Title

Variation in Shrimp Tropomyosin Allergenicity during the Production of *Terasi*, an Indonesian Fermented Shrimp Paste

Authors

Ulfah Amalia[a,b], Yutaka Shimizu[a], and Hiroki Saeki[a*]

Affiliations:

[a]Faculty of Fisheries Sciences, Hokkaido University, Minato 3, Hakodate, Hokkaido 041-8611, Japan

[b]Department of Fisheries Product Technology, Faculty of Fisheries and Marine Science, Universitas Diponegoro, Semarang, 50275, Indonesia

Ulfah Amalia, e-mail: ulfah.amalia@live.undip.ac.id

Yutaka Shimizu, e-mail: Y-SIMIZU@fish.hokudai.ac.jp

Hiroki Saeki, e-mail: saeki@fish.hokudai.ac.jp

*Corresponding author:

Hiroki Saeki

Affiliation: Faculty of Fisheries Sciences, Hokkaido University, Minato 3, Hakodate, Hokkaido 041-8611, Japan. TEL: +81-138-40-5516, E-mail: saeki@fish.hokudai.ac.jp.

Abstract:

Terasi is a fermented shrimp paste in Indonesia. We examined the effect of the Terasi manufacturing process on the abundance of the allergen tropomyosin (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. The processing caused TM degradation, 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 in all meats decreased gradually during manufacturing and nearly completely disappeared in Akiami Terasi. Conclusively, Terasi production is an effective manufacturing process to reduce the IgE-binding ability of TM, and Terasi can be recognized as a low allergenic seafood when produced under an appropriate manufacturing condition.

Keywords: IgE-binding activity, low allergenic seafood, shrimp, Terasi, tropomyosin

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). Tropomyosin (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 & 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 Indonesian National Standards (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 production 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.

2. Materials and methods

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* production. 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 °C

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).

2.2. Sera of shrimp-allergic patients

The sera of six shrimp-allergic patients (P1–P6 in the Table S1) possessing IgE antibodies specific for shrimp TM were used in this study. Sera from two healthy individuals were also used as negative controls. Patient clinical data is listed in Table S1 (Supplementary data). 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 0.2% NaN_3 before being subjected to analysis. These samples were then stored at 4 °C until use. Enzyme-linked immunosorbent assays (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.

2.3. Production 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 production procedure is shown in **Fig. 1**. In detail, whole shrimp were dried at 30 °C 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 °C for 6 h, then covered with plastic food wrap and aluminum foil and incubated at 28 °C 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 °C 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 °C until further use.

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.

2.5. Preparation of shrimp tropomyosin

Shrimp tropomyosin was prepared according to the method employed by Huang et al. (2010) with a slight modification. 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. 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 °C, the supernatant was then boiled at 100 °C for 20 min and cooled to room temperature. The pH was then adjusted to 4.5 with 0.05 M HCl and the solution was kept at 4 °C for 30 min. The isoelectric precipitate thus obtained was collected by centrifugation at 21,000 g for 30 min at 4 °C and was then subjected to ammonium sulfate fractionation at 45–65% saturation. Crude TM was further purified using hydroxyapatite column chromatography (Bio-Rad, Hercules, CA). The protein concentration of purified TM thus obtained was determined using a protein assay rapid kit (Wako Pure Chemical Industries, Ltd.) using bovine serum albumin as a standard.

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 sodium dodecylsulfate (SDS) solution. In brief, each sample was put in a plastic bag and boiled at 98 °C 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 °C 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 °C for future SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The same amount of SDS-treated samples was loaded into each lane of PAGE-gels.

2.7. SDS-PAGE analysis

To investigate the degradation of shrimp TM during the production of Terasi, we visualized protein concentration 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 hours.

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 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 °C 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 °C 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.

2.9. Assessing immunoreactivity using competitive ELISAs

Next, competitive enzyme-linked immunosorbent assays (C-ELISAs) were carried out to investigate changes in the IgE-binding activity of TM contained in shrimp meat after Terasi production. Individual shrimp-allergic patients' sera used in the C-ELISA trials are listed in

Table 1 (Supplementary data). First, we suspended an analytical sample, 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 °C 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 °C overnight. After being washed with TBS-T, each well was coated with blocking buffer (1% casein in TBS-T) at 37 °C 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 °C for 2 h, 70 µl of each protein-serum mixture was placed onto the TM coated ELISA plate and incubated again at 37 °C 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 °C 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 °C 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 absorbance 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 absorbance of each patients' sera without inhibitors, and Y and Z are the absorbance of the patients' sera and healthy individual's serum with the inhibitors, respectively.

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). We used 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."

