dioxide is produced, and organic acids without oxygen, this occurs. Temperature, pH, and substrate concentration are only a few of the environmental factors that influence fermentation. Molds prevalent in soy-based products like shoyu, miso, and tempeh, as well as *Lactobacillus* (lactic acid bacteria) found in milk and wheat, are just a few examples of the microorganisms commonly employed to make fermented food (Pereira *et al.*, 2020).

In term of fermented fishery products, there were a limited report for allergenicity. As confirmed by Kim *et al.* (2008), there was changes in allergenicity of *Saeujeot* (salt-fermented shrimp) after treated by salt concentrations (10%–25%) and temperatures (5–25 ℃) for up to

one year. Moreover, shrimp TM's IgE-binding capacity reduced with longer fermentation times, lower salt concentrations (by 10%), and higher temperatures (by 25 ℃), as a result of protein hydrolysis, which decreased shrimp allergenicity (Zhou *et al.*, 2016). Sardina, common pandora, and shrimp, three species of fish that are frequently consumed in the Fez region of Morocco, were processed using canning, marinating, and fermenting techniques. ELISA results revealed that marinating and fermenting reduced the immunoreactivity of human IgE to fish species, at 69.2% and 64.5%, respectively, of fermented shrimp and sardine showed the greatest reduction in IgG binding (Mejrhit *et al.*, 2018). In fermentation, protein degradation is primarily responsible for the elimination of epitopes and the decrease in allergens. Additionally, metabolites produced during fermentation lessen the IgE sensitization to allergen (Pi *et al.,* 2021).

1.4.3 Backslopping method in fermented products

Fermentation is one of a well-established and the oldest technique for improves the taste, texture, and functional qualities of food by extending its shelf life (Caplice and Fitzgerald, 1999; Bourdichon *et al.*, 2012). Pérez-Díaz *et al.* (2017) reported the fermentation process involves the complex microbiota's metabolism, which includes native microorganism that are typically connected to the main raw materials and/or chosen microorganism introduced as starter cultures. Traditional fermented foods are those that are native to the area and are prepared by the locals using household methods including drying, smoking, germination, and backslopping or spontaneous fermentation, as well as their elders learned ethnic knowledge (Holzapfel, 2002; Akabanda *et al.*, 2014; Sharma and Yaiphathoi, 2020).

Backslopping method is a fermentation technique using a prior successful fermented batch (nature starter) as feedstock for subsequent fermentation steps (Harris, 1998; Leroy and De Vuyst, 2004; Shrivastava and Ananthanarayan, 2014), and no major technical obstacles

introducing to small scale factories. The repeatability and stability of the final products may be impacted by the fermentation's reliance on an undefined microbial population in active condition (Tamang, 2010; Merabti *et al.*, 2019; Albagli *et al.*, 2021). In addition, the study of backslopping application in sourdough fermentation revealed there is no direct connection between a regular sourdough and its associated microbiota (Vuyst *et al.*, 2014) and the presence of microbiota relies on specific condition and procedural points. Parente *et al.* (2016) reported the using of undefined starters portion in the production of traditional cheeses in Italy resulted cheese product with the dynamics of the cultures. Backslopping method, although which is not microbiologically characterized process, proceeds material fermentation due to high proteolytic activity of natural starters, and it is successfully utilized in various fermented foods, such as sourdough bread (Rizello *et al.*, 2016), various beverages (Roos and Vuyst, 2018), and Indonesian fermented milk (Wirawati *et al.*, 2019). Furthermore, the accelerated fermentation with protein digestion may result in the reduction of allergenicity of the final products.

In many developing countries, fermented foods are prized as important dietary components, largely due to their capacity to keep under ambient circumstances as well as for their safety and cultural acceptance (Holzapfel, 2002). As an example in Indonesia, there is an ingredient called *Terasi,* that has the potential to trigger shrimp allergy.

1.5 *Terasi* as an Indonesian seasoning ingredient

1.5.1 Overview

Many marine products are processed in Indonesia as traditional fermented seafood products, the most popular of which is *Terasi. Terasi* is a popular name of Indonesian traditional fermented shrimp paste with unique flavor and excellent nutritional value (Prihanto *et al.*, 2021a; Fan *et al.*, 2017; Faithong *et al.*, 2010). Fermented shrimp paste is thought that it was originated in continental Southeast Asia and known by several names in different regions of

Southeast Asia (Ruddle and Ishige, 2010; Hajeb and Jinap 2012). It is mentioned as *Belacan* (Malaysia), *Ka-pi* (Thailand and Cambodia), *Nga-pi* (Myanmar), *Bagoong-alamang*, *Burong, Dinailan* and *Lamayo* (Philippines), *Mam ruoc* (Vietnam), *Shiokara* (Japan) (Wittanalai *et al.*, 2011, Chaijan and Panpipat, 2012).

Generally, *Terasi* is manufactured by mixing dried small shrimp with salt (10–15% w/w). For *Terasi* manufacture, small shrimp *Acetes japonicus* and *Acetes sibogaesibogae* are typically used as the raw material of this product due to its good appetite (high in umami component), proteolysis, and economical aspects (Omori, 1975; Hajeb and Jinap, 2012). The mixture is grinded and spread out on the ground to dry with sunlight. The salted shrimp meat is solid and subjected to ferment in anaerobe condition at ambient temperature (25–28 ℃) for at least 3 days or longer until the unique aroma has fully established, which also called as natural or spontaneous fermentation (INS, 2016). The final products's appearance of *Terasi* varies from brownish red to dark brown color. *Terasi* is typically a solid paste, but as years progresses, *Terasi* begin to develop with a drier consistency and even in the shape of granules. *Terasi* has a shelf life ranging from a few months up to two years (Phitakpol, 1993; Hajeb and Jinap, 2012). Previous studies reported the different raw material with different endogenous proteases may be attributed the characteristics of *Terasi* (Helmi *et al.,* 2022).

1.5.2 Safety issue of *Terasi* consumption in Indonesia related to shrimp allergy

One aspect of food safety is the prevention of allergic responses to food. Similar towards how *Terasi* is consumed in Indonesia, it is necessary to explore from the viewpoint of food allergy. *Terasi*, which is shrimp-based fermented seafood, is the most popular ingredient in Indonesia (Amalia *et al.*, 2018). Indonesian dishes often include *Terasi* as a food additive in chili sauce and stir-fried vegetables. Although *Terasi* is typically only present in small amounts, its distinctive flavor and odor are highly valued in Indonesian cuisine (Sumardianto *et al.*, 2021).

In Indonesia, the annual production of *Terasi* is approximately 20,000 tons and the mean per capita *Terasi* consumption exceeds 3 kg/year (Statistics Indonesia, 2021). Of the raw materials used in *Terasi*, the potential allergenicity of shrimp TM may remain, but has not been verified at all. Even at extremely low quantities, the same allergenic protein can trigger a severe response in consumers with sensitive immune systems when present in processed foods. This possibility also extends to the foods that contain *Terasi*.

Thus far, there is no feasible technique to ameliorate or abolish the symptoms of shrimp allergy. The best treatment to avoid of shrimp allergy is to remove shrimp from the diet to prevent TM exposure. However, processed foods that use shrimp as a raw material may not provide sufficient information regarding its contents; thus, TM may be accidentally ingested by consumers.

Currently, there are not reports regarding shrimp allergy cases resulting from the consumption of dishes containing *Terasi*. Amalia *et al.* (2023) reported that the appropriate selection of raw material manufacturing conditions may diminish IgE reactivity of shrimp TM, which indicates the significance of *Terasi* manufacture from the viewpoint of food safety. However, the food safety evaluation for *Terasi* cannot using just statistical data, because food allergy cases are not systematically documented in Indonesia (Lee *et al.*, 2013; Prescott *et al.*, 2013) and no studies have been conducted on the distribution of TM in commercial *Terasi* products in Indonesia. With respect to the consumption of *Terasi*, its safety evaluation is a socially important proposition for Indonesia, which has a population of 260 million.

1.5.3 Potential of *Terasi* manufacture in reducing shrimp TM allergenicity

Fermentation process in *Terasi* has little effect on shrimp preservation, and the smaller peptides and amino acids produced can be used as nutrients by microbes. Thus, fermentation is combined with the salt addition or drying to minimize water activity and reduce the activity

of putrefying bacteria (Chaveesak *et al.*, 1993; Lertprakobkit, 2011). It has been demonstrated that *Terasi* flavour increases consumer pleasure and appetite (Ramaekers *et al.*, 2014). Umami flavor in *Terasi* caused by the highly presence of several amino acids, mainly glutamic and aspartic acid, that are known as precursors of umami taste (Lioe *et al.*, 2010). In addition, as reported that *Terasi* has a nutritive value, protein highly source (Sumardianto *et al.*, 2017; Ajifolokun *et al.*, 2019). Several studies revealed that *Terasi* as one of fermented fishery product that contained high GABA (γ-Aminobutyric acid), which has beneficial health as antioxidant, diuresis, tranquilizer, anti-hypertensive, epilepsy treatment and diabetic prevention (Ali *et al.*, 2009; Thwe *et al.*, 2011).

It is reasonable to regard *Terasi* as a significant component of human diet and nutrition worldwide. However, shrimp (*Terasi*'s raw material) is also ranks in the induction of acute (type I) food hypersensitivity, with TM as an allergen (Saeki, 2018).

To date, several studies reported that fermentation reduces food allergenicity, such as in processed milk (Ehn *et al.*, 2005), soybean meal (Yang *et al.*, 2018) and wheat bread (Angelis *et al.*, 2007). Over than the past 10 years, Park *et al.* (2007) and Kim *et al.* (2008) reported a loss of the IgE-binding ability of shrimp TM in *Saeujeuot* (a traditional Korean fermented shrimp). Studies on *Terasi* focused on the microbiology perspective, such as the microbial diversity of fermented shrimp paste (Chukeatirote *et al.*, 2015; Amalia *et al.*, 2018) and the microbial flora appear in a starter that attributing shrimp paste quality (Hua *et al.*, 2020).

It is important to note the starter addition in *Terasi* manufacture, since there were many variations of *Terasi* products that using different raw material and procedures. The employment of starter can promote the consistency of *Terasi* manufacturing as well as the quality of the product. In relation with allergenicity, it might enhance the degradation of protein, which consequently impact on allergenicity reduction. The findings on variation of shrimp TM allergenicity during *Terasi* manufacture can assist in better comprehending TM's behavior as

a major shrimp allergen. *Terasi* manufacturing utilizing an alternate raw material is feasible. It could be beneficial reference for future relevant studies and low allergenic shrimp products in the food industry.

Due to the lack of information related the effect of fermentation on shrimp allergenicity, this dissertation focused on using *Terasi* as a model to evaluate the allergenicity of shrimp TM. The first chapter addresses the background of the study and the current situation of shrimp TM allergenicity. Additionally, it briefly reviews current literature regarding the presence of shrimp TM in many food processing methods, the effect of fermentation on food allergenicity, and the application of backslopping method to improve the quality of fermented products. The second chapter describes the food safety of Indonesian commercial *Terasi* from the viewpoint food allergy, which the sample of *Terasi* was collected from Indonesia. The third chapter investigates the reduction of shrimp TM allergenicity during the manufacture of *Terasi* using three kinds of shrimp species. Meanwhile, the fourth chapter discusses the effect of starter addition into *Terasi* manufacture on the shrimp TM allergenicity. The fifth and sixth chapters provide general discussion and conclusions, respectively.

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CHAPTER II

FOOD SAFETY EVALUATION OF COMMERCIAL *TERASI*, INDONESIAN SHRIMP SEASONING, FROM THE VIEWPOINT OF FOOD ALLERGY

2.1 Introduction

One aspect of food safety is the prevention of allergic responses to food. Nowadays, consumers with food allergies, caregivers of persons with food allergies, food business operators, and responsible authorities remain concerned about food allergies to maintain food safety (FAO, 2020).

Marine products are an important food resource that can address the increase in the world population; however, various marine products, such as fish, shrimp, crab, and shellfish are recognized as food groups that cause allergies. Of these, shrimp, which is an abundant aquaculture product is an excellent source of protein; however, it is considered a major source of food allergy as it is one of the top eight of allergy-causing foods (Lopata *et al.*, 2016; Saeki, 2018). Therefore, shrimp allergy has become an important issue in terms of human health and food safety (Shek *et al.*, 2010; Hossny *et al.*, 2019) when consuming marine products.

TM, one of myofibrillar protein, presents as the core of actin filaments, and contains two identical subunits and a molecular mass of 35–38 kDa. It is well known that TM is a major allergen of aquatic invertebrates such as shrimp (Reese *et al.*, 1997; Rao *et al.*, 1998) and is responsible for 80% of the shrimp allergies (Pomes *et al.*, 2016). Furthermore, TM exhibits broad cross-reactivity as an invertebrate pan allergen (Reese *et al.*, 1999), which indicates that shrimp allergy may also induce other allergies, not only marine invertebrate allergies, but house dust allergies resulting from exposure to mites and cockroaches (Ayuso *et al.*, 2002).

Thus far, there is no feasible technique to ameliorate or abolish the symptoms of shrimp allergy. The best treatment to avoid of shrimp allergy is to remove shrimp from the diet to

prevent TM exposure. However, processed foods that use shrimp as a raw material may not provide sufficient information regarding its contents; and sometimes the use of shrimp is not apparent from the appearance; thus, TM may be accidentally ingested by consumers.

Terasi, which is shrimp-based fermented seafood, is the most popular ingredient in Indonesia. Indonesian dishes often include *Terasi* as a shrimp seasoning in chili sauce and stir-fried vegetables. Although *Terasi* is typically only present in small amounts, its distinctive flavor and odor are highly valued in Indonesian cuisine. The manufacture of *Terasi* consists of drying and grinding dried shrimp, adding water, and fermenting, a step that can last from two days to several weeks (Indonesian National Standards (INS), 2016), with no traces of shrimp appearing in the final product.

In Indonesia, the annual production of *Terasi* is approximately 20,000 tons and the mean per capita *Terasi* consumption exceeds 3 kg/year (Statistics Indonesia, 2022). Of the raw materials used in *Terasi*, the potential allergenicity of shrimp TM may remain, but has not been verified at all. Even at extremely low quantities, the same allergenic protein can trigger a severe response in consumers with sensitive immune systems when present in processed foods. This possibility also extends to the foods that contain *Terasi*. In Japan's allergy labeling system, ppm-level contamination of causative foods (containing more than 10 μg soluble allergen protein/g food) should be labeled in processed foods and shrimp is designated as one of the 28 causative foods (Akiyama and Adachi, 2021).

Amalia *et al.* (2023) reported that the appropriate selection of raw material manufacturing conditions may diminish IgE reactivity of shrimp TM, which indicates the significance of *Terasi* manufacture from the viewpoint of food safety. In addition, currently, there are no reports regarding food allergy cases resulting from the consumption of dishes containing *Terasi*. However, the evaluation of food safety for *Terasi* cannot use just statistical data, because food allergy cases are not systematically documented in Indonesia (Lee *et al.*, 2013; Prescott *et al.*,

2013) and no studies have been conducted on the distribution of TM in commercial *Terasi* products in Indonesia. With respect to the consumption of *Terasi*, its safety evaluation is a socially important proposition for Indonesia, which has a population of 260 million.

In view of these issues, the association between food safety and allergenicity was evaluated by examining the IgE reactivity of shrimp TM contained in *Terasi*. The properties's determination of 20 types of commercially available *Terasi* and the IgE reactivity of the inherent TM. This chapter also discuss the safety of commercially available *Terasi* from the viewpoint of food allergology. Moreover, the correlation between IgE reactivity and the degree of protein hydrolysis in *Terasi* was also analyzed.

2.2 Materials and methods

2.2.1 Materials

Indonesian commercial *Terasi* (CT) is available in 20 different brands and was purchased from local markets in the Central Java province of Indonesia. The CT used in this study were considered representative *Terasi* products that are distributed throughout Indonesia. *Terasi* produced by different manufacturers is available as solid paste, dry blocks, and granules, which conform to standardization (INS, 2016). Each CT was vacuum-packed and stored at 4°C until use.

2.2.2 Sera of shrimp-allergic patients

Sera from six subjects with shrimp allergy (P1–P6) with IgE antibodies specific for shrimp TM were used in this study (**Table 2.1**). Written informed consent was provided by the parents of these patients to their physicians. Patient sera was previously tested by indirect ELISA and exhibited high IgE specificity for the presence of shrimp TM. Negative control (C) serum from

a healthy person with no allergic symptoms was included, and the preliminary indirect ELISA confirmed there no specific IgE responses to TM in C. The ethical review board of the Japan Society of Nutrition and Food Science authorized the use of human sera for the study (No. 90 in 2020). Patient sera was stored frozen (−60°C) and thawed before combining with the same volume of phosphate buffered saline (PBS, pH 7.5) containing 0.02% NaN_3, and stored at 4°C until use.

2.2.3 Chemicals

All chemicals were purchased from Fujifilm Wako Pure Chemical Cooperation (Osaka, Japan) or Kanto Chemical Co., Inc (Tokyo, Japan) unless specified otherwise.

2.2.4 Preparation of TM and anti-TM IgG

2.2.4.1 Preparation of a snow shrimp acetone powder

Shrimp TM was prepared according to the method employed by Huang *et al.* (2010) with a slight modification. To begin with preparation of a snow shrimp acetone powder (**Figure 2.1**): briefly, minced shrimp muscle from white shrimp (*Litopenaeus vannamei*) was washed with a 5-fold weight equivalent of 1 mM sodium bicarbonate and the resulting muscle sample was collected by decantation. After repeating the washing step three times and rinsing samples in cold distilled water three times, the washed meat was then filtered through cotton gauze. Acetone was then added to the filtrate, which was then dried overnight at room temperature. A snow shrimp acetone powder was kept at –60 ℃ until use.

Table 2.1. List of patient sera used in this study

Serum	Sex	Age (year)	Total IgE (IU/mL)	Specific IgE (UA/mL, class)	Symptoms
P1	F	9	238	64.0 (5)	Ur
P2	M	12	558	12.4 (3)	BA, OAS
P3	F	11	1418	80.4 (5)	AD, BA
P4	M	17	172	18.7 (3)	AD
P5	M	4	2904	26.9 (4)	AD
P6	M	7	1640	26 (4)	AD, Ur
C	M	44	–	–	–

P1–P6: Patient's sera (have allergy to shrimp); C: Control serum from healthy individual (have no allergic with shrimp); M: Male; F: Female; IU: International unit; UA: Unit allergy. AD: Atopic dermatitis; BA: Bronchial asthma; OAS: Oral allergy syndrome; Ur: Urticaria; –: No data.

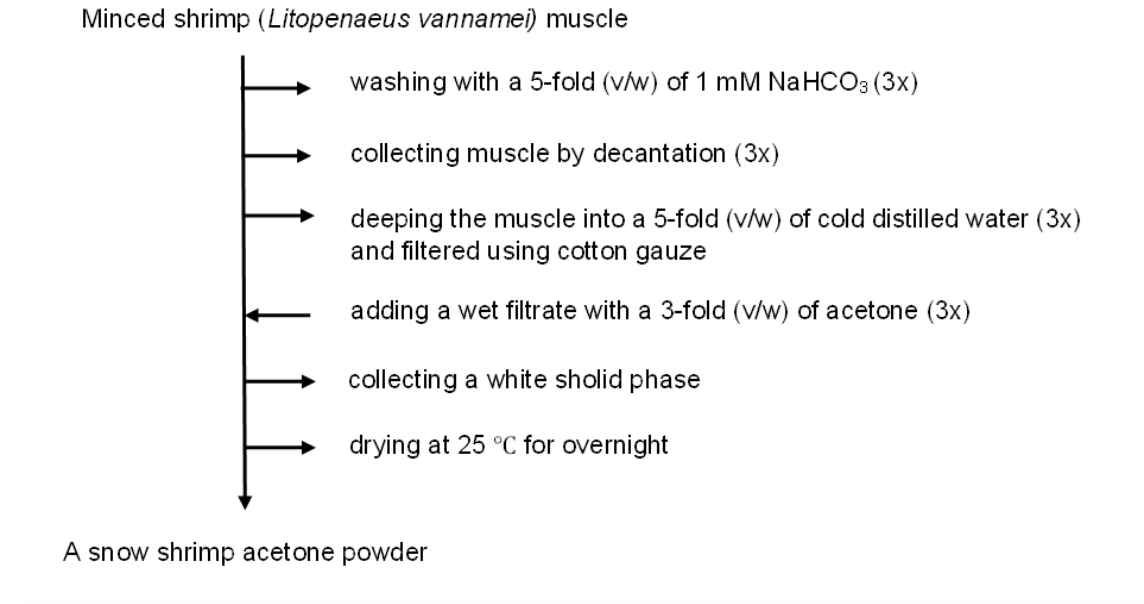

Figure 2.1. Preparation of a snow shrimp acetone powder.

2.2.4.2 Purification of shrimp TM

As shown in **Figure 2.2**, briefly the acetone-dried powder thus obtained was stirred overnight with a 15-fold weight equivalent of 1 M KCl, 10 mM β-mercaptoethanol (ME), and 20 mM Tris-HCl (pH 8.0). After centrifugation at 21,000 *g* for 30 min at 4 ℃, the supernatant was then boiled at 100 ℃ for 20 min and cooled to room temperature. The pH was then adjusted to 4.65 with 0.05 M HCl and the solution was kept at 4 ℃ for 30 min. The isoelectric precipitate thus obtained was collected by centrifugation at 21,000 *g* for 30 min at 4 ℃ and was then subjected to ammonium sulfate fractionation at 35–65% saturation. Changes in SDS-PAGE pattern during TM purification process and fractions collected after saturation process were shown in **Figures 2.3**.

Crude TM was further purified using hydroxyapatite column chromatography (Bio-Rad, Hercules, CA). The crude TM solution was dialyzed three times against 0.05 M potassium-phosphate buffer (pH 6.85, here in after referred to as PPB), and CHT ceramic HA (particle size 20 μm, BIO-RAD, column size : φ1.5 cm × 7 cm), and then the concentration of PPB was increased stepwise in the range of 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, and 1 M. TM was eluted on each step at a flow rate of 30 mL/h. The absorption at 228 and 280 nm of each fraction was measured, and the protein composition of each fraction was examined by SDS-PAGE. The elution pattern and the fractionated protein profile was shown in **Figures 2.4** and **2.5**, respectively. The protein concentration of purified TM (lane 6 in **Figure 2.6**) thus obtained was determined using a protein assay rapid kit (Wako Pure Chemical Industries, ltd.) using bovine serum albumin as a standard.

