Int Aquat Res (2024) 16:245–256
https://doi.org/10.22034/iar.2024.2008753.1701

ORIGINAL RESEARCH

Maillard reaction products derived from salmon frame protein hydrolysate: antioxidant activities in different food model systems, cytotoxicity and bioavailability

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Received: 06 June 2024 / Accepted: 29 August 2024 / Published online: 10 September 2024

Abstract Lipid oxidation is among vital deteriorative reactions associated with quality loss of foods, and some oxidation products have toxicity when consumed. Maillard Reaction Products (MRPs) obtained from salmon frame protein hydrolysate (PH) were determined for antioxidant activities. MRPs exhibited superior antioxidant efficacy in both β-carotene linoleate and lecithin liposome systems to PH ($p<0.05$). MRPs also showed preventive effect toward oxidation in comminuted pork system ($p<0.05$). The antioxidant potential of MRPs was dose-dependent in all systems tested. Based on cytotoxicity toward Caco-2 cells, MRPs up to 10 mg/mL had no toxicity toward cells when the exposure time was up to 48 h ($p>0.05$). In addition, the less absorption of MRPs across the Caco-2 cell monolayer was observed as evidenced by the decreased absorbance at 420 nm in the basolateral side ($p<0.05$). However, small free peptides in MRPs could pass through monolayer effectively. Furthermore, following translocation across the Caco-2 cell monolayer, MRPs exhibited marginally reduced DPPH and ABTS radical scavenging activities, ferric reducing antioxidant capacity and metal chelating activity. Overall, MRPs were therefore a promising safe antioxidant in various food systems, thus improving oxidative stability of treated foods.

Keywords Maillard reaction products . Antioxidant . Cytotoxicity . Bioavailability . Salmon frame

Introduction

Utilization of discards from fisheries industries has gained attention and interest over the past decade. These discards in the form of byproducts or offal, etc. are often employed in manufacturing of silage and fishmeal (Desai et al. 2022). These leftovers have high contents of amino acids, proteins, and other valuable nutrients (Khan et al. 2022). To enhance their market potential, various efforts have been paid till date, including generation of peptides with different bioactivities, recovery of proteins in the form of hydrolysates or isolates, extraction of oil from the head, etc. Hydrolysis, specifically enzymatic hydrolysis, is one amongst the best approaches to produce high valuable products from the discards of fish processing. Recently, different byproducts from salmon (heads, mangled muscles, viscera, skins) were utilised as raw materials for production of protein hydrolysate using enzymatic hydrolysis with the aid of Alcalase. This led to 79 % decrease in the amount of waste sent to landfills, responsible for land and air pollutions (Araujo et al. 2021). Fur-

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thermore, those seafood processing wastes are prone to decomposition due to the presence of endogenous enzymes, thus enhancing autolysis (Nikoo et al. 2023).

Fish and fish-derived products have garnered notable attention due to their numerous health benefits and palatability. Recently, Idowu et al. (2019) produced salmon fame protein hydrolysate (PH) using papain and Alcalase (1-3 % w/w of protein). Higher protein (up to 82%) was recovered and the hydrolysate samples possessed antioxidant activities. Nilsuwan et al. (2021) used a combination of the aforementioned enzymes at different doses to create fat-free hydrolyzed collagen from Sockeye salmon skin with barely detectable fishy odor. Furthermore, hydrolyzed proteins and peptides have been reported to control food oxidation through various mechanisms, including inactivating ROS, scavenging free radicals, chelating pro-oxidative metal ions, reducing lipid hydroperoxides, etc. (Nikoo et al. 2023). In addition, protein hydrolysates from seafood processing wastes showed a variety of bioactivities, biological properties, and functional properties; however, their use is limited in the food industry owing to their strong bitterness. In order to tackle this problem, several debittering techniques have been employed including the utilization of ß-cyclodextrin, chromatographic separation techniques, resins, liposomal encapsulation, plastein reaction, etc. (Sharma et al. 2022, 2023). Recently, Sharma et al. (2024) reported the use of Maillard reaction (10% PH, 4 % glucosamine, 120°C, 60 min) for effective debittering of PH. The aforementioned process could lower the bitterness score from 9.37 to 1.44, and augmented antioxidant activities of resulting Maillard reaction products (MRPs). Maillard reaction takes place by a complex interaction between amino groups of peptides and carbonyl groups of reducing sugars, which lead to the formation of new compounds, such as melanoidins, majorly responsible for flavor development. (Liu et al. 2022a). These complex flavor compounds reduce or mask the bitterness in hydrolysates. Quality of several commercial products is associated with MRPs due to their unique color, aroma and flavor (Liu et al. 2022b). The industrial MRPs include yeast extract, which provides umami flavor and is commonly used in soups, sauces and ready-to-eat meals. Hydrolyzed vegetable proteins provide meaty flavor and are used in seasonings; beef and chicken flavorings provide the cooked or roasted meat flavors and are commonly used in gravies (Singh et al. 2008). The role of MRPs in flavors of other commercial products including Givaudan, Kerry group, Symrise and Mane has been documented (Ziegler 2007). MRPs formed as a result of Maillard reaction have been reported to exhibit robust antioxidant properties due to their functional groups and diverse chemical structure (Vhangani and Wyk 2016). MRPs can enhance the overall shelf life of the product via lowering oxidative deterioration. The antioxidant activity of MRPs involves multiple mechanisms, such as breaking radical chains, scavenging reactive oxygen species, chelating metals and decomposing hydroperoxides. The aforementioned mechanisms collectively inhibit the development of primary and secondary oxidation products (Nooshkam et al. 2019).