2.11. Statistical analysis

All data obtained from 3–4 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 9.2.0 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. Results and discussion

3.1. Evaluation of physicochemical properties of Terasi

Figure 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 Fig. 2 (A), 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 – 50.3%, as shown in Fig. 2 (B). 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 A_W 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 A_W of Terasi can suppress the growth of pathogenic and spoilage microorganisms in the final product (Van-Thuoc et al., 2021). Moreover, controlling A_W can also control the growth of halophilic bacteria that are useful during Terasi production (Pongsetkul et al., 2017). As shown in Fig. 2 (C), the A_W of the products were 0.68 for AT,

286 0.61 for OT, and 0.69 for IT; all of these, including CT (0.73) met the INS. These results clearly
287 illustrate the safety of the Terasi produced in the laboratory in this study.
288
289 *3.2. Protein degradation and the loss of TM during Terasi production*
290 Next, we investigated the changes in whole protein and TM composition of the three types
291 of Terasi prepared in the laboratory using SDS-PAGE analysis and immunoblotting using TM-
292 specific IgGs (**Fig. 3**). CT and native TM were used as references. No protein band and no TM
293 signal were observed for the CT SDS-PAGE and Immunoblotting assays, respectively. In
294 contrast, we found that protein degradation occurred for each of the three types of Terasi
295 produced in the lab, but fragmented proteins still remained in the final product. In addition,
296 only the AT sample showed a complete disappearance of TM signal in the immunoblotting
297 data. These results suggest that higher levels of protein hydrolysis, as well as marked TM
298 degradation, occurred in Terasi produced from Akiami shrimp. Our results agree with previous
299 opinions regarding the digestion of TM into low molecular-weight compounds during
300 fermentation. For example, Pongsetkul et al. (2017) reported such a process occurring in *Acetes*
301 samples used for Kapii (Thai product similar to Terasi) production.
302
303 *3.3. Change in the IgG and IgE-binding abilities of shrimp TM during Terasi production*
304 To investigate TM degradation during the Terasi manufacturing process, we obtained
305 shrimp meat samples after each fermentation-drying step of the Terasi manufacturing process
306 (as described in 3.1). These samples were then subjected to SDS-PAGE analysis followed by
307 immunoblotting using anti-TM rabbit IgG. As shown in Fig. 4, TM was present in raw Akiami
308 shrimp, still existed after the first drying process, but disappeared after the second drying step,
309 after which it was not detectable. In contrast, the OT and IT (Fig. 4) samples showed clear TM
310 blotting signals after each step that remained until the final product. These results agreed with

311 the results of SDS-PAGE analysis (Fig. 3); that is, TM in Akiami was lost during
312 manufacturing, whereas a part of TM of Okiami and Isazaami remained in the final products.
313 However, immunoblotting using IgG (Fig. 4) could not explain the behavior of TM as an
314 allergen during manufacturing.

315 We also examined the reduction in IgE-binding of processed Terasi during manufacturing
316 using C-ELISA to estimate the change in the allergenicity of TM. We measured TM IgE-
317 binding ability in the processed materials by determining the inhibitory effect of the sample
318 against the reaction between IgE and the six shrimp-allergic patients' sera and native TM; here,
319 lower inhibition rate indicated decreased IgE-binding of TM. As shown in Fig. 5, the inhibition
320 rate of the AT and OT shrimp samples decreased gradually during the manufacturing process,
321 indicating that the IgE-binding ability of TM was progressively more impaired with every new
322 stage of the Terasi manufacturing process, regardless of which patients' sera was used for
323 determination of inhibition. However, when the three types of Terasi were compared to each
324 other, AT showed the largest decrease in the inhibition rate as measured by C-ELISA, which
325 showed a high degree of protein degradation, as shown in Fig. 4. Overall, during the
326 manufacturing process the inhibition rates of AT and OT decreased to 17% and 66% in P1;
327 36% and 66% in P2; 27% and 61% in P3; 17% and 33% in P4; 49% and 67% in P5, and 39%
328 and 61% in P6, respectively. The greatest decreases in inhibition rate for both samples were
329 found in P1 and P3, stages which involved the loss of large amounts of IgEs specific to
330 crustaceans (Table S1: Supplementary data). In contrast, the inhibition rate in the IT production
331 process showed a smaller decrease of 66% in P1 and 88% in P3. The fact that the similar trends
332 were observed in all the six patients' sera despite their different specific IgE contents indicates
333 that the most effective in IgE-binding loss was obtained in AT. Shrimp TM of Akiami was
334 significantly diminished in immunoblotting assay (Fig. 4). This indicated that IgE-binding loss
335 in Terasi production 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 Fig. 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 (Fig. 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 Fig. 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 Fig. 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 et al., 2016), and casein in fermented milk (Ahmadova et al., 2013). 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). In a preliminary experiment, the authors 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 (Supplementary figure: S1), indicating that the intrinsic proteolytic activity originated in the raw material. 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 production, 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.

3.4. Sensory evaluation of Terasi

Figure 6 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 Terasis. The color of both the AT and IT Terasis 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 (Fig. 4). In contrast, the texture and taste were rated as clearly different among all Terasis; 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

386 glutamic acid and aspartic acid (Hajeb and Jinap, 2015). According to the results of the sensory
387 evaluation (Fig. 6), AT showed the most advanced proteolysis among the three products and
388 was the “highest quality” Terasi of the three produced in the lab. In addition, the IgE reactivity
389 of TM was greatly reduced during the AT manufacturing process, as described in Fig. 5. This
390 relation between quality and proteolysis in Terasi suggests that Terasi quality may be correlated
391 with the loss of allergenicity. That is, Terasi produced by an ideal manufacturing process with
392 abundant proteolysis would be a low-allergic seafood.