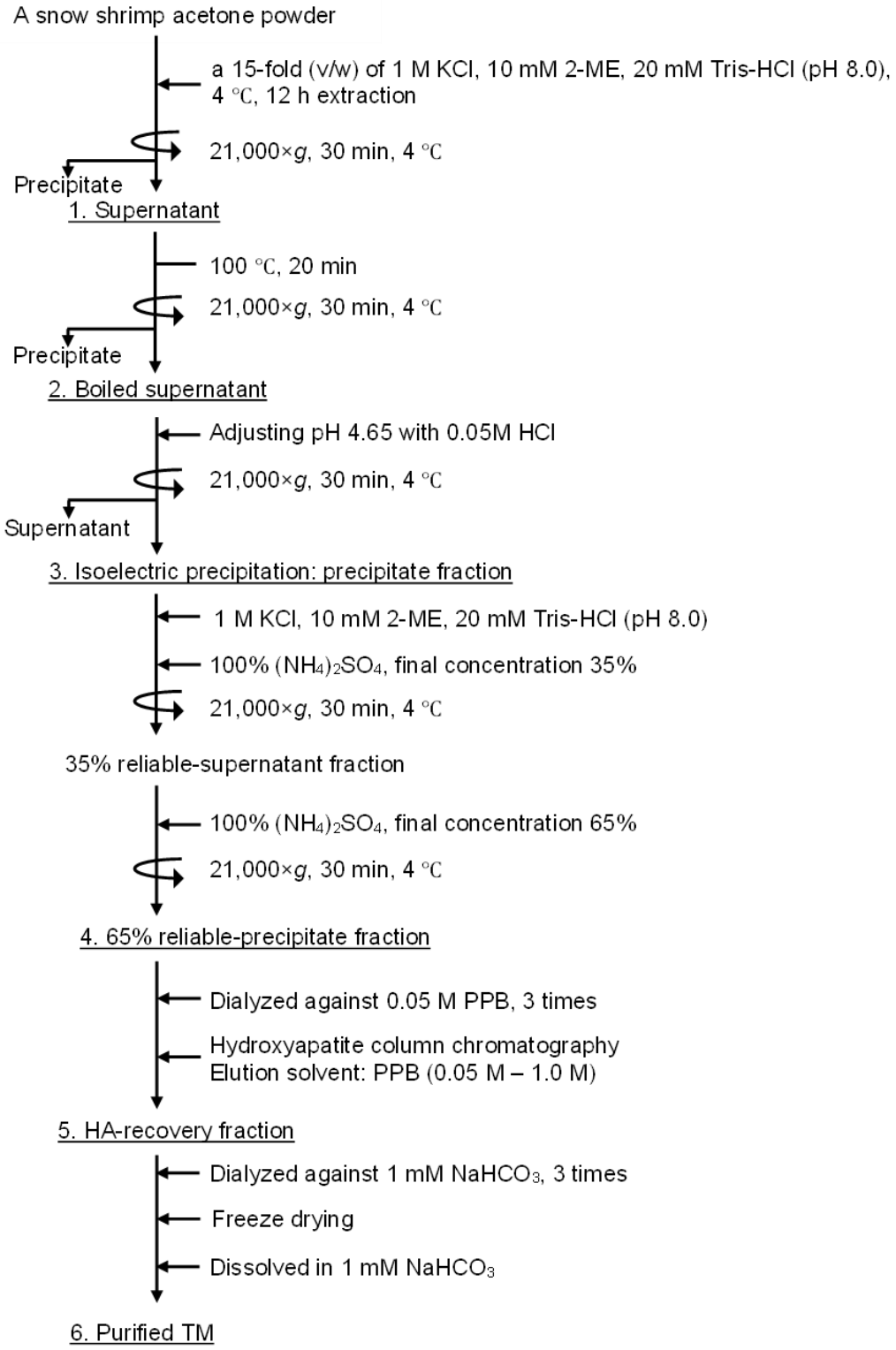

Figure 2.2. Protocol of TM purification using hydroxyapatite column chromatography.

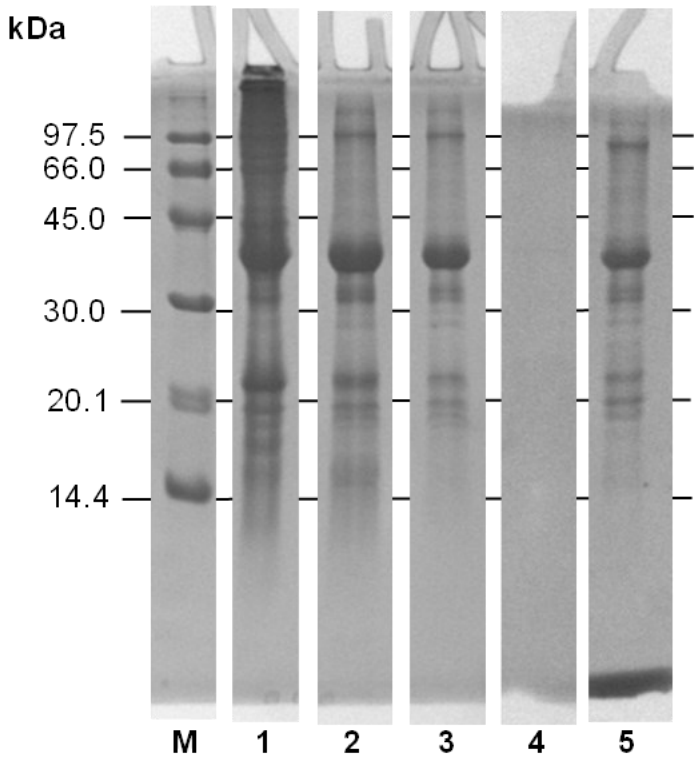

Figure 2.3. Changes in SDS-PAGE pattern during TM purification process. The number of each lane corresponds to Figure 2.2. M: molecular mass marker, TM: tropomyosin.

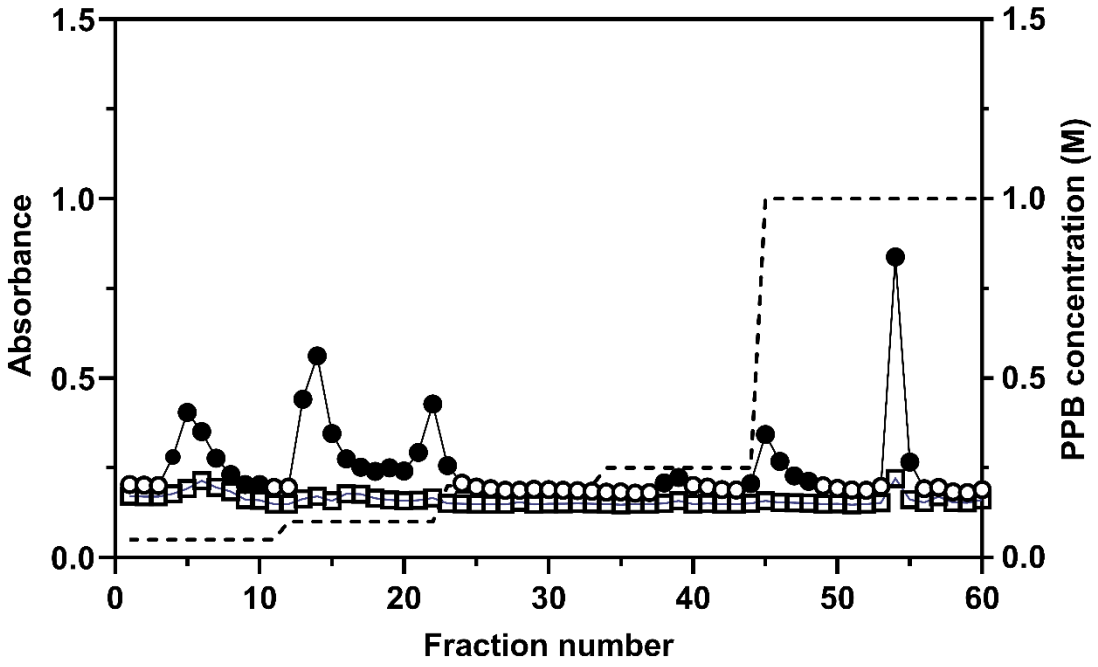

Figure 2.4. Hydroxyapatite column chromatography of ammonium sulfate fractionation (carrier: hydroxyapatite). □: absorbance at 228 nm, ○: absorbance at 280 nm, ●: fractions collected at Figure 2.5. Dotted line: potassium-phosphate buffer (PPB) concentration (0.05–1.0 M).

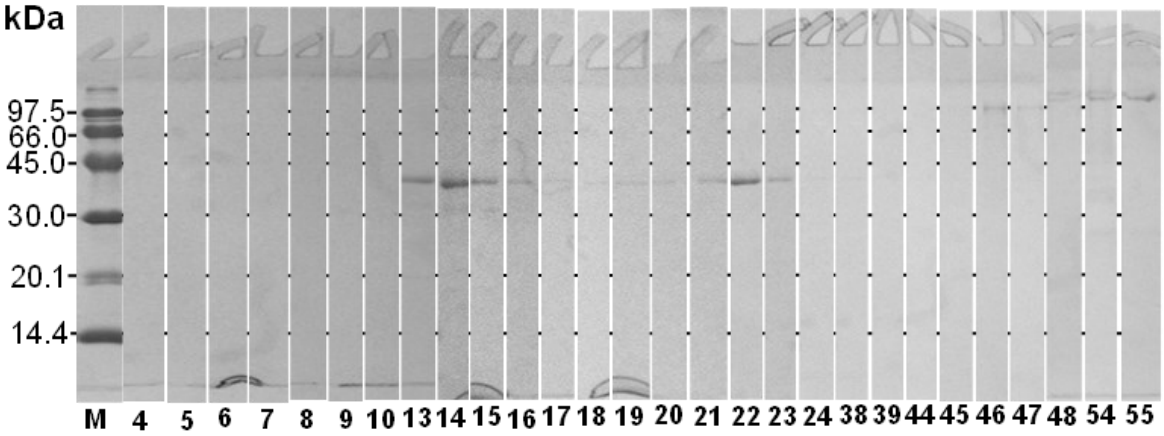

Figure 2.5. SDS-PAGE of protein recovered fractions on hydroxyapatite column chromatography. The lane number correspond to the recovered fractions number shown in Figure 2.4.

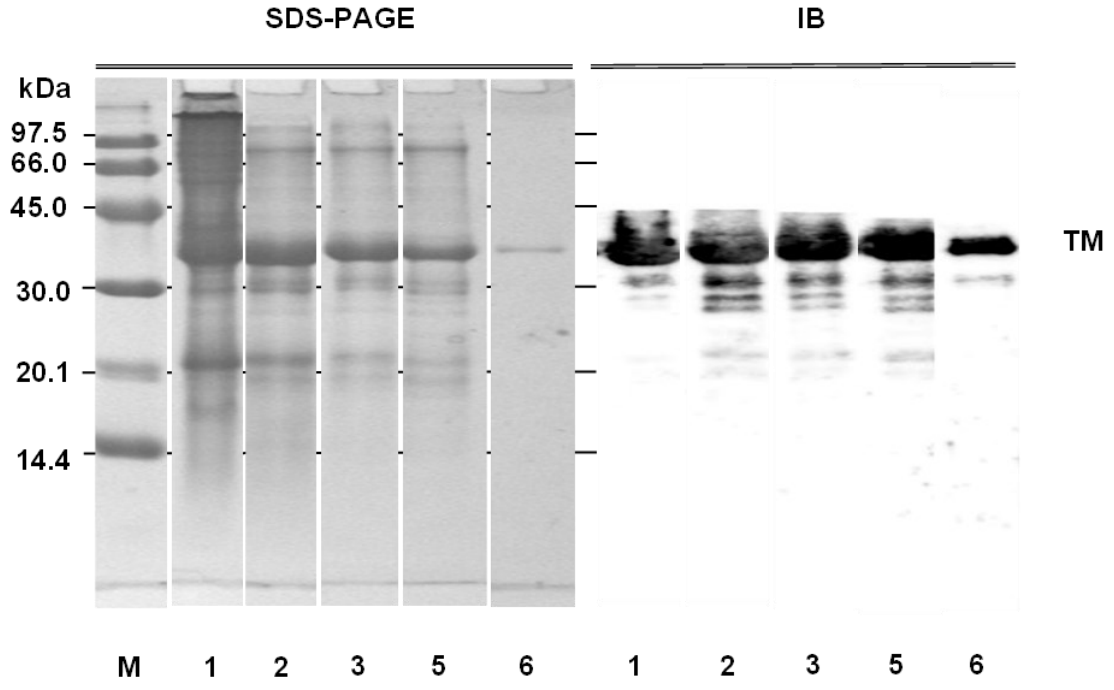

Figure 2.6. Changes in SDS-PAGE pattern and IB responses during TM purification. The number of each lane corresponds to Figure 2.2. M: molecular weight marker, TM: tropomyosin.

2.2.4.3 Preparation of anti-TM IgG

A rabbit (New Zealand White strains, male, 3 months old) was immunized with purified TM according to the method of Shimizu *et al.* (2009) in accordance with the Guidelines Concerning Animal Experiments at Hokkaido University (permission no. 10-0025). Anti-TM IgG antibody (anti-TM IgG) was purified from the immunized rabbit sera using a Protein A HP column (GE Healthcare, Piscataway, NJ, USA), dialyzed against 20 mM Tris-buffer saline (pH 7.5), mixed with 0.1% ProClin 300 (Sigma-Aldrich, St Louis, MO, USA), and stored at 5°C until use.

2.2.5 Physical properties of ICTs

Moisture content, protein content, and water activity (Aw) are used to evaluate the quality of commercial *Terasi* according to the INS (2016). The moisture content was measured using an infrared moisture meter (Model FD-310, Kett Electric Laboratory, Tokyo, Japan). Protein content was measured by the Kjeldahl method, which is based on the AOAC method (AOAC, 2005). Water activity was assayed using an Aw meter (Model 5803, Lufft, Fallbach, Germany) based on the manufacturer's instructions.

2.2.6 Protein profile analysis

2.2.6.1 Preparation of analytical samples

Shrimp muscle, TM, and each CT were placed into a plastic bag and boiled at 98°C for 20 min to inactivate digestive enzymes and 0.1 g of each sample was dissolved in 1.4 mL of 2% SDS solution containing 8 M urea, 2% 2-ME, and 20 mM Tris-HCl (pH 8.0) with a pellet pestle. The samples were heated at 98°C for 2 min and shaken vigorously overnight at room temperature using a shaker (NR-80, TITEC, Tokyo, Japan). After confirming that the sample

was mostly dissolved in the SDS solution, it was centrifugation at 21,000 x *g* for 30 min and stored at −60°C until use.

2.2.6.2 SDS-PAGE

The protein profile of each CT was examined by SDS-PAGE (Laemmli, 1970) using a Compact PAGE device (WSE-1010/25, Atto, Tokyo, Japan) with 4.5% stacking and 10.0% resolving polyacrylamide slab gels. Shrimp muscle and TM were used as controls. The same amount of SDS-treated samples (described in *Preparation of analytical samples*) were loaded into each lane of the gels and prestained molecular mass standards (14–100 kDa; GE Healthcare, Chicago, IL) were included as markers. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) at room temperature for 1 h, followed by destaining in a solution of 7.5% acetic acid and 30% methanol for 2–3 h.

2.2.6.3 Immunoblot analysis of TM

The electrophoresed proteins described in section of SDS-PAGE were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA) using a semidry blotting system (Model AE–8450, Atto, Tokyo, Japan) for 30 min at 68 mA based on the manufacturer's protocol. The membranes were soaked for 1 h at a room temperature in blocking buffer (3% casein dissolved in 150 mM NaCl and 20 mM Tris-HCl, pH 7.5, containing 0.05% Tween-20, referred to as TBS-T). The membrane was then treated with an anti-TM rabbit IgG (diluted 1:20,000 in blocking buffer) overnight at 4°C. After washing six times with TBS-T, TM-positive bands were detected with rabbit antibody using peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA) following incubation at 37°C for 3 h.

After six washes with TBS-T, a Cooled CCD Camera System (Model AE–6981, Atto, Tokyo, Japan) was used to visualize the specific response between the antigen and antibodies.

2.2.7 Determination of protein hydrolysis

Determining the degree of protein hydrolysis was done by the method of Kong *et al.* (2007) with slight modification. The degree of protein hydrolysis in CT is expressed as the percentage of trichloroacetic acid (TCA)-soluble nitrogen to total nitrogen. Four mL of distilled water and 5 mL of 15% (w/v) TCA were added to 1.0 g of each sample, homogenized using a disperser, and maintained at room temperature for 30 min. After centrifugation at 2,400 rpm for 30 min, the supernatant was collected, and the nitrogen content of the TCA soluble and total fractions were measured by the Kjeldahl method (AOAC, 2005) using the Buchi Digest and Distillation System (K–437 & B–324, Switzerland).

2.2.8 Assessing IgE reactivity of ICT

CT was subjected to competitive ELISA (C-ELISA) to examine the IgE reactivity of TM contained in the CT samples. Prior to analysis, CT was suspended in 20-fold (w/v) of 150 mM NaCl, pH 7.5, and immediately heated to 98°C for 2 min, and homogenized using a disperser (Ultra-Turrax T25, IKA, Staufen, Germany). After the addition of 0.05% Tween-20, the pH was adjusted to 7.5 by 2 M NaOH and stored at −20°C until use. For the assay, 10 µl of each CT suspension was diluted by 1,250-fold in 0.1% casein in TBS-T and subjected to C-ELISA based on the method of Amalia *et al.* (2023). Briefly, sera from shrimp-allergic patients or healthy individuals (Table 2) were diluted 50-fold in 0.1% casein in TBS-T and mixed with an equal volume of CT suspension at a final concentration of 100 µg/mL as an inhibitor. After incubation at 37°C for 2 h, each protein-serum mixture was placed into each well of a 96-well

ELISA plate (IWAKI, Tokyo, Japan) previously coated with TM, followed by measurement of the reaction between the specific IgE and TM using β-galactosidase-conjugated anti-human IgE antibody (Biosource International, Camarillo, CA) and 4-methyllumbelliferyl-β-D-galactosidase (Sigma-Aldrich). A reduction in specific IgE reactivity in the patient sera as a result of inhibitor treatment was determined by calculating an inhibition rate using the following formula: inhibition rate (%) = $[(X - Y)/ (X - Z)] \times 100$, where X represents the fluorescence intensity of the patient sera without inhibitor, and Y and Z are the fluorescence intensity of the patient and healthy individual sera with the inhibitors, respectively.

2.2.9 Statistical analysis

The data were analyzed using GraphPad Prism 9. 4. 0 (GraphPad Software Inc., San Diego, CA, USA) and presented as the mean ± standard deviation (SD) of three separate experiments. Statistical analysis of the data was performed using an analysis of variance and Tukey's Multiple Comparisons Test. *P* values <0.05 were considered statistically significant.

2.3 Results and discussion

2.3.1 Physicochemical characteristics of ICTs

Figure 2.7 shows the appearance of the CTs obtained from 20 different manufacturers in Central Java, Indonesia. Generally, *Terasi* manufacture involves repeated fermentation and drying steps, and the processing time requires 1–3 months. The appearance of most CTs was a typical dark brown color. On the other hand, No. 15, 16, and 17 were pale brown in appearance and dried compared with the other CTs, suggesting that they were produced through the typical rapid manufacturing method, which involves high-temperature final drying after repeated fermentation and drying for 2–3 weeks. No.15 exhibited a granulated instead of a pasty appearance, which is common to most CT samples from the dry-oven heating during the final drying step.

The moisture content, protein content, and water activity (Aw) of the CTs are shown in **Figure 2.8** with the INS as a reference. The INS's acceptable range (INS, 2016) is indicated by the dotted lines in the figures. As shown in **Figure 2.8a**, the moisture content of the CTs ranged from 27.4% (No. 14) to 38.6% (No.4), and all CTs were INS compliant.

Figure 2.7. Appearance of CT used in this study.