Although MRPs have desired properties, they also consist of advanced glycation end products (AGEs). These are often regarded as cytotoxic, carcinogenic, or mutagenic (Kuzan 2021). A high consumption of AGEs in the diet has been linked with a higher chance of health risk or diseases like diabetes, etc. (Ribeiro et al. 2019). However, specific conditions such as acidic pH, very high sugar concentrations, high temperature, etc. are related with high production of AGEs (Wei et al. 2018). Thus, by controlling these conditions AGE production can be limited. In addition, natural antioxidants could effectively inhibit AGEs (Jia et al. 2023). Since MRPs are also considered as natural antioxidants, the necessity to assess the equilibrium between reducing AGE formation without compromising antioxidant properties of MRPs is required. The use of synthetic antioxidants such as tertiary butyl hydroquinone, propyl gallate and BHA might affect the liver and induce cancer formation (Vhangani and Van Wyk 2016). The safety of these compounds in the human body is still being investigated. The study aimed to determine the effect of MRPs and protein hydrolysate from salmon frame as antioxidants in different model systems and to elucidate their cytotoxicity and bioavailability.

Materials and methods

Chemicals

All the chemicals used were of analytical grade. Chloroform and ethanol were obtained from RCI Labscan, Bangkok, Thailand. The rest of chemicals were procured from Sigma Aldrich, Inc. (St. Louis, MO, USA).

Preparation of PH and MRPs

PH were prepared as tailored by Sharma et al. (2022). Briefly, frozen salmon frames from *Salmo salar* were thawed overnight in a walk-in cold room, ground using a grinding machine and subjected to hydrolysis using 0.5 % papain (based on dry mince weight) at 40 °C for 5 min. After being centrifuged at 8500 rpm for 20 min, the supernatant was freeze-dried and the powder was packed in zip-lock bags, and stored at -20 °C.

For preparation of MRPs, PH powder was mixed with distilled water (1:8,w/v), as detailed by Sharma et al. (2024). Maillard reaction was carried out using 10% PH containing glucosamine (4 % of PH) at 120 °C in an oil bath for 60 min. Thereafter, the mixture was cooled in iced water. MRPs were freeze-dried and stored as mentioned above.

Both PH and MRPs were tested for their antioxidant efficacy in different model systems.

ß-carotene/linoleic acid system

ß-carotene bleaching assay was carried out as outlined by Vate and Benjakul (2013). ß-carotene (10 mg) was solubilised in chloroform (10 mL). Then, the prepared solution (0.2 mL) was mixed with another solution comprising linoleic acid (20 mg) and Tween 40 (200 mg). Residual chloroform was eliminated via nitrogen purging. Oxygenated deionised water (50 mL) were further added to this emulsion and mixed well. MRPs and PH were added into the system at 100 and 200 mg/mL. During the incubation at 50 °C in dark for 6 h, absorbance at 470 nm (A_{470}) was read every 15 min. As a negative control, 200 µL of deionised water was added instead of the samples. BHA (100 mg/L) was employed as the positive control. The slower decrease of A_{470} during the incubation suggested the ability of samples to prevent oxidation.

Lecithin liposome system

Lecithin liposome system was prepared as detailed by Vate and Benjakul (2013). Lecithin (8 mg/mL) was dispersed in deionised water with the aid of a glass rod, and subsequently sonicated for 30 min using a sonicating bath (Elmasonic S 30H, Elma, Germany). Three mL of sample were mixed thoroughly with 15 mL of lecithin liposome for 2 min, in which final concentrations of 100, 500, 1000 and 2000 mg/L were achieved. Subsequently, cupric acetate solution (0.15 M, 20 µL) was added to start the reaction. The mixture was shaken using a shaker (WNB 14 and SV 1422, Memmert, Germany) at 37 °C in dark at 120 rpm. Thiobarbituric acid reactive substances (TBARS) values of liposome were measured at specified time intervals of 0, 6, 12, 24, 36 and 48 h. Standard curve of malonaldehyde (0 to 3 mg/L) was used for computation of TBARS value, which was reported as milligrams of MDA per mL of liposomes.

Pork mince model system

Antioxidant activity of both MRPs and PH in pork mince model system was tested (Kittiphattanabawon et al. 2012). Ground pork meat (80 g) with fat content of 10 %, was mixed with deionised water containing PH or MRPs powders (100, 200 and 500 mg/kg). BHT (100 mg/kg) was employed as the positive control. The mixture (50 g) was packed in polyethylene bags and stored at 4 °C. TBARS values were measured in all the samples at day 0, 2, 4, 6 and 8 (Mittal et al. 2021b). A standard curve of malonaldehyde was prepared as stated above and the value was reported as milligrams of MDA equivalents per kg of samples.

Simulated *in vitro* gastrointestinal digestion (GID) of MRPs

Procedure outlined by Patil et al. (2023) was employed for the simulated *in vitro* GID of MRPs. Sample (1 g) was added to 10 mL of simulated gastric solution (0.1 M HCl, pH 2.5) containing pepsin (0.04 g/mL) for 1 h, followed by pH adjustment to pH 5.3. Pancreatin and bile salt were dissolved at a concentration of 0.08 g/mL each. The pH was then adjusted to 7.4 before incubation for 2 h. Digestion at all steps was performed at 37°C. The digest was eventually placed in boiling water in order to halt the hydrolysis, cooled, centrifuged and the supernatant was collected for analyses.

Measurement of cytotoxicity

The cytotoxicity of MRPs digest obtained from GID was assessed using the MTT assay with Caco-2 cells (Patil et al. 2022). Caco-2 cells were cultured, exposed to MRPs digest at varying concentrations (1, 5, and 10 mg/mL), followed by dilution and incubation at 37°C in 5% CO_2 for 24 and 48 h. After washing the cell, the absorbance of purple formazan solution was recorded at 595 nm to represent the viable cells.