393

394 **4. Conclusion**

395 This study clearly demonstrated that Terasi manufacturing is an effective processing method
396 for reducing the IgE reactivity of shrimp TM, which was diminished as manufacturing
397 progressed. The selection of Akiami shrimp is the most appropriate of Terasi production in the
398 viewpoint of food quality and safety. This suggests that this traditional Indonesian food can be
399 recognized as a low allergenic seafood. The health benefit thus obtained would be mainly due
400 to the degradation of IgE epitope in TM during processing. Moreover, progressive protein
401 degradation during fermentation had no negative effect on Terasi food quality, suggesting that
402 developing good-tasting Terasi while maintaining low allergenicity is possible.

403

404 **Abbreviations**

405 AT: Akiami Terasi; ELISA: enzyme-linked immunosorbent assay; CT: Indonesian commercial
406 Terasi; IgE: immunoglobulin E; IgG: immunoglobulin G; IT: Isazaami Terasi; ME:
407 mercaptoethanol; OT: Okiami Terasi; PAGE: polyacrylamide gel electrophoresis; SDS:
408 sodium dodecylsulfate; TM: tropomyosin.

409

Credit authorship contribution statement

Ulfah Amalia: Conceptualization, resources, writing-original draft preparation, investigation, data curation, formal analysis. **Yutaka Shimizu:** project administration, methodology, writing-review & editing. **Hiroki Saeki:** Supervision, validation, resources, writing-review & editing, data curation, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to Dr. Kazuhiko Watanabe (Watanabe Kazuhiko Pediatric Clinic, Sapporo, Japan) for providing access to the sera of shrimp-allergic patients. We also thank Dr. Atshushi Yamaguchi (Graduate School of Fisheries Sciences, Hokkaido University) for identifying the species of Okiami shrimp (*Euphasia pacifica*) used in this study. Finally, we would like to thank students from the Graduate School of Fisheries Sciences (Hokkaido University) who participated in the *Terasi* sensory evaluation. This research was carried out by the ordinary budget for education and research supported from Hokkaido University. Universitas Diponegoro, Indonesia, was also acknowledged for PhD scholarship to Ulfah Amalia.

References

Ahmadova, A., El-Ghaish, S., Choiset, Y., Rabesona, H., Drouet, M., Chobert, J-M., Kuliev, A. A., & Haertlé, T. (2013). Modification of IgE binding to β- and αS1-caseins by proteolytic activity of *Lactobacillus helveticus* A75. *Journal of Food Biochemistry*, *37*, 491–500. https://doi.org/10.11/j.1745-4514.2012. 00664.x

Akonor, P.T., Ofori, H., Dziedzoave, N. T., & Kortei, N. K. (2016). Drying characteristics and physical and nutritional properties of shrimp meat as affected by different traditional drying techniques. *International Journal of Food Science*, Article ID 7879097, 5 pages http://dx.doi.org/10.1155/2016/7879097

Angelis, M. D., Rizello, C.G., Scala, A., Simone, C. D., Farris, G. A., Turrini, F., & Gobbett, M. (2007). Probiotic preparation has the capacity to hydrolyze proteins responsible for wheat allergy. *Journal of Food Protection*, *70*, 135–144. http://doi.org/10.4315/0362-028X-70.1.135

Anh, D.B.Q., Mi, N. T.T., Huy, D. N. A., & Hung, P. V. (2015). Isolation and optimization of growth condition of *Bacillus* sp. from fermented shrimp paste for high fibrinolytic enzyme production. *Arabian Journal for Science and Engineering*, *40*, 23–28. https://doi.org/10.1007/s13369-014-1506-8

Ambarita, M.T.D., Raes, K., & Bruno, D. M. (2019). The sensory acceptance and profile of Indonesian sambal-*Terasi*: impact of *Terasi* type and concentration. *International Journal of Gastronomy and Food Science*, *16*, 100149. https://doi.org/10.1016/j.ijgfs.2019.100149

AOAC. (2005). Official Methods of Analysis of International, 18th ed. AOAC International, Gaithersburg, MD, USA.

454 Bloom, K.A., Huang, F. R., Bencharitiwong, R., Bardina, L., Ross, A., Sampson, H. A., &
455 Nowak-Wezgrzyn, A. (2015). Effect of heat treatment on milk and egg proteins
456 allergenicity. *Pediatric Allergy and Immunology*, *25*, 740–746.
457 https://doi.org/10.1111/pai.12283

458 Brandt, O., M., Fujimura, R., W., and Finlayson, B., J. (1993). Use of *Neomysis mercedis*
459 (Crustacea: Mysidacea) for Estuarine Toxicity Tests. *Transactions of the American*
460 *Fisheries Society*, *122*, 279–288.

461 Breiteneder, H & Clare Mills, E. N. (2005). Plant food allergen-structural and functional
462 aspects of allergenicity. *Biotechnology Advances*, *23*, 395–399.
463 https://doi.org/10.1016/j.biotechadv.2005.05.004

464 Bu, G., Luo, Y., Chen, F., Liu, K, & Zhu, T. (2013). Milk processing as a tool to reduce cow's
465 milk allergenicity: A mini review. *Dairy Science and Technology*, *93*, 211–223.
466 https://doi.org/10.1007/s13594-013-0113-x

467 Chan, T. Y. (1998). Shrimps and Prawns in Carpenter, K. E. and Niem, V. H. (Ed.), FAO
468 Species identification guide for gishery purposes the living marine resources of the
469 Western Central Pacific Volume 2. Cephalopod, crustaceans, holothurians and sharks (pp.
470 851–971). Rome: Food and agriculture organization of the United Nations.