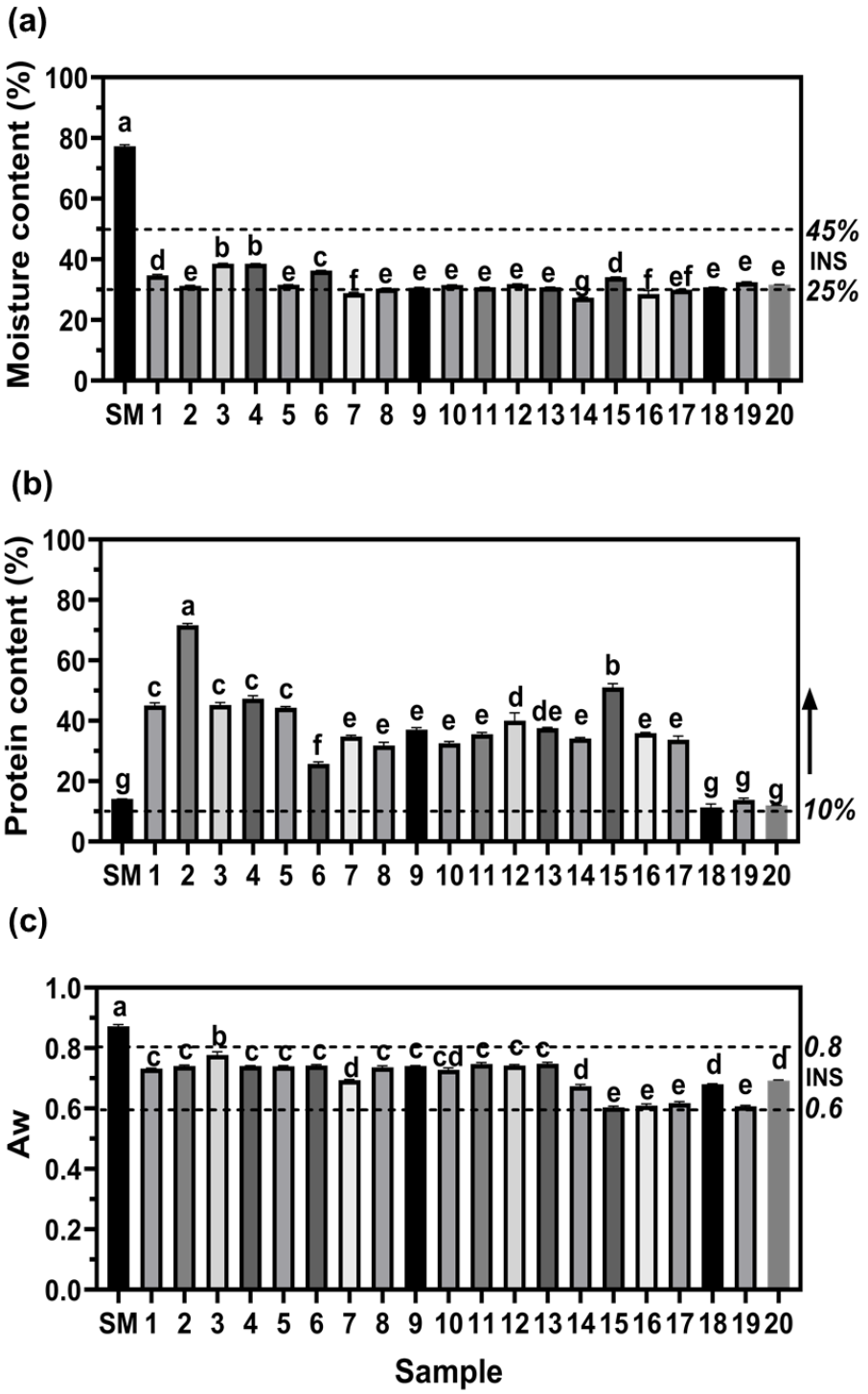

Figure 2.8. Proximate composition and water activity of CT(a) Moisture content, (b) Protein content, (c) Water activity. Values are the mean ± standard deviation (n = 3). The INS's acceptable range (INS, 2016) is indicated by the dotted lines in the figure. Different lowercase letters on the bar charts indicate significant differences ($p < 0.05$, Tukey's Multiple Comparison test).

As shown in **Figure 2.8b**, the protein content ranged from 11.3% (No. 19) to 71.7% (No. 2) for the 20 CTs. All samples met the protein content standard of the INS and were markedly higher compared with that of the INS, except for No. 18, 19, and 20. The low protein content of the three CTs may have resulted from the relative reduction in protein content caused by the addition of carbohydrates as indicated on the product packaging. In contrast, all other CTs exhibited a 3-fold increase in protein concentration compared with SM, indicating that the *Terasi* manufacturing processes effectively concentrate protein.

Figure 2.8c shows that the Aw of all CTs is compliant with the INS standard (2016). Van-Thuoc *et al.* (2021) noted that appropriately maintaining the Aw of *Terasi* can suppress the growth of pathogenic and spoilage microorganisms in the final product. This may be done with the addition of high salt concentration as reported by Cai *et al.* (2017). In addition, adjusting Aw to an intermediate level can limit the development of halophilic bacteria that are necessary for the manufacture of *Terasi* (Pongsetkul *et al.*, 2017). The results shown in Figure 2.8c confirmed the safety of the CT samples with respect to food preservation.

2.3.2 Protein profile

The degree of protein hydrolysis and the protein composition of CT were examined by measuring TCA-water soluble nitrogen, SDS-PAGE analysis, and immunoblotting with anti-TM IgG. Shrimp muscle proteins are digested by endogenous proteases and fermenting bacteria during *Terasi* production (Sun *et al.*, 2014; Kleekayai *et al.*, 2016; Helmi *et al.*, 2022a). Furthermore, it was found that protein degradation in *Terasi* manufacture results in a loss of TM, the major allergen in shrimp muscle. Indeed, as shown in **Figure 2.9a**, all CTs showed an increase in the degree of protein hydrolysis compared with shrimp muscle. However, the degree of protein hydrolysis in the CT samples was different from one another and ranged from 39.3%

(No. 15) to 80.2% (No. 5). This characteristic was unrelated to the protein content **(Figure 2.8b**).

Even sample No. 18, 19, and 20, which had low protein content because of the addition of carbohydrates, exhibited protein hydrolysis that was comparable to the other CTs. The results of SDS-PAGE (**Figure 2.9b**) indicated that shrimp myofibrillar proteins, such as myosin, actin, and TM, disappeared from all of the CTs. In addition, no TM signal was observed in immunoblotting assay using anti-TM IgG as shown in **Figure 2.9c**, which suggests that TM was hydrolyzed to small fragments of at least <14.4 kDa.

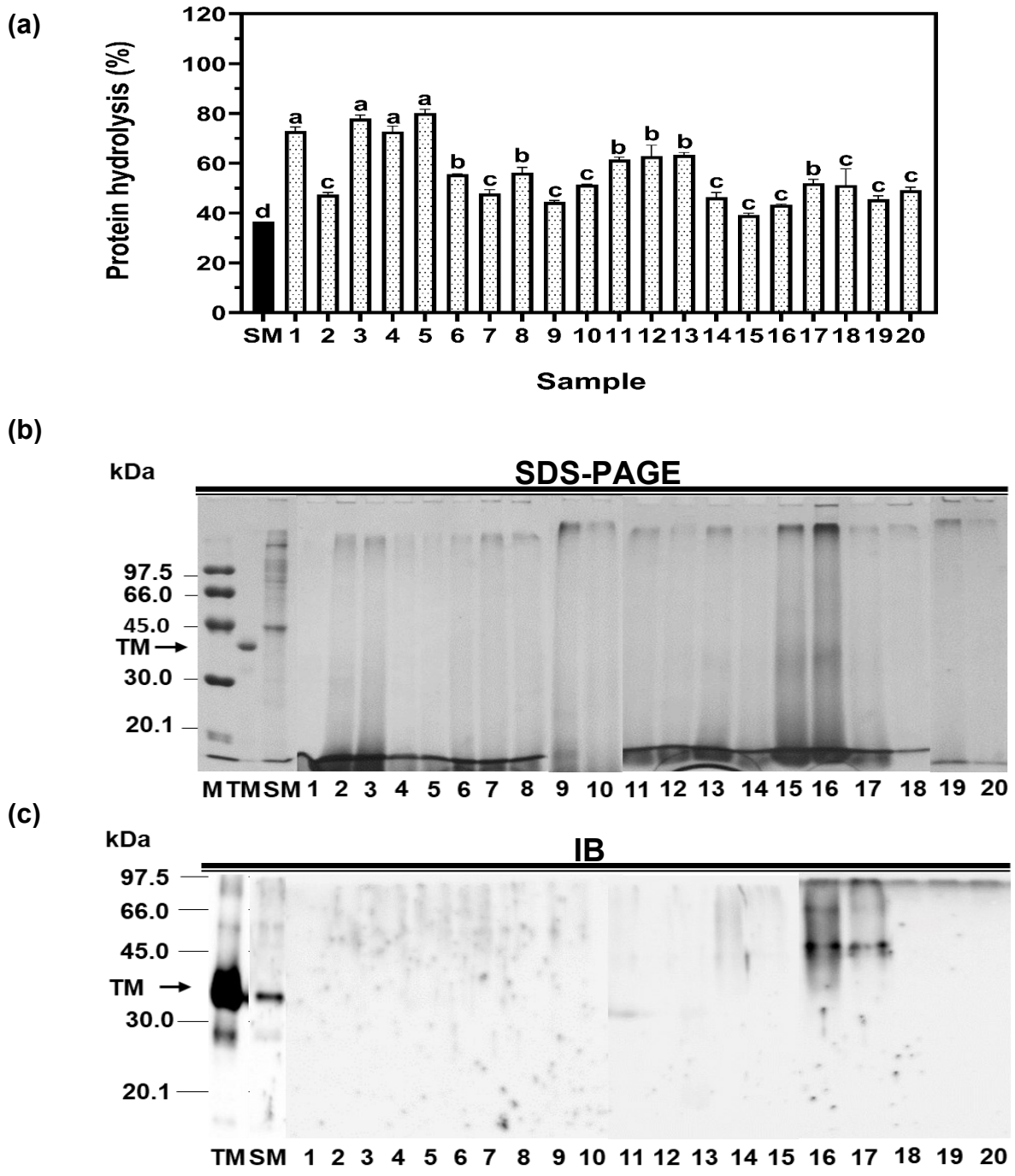

Figure 2.9. Protein profile and immunoblotting results. (a) The degree of protein hydrolysis, values represent the mean ± standard deviation (n = 3). Different lowercase letters on the bar charts indicate a significant difference ($p < 0.05$, Tukey's Multiple Comparison test). (b) Composition of the digested proteins by SDS-PAGE. (c) Immunoblot assays using anti-TM rabbit IgG. M: low molecular mass marker; TM: shrimp tropomyosin, SM: shrimp muscle, No. 1–20: CT samples.

2.3.3 IgE-binding ability of ICT to shrimp TM

To evaluate food safety of each CT from an allergy perspective, the CTs were subjected to a C-ELISA using sera from six patients with shrimp allergies. The IgE-binding capacity of each CT was examined by measuring the inhibitory effect of CT on the reaction between the specific IgE the patients and native TM (**Figure 2.10**). A decrease in the inhibition rate indicates a loss of IgE-binding ability of shrimp TM in the CTs.

As shown in **Figure 2.10**, shrimp muscle protein (referred as to SM in the figure) as a raw material had almost the same inhibition rate as that of TM in the patient. In contrast, the inhibition rate of all CTs was consistently lower compared with that of shrimp muscle protein and TM. That is, despite the patient sera showing different specific IgE levels as listed in **Table 2.1**, the decreased tendency of the inhibition rates for all CTs was similar using different sera. Considering that all CT manufacturing methods resulted in increased protein concentration as shown in **Figure 2.8b**, the result of **Figure 2.10** suggests that *Terasi* manufacturing contributes to improve safety of shrimp as an allergy-causing food source.

In some CTs, the inhibition rate varied depending on the patient serum used in the C-ELISA. In particular, the inhibition rate of No. 9 varied greatly from 22% in P2 to 71% in P6. This difference may result from patient-to-patient differences in the binding site of the IgE TM fragment contained in the blood. Thus, the difference in the inhibition rate of CTs among patient sera reflects the reactivity between the IgEs in the patient's blood and the residual IgE-binding cite in TM, which remained undegraded during the manufacture of *Terasi*. Therefore, I propose that a mixture of multiple patient sera in the C-ELISA should be used to assess the safety of CT based on IgE reactivity.

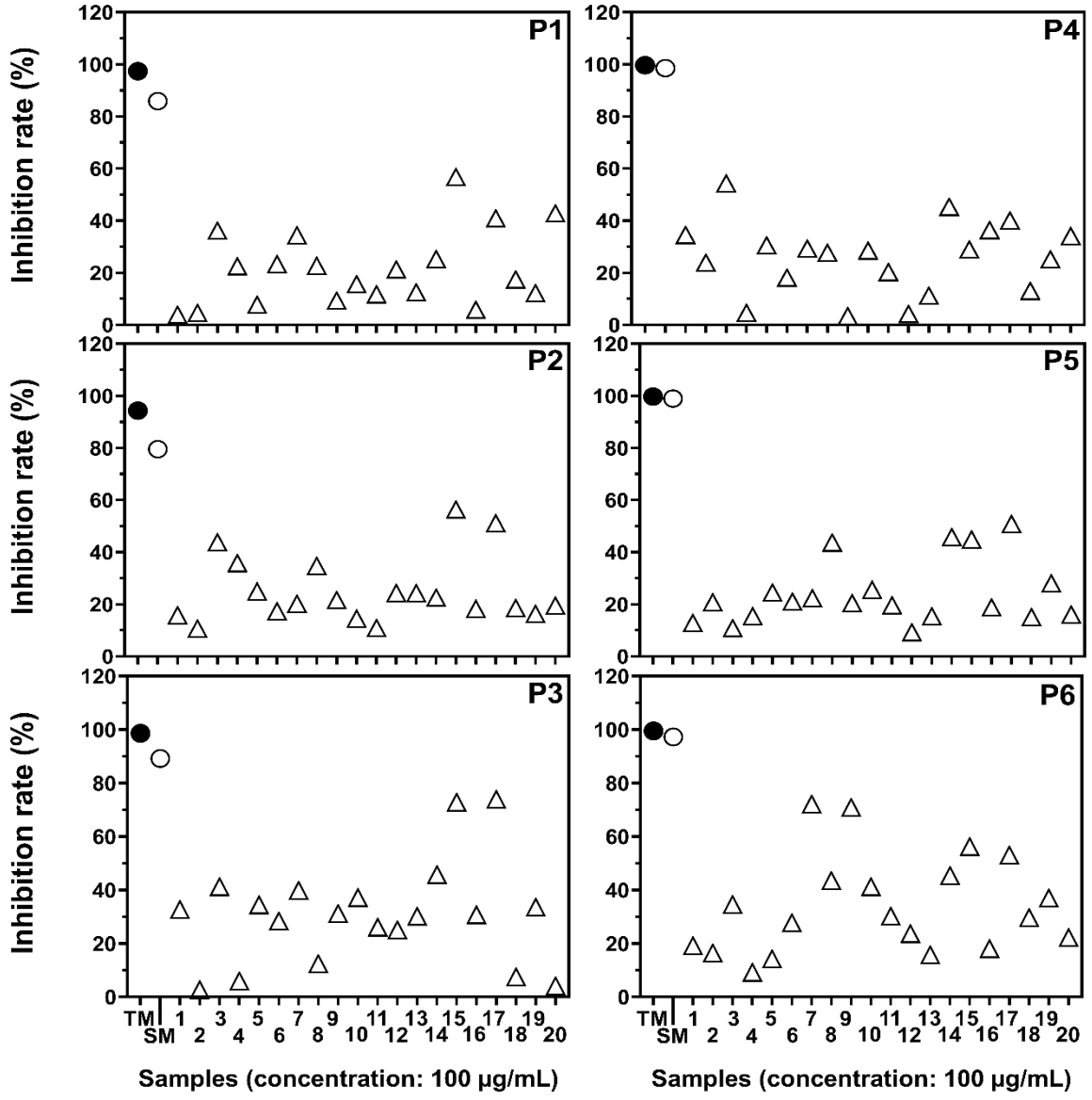

Figure 2.10. IgE reactivity of shrimp TM in a series of CT samples. TM (●: shrimp tropomyosin), SM (○: shrimp muscle), and CTs (Δ: No. 1–20) were mixed with sera from shrimp-allergic patients (P1–P6) and subjected to C-ELISA.

2.3.4 The correlation between the degree of protein hydrolysis and IgE reactivity in *Terasi*

Structural degradation of allergenic proteins is accompanied by the destruction of IgE-binding sites, so digested proteins are generally less allergenic compared with native proteins. The present study demonstrated that the degradation of shrimp TM during *Terasi* manufacture induced a decrease in IgE reactivity (Amalia *et al.*, 2023).

Based on the data in **Figure 2.9a** and **Figure 2.10**, we examined the relationship between protein hydrolysis and IgE reactivity in the CT samples. The results are shown in **Figure 2.11**. In all patient sera, no clear relationship was observed between protein hydrolysis and IgE reactivity in *Terasi*. Thus, it is clear that the degree of protein hydrolysis assessed by TCA-soluble nitrogen cannot be used for assessing the safety of *Terasi*.

As reported by Helmi *et al.* (2022b), the degree of protein decomposition in *Terasi* is closely associated with the salt content of the final product. Products with a high salt content tend to have a low degree of protein decomposition.

In fact, the 20 types of CT used in this experiment also showed a statistically negative correlation between the degree of proteolysis and salt concentration in the final product (**Figure 2.12**); however, considering the results of Figure 2.11, the salt concentration of the final product cannot be used as an index for assessing the allergic potential of *Terasi*.

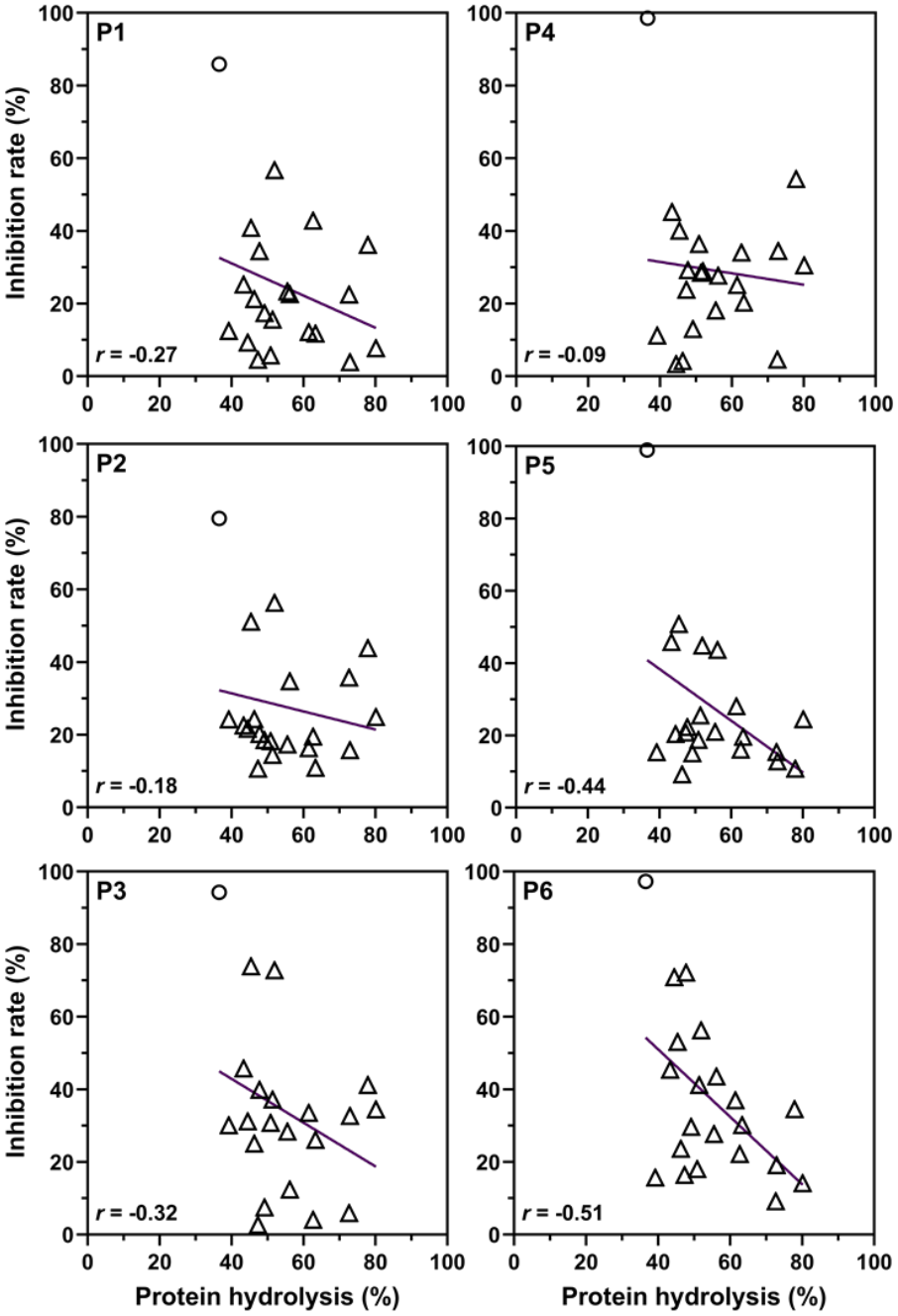

Figure 2.11. Relationship between the degree of protein hydrolysis with IgE reactivity of shrimp TM in CT.

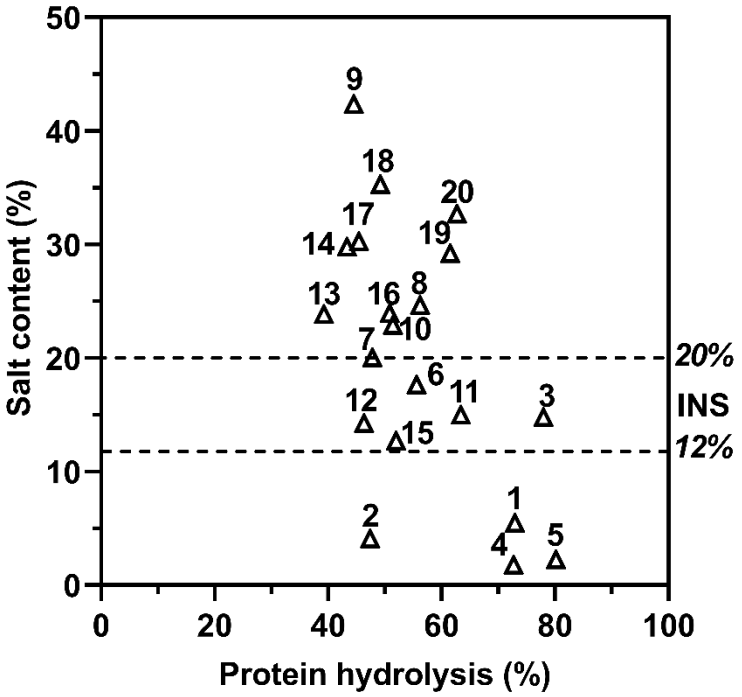

Figure 2.12. The correlation between salt content and protein hydrolysis in the CTs.

2.4 Conclusion

This study demonstrated that a decrease in IgE-binding ability, an index of allergenicity, occurred in 20 types of commercial *Terasi*, indicating the effectiveness of *Terasi* manufacturing at reducing the allergenicity of shrimp. All of the CT samples tested were less allergenic than shrimp muscle; however, the degree of decrease in IgE reactivity varied among the *Terasi* products and the potential hazard as an allergy causative food was not completely eliminated. C-ELISA using pools of sera from multiple shrimp-allergic patients should be effective for the safety evaluation of *Terasi*, because individual differences were observed in the TM binding capacity of patient serum IgE. Measurement of the degree of protein hydrolysis and salt concentration of the final products were not effective as judgment criteria.