Transportation studies

Cell culture

Caco-2 cells, derived from human colon adenocarcinomas, were cultured in Dulbecco's Modified Eagle Medium (DMEM) in the presence of 1% antibiotic-antimycotic and 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. Upon reaching 80-90% confluency after approximately 10-12 passages, the cells were detached with 0.25% trypsin-EDTA and then sub-cultured. Caco-2 cells were seeded onto 12-well Transwell inserts (0.4 µm pore size, 12 mm diameter; Corning Costar, Corning, NY, USA) with a density of 1.2×10^5 cells/cm^2. It took 25-31 days for full differentiation. The culture medium was replaced with fresh medium every other day. The integrity of cell monolayers was assessed with the aid of a Millicell ERS device (Millipore, Billerica, MA, USA).

Apical-to-basolateral transport studies

The protocol outlined by Patil et al. (2022) was used for the transport study. Prior to the study, Caco-2 monolayer was rinsed with Hanks' Balanced Salt Solution (HBSS) to eliminate any residual culture media. MRPs digest was transferred to apical (AP) chamber. The permeates in the basolateral (BL) side were collected for measurement of α-amino group content representing small peptides or free amino acids using TNBS (2,4,6-trinitrobenzenesulfonic acid) as reagent, and L-leucine (0.5 to 5.0 mM) as standard (Patil et al. 2022). A_{420} of the samples was read. Permeation was computed as follows:

$$\text{Permeation (\%)} = \frac{P_{\text{basolateral}} - P_{\text{blank}}}{P_{\text{apical}}} \text{X } 100$$

where P_{apical} represents the initial peptide/amino acid amounts present in AP-side, $P_{basolateral}$ represents the peptide/amino acid amounts detected in the BL-side after the designated time of transport, and P_{blank} indicates the blank in the BL-side at the specified transport time.

The samples collected from the AP-side and their corresponding Caco-2 cell permeates in the BL-side were also determined for apparent absorbance recovery and antioxidant activities. Apparent absorbance recovery was calculated as per the protocol outlined by Cueva et al. (2017). A_{420} was read in both AP and BL chambers after different transportation times. Necessary dilutions were made before measurement. The apparent absorbance recovery (%) was calculated using the above equation, except A_{420} was used instead of P.

Antioxidant activities (DPPH and ABTS radical scavenging activities, ferric reducing antioxidant power (FRAP) and metal chelating activity (MCA)) in both AP and BL sides were examined as outlined by Sharma et al. (2023). Activities were reported as mmol Trolox equivalent (TE)/g of digest, except for MCA, which was reported as micromoles of EDTA equivalent/g sample.

Statistical analysis

A completely randomised design (CRD) was employed throughout the study. The experiments and analyses were carried out in triplicate. The data underwent analysis of variance (ANOVA), and mean comparisons were conducted using Duncan's multiple range test. Pair comparisons were done using the t-test (Mittal et al. 2021a). The Statistical Package for Social Science (SPSS) version 22.0 for Windows, developed by SPSS Inc. in Chicago, IL, USA, was utilised for the statistical analysis.

Results and discussion

Antioxidative effect of MRPs in varying model systems

ß-carotene/linoleic acid system

Capacity of MRPs in preventing the oxidation of linoleic acid as indicated by lowering the bleaching of ß-carotene is shown in Figure 1. Free radicals within the system induce bleaching of ß-carotene proportionally (Benchikh et al. 2022). Generally, radicals caused by the elimination of hydrogen from diallylic methylene groups present in linoleic acid were generated in the system (Tagrida and Benjakul 2020). Subsequently, the resultant free radicals will ultimately accelerate oxidation of the double bonds present in highly unsaturated ß-carotene molecule, which results in color alteration from orange to colorless (Kittiphattanabawon et al. 2012). Antioxidants could mitigate the oxidation of β-carotene triggered by radicals or hydroperoxides. The systemic oxidation of ß-carotene due to the oxidation process is indicated by the decrease in A_{470}. The use of 100 mg/L of the synthetic antioxidant BHA substantially delayed the decline in A_{470}. Overall, the different samples exhibited degradation of β-carotene at varying rates. However, after 75 min, β-carotene underwent degradation to a lower degree. Amongst all the samples, the rate of degradation was highest in the control as evidenced by the highest decrease in A_{470}. This phenomenon might be due to the absence of antioxidant compounds in the control sample, allowing free radicals to degrade β-carotene in the system.

The inclusion of PH in the system did not exhibit a significant inhibitory effect on retaining A_{470} compared to MRPs. MRPs with augmenting concentrations demonstrated a notable reduction in β-carotene oxidation. However, PH had poor efficacy in maintaining $A_{470.}$ An enhanced antioxidant capacity was noted with higher concentrations of MRPs (200 mg/L). Thus, MRPs could act as natural antioxidants in food systems (Sharma et al. 2024). Antioxidants present in MRPs might potentially scavenge the hydroperoxides formed within the system (Laouicha et al. 2020)frequently used in traditional folk medicine to treat diabetes and arterial hypertension. This study aims to evaluate the phenolic composition and antioxidant activity of ethyl acetate (EA.E. MRPs derived from proteinaceous byproducts of smooth hound viscera using sucrose exhibited improved taste characteristics with reduced content of bitter amino acids and enhanced antioxidant properties. This was evidenced by their increased reducing power and effectiveness in preserving β-carotene in comparison to unconjugated proteins (Abdelhedi et al. 2017). Huang et al. (2012) discovered