471 Chukeatirote, E., Arfarita, N., Niamsup, P., & Kanghae, A. (2015). Phenotypic and genetic
472 characterisation of Bacillus species exhibiting strong proteolytic activity isolated from
473 Terasi. An Indonesian fermented seafood product. *Journal of Northeast Agricultural*
474 *University*, *22*, 15–22. https://doi.org/10.1016/S1006-8104(16)30014-9

475 Ehn, B. M., Allmere, T., Telemo, E., Bengtsson, U & Ekstrand, B. (2005). Modification of IgE
476 binding to â-lactoglobulin by fermentation and proteolysis of cow's milk. *Journal of*
477 *Agricultural and Food Chemistry*, *53*, 3743−3748. https://doi.org/10.1021/jf048121w

478 Fan, Y., Yin, L., Xue, Y., Zhaojie, L., Hu, H., & Chang, X. (2017). Analyzing the flavor
479 compounds in Chinese traditional fermented shrimp pastes by HS-SPME-GC/MS and
480 electronic nose. *Journal of Ocean University of China*, 16, 311–318.
481 https://doi.org/10.1007/s11802-017-3194-y

482 FAO [Food Agricultural Organization]. (2021). Overview: Major Trends and Issues in *FAO*
483 *Yearbook. Fishery and Aquaculture Statistics 2019.* Retrieved from
484 https://www.fao.org/3/cb7874t/cb7874t.pdf. Accessed December 28, 2021

485 Fu, L., Wang, J., Ni, S., Wang, C., & Wang, Y. (2018). Identification of allergenic epitopes
486 and critical amino acids of major allergens in Chinese shrimp (*Penaeus chinensis*) by
487 immunoinformatics coupled with competitive-binding strategy. *Journal of Agricultural and*
488 *Food Chemistry*, *66*, 2944−2953. https://doi.org/10.1021/acs.jafc.7b06042

489 Hajeb, P., & Jinap, S. (2012). Fermented shrimp products as source of umami in Southeast
490 Asia. *Journal of Nutrition and Food Science S10:006.* https://doi.org/10.4172/2155-
491 9600.S10-006

492 Hajeb, P., & Jinap, S. (2015). Umami taste components and their sources in Asian foods.
493 *Critical Reviews of Food Science and Nutrition*, *55*, 778–791. https://doi.org/10.1080/
494 10408398.2012.678422

495 Hua, Q., Gao, P., Xu, Y., Xia, W., Sun, Y., & Jiang, Q. (2020). Effect of commercial starter
496 cultures on the quality characteristics of fermented fish-chili paste. *LWT - Food Science and*
497 *Technology*, *122*, 109016. https://doi.org/10.1016/j.lwt.2020.109016

498 Huang, Y. Y., Liu, G. M., Cai, Q., F., Weng, W. Y., Maleki, S., Su, W., J., & Cao, M. J. (2010).
499 Stability of major allergen tropomyosin and other food proteins of mud crab (Scylla serrata)
500 by in vitro gastrointestinal digestion. *Food and Chemical Toxicology*, *48*, 1196–1201.
501 https://doi.org/10.1016/j.fct.2010.02.010

502 Indonesian National Standard [INS]. (2016). SNI 2716: 2016 Shrimp Paste. ICS 67.120.30.
503 SNI 2716:2016 (*In Indonesian language*).

504 Kim, S. M., Park, J. G., Kim K. B. W. R., Saeki, H., Nakamura, A., Lee, J. W., Byun, M. W.,
505 & Ahn, D. H. (2008). Changes in the allergenicity of saeujeot by fermentation. *Food*
506 *Science and Biotechnology*, *17*, 919–924.

507 Kobayashi, T., Kajiwara, M., Wahyuni, M., Kitakado, T., Hamada-Sato, N., Imada, C., &
508 Watanabe, E. (2003). Isolation and characterization of halophilic lactic acid bacteria
509 isolated from 'terasi' shrimp paste: a traditional fermented seafood product in Indonesia.
510 *Journal of General and Applied Microbiology*, *49*, 279–86.
511 https://doi.org/10.2323/jgam.49.279

512 Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of
513 bacteriophage T4. *Nature*, *227*, 680-685.

514 Lee, A. J., Thalayasingam, M., & Lee, B. W. (2013). *Food allergy in Asia: how does it*
515 *compare? (Current Review). Asia Pacific Allergy*, *3*, 3-14.
516 https://doi.org/10.5415/apallergy.2013.3.1.3

517 Lv, X., Li, Y., Cui, T., Sun, M., Bai, F., Li, X., Li, J., & Yi, S. (2020). Bacterial community
518 succession and volatile compound changes during fermentation of shrimp paste from

519 Chinese Jinzhou region. *LWT-Food Science and Technology*, *122*, 108998.
520 https://doi.org/10.1016/j.lwt.2019.108998.