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CHAPTER III

REDUCTION OF SHRIMP TROPOMYOSIN ALLERGENICITY DURING THE *TERASI* MANUFACTURING WITH APPRORIATE RAW MATERIAL

3.1 Introduction

Shrimps, including prawns, are one of the most important types of highly productive seafood. Together, they accounted for approximately 16 percent of the total value of internationally traded fish products in 2019 (FAO, 2021). However, marine crustaceans such as shrimp and crab often induce food allergies, and both shrimp and prawn have been designated as allergy-causing foods by the food labeling systems of many countries (Saeki, 2018). TM is a major allergen present in shrimp (Reese *et al.*, 1997; Rao *et al.*, 1998) and is classified as a myofibrillar protein composed of two identical subunits with a molecular mass of 35–38 kDa (Shanti *et al.*, 1993). TM has been called a "pan-allergen," since its IgE-cross reactivity is widely established among invertebrates such as crustaceans, shellfish, squid, and octopus (Reese *et al.*, 1999). Food processing can affect the allergenicity of processed foods by reducing the IgE-binding ability of allergenic proteins contained in raw food. For some food allergens, such as 2S albumin present in nuts (Breiteneder and Clare Mills, 2005), casein in cow milk (Bu *et al.*, 2013), and ovomucoid in white egg (Bloom *et al.*, 2015), thermal processing results in only a limited reduction in their immunoreactivity. However, TM is a relatively heat-stable protein, and simple heat treatments such as boiling and steaming only slightly affect its capacity to bind IgE, indicating that it is difficult to reduce allergenicity by heat treatment alone (Usui *et al.*, 2013). Indonesia is the world's third largest producer of shrimp next after China and India. Shrimp is consumed daily both as a foodstuff and for further processing. *Terasi*, the subject of this research, is one of the most popular food ingredients in Indonesia. *Terasi* is a naturally fermented shrimp paste produced from small shrimp, mainly

Acetes japonicus, and is an indispensable ingredient in many Indonesian dishes due its unique flavor. The annual production of *Terasi* in Indonesia is approximately 20,000 tons (SI, 2021) and the mean per capita *Terasi* consumption is more than 3 kg/year (SI, 2021). The manufacturing of *Terasi* consists of drying shrimp, grinding dried shrimp, adding water, and finally fermentation, a step that can last from two days to several weeks. These three processes are repeated until the *Terasi* product meets INS for food quality (INS, 2016). The most important chemical reaction in the production of *Terasi* is the enzymatic degradation of proteins during fermentation (Hajeb and Jinap, 2012). Protein hydrolysis during fermentation is caused by endogenous proteases in shrimp and proteases from halophilic bacteria, and these proteases affect food quality of shrimp paste such as texture and taste (Pongsetkul *et al.*, 2017). Furthermore, volatile low molecular compounds (such as aldehydes, organic acids, and amines) generated by fermentation may contribute to the distinctive aroma of fermented shrimp paste (Ambarita *et al.*, 2019). As of 2022, the food allergy labeling system is underdeveloped and no published epidemiological data for shrimp allergies exists for Indonesia (Lee *et al.*, 2013) despite widespread daily consumption of both shrimp and *Terasi*. It is therefore apparent that understanding the low allergenicity of shrimp TM contained in *Terasi* is very important for further discussions of the safety of shrimp consumption in Indonesia and neighboring countries that use *Terasi*. Recently, several studies have reported that fermentation reduces food allergenicity. Decreased food allergen IgE reactivity has been found in processed milk (Ehn *et al.*, 2005), soybean meal (Yang *et al.* 2018), and wheat bread (Angelis *et al.*, 2007). Furthermore, Park *et al.* (2007) and Kim *et al.* (2008) reported a loss of the IgE-binding ability of shrimp TM present in *Saeujeot*, a traditional Korean seasoning that is produced by self-fermentation with salt and pickled vegetables. Other studies have focused on the microbial diversity isolated from fermented shrimp paste products (Chukeatirote *et al.*, 2015) and the microbial flora present in a starter, which affecting shrimp paste quality (Akonor *et al.*, 2016;

Hua *et al.*, 2020). However, so far, no studies have investigated the effect of the *Terasi* manufacturing process on the behavior of shrimp TM as food allergen. The objective of this study is to characterize factors inducing low allergenicity in *Terasi* manufacture by investigating the biochemical behavior of TM and the change in TM allergenicity caused by the manufacturing process. The changes in the composition, degree of protein digestion, and biochemical behavior of TM (including structure and specific immunoglobulin-binding ability) were examined after each manufacturing step. In addition, this study also discusses the relationship between the loss of TM IgE-binding ability after manufacturing and the quality of *Terasi*.

3.2 Materials and methods

3.2.1. Materials

Three species of small shrimp, Akiami (*Acetes japonicus*), Okiami (*Euphasia pacifica*), and Isazaami (*Neomysis awatchensis*) were used as raw input material for *Terasi* manufacture. Akiami shrimp (*Acetes japonicus*) is widely distributed in the Indo-West Pacific region from the Persian Gulf to Japan and Indonesia (Chan, 1998) and is used as a raw material of Indonesian *Terasi* (Hajeb and Jinap, 2012). Furthermore, Okiami shrimp (*Euphausia pacifica*), euphausiid, living in the northern Pacific Ocean, has enormous abundant availability around Japan (Nicol and Endo, 1999) and is probably a novel raw material of *Terasi*. Then, Isazaami shrimp (*Neomysis awatchensis*) was selected as a representative of small shrimps living in the estuarine (Brandt *et al.*, 1993). All shrimp samples were purchased from a wholesale fish market at Hakodate (Hokkaido, Japan), and stored at −25 °C until use. *Terasi* produced for the Indonesian commercial market was obtained from local food markets in Pati city (Central Java Province, Indonesia). These *Terasi* samples were stored at room temperature in a sterile

polypropylene plastic bag for transport to the laboratory, where they were then stored at −25 ℃ until further use. All chemicals with no description in this manuscript were purchased from Kanto Chemical Co., Inc (Tokyo, Japan) or Fujifilm Wako Pure Chemical Cooperation (Osaka, Japan).

3.2.2 Sera of shrimp-allergic patients

The sera of six shrimp-allergic patients (P1–P6) possessing IgE antibodies specific for shrimp TM were used in this chapter as the same as list of patients' sera in Chapter II (listed in **Table 2.1**). The patients' sera were stored at −60 ℃ until use, and thawed sera were mixed with the same volume of phosphate buffered saline (pH 7.5; PBS) containing 0.2% NaN_3 before being subjected to analysis. These samples were then stored at 4 ℃ until use. ELISAs using purified shrimp TM confirmed that all sample sera contained the specific IgE. The use of human sera in the study was approved by the ethics review board of the Japan Society of Nutrition and Food Science (No. 90 in 2020), and all patients provided written informed consent to their doctor prior to providing serum samples.

3.2.3 The manufacture of *Terasi* on a laboratory scale

Terasi was produced according to the method specified by Indonesian National Standards (SNI 2716:2016) (INS, 2016) with a slight modification. According to the traditional procedure, *Terasi* should be prepared from crushed whole shrimp, including the carapace. An overview of the *Terasi* manufacture procedure is shown in **Figure 3.1**. In detail, whole shrimp were dried at 30 ℃ for 6 h until their moisture content level was < 30%. Next, whole dried shrimp were mixed and ground in a mortar and pestle with 15% (w/w) NaCl at a final concentration and a half weight of distilled water (for the first grinding step). Ground shrimps were then dried again

at 30 ℃ for 6 h, then covered with plastic food wrap and aluminum foil and incubated at 28 ℃ for 48 h (the first fermentation step). After the addition of 50% (w/w) distilled water, the fermented shrimp was ground again and subjected to a second fermentation step at 28 ℃ for 24 h. This drying and grinding cycle was performed three times in total, with two fermentation cycles. Following the last fermentation step, the fermented shrimp paste was added to rectangular forms of 2 cm × 1 cm × 0.5 cm in dimension and was then subjected to a fourth drying step that took place overnight. The final *Terasi* product was thereafter tightly wrapped in plastic film and stored at 4 ℃ until further use.

3.2.4 Physical properties of *Terasi*

Moisture content, protein content, and water activity (Aw) are key physical properties used to evaluate the quality of commercial *Terasi* according to the INS (2016). The moisture content of our *Terasi* was measured using an infrared moisture meter (Model FD-310, Kett Electric Laboratory, Tokyo, Japan). Protein content was measured via the Kjeldahl method, which is based on the AOAC method (AOAC, 2005). Finally, water activity was assayed using an Aw meter (Model 5803, Lufft, Fallbach, Germany) according to the manufacturer's instructions.

3.2.5 Preparation of shrimp TM and anti-TM IgG

Shrimp TM was purified from white shrimp (*Litopenaeus vannamei*) as described in Chapter II and stored at −60°C. Protein concentration was measured using a rapid protein assay kit (Wako Pure Chemical Industries, ltd) with bovine serum albumin as a standard.

A rabbit (New Zealand White strains, male, 3 months old) was immunized with purified TM according to the method of Shimizu *et al.*, (2009) in accordance with the Guidelines Concerning Animal Experiments at Hokkaido University (permission no. 10-0025). Anti-TM

IgG antibody (anti-TM IgG) was purified from the immunized rabbit sera using a Protein A HP column (GE Healthcare, Piscataway, NJ, USA), dialyzed against 20 mM Tris-buffer saline (pH 7.5), mixed with 0.1% ProClin 300 (Sigma-Aldrich, St Louis, MO, USA), and stored at 5°C until use.

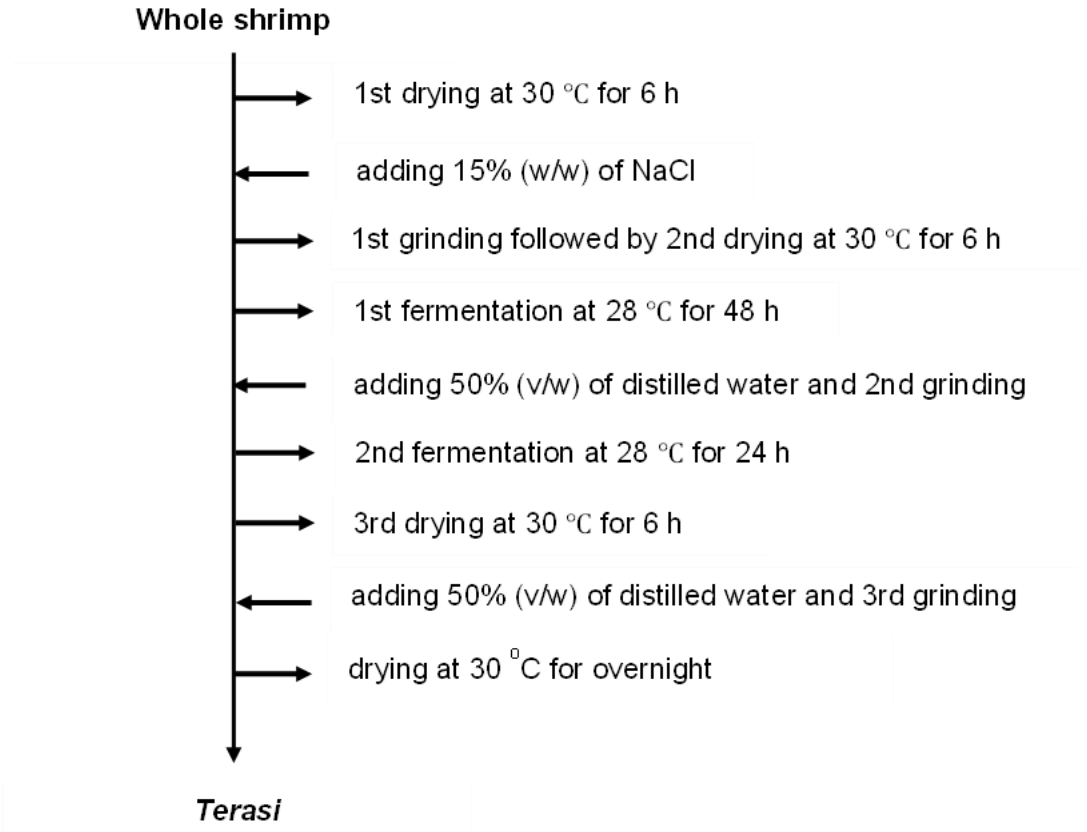

Figure 3.1. Outline of *Terasi* manufacturing process in the laboratory.

3.2.6 SDS treatment of shrimp during manufacturing

Processed shrimp meat/paste and the final *Terasi* products produced in the laboratory were dissolved in a SDS solution. In brief, each sample was put in a plastic bag and boiled at 98 ℃ for 20 min to inactivate digestive enzymes. Additionally, since the moisture of shrimp meat/paste sampled from each manufacturing step differed, the samples were diluted with distilled water to correct the difference in water content followed by keeping the total dry matter content constant (corresponding to 0.72 mg/mL protein content). Then, 0.1 g of each sample was dissolved in 1.4 mL of 2% SDS solution containing 8 M urea, 2% ME, and 20 mM Tris-HCl (pH 8.0) followed quickly by rapid heating at 98 ℃ for 2 min. Samples were then stirred vigorously and continuously overnight at room temperature. After centrifugation at 21,000 *g* for 30 min, the near-dissolved samples were stored at −60 ℃ for future SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The same amount of SDS-treated samples was loaded into each lane of PAGE-gels.

3.2.7 SDS-PAGE analysis

To investigate the degradation of shrimp TM during the production of *Terasi*, protein concentration visualized using SDS-PAGE (Laemmli, 1970). Here, SDS-PAGE was performed using a Compact PAGE apparatus (WSE-1010/25, Atto, Tokyo, Japan) with a 10%-polyacrylamide separation gel and a 4.5%-polyacrylamide concentration gel. A pre-stained molecular mass standard (14–100 kDa; GE Healthcare, Chicago, IL) was used as a protein marker. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) at room temperature for 1 h. Gels were then destained with a 7.5% acetic acid and 30% methanol solution for 2–3 h.

3.2.8 Immunoblotting analysis

Analytical samples from each step were treated as the same as SDS treated samples (produced by the methods described in section **3.2.6**) were subjected to Laemmli SDS-PAGE before being transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA) using a semidry blotting system (Atto, Tokyo, Japan). The membrane was then soaked in a blocking buffer (3% casein dissolved in 150 mM NaCl and 20 mM Tris-HCl (pH 7.5) containing 0.05% Tween-20 (TBS-T)) at room temperature for 1 h. Membranes were then incubated with an anti-TM rabbit antibody (diluted 1: 20,000 in blocking buffer) at 4 ℃ overnight. After washing six times with TBS-T, TM reaction with rabbit antibodies was detected by a second antibody reaction of peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad, Hercules, CA) at 37 ℃ for 3 h. After another six washes with TBS-T, we used an Enhanced Chemiluminescence photosystem (Amersham ECL, GE Healthcare) to visualize the specific reaction between the antigen and antibodies.

3.2.9 Assessing immunoreactivity using competitive ELISAs

Next, competitive ELISA (C-ELISAs) were carried out to investigate changes in the IgE-binding activity of TM contained in shrimp meat after *Terasi* manufacture. Individual shrimp allergic patients' sera used in the C-ELISA trials are listed in **Table 2.1** (Chapter II). First, the suspension of analytical sample, which were raw shrimp meat, processed meat sampled from the first, the second, and the third drying process, and the final *Terasi* product in 20-fold weight of 150 mM NaCl (pH 7.5). These samples were then heated at 98 ℃ for 2 min then homogenized using a disperser (Ultra-Turrax T25, IKA, Staufen, Germany). Next, we added 0.05% of Tween-20, adjusted the pH to 7.5, and diluted samples with 0.1% casein in TBS-T to a final protein concentration of 2 μg/mL. Sample suspensions thus obtained were subjected to C-ELISA as described below. A 96-well ELISA plate (IWAKI, Tokyo, Japan) was coated with

2.5 μg/mL TM (100 μL/well) dissolved in 50 mM carbonate buffer (pH 9.6) before being incubated at 4 ℃ overnight. After being washed with TBS-T, each well was coated with blocking buffer (1% casein in TBS-T) at 37 ℃ for 1.5 h. Simultaneously, 125 μL samples of shrimp-allergic patients' or healthy individuals' sera was diluted 50-fold in 0.1% casein in TBS-T. These samples were then mixed with an equal volume of an inhibitor at a 100 μg/mL dilution in 0.1% casein solution. After incubation at 37 ℃ for 2 h, 70 μL of each protein-serum mixture was placed onto the TM coated ELISA plate and incubated again at 37 ℃ for 2 h. The plate was then washed with TBS-T containing 1 M NaCl and 100 μL/well of β-galactosidase-conjugated anti-human IgE antibody (Biosource International, Camarillo, CA) diluted in blocking buffer (1:2000) was added to each well; plates were then incubated at 37 ℃ for 1.5 h. Plates were then washed again in TBS-T containing 1 M NaCl, and the enzyme-substrate reaction was performed by adding 100 μL/well of 0.1 mg/mL 4-methyllumbelliferyl-β-D-galactosidase (Sigma-Aldrich) in 0.1 M phosphate buffer containing 1 mM MgCl (pH 7.8); plates were then incubated at 37 ℃ for overnight. The reaction was terminated by adding 100 μL/well of 0.1 M glycine-NaOH (pH 10.3). The enzyme reaction was visualized by measuring the fluorescence intensity at 450 nm using a microplate reader (MTP-100, Corona Electric, Ibaraki, Japan). The loss of specific IgE-binding ability in the patients' sera resulting from the treatment with the inhibitors was represented by calculating an inhibition rate using the following formula: inhibition rate (%) = ((X − Y)/ (X − Z)) × 100, where X represents the fluorescence intensity of each patients' sera without inhibitors, and Y and Z are the fluorescence intensity of the patients' sera and healthy individual's serum with the inhibitors, respectively.

3.2.10 Sensory evaluation of *Terasi* produced on a laboratory scale

Sensory evaluation of *Terasi* produced on a laboratory scale was performed according to the specifications of the INS (2016). A score sheet for evaluating appearance, aroma, texture, and taste of the three kinds of *Terasi* produced. Sixteen semi-trained panelists—consisting of ten male and six female Cambodian, Indonesian, Japanese, Thai, and Vietnamese people, aged 21–44—evaluated the laboratory-produced *Terasi*. After explaining the procedure, they agreed in writing to voluntarily participate in this experiment. Before the evaluation process, a brief explanation specific to each shrimp paste was conducted and a commercial Indonesian *Terasi* product was offered to inform the panels. During the evaluation, the panel was asked to give a score to the physical characteristics of the lab-produced *Terasi* (by its appearance, aroma, texture, and taste), which were coded using random letters. Mineral water was served to neutralize the palate after tasting each sample. The score scale was defined from 5 to 9, where five represented "non-preferable" and nine represented "most preferable.

3.2.11. Statistical analysis

All data obtained from 3 to 4 independent replicate experiments were expressed as mean ± standard deviation. Analyses of variance followed by Tukey's Multiple Comparison tests were used to estimate the statistical significance of differences between means ($p < 0.05$). All data were processed using GraphPad Prism 9. 4. 0 for Windows (GraphPad Software, San Diego, California, USA) except sensory evaluation data, which was analyzed using SPSS Statistics for Windows, version 16.0 (IBM SPSS Inc., Chicago, Illinois, USA).

3.3 Results and discussion

3.3.1 Evaluation of physicochemical properties of *Terasi*

Figure 3.2 presents the moisture content, protein content, and water activity of the three types of lab-produced *Terasi*, with a typical Indonesian commercial *Terasi* included as a reference. The dotted lines in the figure show the acceptable range according to the INS (2016). The moisture content of each raw shrimp material, for Akiami, Okiami, and Isazaami shrimps was 77.3%, 74.5%, and 75.5%, respectively. Akiami and Isazaami shrimps had a softer texture than Okiami shrimp, which were covered in a thick carapace. As shown in **Figure 3.2a**, the moisture of the final product was 25.3% for Akiami *Terasi* (AT), 13.5% for Okiami *Terasi* (OT), and 21.1% for Isazaami *Terasi* (IT). The moisture content of AT was similar to that of Indonesian commercial *Terasi* (CT), which was consistent with the INS. On the other hand, OT showed a significantly lower moisture content than AT, IT, or CT. This is due to the fact that Okiami (a kind of krill) contains a higher proportion of its mass as carapace (approximately 30%) and this adversely impacts its ability to produce a moist product (Storebakken, 1988). The whole shrimp protein content was 13.0% for Akiami, 16.1% for Okiami, and 18.7% for Isazaami. In the final *Terasi* product, the protein content ranged from 44.8 to 50.3% (**Figure 3.2b**). These data are significantly higher than the INS for various processed products such as *Terasi*. Thus, shrimp protein was concentrated through the manufacturing process, resulting in lab-produced *Terasi* becoming a rich source of protein. The National Standard for *Terasi* Aw is 0.60 – 0.80; this is the most important factor for insuring food safety because *Terasi* is generally distributed at room temperature. It is known that properly maintaining the Aw of *Terasi* can suppress the growth of pathogenic and spoilage microorganisms in the final product (Van-Thuoc *et al.*, 2021). Moreover, controlling Aw can also control the growth of halophilic bacteria that are useful during *Terasi* production (Pongsetkul *et al.*, 2017). As shown in **Figure 3.2c**, the Aw of the products were 0.68 for AT, 0.61 for OT, and 0.69 for IT; all of these,

including CT (0.73) met the INS. These results clearly illustrate the safety of the *Terasi* produced in the laboratory in this study.

3.3.2 Protein degradation and the loss of TM during *Terasi* manufacture

Next, the investigation of the changes in whole protein and TM composition of the three types of *Terasi* prepared in the laboratory using SDS-PAGE analysis and immunoblotting using TM-specific IgGs (**Figure 3.3**). CT and native TM were used as references. No protein band and no TM signal were observed for the CT SDS-PAGE and Immunoblotting assays, respectively. In contrast, it is found that protein degradation occurred for each of the three types of *Terasi* produced in the lab, but fragmented proteins still remained in the final product. In addition, only the AT sample showed a complete disappearance of TM signal in the immunoblotting data. These results suggest that higher levels of protein hydrolysis, as well as marked TM degradation, occurred in *Terasi* produced from Akiami shrimp. These results agree with previous opinions regarding the digestion of TM into low molecular-weight compounds during fermentation. For example, Pongsetkul *et al.* (2017) reported such a process occurring in *Acetes* samples used for *Kapii* (Thai product similar to *Terasi*) production.