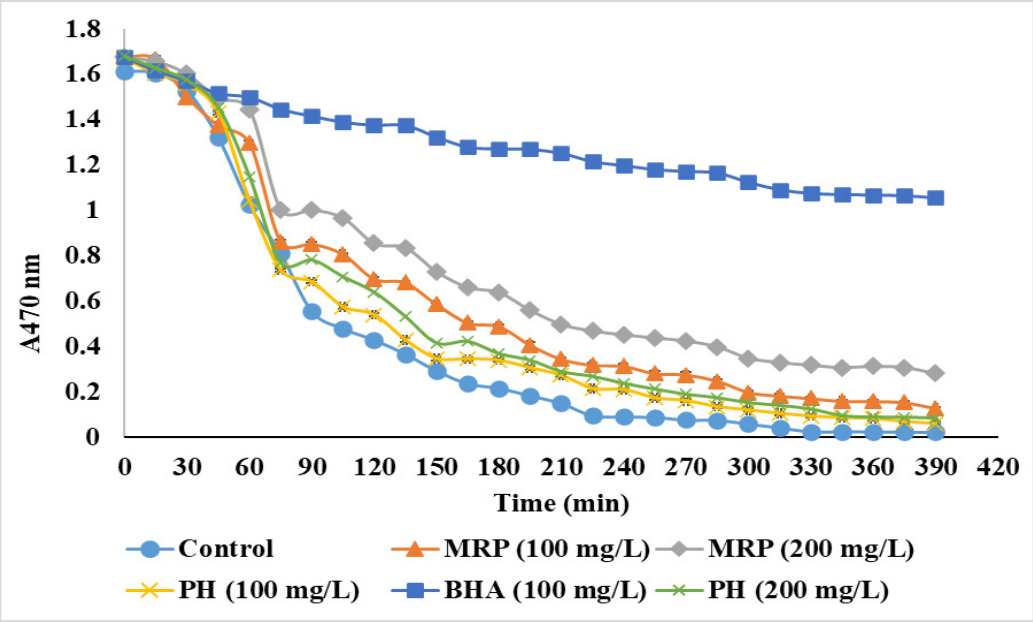

Fig. 1 Degradation rate in ß-carotene bleaching assay in the absence or presence of protein hydrolysate (PH) or Maillard reaction products (MRP) at different concentrations. Vertical bars in the curve represent standard deviation (n=3)

that the glycation of low molecular weight (LMW) peptides using glucose and maltose could significantly enhance the reducing power and antiradical activity of ovalbumin hydrolysates than peptides alone. The formation of these cross-linked products might augment antioxidant properties. Additionally, according to Liu et al. (2014), the Maillard reaction could serve as an effective method for improving antioxidant activities of protein hydrolysates. Therefore, MRPs were able to mitigate the oxidation of β-carotene induced by radicals generated within the tested system.

Lecithin liposome system

The capacity of PH and MRPs at varying concentrations to impede oxidation in the lecithin liposome system is illustrated in Figure 2a. Both PH and MRPs inhibited oxidation in the system during a 48-h incubation period. However, the protective effect was more pronounced with the use of MRPs, compared to PH at the same concentration ($p<0.05$). Additionally, BHA (100 mg/L) exhibited a higher preventative effect, as evidenced by lower TBARS values in the systems. Notably, the control sample (devoid of PH, MRPs, or BHA) displayed the greatest upsurge in TBARS after 24 h, indicating that more extensive oxidation process occurred. Without antioxidants, lecithin could undergo oxidation rapidly. Liposomes serve as suitable lipid models for assessing antioxidative activity in lipid-based foods or lipoprotein comprising phospholipids (Vate and Benjakul 2013). Notably, the sample supplemented with MRPs at 2000 mg/L demonstrated less TBARS values than that incorporated with BHA at 100mg/L. The result revealed the dose-dependent nature of antioxidant activity of PH or MRPs in the lecithin liposome system. Moreover, hydrophilic domains of peptides or proteins found in PH or MRPs plausibly migrated and got localized in the liposome's head section of lecithin, where they inhibited oxidation by scavenging free radicals. Moreover, owing to their metal-chelating ability, MRPs could effectively chelate Cu^{2+}, a known prooxidant in the system. As a consequence, liposome oxidation was impeded more efficiently than PH. Vate and Benjakul (2013) also observed the dose dependent nature of antioxidant activity when melanin free ink from splendid squid was added at different concentrations (100-500 mg/L). The sample with the highest squid ink concentration (500 mg/L) showed the lowest TBARS value. Moreover, similar re-