521 Nicol, S., & Endo, Y. (1999). Review: Krill fisheries: Development, management and
522 ecosystem implications. *Aquatic Living Resources*, *12*, 105–120.

523 Park, J.G., Saeki, H., Nakamura, A., Kim, K. B. W. R., Lee, J. W., Byun, M. W., Kim, S. M.,
524 Lim, S. M., & Ahn, D. H. (2007). Allergenicity changes in raw shrimp (*Acetes japonicus*)
525 and *saeujeot* (salted and fermented shrimp) in cabbage *kimchi* due to fermentation condition.
526 *Food Science and Biotechnology*, *16*, 1011–1017.

527 Pongsetkul, J., Benjakul, S., Vongkamjan, K., Sumpavapol, P., & Osaka, K. (2017).
528 Microbiological and chemical changes of Shrimp Acetes vulgaris during Kapi production.
529 *Journal of Food Science and Technology*, *54*, 3473–3482. https://doi.org/10.1007/s13197-
530 017-2804-4

531 Rao, S. P. V., Rajagopal, D., & Ganesh, K. A. (1998). B- and T-cell epitopes of tropomyosin,
532 the major shrimp allergen. *Allergy*, *53*, 44–47.

533 Reese, G., Jeoung, B. J., Daul, C. B., & Lehrer, S. B. (1997). Characterization of recombinant
534 shrimp allergen pen a 1 (tropomyosin). *International Archive of Allergy and Immunology*,
535 *113*, 240–242. https://doi.org/10.1159/000237558

536 Reese, G., Ayusho, R., & Lehrer, S. B. (1999). Tropomyosin : an invertebrate pan-allergen.
537 *International Archives of Allergy and Immunology*, *119*, 247–258.
538 https://doi.org/10.1159/000024201

539 Saeki, H. (2018). Seafood allergy. In M. L. Bari & K. Yamazaki (Eds.), Seafood Safety and
540 Quality (pp. 201–223). CRC Press.

541 Seo, S. H., & Cho, S. J. (2016). Changes in allergenic and antinutritional protein profiles of

soybean meal during solid-state fermentation with *Bacillus subtilis*. *LWT-Food Science and Technology*, *70*, 208–212. https://doi.org/10.1016/j.lwt.2016.02.035

Shanti, K. N., Martin, B. M., Nagpal, S., Metcalfe, D. D., & Rao, P. V. (1993). Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *Journal of Immunology (Baltimore, Md.: 1950), 151,* 5354–5363. http://doi.org/10.1111/j.1440-1592.2004.00344.x

Statistic Indonesia [SI]. (2021). *Terasi consumption.* (1) Retrieved from https://www.bps.go.id/indicator/5/2090/1/rata-rata-konsumsi-perkapita-seminggu-di-daerah-perkotaan-dan-pedesaan-menurut-komoditi-makanan-dan-golongan-pengeluaran-per-kapita-seminggu. html. (2) Retrieved from https://www.bps.go.id/indikator/view_data_pub/0000/api_pub/dDZNdkh0dEUwRENsOVhnL1phWTZKUT09/da_05/2. Accessed December 29, 2021 (*In English* available)

Storebakken, T. (1988). Krill as apotential feed source for salmonids. *Aquaculture*, *70*, 193–205. https://doi.org/10.1016/0044-8486(88)90096-8

Usui, M., Harada, A., Ishimaru, T., Sakumichi, E., Saratani, F., Minami, C. Azakami, H., Miyasaki, T., & Hanaoka, K. (2013). Contribution of structural reversibility to the heat stability of the tropomyosin shrimp allergen. *Bioscience, Biotechnology, and Biochemistry*, *77*, 948–953. https://doi.org/10.1271/ bbb.120887

Van-Thuoc, D., Loan, T. T., & Tra, N. T. (2021). Accumulation of ectoines by halophilic bacteria isolated from fermented shrimp paste: an adaptation mechanism to salinity, temperature, and pH stress. *Current Microbiology, 78*, 2355–2366. https://doi.org/10.1007/s00284-021-02481-1

Yang, A., Zuo, L., Cheng, Y., Wu, Z., Li, X., Tong, P., & Chen, H. (2018). Degradation of major allergens and allergenicity reduction of soybean meal through solid-state

566 fermentation with microorganisms. *Food and Function*, *9*, 1899–909.
567 https://doi.org/10.1039/C7FO01824J

568

Figure captions

Fig. 1. Outline of Terasi manufacturing process in the laboratory.

Fig. 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 charts indicate statistically significant differences at each *Terasi* were assessed by the Tukey-Multiple Comparison at $p<0.05$.

Fig. 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: Fig. 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.

Fig. 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

590 (2G), second fermentation (2F), third drying (3D), third grinding (3G), and final product (T:
591 Terasi).

592

593 **Fig. 5. Change in the IgE-binding ability of shrimp tropomyosin during Terasi**
594 **production.**

595 Samples of raw material (R), processed meat from the 1st drying (1D), 2nd drying (2D), 3rd
596 drying process (3D), and the final product (T) taken during the Terasi manufacturing process
597 for AT (Δ); OT (○), and IT (◊) shrimp types, as described in Fig. 4. Each sample was mixed
598 with shrimp-allergic patients' sera (P1-P6) and subjected to C-ELISA analysis. AT: Akiami
599 Terasi; OT: Okiami Terasi; IT: Isazaami Terasi; and TM: tropomyosin as an inhibitor control.