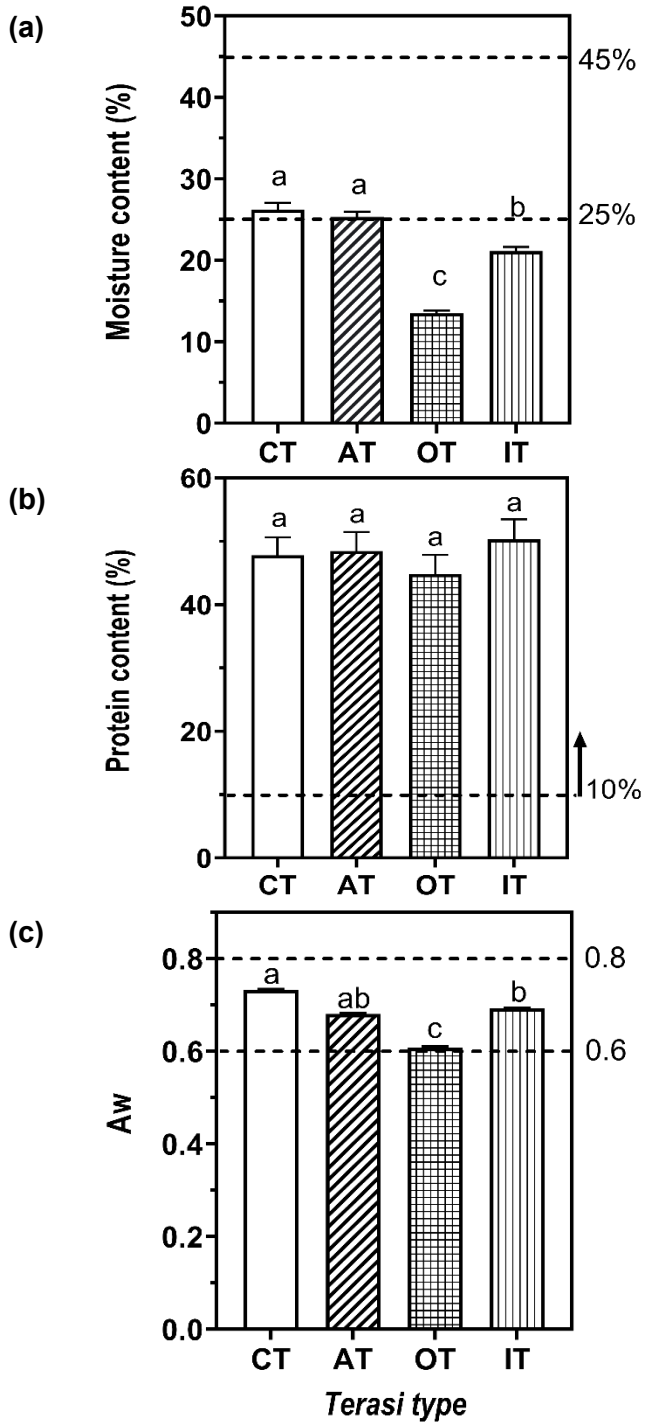

Figure 3.2. Proximate composition and water activity of *Terasi* produced. *Terasi* produced from three kinds of shrimp (Akiami (AT), Okiami (OT), and Isazaami (IT)) as well as Indonesian commercial Terasi (CT), were examined to determine their moisture content (a), protein content (b), and water activity (c). Values are reported as the mean of four replicate groups and error bars indicate standard deviation. Different lowercase letters on the bar charta indicate statistically significant differences at at each *Terasi* were assessed by the Tukey's Multiple Comparison tests at $p < 0.05$.

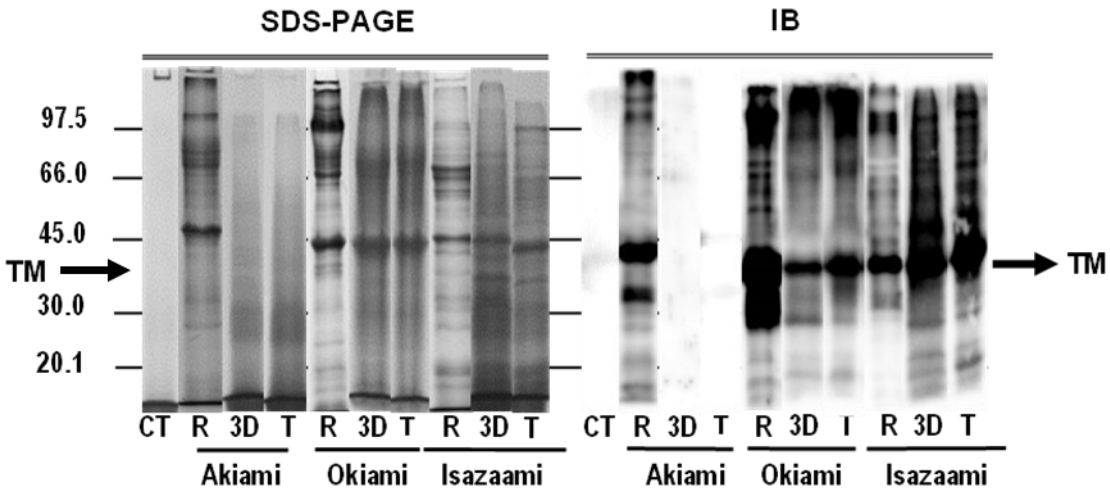

Figure 3.3. Protein degradation of shrimp meat during the *Terasi* manufacturing process. Shrimp meats processed were sample from the *Terasi* manufacturing process. Stages examined included: raw material (R), third drying process (see: Figure 3.1.) (3D), and final product (T). CT is the Indonesian commercial *Terasi*, used here as reference. Samples were subjected to SDS-PAGE and immunoblot assays using anti-TM rabbit IgG.

3.3.3 Change in the IgG and IgE-binding abilities of shrimp TM during *Terasi* manufacture

To investigate TM degradation during the *Terasi* manufacturing process, we obtained shrimp meat samples after each fermentation drying step of the *Terasi* manufacturing process (as described in **3.2.3**). These samples were then subjected to SDS-PAGE analysis followed by immunoblotting using anti-TM rabbit IgG. As shown in **Figure 3.4**, TM was present in raw Akiami shrimp, still existed after the first drying process, but disappeared after the second drying step, after which it was not detectable. In contrast, the OT and IT (**Figure 3.4**) samples showed clear TM blotting signals after each step that remained until the final product. These results agreed with the results of SDS-PAGE analysis (**Figure 3.3**); that is, TM in Akiami was lost during manufacturing, whereas a part of TM of Okiami and Isazaami remained in the final products.

It is believed that the differences of proteolytic activity induced by natural proteases found in each shrimp affected the band TM appearance in the *Terasi* final products. However, the results indicate that fermentation step plays a greater role in increasing protease activity with the support of microorganism that survive during the manufacture of *Terasi.* Although the diversity of microorganism contained in *Terasi* was not examined in detail in this study, Phewpan *et al*. (2020) mentioned the differences in microorganism community in shaping the typically characteristics of fermented shrimp paste products starting from raw shrimp material, during fermentation, and until the final products. In this study, the highest degree of protein hydrolysis in AT, 34% compared to OT, 19.2% and IT, 20.4% can be proven as a reason why the TM band disappeared completely in AT. Indeed, Pongsetkul *et al.*, (2017) stated proteolysis generated from raw material is an important biological event that occurs as well as proteases resulted from microorganism activity during *Kapi* (Thai shrimp paste) fermentation. However, immunoblotting using IgG (**Figure 3.4**) could not explain the behavior of TM as an allergen

during manufacturing. The reduction in IgE-binding of processed *Terasi* during manufacturing examined using C-ELISA to estimate the change in the allergenicity of TM.

TM IgE-binding ability in the processed materials was measured by determining the inhibitory effect of the sample against the reaction between IgE and the six shrimp-allergic patients' sera and native TM; here, lower inhibition rate indicated decreased IgE-binding of TM. As shown in **Figure 3.5**, the inhibition rate of the AT and OT shrimp samples decreased gradually during the manufacturing process, indicating that the IgE-binding ability of TM was progressively more impaired with every new stage of the *Terasi* manufacturing process, regardless of which patients' sera was used for determination of inhibition. However, when the three types of *Terasi* were compared to each other, AT showed the largest decrease in the inhibition rate as measured by C-ELISA, which showed a high degree of protein degradation. Overall, during the manufacturing process the inhibition rates of AT and OT decreased to 17% and 66% in P1; 36% and 66% in P2; 27% and 61% in P3; 17% and 33% in P4; 49% and 67% in P5, and 39% and 61% in P6, respectively. The greatest decreases in inhibition rate for both samples were found in P1 and P3, stages which involved the loss of large amounts of IgEs specific to crustaceans (**Table 2.1** in Chapter II).

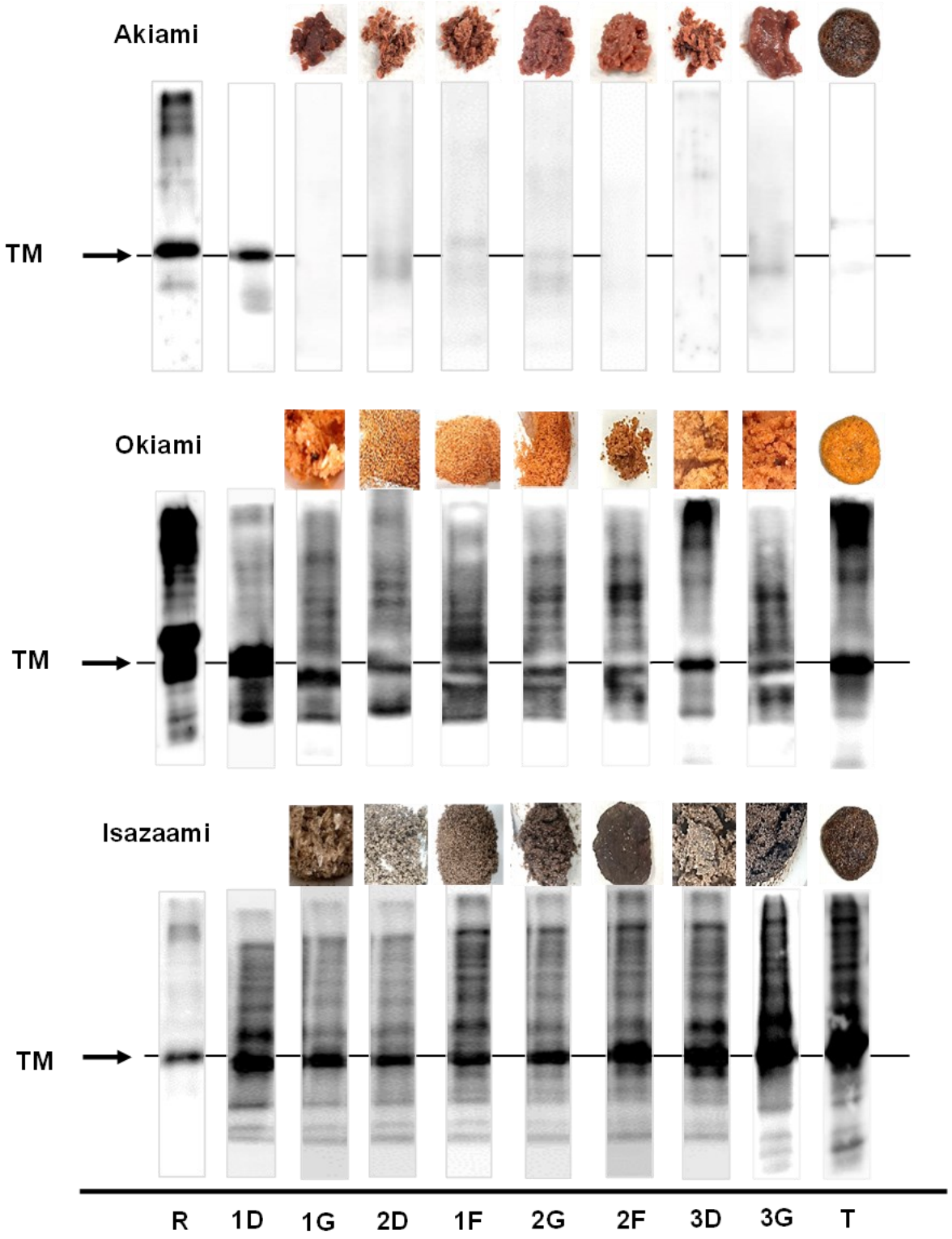

Figure 3.4. TM degradation during *Terasi* manufacturing process was monitored by immunoblotting using anti-TM IgG. Shrimp meat was sampled from the following manufacturing steps: raw material (R), first drying (1D), first grinding (1G), second drying (2D), first fermentation (1F), second grinding (2G), second fermentation (2F), third drying (3D), third grinding (3G), and final product (T: *Terasi*).

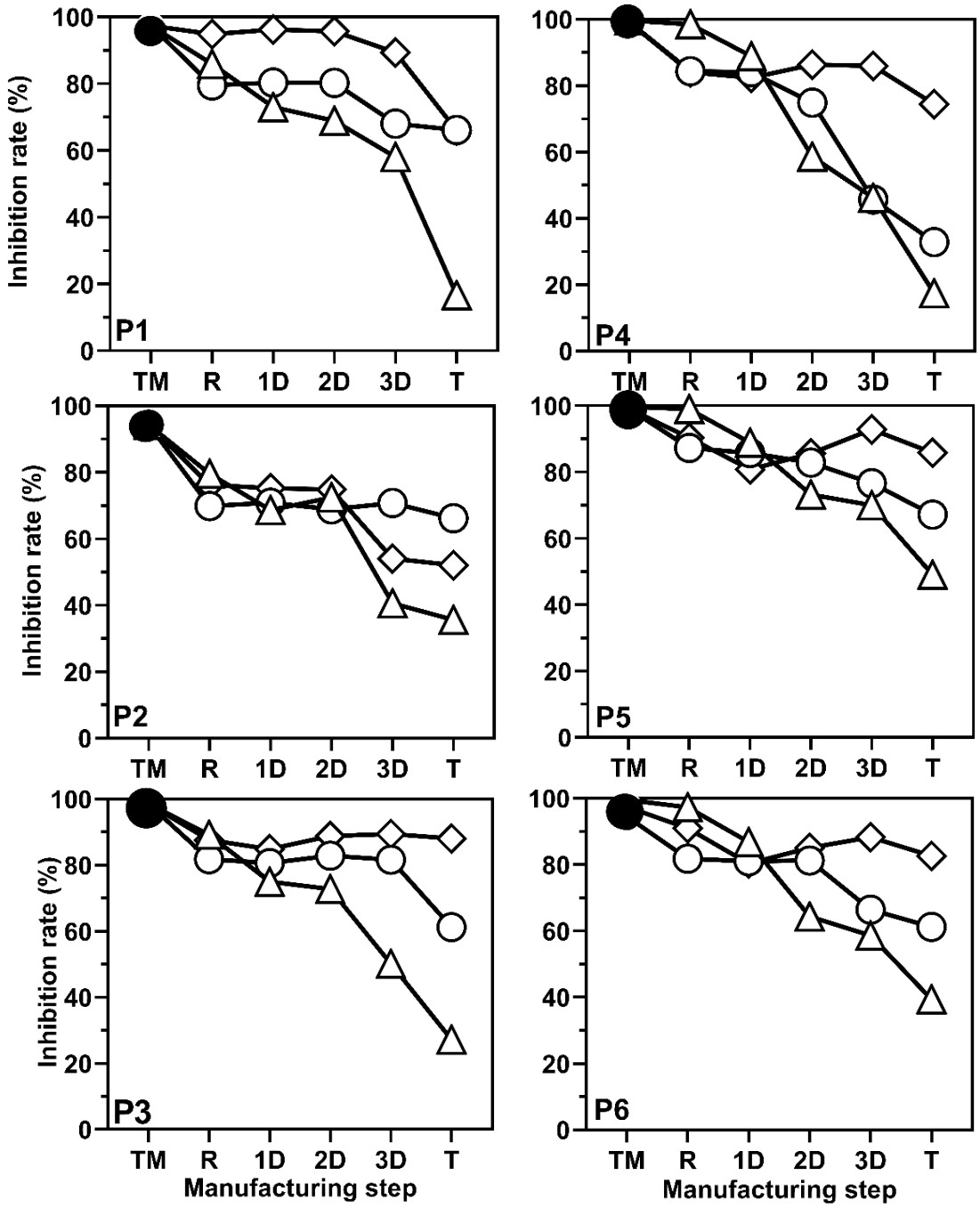

Figure 3.5. Change in the IgE-binding ability of shrimp tropomyosin during *Terasi* manufacture. Samples of raw material (R), processed meat from the 1st drying (1D), 2nd drying (2D), 3rd drying process (3D), and the final product (T) taken during the *Terasi* manufacturing process for AT (Δ); OT (○), and IT (◊) shrimp types, as described in Fig. 3.6. Each sample was mixed with shrimp-allergic patients' sera (P1-P6) and subjected to C-ELISA analysis. AT: Akiami *Terasi*; OT: Okiami *Terasi*; IT: Isazaami *Terasi*; and TM: tropomyosin as an inhibitor control.

In contrast, the inhibition rate in the IT production process showed a smaller decrease of 66% in P1 and 88% in P3. The fact that the similar trends were observed in all the six patients' sera despite their different specific IgE contents indicates that the most effective in IgE-binding loss was obtained in AT. Shrimp TM of Akiami was significantly diminished in immunoblotting assay (**Figure 3.4**). This indicated that IgE-binding loss in *Terasi* manufacture is closely related to the fermentation step inducing protein degradation. Additionally, it is presumed that the following factors are involved in the strong degradation of Akiami TM: strong endogenous protease and/or microbial flora having a high protein assimilation activity. As shown in **Figure 3.4**, the immunoblotting did not detect structural changes in TM for the AT samples because it degraded to small peptides. However, the results of C-ELISA (**Figure 3.5**) indicates that a series of IgE-binding epitopes in shrimp TM (Fu *et al.*, 2018) were degraded as the manufacturing process progressed. Basically, no critical step was found in **Figure 3.5** that contributed to the loss of TM IgE-binding activity common to all kinds of *Terasi* and patient sera. Furthermore, these behavior of TM as food allergen was not detected by the immunoblotting shown in **Figure 3.4**, which shows the limits of electrophoretic analysis in food safety assessment. It has been reported that protein hydrolysis during fermentation reduced the allergenicity of processed foods other than shrimp. Examples include β-lactoglobulin in yogurt (Ehn *et al.*, 2005), β-conglycinin in soybean subjected to solid state fermentation (Seo and Cho, 2016), and casein in fermented milk (Ahmadova *et al.*, 2013). As in these fermented foods, the degradation of shrimp TM in *Terasi* manufacture may involve interactions between endogenous proteases and degradation by environmental microorganisms (Anh *et al.*, 2015; and Lv *et al.*, 2020). In a preliminary experiment, it is found that myosin, a major myofibrillar protein present in whole shrimp homogenate disappeared during storage at room temperature. However, this loss was suppressed in shrimp meat heated in boiling water (**Figure 3.6**), and the intrinsic proteolytic activity originated in the raw material.

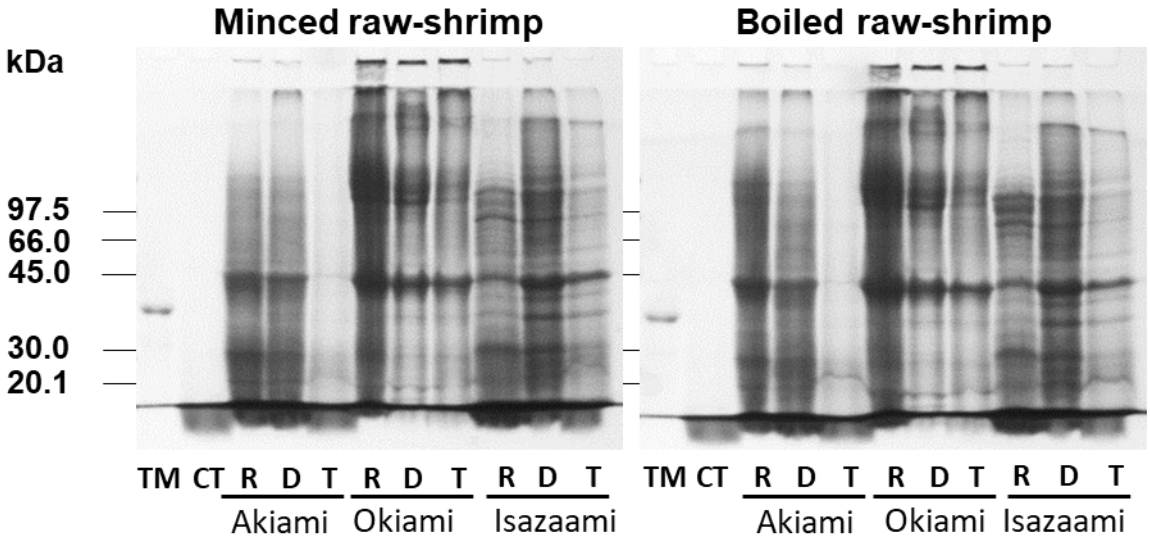

Figure 3.6. Preliminary experiment to compare the effect of heating treatment on SDS-PAGE of protein pattern in raw shrimp as a material of *Terasi*. TM: tropomyosin, R: raw shrimp, D: dried shrimp, T: *Terasi*.

Taken together, our results suggest that endogenous proteases may play an important role during the manufacturing process but also that an appropriate selection of microorganisms may contribute to TM degradation during *Terasi* manufacture, as stated on the previous study that *Bacillus* spp (69% of 117 isolates) play a role in the proteolytic activity of *Terasi* (Chukeatirote *et al.*, 2015). Also, lactic acid and halophilic bacteria are crucial in the fermentation of Indonesian *Terasi* (Kobayashi *et al.*, 2003). Together, these processes can help develop high-quality *Terasi* that presents low allergenicity.