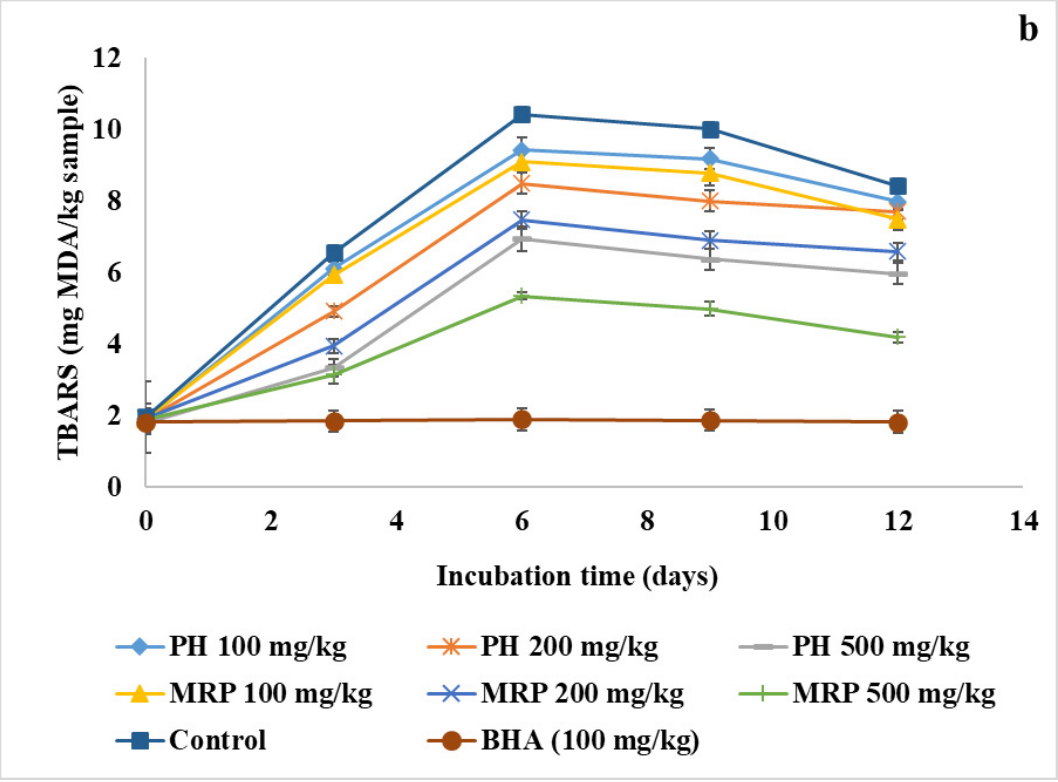

Fig. 2 Changes in thiobarbituric acid reactive substances (TBARS) in (a) lecithin liposome system and (b) pork mince system in the absence or presence of protein hydrolysate (PH) or Maillard reaction products (MRP) at different concentrations. Vertical bars in the curve represent standard deviation (n=3)

sults were obtained by Lertittikul (2005) where MRPs derived from porcine plasma protein with 20% degree of hydrolysis (DH) using 2% glucose at pH 12 upon heating at 100 °C for 2 h displayed the highest antioxidant activity in a lecithin liposome system. Therefore, these findings demonstrated the superior antioxidant activity of MRPs compared to PH in the lecithin liposome system, thereby underscoring their potential as natural antioxidants for lipid-based foods or lipoprotein rich foods.

Pork mince model system

Lipid oxidation in comminuted pork added with PH and MRPs at 100, 200 and 1000 mg/kg, or BHA (100 mg/kg) was monitored over a 12-day storage period at 4°C as illustrated in Figure 2b. Notably, TBARS levels in comminuted pork, both with and without antioxidants, displayed an upward trajectory with increasing storage duration up to 6 days ($P<0.05$). Subsequently, a slight decline in TBARS levels was found up to the end of storage ($P<0.05$). This phenomenon can be attributed to the depletion of oxidation products, via the evaporation of low molecular weight volatile compounds. Notably, hydroperoxides produced from lipid oxidation are known for their inherent instability and subsequent decomposition into alcohols, aldehydes, ketone, acids and other compounds, detectable by the TBARS assay (Kittiphattanabawon et al. 2012). However, some compounds, particularly aldehydes, could bind with proteins and were not react with TBA (Shakoor et al. 2022). Moreover, comminuted meat systems containing higher concentrations of PH or MRPs exhibited significantly lower TBARS values throughout the storage duration ($P<0.05$), whereas the control sample had the highest TBARS value during 12 days storage. An initial increase in TBARS values up to 7 days, followed by a subsequent decline in TBARS levels until the end of storage was also reported by Kittiphattanabawon et al. (2012). At the same concentrations of PH and MRPs, the latter showed superior efficacy in attenuating lipid oxidation to the former. This heightened efficiency of MRPs in mitigating lipid oxidation, mainly attributed to its inherent antioxidant properties, including radical scavenging activity and chelating ability towards prooxidants such as Fe^{2+}. Sivaraman et al. (2016) also observed the development of secondary lipid oxidation products in minced sardine meat treated with squid protein hydrolysates (SPH) during iced storage. The results revealed an increase in TBARS levels up to day 8, followed by a subsequent decline. This increase in TBARS indicated the decomposition of primary products into secondary products, particularly aldehydes. The subsequent decrease in TBARS values after day 8 may be attributed to the breakdown of aldehydes into volatile compounds. Similar findings were also reported, where TBARS values increased at a slower rate in minced meat model systems containing SPH, compared to the control (Sivaraman et al. 2016). In addition, gelatin hydrolysates derived from blacktip shark skin, prepared with papain, effectively reduced lipid oxidation in a comminuted pork meat model system when used at concentrations of 500 and 1,000 ppm (Kittiphattanabawon et al. 2012). In the present study, MRPs exhibits a more pronounced capacity of inhibiting lipid oxidation, compared to PH, thereby contributing to its enhanced efficacy in preserving lipid quality of ground pork during storage. However, it is noteworthy that BHA (100 mg/kg) exhibited superior efficacy in mitigating lipid oxidation, compared to both MRPs and PH.

Cell cytotoxicity

Cytotoxicity of MRPs digest after simulated *in vitro* gastrointestinal (GI) digestion toward Caco-2 cells after 24 and 48 h is depicted in Figure 3. Notably, no discernible cytotoxic effects of MRPs were observed on the Caco-2 cells, regardless of concentrations tested ($p>0.05$). In addition, the exposure durations (24 and 48 h) had no impact on Caco2-cells ($p>0.05$). These findings revealed the biocompatibility and non-toxic attributes of MRPs towards epithelial cell lines. Consequently, the MRPs exhibited the promise for applications as antioxidant in food due to the lack of toxicity.