600

601 **Fig. 6. Food quality of Terasi produced in the laboratory.**

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

607

608

609

610

611

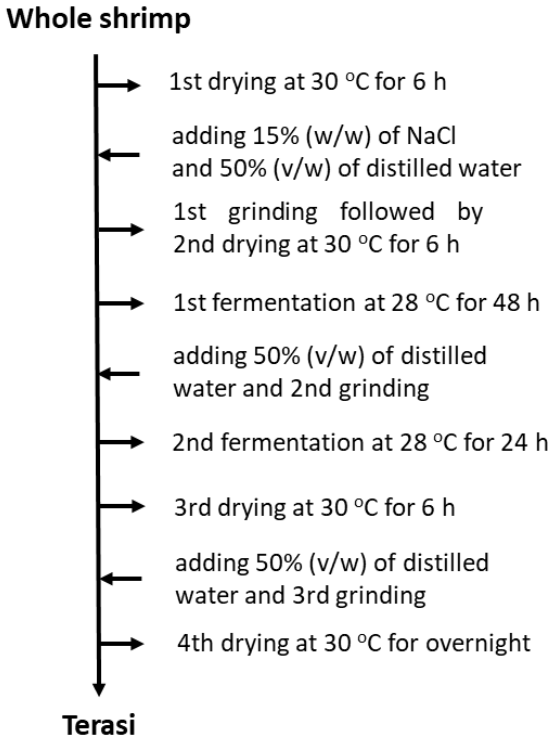


Fig. 1. (Amalia et al.)

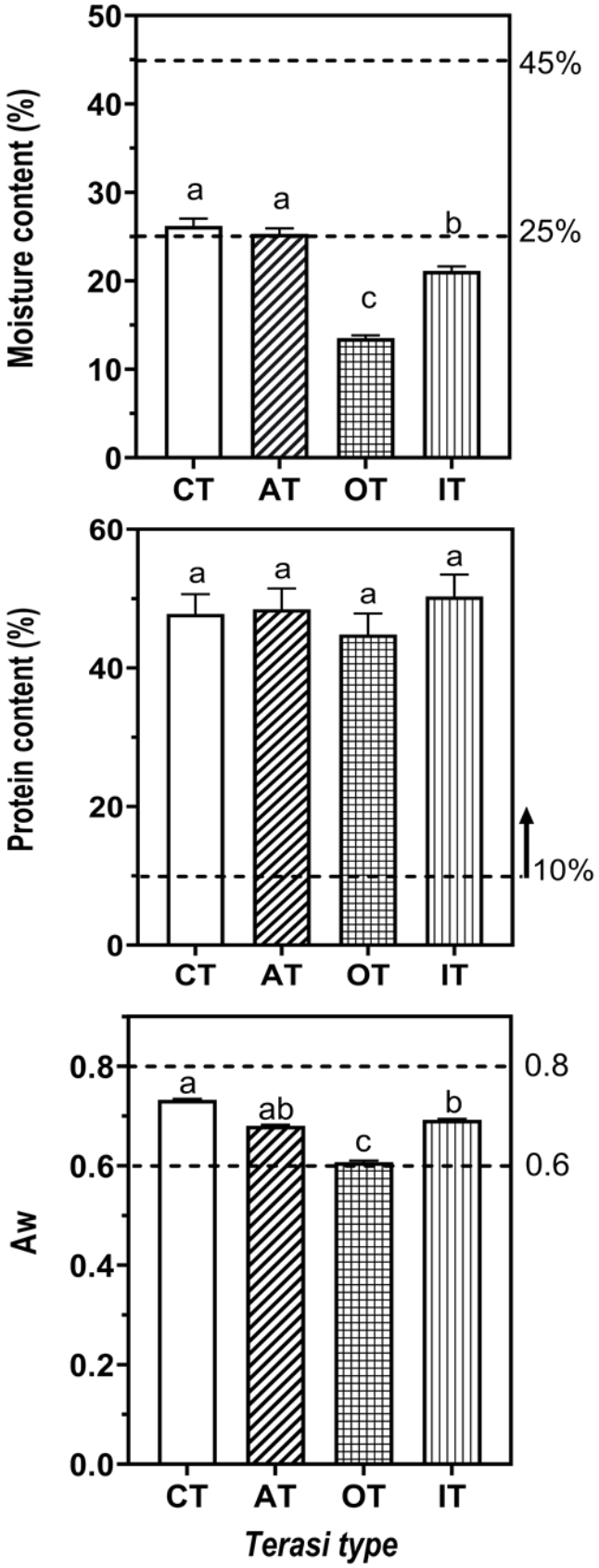


Fig. 2. (Amalia et al.)