3.3.4 Sensory evaluation of *Terasi*

Figure 3.7 shows the sensory evaluation of *Terasi* produced in the laboratory. Our data set contains the average score of the three types of *Terasi* as evaluated by 16 panelists and is summarized by a spider chart with four specifications indicating the quality of the *Terasi*. Significant differences in appearance and aroma between the AT and OT samples were recognized by the panelists but they observed no significant differences between the AT and IT samples. According to homogeneous subset testing, the data suggest that the panelists reported that the three types of *Terasi* each had specific food characteristics. Overall, the panelists preferred the appearance of the AT and IT samples (score: 7.6 for both) over the OT sample (score: 5.5), and that they preferred the aroma of the AT sample (7.6) over the OT (5.5) and IT (7.3) samples. The appearance of the OT *Terasi* was clearly different from that of the AT and IT *Terasi*s. The color of both the AT and IT *Terasi*s was dark gray-brown, which is a common color for these varieties, while the OT *Terasi* showed a dark orange color, which was likely derived from the presence of carotenoids (**Figure 3.4**). In contrast, the texture and taste were rated as clearly different among all *Terasi*s; the AT sample was rated as the most preferable with respect to homogeneity and extensibility, and it was also rated as having the clear umami and shrimp tastes characteristic of *Terasi*. The selection of raw shrimp inputs and

processing conditions are both important factors affecting the generation of specific *Terasi* aromas (Fan *et al.*, 2017). Furthermore, the umami taste and the specific flavor of *Terasi* is determined by protein degradation during the fermentation process, in which shrimp proteins are digested, yielding amino acids such as glutamic acid and aspartic acid (Hajeb and Jinap, 2015). According to the results of the sensory evaluation (**Figure 3.7**), AT showed the most advanced proteolysis among the three products and was the "highest quality" *Terasi* of the three produced in the lab. In addition, the IgE reactivity of TM was greatly reduced during the AT manufacturing process, as described in **Figure 3.5**. This relation between quality and proteolysis in *Terasi* suggests that *Terasi* quality may be correlated with the loss of allergenicity. That is, *Terasi* produced by an ideal manufacturing process with abundant proteolysis would be a low-allergic seafood.

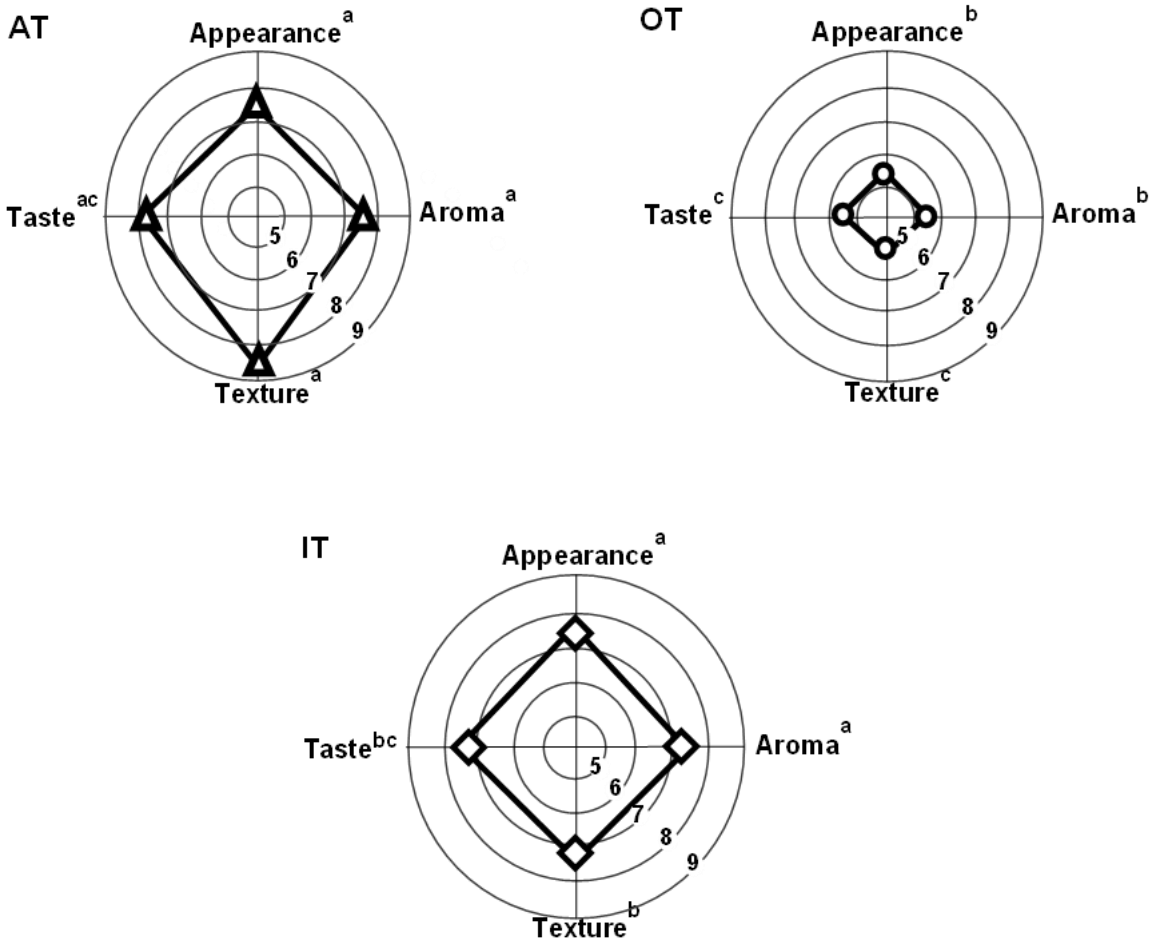

Figure 3.7. Food quality of *Terasi* manufactured in the laboratory. Sensory evaluation data characterizing the food quality of *Terasi* manufactured from three kinds of shrimp, AT: Akiami *Terasi*; OT: Okiami *Terasi*; IT: Isazaami *Terasi*. Values are reported as the mean of three independent groups. Different lowercase letters on the spider charts indicate statistically significant differences as each *Terasi* were assessed by the Tukey-Multiple Comparison at $p < 0.05$.

3.4. Conclusion

This study clearly demonstrated that *Terasi* manufacturing is an effective processing method for reducing the IgE reactivity of shrimp TM, which was diminished as manufacturing progressed. The selection of Akiami shrimp is the most appropriate of *Terasi* manufacture in

the viewpoint of food quality and safety. This suggests that this traditional Indonesian food can be recognized as a low allergenic seafood. The health benefit thus obtained would be mainly due to the degradation of IgE epitope in TM during processing. Moreover, progressive protein degradation during fermentation had no negative effect on *Terasi* food quality, suggesting that developing good-tasting *Terasi* while maintaining low allergenicity is possible.

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CHAPTER IV

EFFECT OF VARIOUS STARTER USED IN *TERASI*, INDONESIAN SHRIMP SEASONING, ON SHRIMP ALLERGENICITY

4.1 Introduction

Terasi is a naturally fermented shrimp paste and have been consumed in Indonesia as one of popular food seasoning, which offers unique aroma, taste and desirable characteristics to Indonesian dishes. *Terasi* manufacturing process consist of drying, salting, and fermenting at room temperature for 3 days to two months (Mizutani *et al.*, 1987; Kobayashi *et al.*, 2003), which is one of the convenient ways to impart processed foods superior shelf life to with less energy compared to other food processing methods such as retort, spray-drying, and freeze-drying (Tamang *et al.*, 2020). *Terasi* can be stored at room temperature for 6 months up to two years (Faithong *et al.*, 2010; Hajib and Jinap, 2012; Cai *et al.*, 2015).

The manufacture of *Terasi* is generally carried out by a home–scale industry with a natural fermentation method (Aryanta, 2000; Hajib and Jinap, 2012), and the fermentation period varies according to family traditions in making *Terasi* that are passed down from generation to generation (Ambarita, 2016). As a result, there are many variations of *Terasi* products distributed in local markets, especially in terms of appearances and nutritional contents (Sato *et al.*, 2022), and some of Indonesian commercial *Terasi* deviated from the standardization required by INS (2016).

Chapter III reported that shrimp TM, which is a major allergen of crustacean, was digested through the *Terasi* manufacturing process, and the loss of TM induced the reduction in the specific IgE-binding ability of *Terasi*. The final conclusion in Chapter III was *Terasi* can be recognized as a low allergenic seafood when produced under an appropriate manufacturing condition.

Protein degradation in *Terasi* manufacture proceeds by spontaneous fermentation involving microorganisms and endogenous enzymes attached to raw materials (Phitakpol, 1993; Pongsetkul *et al.*, 2015; Kleekayai *et al.*, 2015). Therefore, the allergenicity of the final product is likely to depend on the raw materials. In fact, the authors showed that *Terasi* made from Akiami shrimp (*Acetes japonicus*), which is a common raw material in Indonesia (Hajeb and Jinap, 2012), was less allergenic than those of *Terasi* from other shrimps (Isazaami shrimp, *Neomysis awatchensis* and Okiami shrimp, *Euphasia pacifica*) (Amalia *et al.*, 2023). However, in order to strictly ensure the food safety of *Terasi*, it is necessary to develop a novel food processing technology that effectively promotes TM degradation in the final products without depending on the type of raw material.

Backslopping method is a fermentation technique using a prior successful fermented batch (nature starter) as feedstock for subsequent fermentation steps (Harris, 1998; Leroy and De Vuyst, 2004; Shrivastava and Ananthanarayan, 2014), and no major technical obstacles introducing to small scale factories. Backslopping method, although which is not microbiologically characterized process, proceeds material fermentation due to high proteolytic activity of natural starters, and it is successfully utilized in various fermented foods, such as sourdough bread (Rizello *et al.*, 2016), various beverages (Roos and Vuyst, 2018), and Indonesian fermented milk (Wirawati *et al.*, 2019). Furthermore, the accelerated fermentation with protein digestion may result in the reduction of allergenicity of the final products. Thus, we are focusing on the backslopping method using the natural starter for progressing degradation of shrimp TM during *Terasi* manufacture.

The objective of this study is to clarify the contribution of the backslopping method on producing *Terasi* with low allergenicity independent of the type of raw materials. In this study, Isazaami shrimp was selected as the experimental material, that could not produce a final product with reduced allergenicity. As a starter for the backslopping method, a series of *Terasi*

products with different levels of protein digestion were then mixed with the Isazaami shrimp, and the effect of the natural starter on the reduction of potential allergenicity of the final products was examined by monitoring changes in protein characteristics and in specific IgE-binding ability during the *Terasi* manufacture.

4.2 Material and Methods

4.2.1 Raw materials of *Terasi* manufacture

Small shrimp, Isazaami (*Neomysis awatchensis*) was used as a material for *Terasi* manufacture. Raw Isazaami shrimp was purchased from a wholesale fish market at Hakodate city (Hokkaido, Japan), and stored at −25 °C until use.

4.2.2 Chemicals

All chemicals with no description in this chapter were purchased from Kanto Chemical Co., Inc (Tokyo, Japan) or Fujifilm Wako Pure Chemical Cooperation (Osaka, Japan).

4.2.3 Sera of shrimp-allergic patients

The sera of six shrimp-allergic patients (P1-P6) (**Table 2.1** in Chapter II) possessing IgE antibodies specific for shrimp TM were used in this study. Sera from healthy individuals were also used as negative controls. The patients' sera were stored at −60°C until use, and thawed sera were mixed with the same volume of phosphate buffered saline (pH 7.5; PBS) containing 0.02% NaN_3 before being subjected to analysis. These samples were then stored at 4 °C until use. ELISAs using purified shrimp TM confirmed that all sample sera contained the specific IgE. The use of human sera in the study was approved by the ethics review board of the Japan

Society of Nutrition and Food Science (No. 90 in 2020), and all patients provided written informed consent to their doctor prior to providing serum samples.

4.2.4 Starter used in the *Terasi* manufacture

Three types of starters were prepared for manufacturing *Terasi* using the backslopping method as follows: (1) Indonesian commercial *Terasi* (CT), was obtained from local food markets in Pati city (Central Java Province, Indonesia); (2) *Terasi* produced from Akiami shrimp (AT) produced in the previous work (Amalia *et al.*, 2023), (3) AT heated at 98 °C for 20 min (referred as to HAT), in which the proteolytic and microbial activities were eliminated. These starters were then mixed with Isazaami dried shrimp [starter : dried Isazaami shrimp (1 : 2 w/w)] and other ingredients for *Terasi* manufacture as described below.

4.2.5 Manufacture of *Terasi* using starters

Terasi was produced from dried Isazaami shrimp mixed with the starter according to the method specified by Indonesian National Standards No. 2716:2016 (INS, 2016) with a slight modification. An overview of the *Terasi* manufacture procedure without and with starter are shown in **Figure 4.1**. The difference between the two *Terasi* samples is the number of drying processes: IT has four, while Isazaami *Terasi* with starter has three. The 2D step in IT aimed to create a semi-solid paste before the 1st fermentation step since only a little salt is required and prevented the increase of the potential for the overpopulation of undesirable microorganisms during fermentation (Ishige, 1993; Hajeb and Jinap, 2012). The processes afterward (from 1st fermentation to the final product) were the same between two *Terasi*. In detail, whole raw Isazaami shrimp was dried at 30 °C for 6 h until their moisture content level was less than 30% (refereed as to the 1st dried shrimp: 1D), which was mixed and ground with

50% (w/w) each starter (CT, AT, and HAT), 15% (w/w) NaCl, and 50% (w/w) in a mortar. Ground shrimp thus obtained (first grinding step, 1G) was placed in a small container covered with plastic food wrap and aluminium foil and incubated at 28 °C for 48 h as a first fermentation step (1F). After addition of 50%(w/w) distilled water, the fermented shrimp was ground again (2G) and subjected to a second fermentation step at 28 °C for 24 h (2F). This drying and grinding cycle was totally performed three times, including two fermentation cycles. Following 2F, the fermented shrimp paste was formed into oval shapes and was then subjected to a third drying step at 30 °C for overnight. As described above, the *Terasi* products manufactured using the backslopping method were thereafter tightly wrapped in a plastic film and stored at 4 °C until further use.

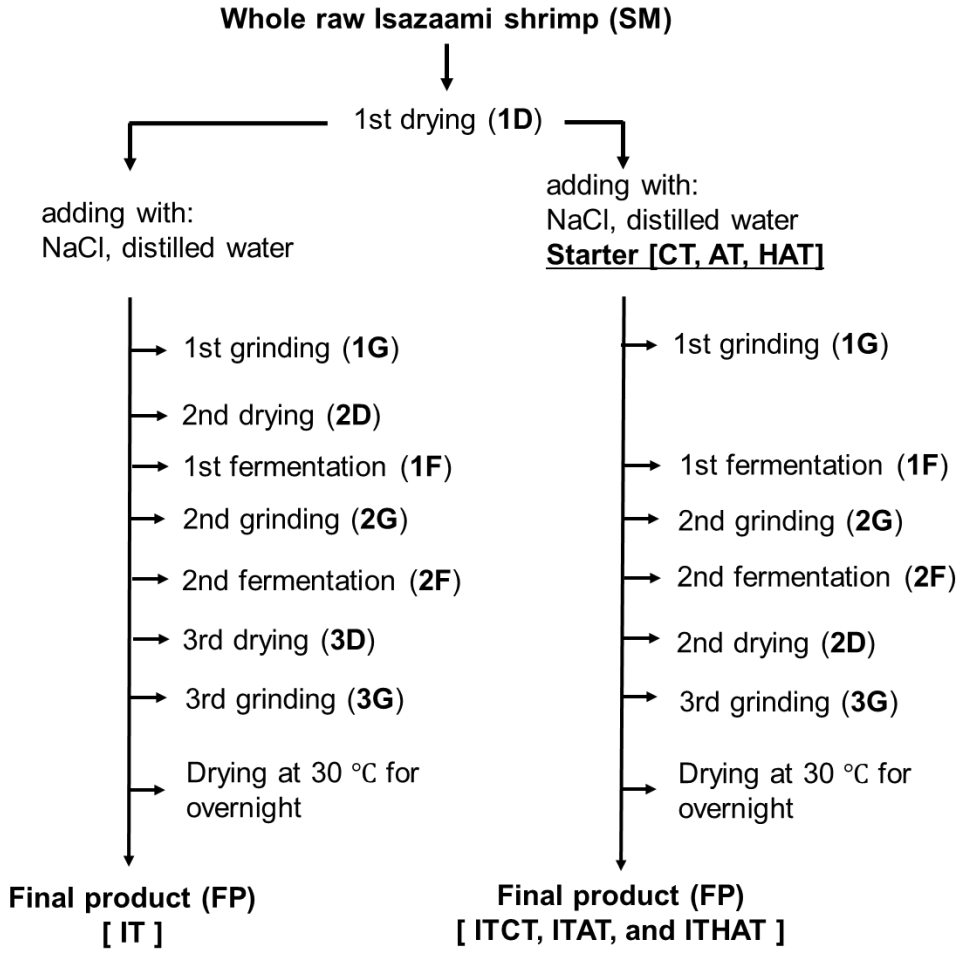

Figure 4.1. Overview of *Terasi* manufacture. Various types of *Terasi* used as starters in this study (CT: Indonesian commercial *Terasi*; AT: Terasi produced from Akiami shrimp (AT), in previous work (Amalia *et al.*, 2023); HAT: Akiami *Terasi* heated at 98 ℃ for 20 min). The final product of *Terasi*: IT: Isazaami *Terasi* without starter, ITCT: Isazaami *Terasi* with CT; ITAT: Isazaami *Terasi* with AT, and ITHAT: Isazaami *Terasi* with HAT.

4.2.6 Purification of shrimp TM

Shrimp TM as a standard for each assay was purified from white shrimp (*Litopenaeus vannamei*) as the same manner as previously studied (Amalia *et al.*, 2023) and kept at –60 °C until used. Protein concentration was measured using a rapid protein assay kit (Wako Pure Chemical Industries, ltd) with bovine serum albumin as a standard.

4.2.7 Physical properties of *Terasi*

The moisture content was measured using an infrared moisture meter (Model FD-310, Kett Electric Laboratory, Tokyo, Japan). Protein content was measured by the Kjeldahl method based on the AOAC pocedure (AOAC, 2005). Water activity was assayed using an Aw meter (Model 5803, Lufft, Fallbach, Germany) according to the manufacturer's instruction.

4.3.8 Determination of protein hydrolysis

The degree of protein hydrolysis of each sample was examined by the method of Kong *et al.* (2007) with slight modification. Four mL of distilled water and 5 mL of 15% (w/v) TCA were added to 1.0 g of each sample, homogenized using a disperser, and kept at room temperature for 30 min. After collecting the centrifugal supernatant at 2,400 *g* for 30 min, and the nitrogen content of the supernatant (TCA-soluble fraction) and total fraction were measured by the Kjeldahl method (AOAC, 2005) using the Buchi Digest and Distillation System (K–437 & B–324, Buchi, Flawil, Switzerland). The degree of protein hydrolysis in *Terasi* is expressed as the percentage of TCA-soluble nitrogen to total nitrogen.

4.2.9 Protein profile analysis

4.2.9.1 Preparation of analytical samples

Terasi sample in each manufacturing step was placed into a plastic bag and boiled at 98°C for 20 min to inactivate digestive enzymes, and 0.1 g of each sample was dissolved in 1.4 mL of 2% SDS solution containing 8 M urea, 2% 2-ME, and 20 mM Tris-HCl (pH 8.0) with a pellet pestle. The samples were heated at 98°C for 2 min and shaken vigorously overnight at room temperature using a laboratory shaker (NR-80, TITEC, Tokyo, Japan). After confirming that the sample was mostly dissolved in the SDS solution, it was centrifugation at 21,000 *g* for 30 min and stored at −60°C until use.

4.2.9.2 SDS-PAGE

The protein profile of each sample was examined by SDS-PAGE (Laemmli, 1970) using a Compact PAGE device (WSE-1010/25, Atto, Tokyo, Japan) with 4.5% stacking and 10.0% resolving polyacrylamide slab gels. TM was used as controls. The same amount of SDS-treated samples described in 4.2.9.1 were loaded into each lane of the gels and prestained molecular mass standards (14–100 kDa; GE Healthcare, Chicago, IL) were included as markers. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) at room temperature for 1 h, followed by destaining in a solution of 7.5% acetic acid and 30% methanol for 2–3 h.

4.2.10 Assessing IgE reactivity of *Terasi* using starters

Each sample was subjected to C-ELISA to examine the IgE reactivity of TM contained in the samples. Prior to analysis, sample was suspended in 20-fold (w/v) of 150 mM NaCl, pH 7.5, and immediately heated to 98°C for 2 min, and homogenized using a disperser (Ultra-

Turrax T25, IKA, Staufen, Germany). After the addition of 0.05% Tween-20, the pH was adjusted to 7.5 by 2 M NaOH and stored at −20°C until use. For the assay, 10 µl of each sample suspension was diluted by 1,250-fold in 0.1% casein in TBS-T and subjected to C-ELISA based on the method of Amalia *et al.* (2023). Briefly, sera from shrimp-allergic patients and healthy individual were diluted 50-fold in 0.1% casein in TBS-T and mixed with an equal volume of *Terasi* suspension at a final concentration of 100 µg/mL as an inhibitor. After incubation at 37°C for 2 h, each protein-serum mixture was placed into each well of a 96-well ELISA plate (IWAKI, Tokyo, Japan) previously coated with TM, followed by measurement of the reaction between the specific IgE and TM using β-galactosidase-conjugated anti-human IgE antibody (Biosource International, Camarillo, CA) and 4-methyllumbelliferyl-β-D-galactosidase (Sigma-Aldrich). A reduction in specific IgE reactivity in the patient sera as a result of inhibitor treatment was determined by calculating an inhibition rate using the following formula: Inhibition rate (%) = [(X − Y)/ (X − Z)] × 100, where X represents the fluorescence intensity of the patient sera without inhibitor, and Y and Z are the fluorescence intensity at 450 nm of the patient and healthy individual sera with the inhibitors, respectively. When *Terasi* as an inhibitor almost inhibited the reactivity of TM and patient IgE, the calculated value of the inhibition rate sometimes became 0 or less. In this case, it was determined that the sample did not have IgE reactivity, and the inhibition rate was quantified as 0.

4.2.11 Statistical analysis

The data were analyzed using GraphPad Prism 9. 4. 0 (GraphPad Software Inc., San Diego, CA, USA) and presented as the mean ± standard deviation (SD) of three separate experiments. Statistical analysis of the data was performed using an analysis of variance and Tukey's Multiple Comparisons Test. p values < 0.05 were considered statistically significant.