Transportation studies

Permeability of free peptides or amino acids

The transportation of free peptides in MRPs digest at varying concentrations (1 and 5 mg/mL) from the AP through the corresponding Caco-2 cell monolayer, in which permeates were collected in the

BL side at various time intervals (1, 2, and 4 h) is illustrated in Figure 4. Permeability percentages of MRPs (majorly free small peptides with available amino acid at N-terminal) predominantly ranged from 10% to 100%. Several pathways have been documented for the movement of peptides across the human intestinal epithelium, which encompass the proton-coupled peptide transporter 1 (PepT1)-mediated transport pathway, the paracellular pathway facilitated by tight junctions (TJs), and the vesicle mediated transcytosis pathway (Patil et al. 2022). Varying permeability of free peptides was observed when MRPs at different concentrations were tested. Notably, permeability increased with higher MRPs digest concentrations and duration of transportation ($p < 0.05$) (Figure 4). This indicated the presence of more free small peptides or amino acids in the sample which could easily pass through the Caco2-cell monolayer. Similar trends were observed for Gln-Ile-Gly-Leu-Phe (QIGLF), an ACE-inhibitory peptide, in which permeability increased over time (Ding et al. 2014). At 4 h, MRPs digests at a concentration of 5 mg/mL exhibited the highest permeability (up to 100%), compared to digest having 1 mg/mL concentration ($p<0.05$). This variation in permeability might be determined by digest concentrations. High content of digest could facilitate the migration through the monolayer via diffusion (Ding et al. 2021). MRPs still contained smaller peptides with free N-terminal. PepT1 primarily transports dipeptides and some tripeptides, while oligopeptides with more than three amino acids can traverse the Caco-2 cell monolayer via paracellular tight junctions (TJs) (Ding et al. 2014). Transcytosis is the main transport mechanism for peptides exceeding ten amino acids in length (Xu et al. 2023). TJs consist of small pores, facilitating the passage of water-soluble molecules with low molecular weights (Xu et al. 2023). Therefore, sample permeation through Caco-2 cell monolayers was governed by transportation time and digest concentrations.

Apparent absorbance (A_{420}) recovery

A_{420} is one of the most commonly used parameters to monitor the progress of MR. It is measure of formation of colored compounds of MRPs formed at later stages (Sharma et al. 2024). MRPs, derived from PH, primarily comprise Amadori products and hydroxymethyl furfurals (HMF), which were efficiently hydrolyzed by α-glucosidase activity expressed by Caco-2 cells (Seidowski et al. 2009).

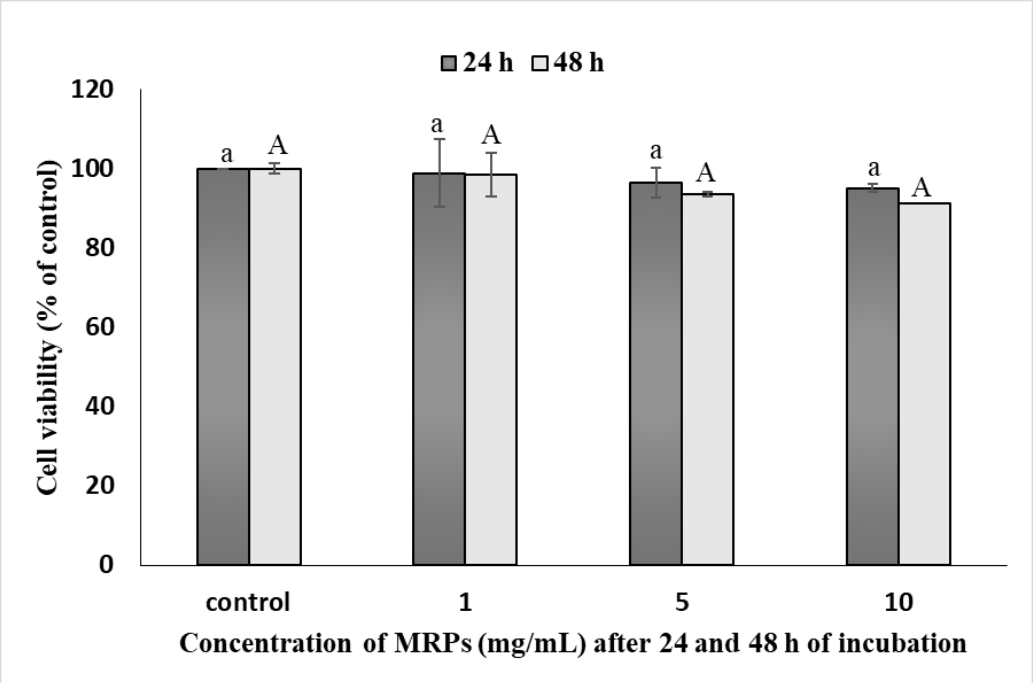

Fig. 3 Effect of MRP digest at different concentrations on cell viability of Caco-2 cells after 24 h and 48 h of incubation. Different lowercase or uppercase letters on the bars within the same incubation time denote significant differences ($p<0.05$). Vertical bars represent standard deviation (n=3)

A consistent decrease in A_{420} was observed in BL chamber in comparison to AP chamber over the course of transportation time ($p<0.05$) (Table 1). This indicated the partial transport of MRPs across the monolayer, in which a portion of MRPs reached the BL chamber. The slight decrease in A_{420} in AP chamber might be due to polymerization of the peptides induced by Maillard reaction. Additionally, the other plausible cause could be absorption of peptides by Caco-2 cells, contributing to the decreased $A_{420.}$ Furthermore, sequestration or cellular uptake of the MRPs by cells might lead to decreased A_{420} in BL chamber. (Ruiz-Roca et al. 2008) also observed the reduced content of MRPs (Glucose-lysine heated mixture) in solutions collected from BL chambers. These results aligned with the findings of metabolic transit studies conducted in rats. Almost 30 % of low molecular weight compounds, including MRPs were absorbed, whereas high molecular weight compounds were absorbed to lesser extent (Ruiz-Roca et al. 2008).