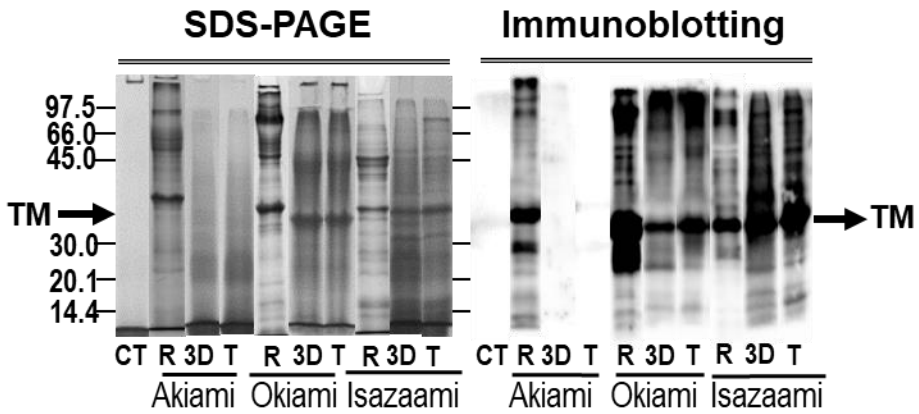


Fig. 3. (Amalia et.al)

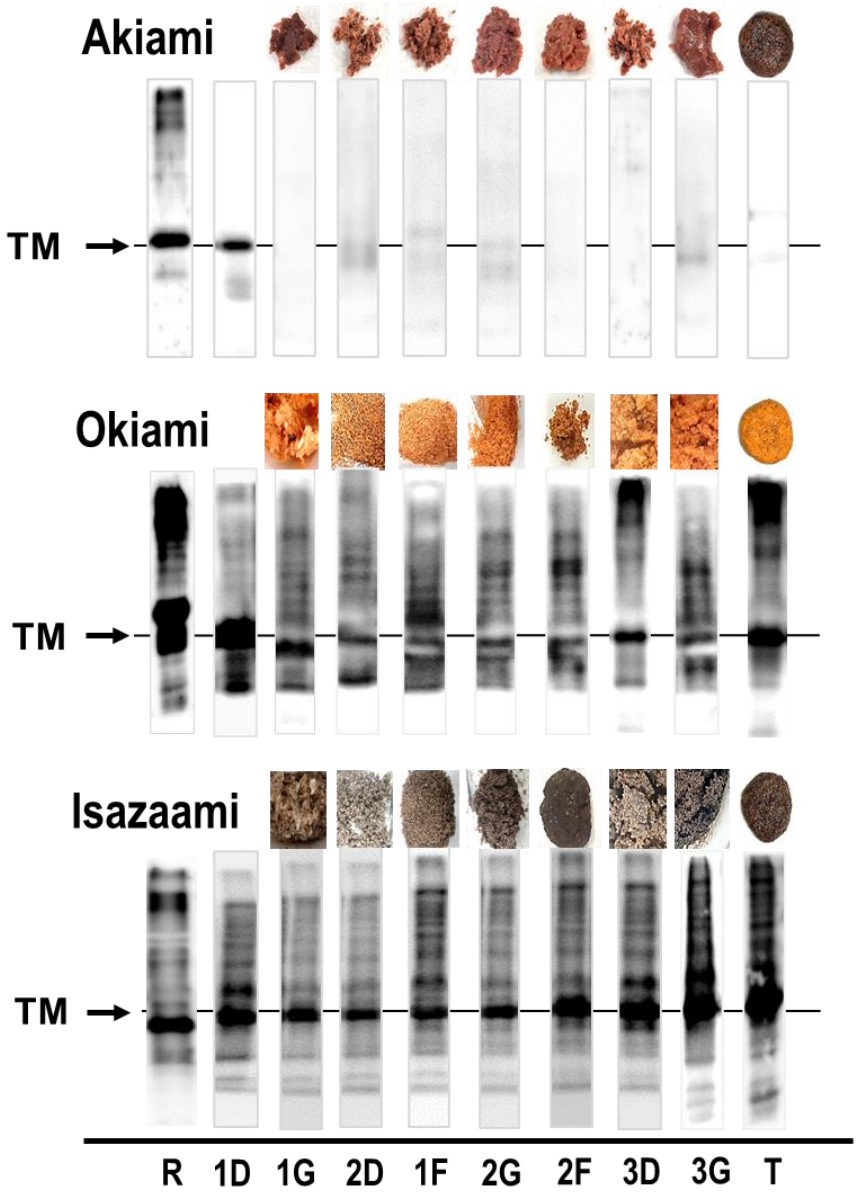


Fig. 4. (Amalia et.al)