4.3 Results and Discussion

4.3.1 Physicochemical properties of *Terasi* with starter

The physicochemical properties of *Terasi* prepared by the backslopping method were investigated. **Figure 4.2a** shows the moisture content of *Terasi* made by the backslopping methods using starters, in which the data of the starters are also listed. The moisture content of IT, Isazaami *Terasi* without starter was lower than that of the starters (CT, AT, and HAT), and that of ITs produced by the backslopping method using the starters (ITCT, ITAT, and ITHAT, respectively). There were no differences in both moisture and protein contents among three types of starters, but the moisture content of *Terasi* was increased by AT and HAT and diminished by CT. As a result, except for ITCT, the moisture content of *Terasi* produced using the backslopping method was matched with Indonesian national standardization of moisture content ranges from 25–45% (INS, 2016).

In protein content (**Figure 4.2b**), IT did not differ from starter and its *Terasi* product except for ITAT whose protein content was the highest (56.3%) among samples. As a result, there was a little effect of the starters on the protein contents of the final products, and all of *Terasi* produced using the backslopping method were fulfil in the minimum requirement of protein content according to standardization, which is 10% (INS, 2016). Since *Terasi* is often distributed at room temperature, the INS requires *Terasi* to have a water activity (Aw) of 0.6 to 0.8 (INS, 2016) to ensure food safety. As shown in **Figure 4.2c**, all the *Terasi* products produced in this study showed its Aw at range of 0.68 to 0.74, indicating that the backslopping method did not adversely affect the shelf life of *Terasi* in the viewpoint of food hygiene.

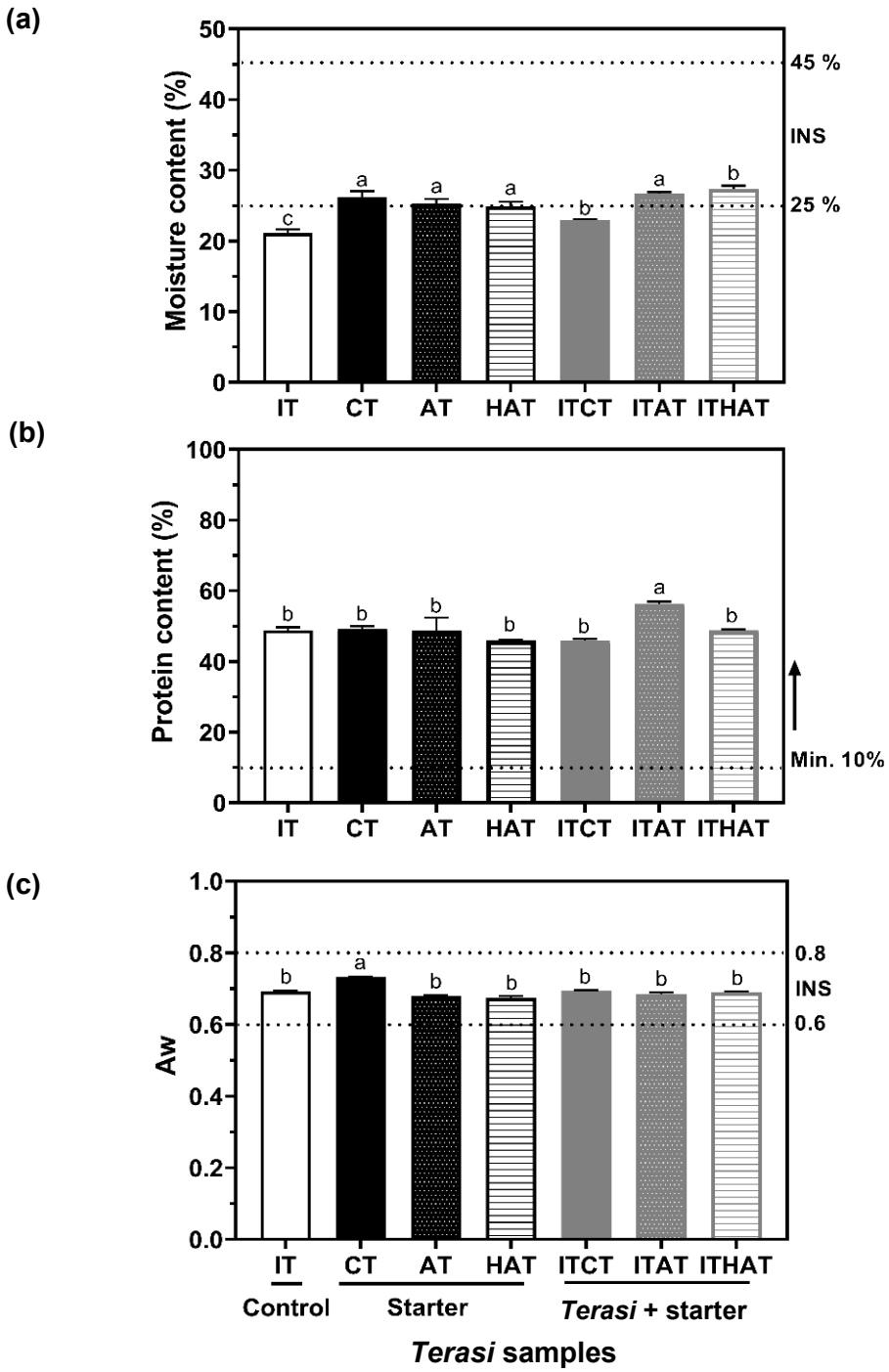

Figure 4.2. Proximate composition and water activity of *Terasi*. (a) Moisture content, (b) Protein content, (c) Water activity. Values are the mean ± standard deviation (n = 3). Different lowercase letters on the bar charts indicate significant differences ($p < 0.05$, Tukey's Multiple Comparison test).

4.3.2 Effect of starter addition on protein hydrolysis during *Terasi* manufacture

Next, we investigated the degree of protein hydrolysis during *Terasi* manufacture by measuring TCA-water soluble nitrogen. As shown in **Figure 4.3**, the degree of protein hydrolysis during manufacture in *Terasi*s with starter (ITCT, ITAT, and ITHAT) was significantly increased compared to *Terasi*s without starter (IT) ($p < 0.05$). Isazaami *Terasi* with HAT (ITHAT) had the highest level of protein hydrolysis, which is 39.4%, followed by ITCT (36.3%) and ITAT (31.1%). Observing the changes in the degree of protein hydrolysis during the manufacturing process, it showed an increasing tendency with the progress of *Terasi* manufacture, and a marked increase was observed especially in the fermentation process (1F and 2F). These results indicate that the use of the starters accelerate the fermentation and enhances the degradation of proteins contained in *Terasi*. The reason why fermentation is promoted by the starter may be the involvement of the enzyme activity derived from the microorganisms contained in the starters, or the starter, which is already a decomposition product, may have become an assimilatory nutrient for the fermentation microorganisms. Indeed, shrimp muscle proteins are easily digested by endogenous proteases and fermenting bacteria during *Terasi* production (Sun *et al.* 2014; Kleekayai *et al.* 2016; Helmi *et al.* 2022), and the variations of bacterial community composition during fermentation of shrimp paste which impacted on the quality and safety of shrimp paste (Lv *et al.*, 2020; Li *et al.*, 2022).

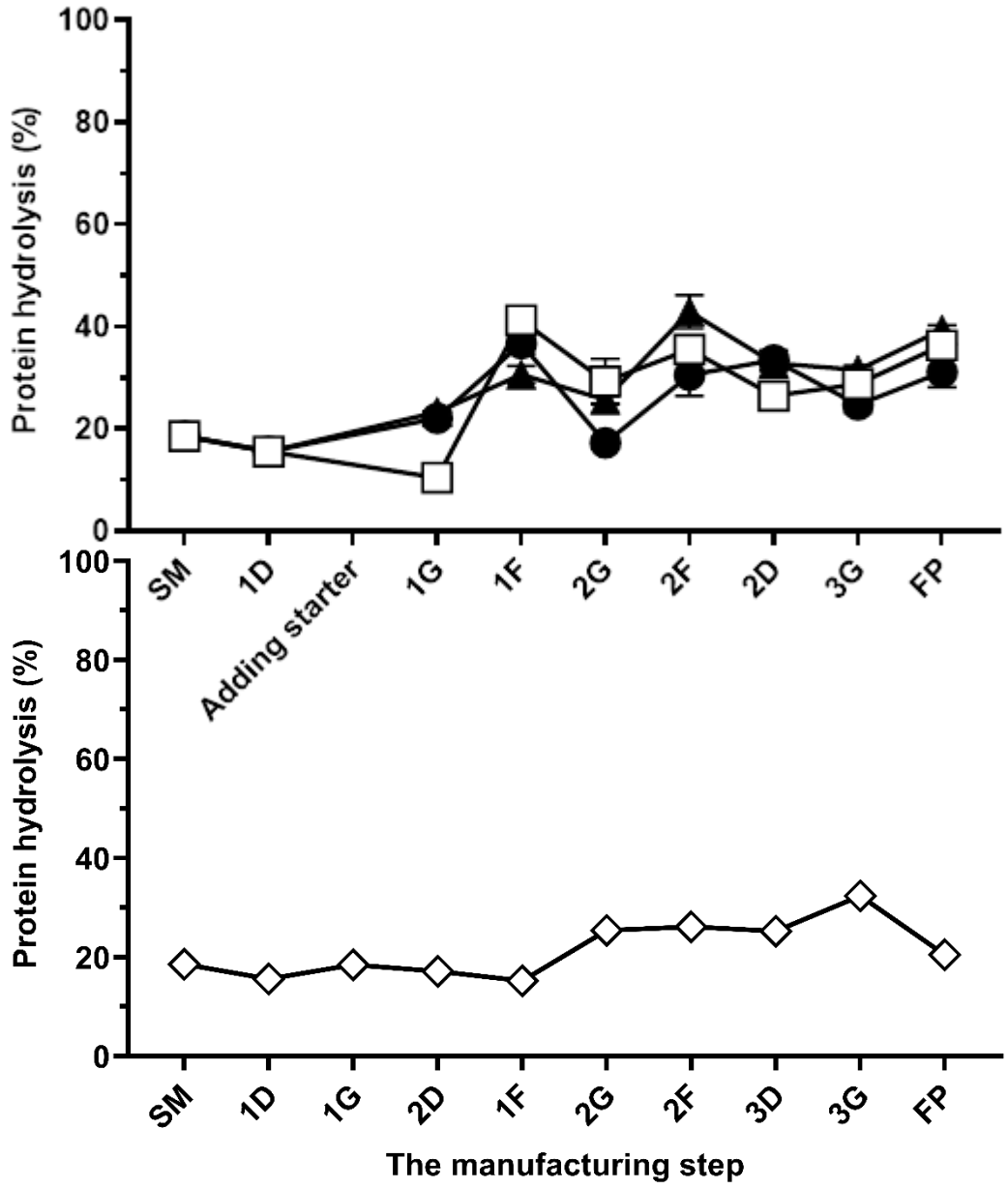

Figure 4.3. The influence of various starter on the degree of protein hydrolysis during *Terasi* manufacture. IT (◊): Isazaami *Terasi* without starter, ITCT (□): Isazaami *Terasi* with CT; ITAT (●): Isazaami *Terasi* with AT; and ITHAT (▲): Isazaami *Terasi* with HAT. Values are the mean ± standard deviation (n = 3) ($p < 0.05$, Tukey's Multiple Comparison test).

4.3.3 Effect of starter on protein-profile change during *Terasi* manufacture

Protein is the most abundant nutrient in *Terasi* as shown in **Figure 4.2b**, and it is important to clarify the behavior of allergens contained in the protein fraction in the manufacturing process for understanding the food safety of *Terasi*. Therefore SDS-PAGE assay was carried out to investigate the effect of the starters on the protein breakdown in *Terasi* during the manufacturing process.

In the production of *Terasi* from Isazaami shrimp meat (SM) without any starter (**Figure 4.4a**), the major allergen TM (about 35 kDa) was clearly observed in all manufacturing steps from SM to the final product (FP), indicating that TM almost remained unchanged during the drying and fermentation process in the *Terasi* manufacture. On the other hand, degradation of various muscle protein was found in the test samples mixed with CT, AT, and HAT as starters (**Figures 4.4b**, **4.4c**, and **4.4d**, respectively). That is, TM shown in SM and 1D disappeared markedly at the early stage of the manufacturing step, when the starters were mixed with the shrimp muscle. That is, the intact TM degraded rapidly (between 1D and 1G) with the addition of starter and was completely absent at 1F. In the final result, although a slight difference was found in the SDS-PAGE patterns among the final products, the intact TM band was no longer observed in all *Terasi* produced using the backslopping method. The disappearance of TM in the *Terasi* manufacture using starters shown in **Figure 4.4** are presumed to be due to the presence of substrate from Isazaami shrimp that can induced the activity of microorganism contained in starter to break down the protein including TM as allergenic protein.

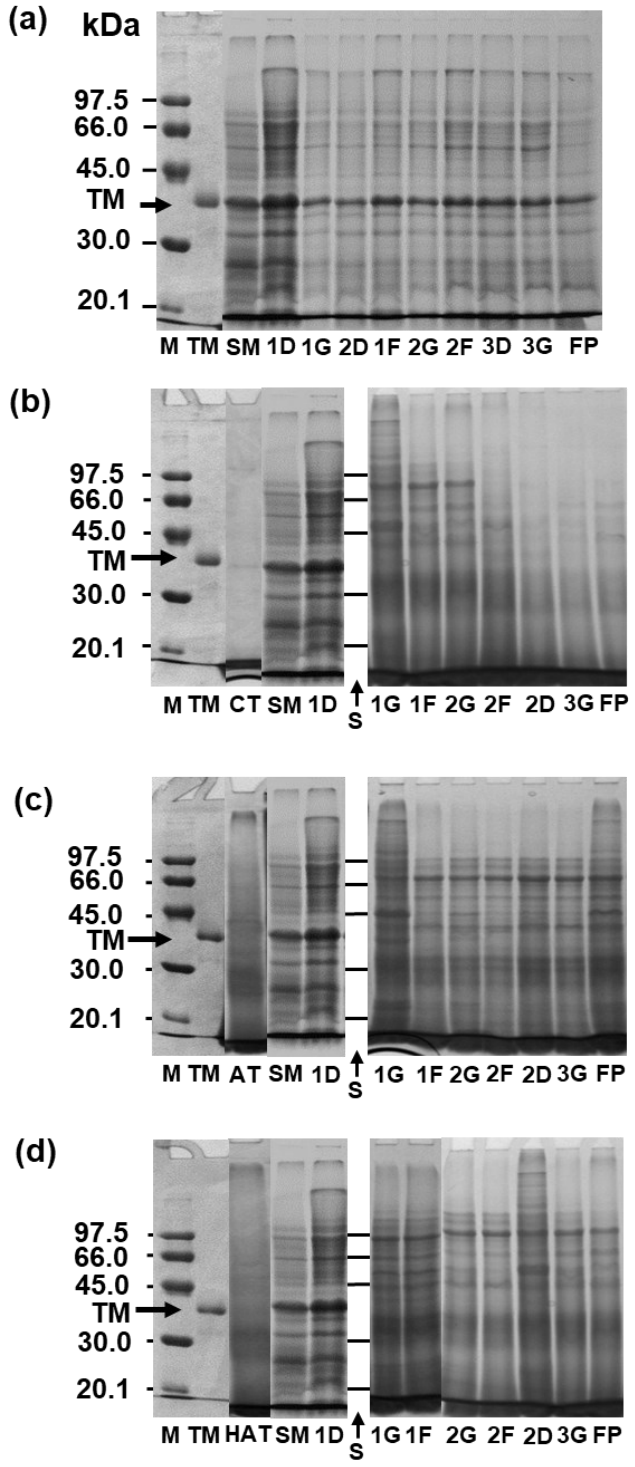

Figure 4.4. Change in protein profile during *Terasi* manufacture. *Terasi* was produced from Isazaami shrimp with various starters: CT (b), AT (c), and HAT (d), and each final product was ITCT, ITAT, and ITHAT, respectively. As a negative control, *Terasi* without addition of starter (IT) was also examined (a). The processed material from each manufacturing step described below was subjected to SDS-PAGE. M: Molecular mass marker. TM: shrimp tropomyosin, SM: Isazaami shrimp muscle, S: starter addition between 1D and 1G, and each manufacturing step was shown in Figure 4.1.

4.3.4 IgE-binding ability of *Terasi* with starter

To better understanding of shrimp TM's allergenicity in comprehensive manner, an investigation was carried out on the IgE reactivity of each *Terasi* by using serum from six individuals with shrimp allergy. The samples were chosen up from four steps during *Terasi* manufacture: (1) raw material or shrimp muscle of Isazaami (SM); (2) first grinding step (1G); (3) drying (D) after last fermentation step (2D for *Terasi* with starter, that are ITCT, ITAT, and ITHAT; and 3D for *Terasi* without starter, IT) and (4) final product (FP). Although not all steps, the selection of samples at certain of steps is thought to have been a key milestone in the manufacture of *Terasi*. Also, purified TM was used as a control to examine the extent toward which changes in the allergenicity of shrimp TM in the manufacture of *Terasi* using a starter had affected the product. Analytical samples were then performed to a C-ELISA, where the measurement of IgE reactivity was examined by determining the inhibitory effect of *Terasi* on the reaction between the specific IgE the patients and purified TM. The protein mixture of SM was also used as a control. A decrease in the inhibition rate indicates a loss of IgE-binding ability of shrimp TM in the *Terasi*s.

Figure 4.5 showed that SM protein had similar inhibition rate with purified TM in all patients (P1–P6), which demonstrating that TM contained in SM is capable of binding specific patient's IgE potently as native TM. In addition, it was confirmed in all patient's sera that the IgE-binding ability of the shrimp meat without starter (IT) was hardly lost in the *Terasi* manufacturing process.

When SM was processed according to the process shown in **Figure 4.1**, the addition of AT and HAT to shrimp muscle led to a marked decrease in the inhibition rate as the manufacturing process progressed. Although the decreasing degree differed among patients' sera, their marked decreased due to the progress of raw material processing was observed for all sera and reached 0–30% at the final product (FP). On the other hand, the average's degree of decrease in

inhibitory rate in CT-added shrimp muscle (ITCT) was smaller (10.1%) than that of ITAT (69.4%) and ITHAT (99.9%), and the tendency was almost the same as that of shrimp muscle without starter (IT) (14.7%).

In **Figure 4.4**, the degree of protein hydrolysis of the starter adding *Terasi* products (ITCT, ITAT, and ITHAT) was higher than that of IT (no starter), whereas the inhibition rate of ITCT was similar to that of IT, as in **Figure 4.5**. In contrast, ITAT and ITHAT, whose degree of protein hydrolysis was similar to that of ITCT, showed a very strong decrease in inhibition rate during the manufacturing process; which indicates that the marked loss (degradation) of the IgE-binding site of TM in the raw materials occurred due to the addition of AT and HAT during each manufacturing process, regardless of the hydrolysis rate.

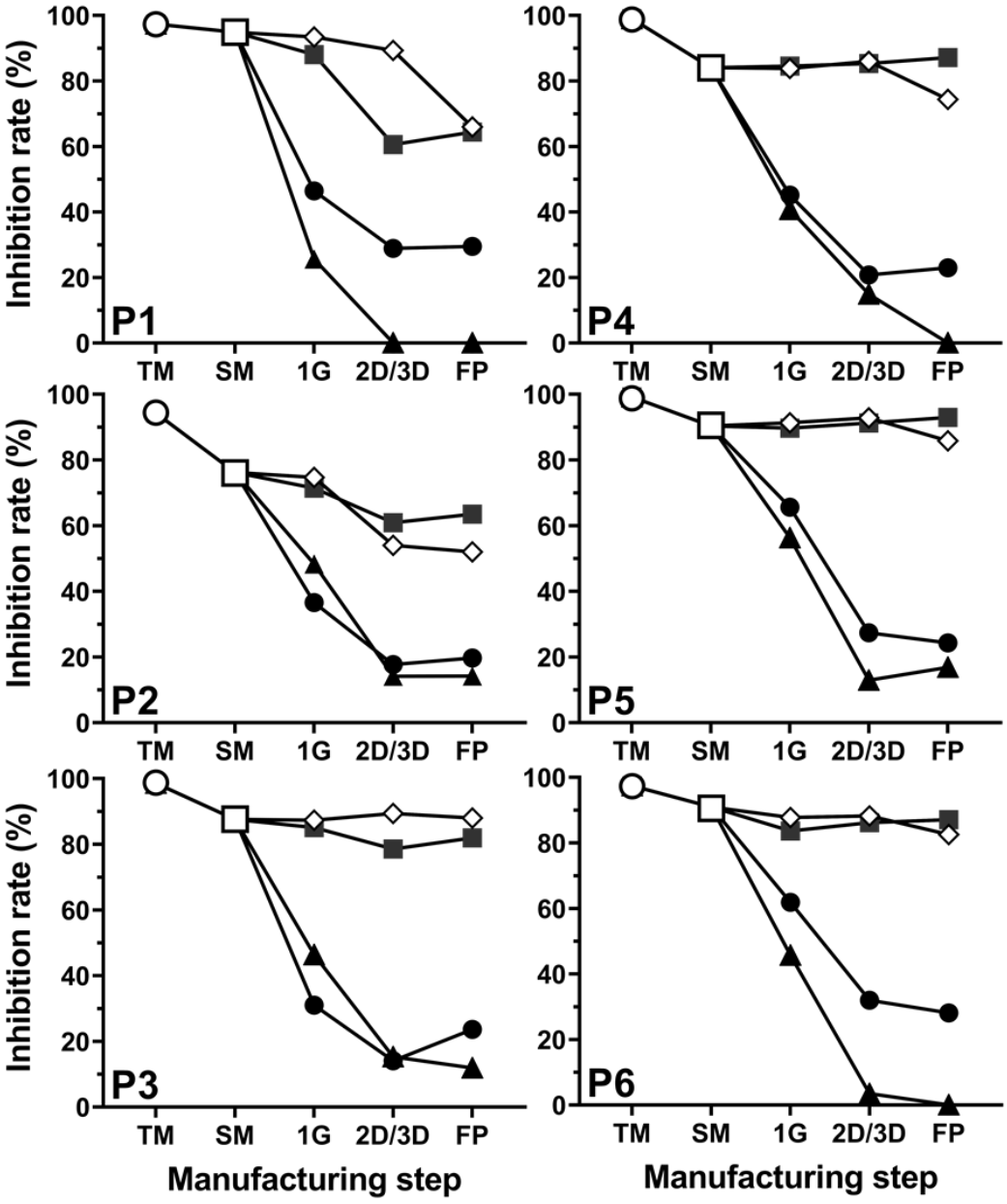

Figure 4.5. IgE reactivity of shrimp TM during the manufacturing of *Terasi* with starter. TM (○: shrimp tropomyosin), SM (□: shrimp muscle), 1G: 1st grinding, 3D: 3rd drying after the last fermentation step for IT (◊), 2D: 2nd drying after the last fermentation step for ITCT (■), ITAT (●) and ITHAT (▲), FP: the final product. All samples were mixed with sera from shrimp-allergic patients (P1–P6) and subjected to C-ELISA.