Antioxidant activity

The antioxidant capacities of MRPs in both AP and BL sides as a function of permeation time are delineated in Table 2. After 1 h, the digest in AP side exhibited the higher DPPH-RS (4.13 μmol TE/g), ABTS-RS (4.13 μmol TE/g), FRAP (0.64 μmol TE/g) than those in BL side ($p < 0.05$), except for MCA which showed no difference between AP and BL ($p>0.05$). After 4 h, all activities tested by 4 assays had the decreased activities. Generally, peptides of varying molecular weights typically exhibit differential capacities for scavenging DPPH and ABTS radicals (Patil et al. 2022). Peptides present in MRPs typically serve as hydrogen donors, thereby interacting with radicals to convert them into more stable compounds. As a result, the radical chain reaction was arrested (Nalinanon et al. 2011). Decreases in the antioxidant activities of MRPs digest were noted after 4 h for all assays, except for MCA which had no marked changes. Upon passage through the Caco-2 cell monolayer, ABTS-RSA, DPPH-RSA and FRAP exhibited lower activities for MRPs digests. Zhang et al. (2018) and Patil et al. (2022) indicated alterations in the antioxidant activities of the permeates following absorption by Caco-2 cells. These results implied that some peptides or MRPs were modified or degraded during absorption and passage through the Caco-2 cell monolayer, which

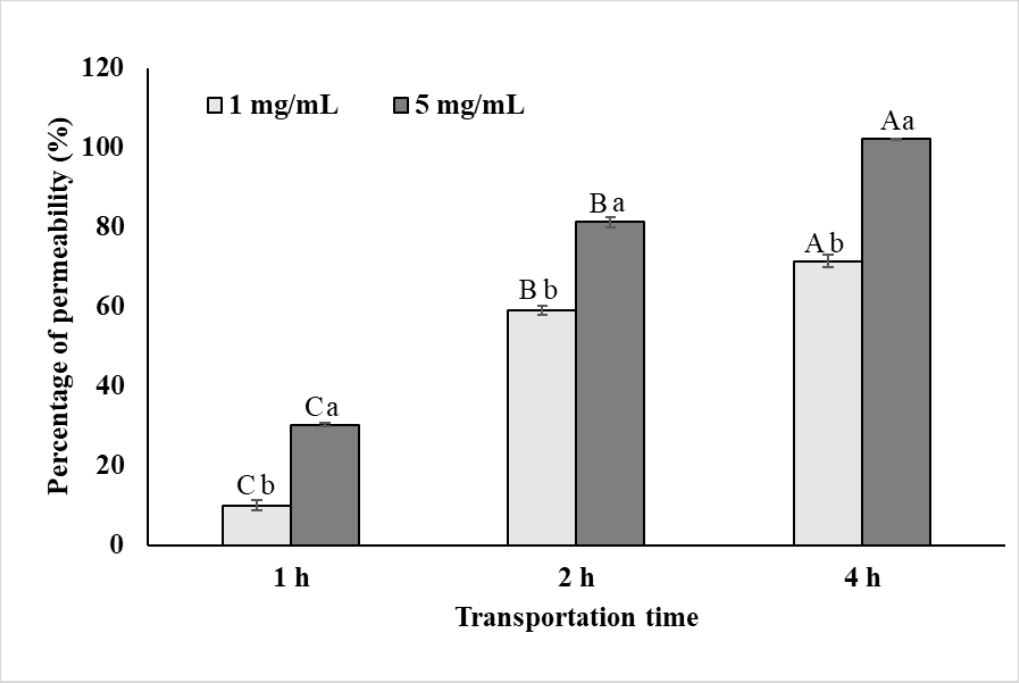

Fig. 4 Percentage of permeation of free peptides/amino acids in MRP digest at different concentrations (1 and 5 mg/mL) across Caco-2 monolayer. Different lowercase on the bars within same transportation time denote significant differences ($p<0.05$). Different uppercase letters on the bars within the same concentration denoted significant differences ($p<0.05$). Vertical bars represent standard deviation (n=3)

Table 1 Absorbance (A_{420}) recovery of MRP digest across Caco-2 cell monolayer after different incubation times

Time (h)	A_{420} (1 mg/mL)			A_{420} (5 mg/mL)		
	Apical	Basolateral	Recovery (%)	Apical	Basolateral	Recovery (%)
1	0.95 ± 0.03^{a}	0.29 ± 0.01^{a}	29.79 ± 1.08^{a}	1.18 ± 0.04^{a}	0.46 ± 0.01^{a}	38.70 ± 1.94^{a}
2	0.87 ± 0.07^{ab}	0.22 ± 0.01^{b}	25.37 ± 1.93^{b}	1.13 ± 0.04^{a}	0.32 ± 0.01^{b}	29.83 ± 1.35^{b}
4	0.82 ± 0.01^{b}	0.18 ± 0.01^{c}	21.74 ± 0.05^{c}	0.97 ± 0.01^{b}	0.23 ± 0.01^{c}	23.21 ± 0.88^{c}

Data were expressed as mean ± SD (n=3). Different superscripts within the same column indicates significant differences ($p<0.05$).