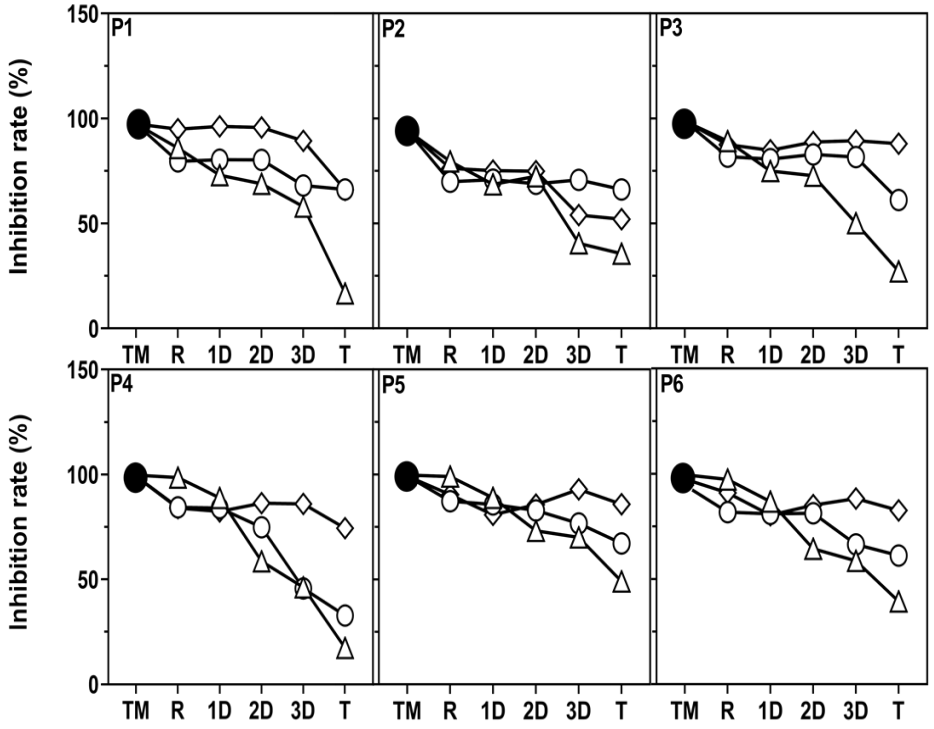


Fig. 5. (Amalia et.al)

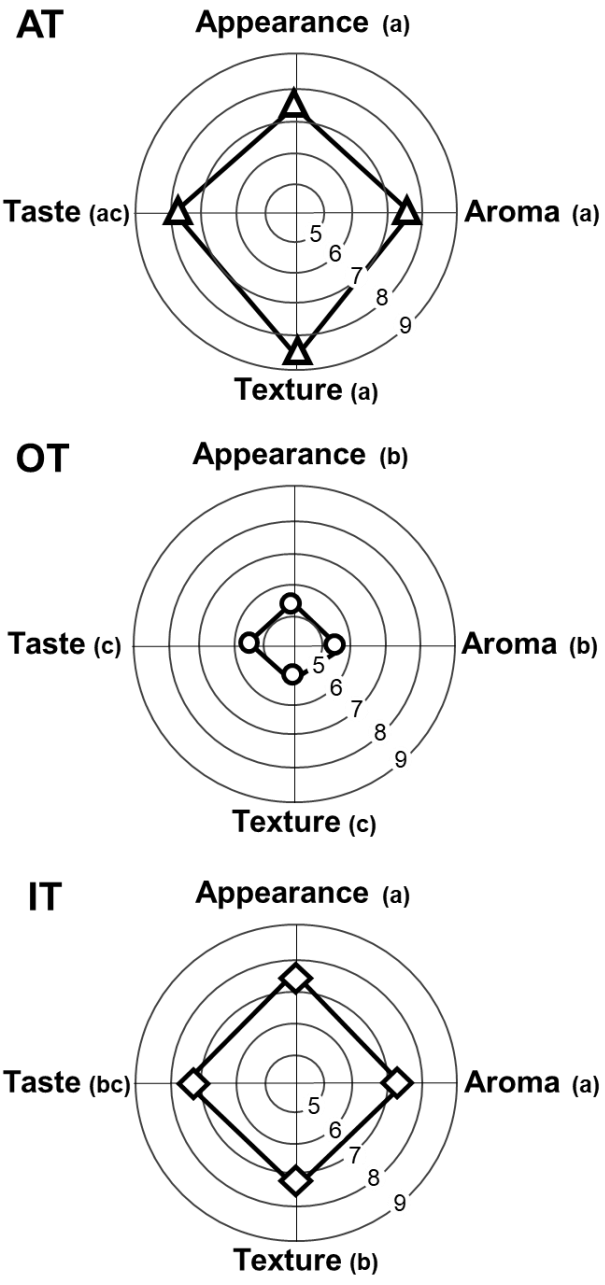
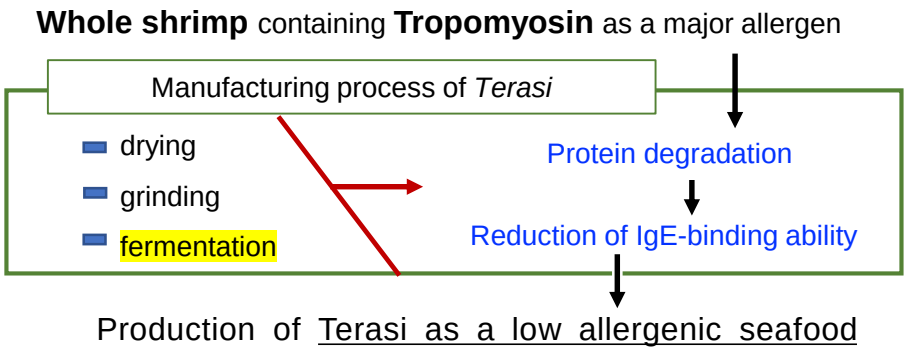


Fig. 6. (Amalia et.al)



Graphical abstract (Amalia et al.)