In the effect of the starter addition shown in **Figure 4.5** (particularly, P2, P4, and P5), HAT which suffered from strong heat stress resulted in the same decrease in IgE-binding ability of *Terasi* as that of AT, which decreased mainly between 1G and 2D, including the fermentation process. These results suggest that AT and HAT acted as a nutrient source for fermentation microorganisms rather than as a source of proteolytic enzymes and microorganisms, suggesting that HAT may have promoted microbial fermentation in the *Terasi* manufacturing process. As previously studied the proteolytic activities of bacteria were primarily responsible for their contribution to the fermentation of shrimp sauce (Duan *et al.*, 2016).

As the highlight of this section, the most effective decrease in the allergenicity of TM was found in ITHAT, compared to other *Terasi*; the IgE-binding ability of TM was diminished in all of the C-ELISA, and completely disappeared in the sera of P1, P4, and P6. The results of **Figure 4.5** indicates that the combination between Isazaami shrimp with HAT as a starter (of the backslopping method) is an effective manner to produce excellent ingredients of *Terasi* as a low-allergenic seafood.

4.3.5 Relationship between the IgE-binding ability and the degree of protein hydrolysis in *Terasi* with starter

Generally, digested proteins are less allergenic than native proteins since structural breakdown of allergenic proteins is associated with the loss of IgE-binding sites. However, ITCT that showed clear degradation of all proteins (**Figure 4.3**) and the loss of TM by SDS-PAGE analysis (**Figure 4.4**) did not significantly lose IgE-binding ability as the same as in IT (**Figure 4.5**). This would indicate that the IgE-binding domain of TM is not destroyed in IT and ITCT. Given this fact, the correlation between IgE-binding ability and protein hydrolysis was evaluated in ITAT and ITHAT after omitting the CT and ITCT.

The combination between the protein hydrolysis data of *Terasi*'s final product (**Figure 4.3**) with the data **Figure 4.5**. are illustrated in **Figure 4.6**, which indicated that the enhanced degree of digestion due to AT and HAT shown in **Figure 4.3** contributes to the low allergenicity of *Terasi* products. That is, a strong negatively correlation was found between the protein hydrolysis and IgE reactivity in *Terasi* in all patient sera. This result indicates that the degradation of the IgE-binding domain in TM could be evaluated by measuring the hydrolysis reaction of the total proteins in case of the starter-added *Terasi*. It is probable that the measurement of the whole protein hydrolysis is effective for allergenicity evaluation of protein hydrolysates such as *Terasi* under certain conditions, and it could be used as a screening index. Protein hydrolysates have various benefits over intact proteins, including better solubility, in addition to having less antigenicity (Yang *et al.*, 2020).

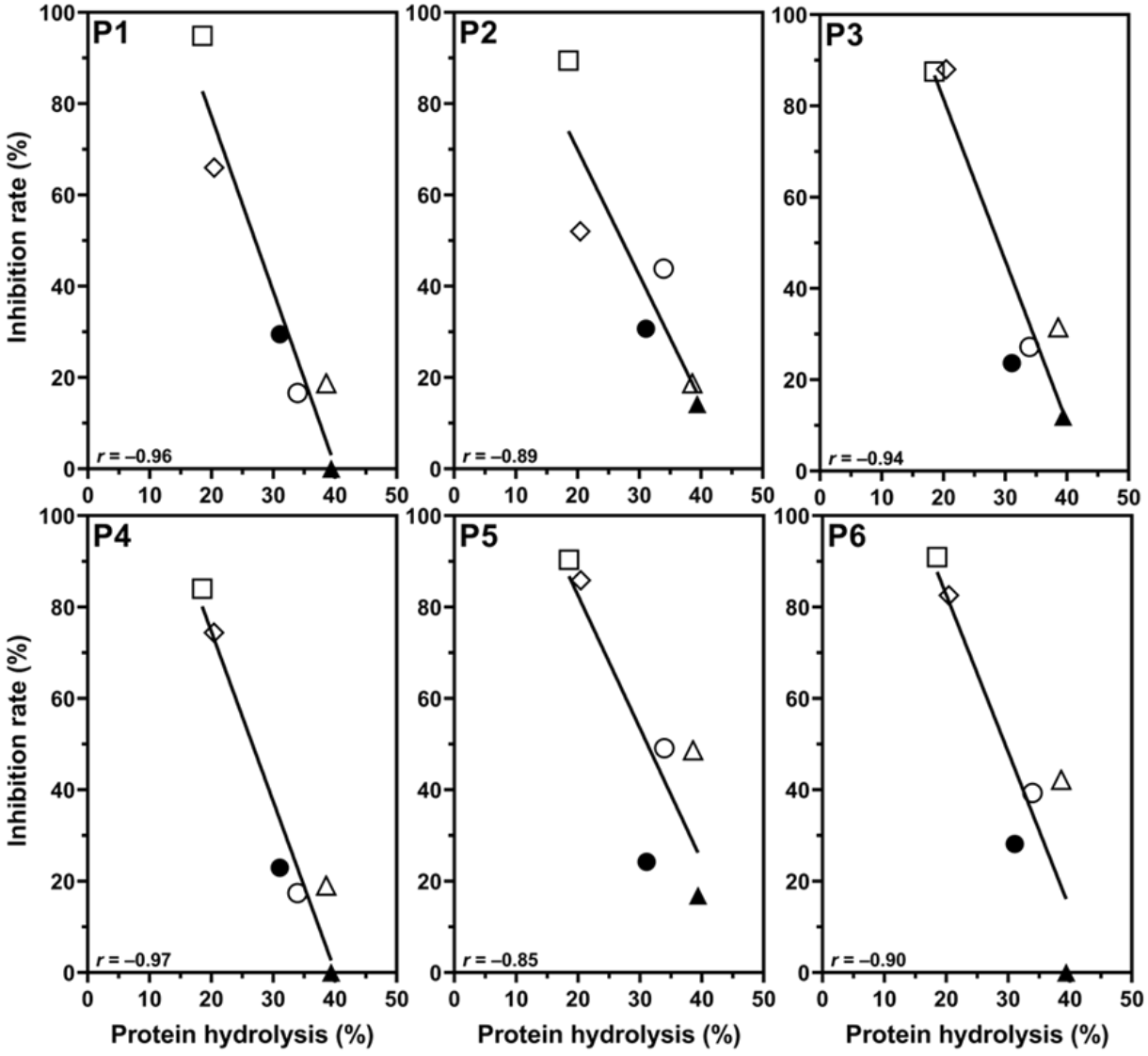

Figure 4.6. Relationship between the degree of protein hydrolysis with IgE reactivity of shrimp TM in Terasi with starter. SM (□: shrimp muscle), IT (◊), AT (○), HAT (Δ), ITAT (●), and ITHAT (▲). r value indicates the Pearson correlation coefficient (r < –0.5: strong negative correlation; r > 0.5: strong positive correlation). P1–P6: shrimp-allergic patients' sera.

4.4. Conclusion

This chapter demonstrated that backslopping method using *Terasi* products is an effective manner to produce low allergenic *Terasi* by inducing reduction of IgE-binding ability of TM. Addition of the starter accelerated the fermentation of the raw material, effectively promoting the degradation of the shrimp protein and the reduction of the IgE-binding ability of TM. However, the backslopping effect was dependent on the type of *Terasi* used as a starter, and the commercial *Terasi* used in this study did not contribute to the allergenicity reduction of the final product. Interestingly, *Terasi* added as a starter would have acted primarily as a nutrient source to promote microbial fermentation rather than as a source of fermenting microorganisms and endogenous proteases.

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CHAPTER V

GENERAL DISCUSSION

Food allergy have been a major global dietary concern. A dramatic rise in the prevalence of food allergies over the past few years has left 10% of the population at risk (Seth *et al.*, 2020). Various allergic symptoms, such as nausea, vomiting, diarrhea, flushing, urticaria, angioedema, acute bronchospasm, hypotension, dizziness, and occasionally lethal manifestations like anaphylactic shock, can result from food allergies (Florsheim *et al.*, 2021; Renz *et al.*, 2018). One of the main causes of serious allergic responses is shrimp allergy, and this has the effect of increasing the use of healthcare (Ross *et al.*, 2008; FAO/WHO, 2021). Only 42% of adults and 61% of children with shrimp allergy reported getting a doctor-confirmed diagnosis, despite the fact that over 50% of patients with shrimp allergy suffer at least one lifetime emergency department visit connected to a food allergy (Gupta *et al.*, 2019; Wang *et al.*, 2020). The majority of causative of shrimp allergy is TM, an actin-binding, heat-stable protein present in both muscle and non-muscle cells (Shanti *et al.*, 1993; Faber *et al.*, 2017). Given the potentially fatal consequences of inadvertent exposure, the lack of medical confirmation of shrimp allergy is troubling. The sole management options at this time are to prevent severe reactions and use adrenaline to treat them when they do occur (Davis *et al.*, 2020). Avoidance, meanwhile, can be challenging and necessitates severe dietary restrictions (Nguyen *et al.*, 2022).

In order to reduce the immunoreactivity of shrimp TM, there was the most recent research on innovative shrimp processing techniques, such as high-frequency ultrasound, pulsed light, cold plasma, fermentation, enzymatic hydrolysis, microwave, and pressure and a mix of different processing techniques (Dong and Raghavan, 2022). Among them, information on fermented shrimp products is relatively less than those of processing method; therefore, the present study is mainly focused on using *Terasi,* Indonesian traditional fermented shrimp paste

as an evaluation model from food allergy's perspective. In addition, *Terasi* is used as a seasoning ingredient in some dishes which easily leads to accidentally consume for people suffer from shrimp allergy. It's difficult to avoid hidden allergic substances, and shrimp as a food allergen possesses significant hazards to human health (Nonthawong *et al.*, 2019). As of 2022, despite extensive daily consumption of both shrimp and *Terasi*, Indonesia has no published epidemiological data regarding shrimp allergies (Lee *et al.*, 2013) and the food allergy labeling system is still in its infancy. It is evident from the previous that understanding the low allergenicity of shrimp TM present in *Terasi* is crucial for further talks regarding the safety of shrimp intake in Indonesia and neighboring countries that use a similar typical product as *Terasi*.

Three studies were carried out in this thesis; the first study (**Chapter II)** which is to evaluate the presence and safety of Indonesian commercial *Terasi* products (CTs) in the viewpoint of food allergy, continuing with the second study (**Chapter III**) the allergenicity change of shrimp TM during the *Terasi* manufacturing process, and the third study (**Chapter IV**) described that the backslopping method using a *Terasi* products as a starter is the effective method for reduction of the allergenicity in *Terasi* products.

5.1 Food safety evaluation of Indonesian commercial *Terasi*

Chapter II used 20 different brands of CTs collected from markets in Indonesia. All CTs met the Indonesian National Standardization (INS, 2016) of *Terasi* for moisture content and water activity (**Figures 2.4a** and **2.4c**). Due to protein content (**Figures 2.4b**), CTs were markedly higher compared with that of the INS, except for No. 18, 19, and 20. As compared to shrimp muscle, all CTs showed an increase in the degree of protein hydrolysis (**Figure 2.5a**), and TM band was disappeared in SDS-PAGE and immunoblotting results (**Figures 2.5b** and **2.5c**) that indicate protein completely degraded to small fragments of at least <14.4 kDa in

Terasi manufacture. These results might be related to the digestion of shrimp muscle proteins by endogenous proteases and fermenting bacteria during *Terasi* production (Sun *et al.* 2014; Kleekayai *et al.* 2016; Helmi *et al.* 2022).

The most important finding in the allergenicity survey is that the decrease in IgE-binding ability was confirmed in 20 types of commercial *Terasi*, and it clearly indicates the effectiveness of *Terasi* manufacturing at reducing the allergenicity of shrimp (**Figure 2.6**). In addition, it was suggested that it is necessary to use a mixture of serum from multiple patients, because the reactivity to the IgE TM fragment contained in the serum of shrimp-allergic patients differs among patients. On the other hand, it should be emphasized that a simple protein degradation study (**Figure 2.7**) is utterly meaningless for assessing the allergenicity of the final products. Considering the diversity of *Terasi* commercial products, it is necessarily needed that such allergenicity assessments need to be expanded regionally.

5.2 The allergenicity change of shrimp tropomyosin during the *Terasi* manufacture

Chapter III investigated the crucial step decreasing IgE reactivity of shrimp TM during the *Terasi* manufacturing process. Since the raw material and the protocol of the commercial *Terasis* in Chapter II are unknown, three kinds of shrimps (Akiami, Okiami, and Isazaami) were subjected to *Terasi* manufacture under the controlled laboratory scale. The three types of final *Terasis* products (AT: Akiami *Terasi*; OT: Okiami *Terasi*; and IT: Isazaami *Terasi*) were met the quality according to INS (2016) for moisture and protein content, and water activity (**Figures 3.2a**, **3.2b**, and **3.2c**), illustrating food safety of the *Terasi* produced in the laboratory.

The gradual degradation of shrimp TM during *Terasi* manufacture (from raw, dried to the final product) was observed in all *Terasis*, but fragmented TM having the specific IgE-binding ability still remained in the final product. When compared with three types of TM final products, only AT showed a complete disappearance of TM signal in the immunoblotting data (**Figures**

3.3 and 3.4). These results indicate that higher levels of protein hydrolysis, as well as marked TM degradation, occurred in *Terasi* produced from Akiami shrimp. Our results agree with previous opinions regarding the digestion of TM into low molecular-weight compounds during fermentation. For example, Pongsetkul *et al.* (2017) reported such a process occurring in *Acetes* samples used for *Kapii* (Thai product similar to *Terasi*) production. This immunoblotting is useful to describe the behavior of TM during *Terasi* manufacture. However, small size of TM fragments probably remained in the *Terasi* and cannot detected by immunoblotting. Therefore, C- ELISA was performed to assess the potential allergenicity of *Terasi* final product.

Chapter III (**Figure 3.5**) confirmed that the reduction in IgE-binding of raw materials and processed *Terasi* during manufacturing process using C-ELISA can lead to an understanding of the manufacturing process that contributes to the allergenicity of the final product. This result supports the validity of Chapter II discussing the potential allergenicity of product *Terasi in* terms of IgE reactivity. On the other hand, the results of C-ELISA indicated that IgE-binding loss in *Terasi* manufacture is closely related to the fermentation step inducing protein degradation, but no critical manufacturing step was found to contribute to the loss of TM IgE-binding activity. It is presumed that the following factors, strong endogenous protease and/or microbial flora having a high protein assimilation activity, are involved in the strong degradation of Akiami TM, and they could continuously reduce the allergenic properties of TM during the manufacturing process.

Based on the sensory evaluation (**Figure 3.6**), it is found that AT is the most preferred by the panelist in overall physical properties especially in taste and specific flavor over than IT and OT. Previous studies reported the the different raw material with different endogenous proteases may be attributed the characteristics of *Terasi* (Helmi *et al.,* 2022). The selection of raw shrimp inputs and the processing conditions are both important factors affecting the generation of specific *Terasi* aromas (Fan *et al.*, 2017). Overall, the finding of this second study,

AT has the lowest allergenicity of shrimp TM followed by OT and IT. Akiami (*Acetes japonicus*) is one of the representative shrimps used for *Terasi* manufacture in Indonesia (Chan, 1998). Therefore, the results of this sensory evaluation show that the *Terasi* manufactured on a laboratory scale is of comparable quality to commercial products, which indicates that the discussion in Chapter III can be implemented in the Indonesian seafood industry as a feasibility study.

It has been reported that protein hydrolysis during fermentation reduced the allergenicity of processed foods other than shrimp (El-Mecherfi *et al.*, 2020; Pi *et al.*, 2022). As in these fermented foods, the degradation of shrimp TM in *Terasi* production may involve interactions between endogenous proteases and degradation by environmental microorganisms (Anh *et al.*, 2015; and Lv *et al.*, 2020). Therefore, the next research step examined the effect of the use of fermentation starters in *Terasi* manufacture on the potential allergenicity of *Terasi* products, with understanding the combination between the raw material including its endogenous proteases and the environment created by microorganism.

5.3 Reducing potential allergenicity of *Terasi* products through the manufacturing improvement

Chapter IV revealed that the backslopping method, which adds the final fermentation product, contributes to the production of less potentially allergenic *Terasi*. The addition of selected appropriate starter accelerated the fermentation of the raw material and enhanced the degradation of proteins (**Figure 4.3**), followed by leading to the reduction of the IgE-binding ability (**Figure 4.5**) of TM in the final product. Such properties change in *Terasi* manufacture is due to the appropriate starter added to the material. Generally, in the backslopping method, the fermentation process (in the context of food microbiology) uses starter (in the form of bacteria, yeast, and mould) to augment protease enzyme activity, which ultimately improves

the nutrition and flavor of the finished product. However, even if the starter AT was heated (HAT), no difference was observed in the IgE reactivity-reducing effect (allergenicity) of the final product between the two starters (**Figure 4.5**). The reason why fermentation is promoted by the starter may be the involvement of the enzyme activity derived from the microorganisms contained in the starters, or the starter, which is already a decomposition product, may have become an assimilatory nutrient for the fermentation microorganisms. Indeed, shrimp muscle proteins are easily digested by endogenous proteases and fermenting bacteria during *Terasi* production (Sun *et al.* 2014; Kleekayai *et al.* 2016; Helmi *et al.* 2022), and the variations of bacterial community composition during fermentation of shrimp paste which impacted on the quality and safety of shrimp paste (Lv *et al.*, 2020; Li *et al.*, 2022). The reason why no difference in the starter effect between AT and HAT would be that they acted as supplement nutrients for fermenting microorganism to hydrolyse protein contained in shrimp during fermentation.

As shown in **Figure 4.6**, the loss of the IgE-binding ability of the products was able to evaluate by measuring the total protein hydrolysis in case of the starter-added *Terasi*. Therefore, although the use is limited, it is probable that the measurement of the whole protein hydrolysis is effective for allergenicity evaluation of protein hydrolysates such as *Terasi* under certain conditions, and it could be used as a screening index. Indeed, previous study stated that comparing one processing procedure to another, the combined processing treatment typically performs better at reducing food immunoreactivity (Sun *et al.*, 2022). Conclusively, the results of Chapter IV clearly demonstrate the effectiveness of the backslopping method for reducing potential allergenicity of *Terasi* product.

The following are unprecedented new findings obtained in this study, which provide scientific benefits in the field of food science in Indonesia:

a. The food allergenicity based on shrimp TM was found in Indonesian commercial *Terasi* products, which is a new information in the food research in Indonesia.
b. Akiami (*Acetes japonicus*) as shrimp raw material, in addition to manufacture high-quality Akiami *Terasi*, might even reduce shrimp allergy risk more effectively than other two shrimps (Okiami and Isazaami). This finding pointed out that appropriate selection of raw materials is important for making *Terasi* with low allergenicity.
c. It covers in great detail, to begin with the unchanges of Isazaami *Terasi*'s allergenicity and ending with the discovery of a way to combine Isazaami shrimp with an appropriate starter to create a high quality Isazaami *Terasi* products and also a low-allergenic seafood product as the same as Akiami *Terasi*. Thus, the backslopping method in *Terasi* manufacture is a workable approach to counter shrimp allergy.

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CHAPTER VI

GENERAL CONCLUSIONS AND PROSPECTIVE

This research aims to evaluate the safety of Indonesian commercial *Terasi* in the viewpoint of food allergy, changes of shrimp TM allergenicity during *Terasi* manufacture, and the effect of starter addition in *Terasi* manufacture on reduction of protein allergenic contain in shrimp. In light of these three attributes, the finding's conclusions can be drawn as follows:

1. Although the degree of decrease in IgE reactivity varied among the *Terasi* products and the potential hazard as an allergy causative food was not completely eliminated, the present study provide clearly data that a decrease in IgE-binding ability, an index of allergenicity, occurred in 20 types of commercial *Terasi*, indicating the effectiveness of *Terasi* manufacturing at reducing the allergenicity of shrimp.
2. The different raw shrimps as a main material in *Terasi* manufacture showed varying degradation of shrimp TM as a major allergen in shrimp. The important thing is the selection of Akiami shrimp is the most appropriate of *Terasi* manufacture in the viewpoint of food quality and safety, and progressive protein degradation during fermentation had no negative effect on *Terasi* food quality, suggesting that developing good-tasting *Terasi* while maintaining low allergenicity is possible.
3. This study demonstrated that specific starter in *Terasi* manufacturing via backslopping method is an effective processing method to accelerate the protein hydrolysis, and subsequently reduce the shrimp TM allergenicity. However, the effect was dependent on the type of *Terasi* used as a starter, and the commercial *Terasi* used in this study did not contribute to the allergenicity reduction of the final product. Interestingly, *Terasi*, added as a starter, would have act as a nutrient source to promote microbial fermentation rather than as a source of fermenting microorganisms and endogenous proteases. In

future, the manufacture of *Terasi* with stable quality at industrial scale need a consideration since traditional products do not always exhibit same physicochemical and microbial condition, such as using pure identified bacterial strains and/or suitable commercial proteinases.

In conclusions, *Terasi* manufactured using proper raw materials and techniques can be selected as a low allergenic seafood. It can be added to foods to boost consumer preferences for food products while also providing several health benefits to humans, particularly as a source of protein.