Table 2 Antioxidant activities of MRP digest before transport (apical) and after transport (basolateral) as a function of time

Time	1 h		2 h		4 h	
Antioxidant activity	Apical	Basolateral	Apical	Basolateral	Apical	Basolateral
DPPH-RSA*	4.13 ± 0.38^{a}	3.77 ± 0.25^{bc}	4.03 ± 0.06^{ab}	3.56 ± 0.12^{cd}	3.6 ± 0.15^{cd}	3.3 ± 0.10^{d}
ABTS-RSA*	4.13 ± 0.21^{a}	3.6 ± 0.10^{bc}	4.07 ± 0.25^{a}	3.33 ± 0.21^{cd}	3.7 ± 0.10^{b}	3.16 ± 0.16^{d}
FRAP*	0.64 ± 0.09^{ab}	0.41 ± 0.07^{c}	0.75 ± 0.09^{a}	0.51 ± 0.11^{bc}	0.59 ± 0.09^{ab}	0.39 ± 0.11^{c}
MCA**	0.68 ± 0.14^{ab}	0.54 ± 0.10^{bc}	0.85 ± 0.08^{a}	0.61 ± 0.10^{bc}	0.7 ± 0.08^{ab}	0.48 ± 0.10^{c}

Data were expressed as mean ± SD (n=3). Different superscripts within the same row indicates significant differences ($p<0.05$). RSA: radical scavenging activity; DPPH: 1,1-diphenyl-2-picrylhydrazine; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, FRAP: ferric reducing antioxidant power, MCA: metal chelating activity, TE: Trolox equivalent; EE: ethylenediaminetetraacetic acid equivalent. *μmol TE g^{-1}; ** μmol EE g^{-1}

led to a partial reduction in the antioxidant activity of all samples. It was noted that the decreases in both DPPH-RS and ABTS-RS were also observed in AP side. This might be due to instability of some antioxidative compounds in MRPs after 4 h. Further polymerisation of some antioxidative compounds might lower antioxidant activity of MRPs digest. Notably, peptides or MRPs present in BL side retained antioxidant activities after translocation across the Caco-2 cell monolayer to some extent, implying the persistence of antioxidant activity in transported peptides or MRPs.

Conclusions

Superior antioxidant efficacy of MRPs was observed to PH when tested using various model systems. MRPs exhibited a greater ability to prevent the development of secondary oxidation products, as evidenced by TBARS analysis in both pork mince and lecithin liposome systems. Additionally, MRPs demonstrated a lesser degradation of β-carotene in the β-carotene linoleate system, further emphasizing their potent antioxidant properties. Importantly, no cytotoxicity was observed in MRPs towards Caco-2 cells suggesting their safety for consumption. Decrease in A_{420} over time in BL chamber suggested the partial transportation of MRPs across the Caco-2 monolayer, while free peptides or amino acids could pass through effectively. Furthermore, antioxidative compounds in BL side retained antioxidant activities following translocation across the Caco-2 cell monolayer, highlighting the sustained antioxidant activities of MRPs to high degree. Thus, MRPs hold promise as effective antioxidants in food systems.

Author contribution Kartik Sharma: Data curation, Investigation, Methodology, Writing – original draft. Phutthipong Sukkapat: Conceptualization, Data curation, Prabjeet Singh: Writing – review & editing, Jirakrit Saetang: conceptualization, editing, Soottawat Benjakul: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Ethical guidelinesn Ethics approval was not required for this research.

Acknowledgement This project was funded by Prince of Songkla University (PSU) president scholarship for Kartik Sharma, Prachayacharn grant from PSU (Grant No: AGR6602079N). Agricultural Research Development Agency (ARDA), Thailand also supported this research and are gratefully acknowledged.

Competing interests The authors declare that they have no competing interests.

References

Abdelhedi O, Mora L, Jemil I, Jridi M, Toldra F, Nasri M, Nasri R (2017) Effect of ultrasound pretreatment and Maillard reaction on structure and antioxidant properties of ultrafiltrated smooth-hound viscera proteins-sucrose conjugates. Food Chem 230:507–515.

https://doi.org/10.1016/j.foodchem.2017.03.053
Araujo J, Sica P, Costa C, Marquez MC (2021) Enzymatic hydrolysis of fish waste as an alternative to produce high value-added products. Waste Biomass Volari 12:847–855. https://doi.org/10.1007/s12649-020-01029-x
Benchikh F, Benabdallah H, Amira H, Mamache W, Amira S (2022) Free radical scavenging, reducing power and lipid peroxidation inhibition activities of *M. communis* berries methanol Extract and its fractions. Turkish J Agric - Food Sci Technol 10:760–765. https://doi.org/10.24925/turjaf.v10i4.760-765.5060
De la Cueva S, Seiquer I, Mesías M, Rufian-Henares JA, Delgado-Andrade C (2017) Evaluation of the availability and antioxidant capacity of Maillard compounds present in bread crust: studies in Caco-2 cells. Foods 6:5. https://doi.org/10.3390/foods6010005
Desai AS, Brennan M, Gangan SS, Brennan C (2022) Utilization of fish waste as a value-added ingredient: sources and bioactive properties of fish protein hydrolysate. In: Sustainable Fish Production and Processing. Elsevier, pp 203–225
Ding L, Zhang Y, Jiang Y, Wang L, Liu B, Liu J (2014) Transport of egg white ACE-inhibitory peptide, Gln-Ile-Gly-Leu-Phe, in human intestinal Caco-2 cell monolayers with cytoprotective effect. J Agric Food Chem 62:3177–3182. https://doi.org/10.1021/jf405639w
Ding X, Hu X, Chen Y, Xie J, Ying M, Yang Y, Yu Q (2021) Differentiated Caco-2 cell models in food-intestine interaction study: Current applications and future trends. Trends Food Sci Technol 107:455–465. https://doi.org/10.1016/j.tifs.2020.11.015
Huang X, Tu Z, Xiao H, Wang H, Zhang L, Hu Y, Niu P (2012) Characteristics and antioxidant activities of ovalbumin glycated with different saccharides under heat moisture treatment. Food Res Int 48:866–872. https://doi.org/10.1016/j.foodres.2012.06.036
Jia W, Guo A, Zhang R, Shi L (2023) Mechanism of natural antioxidants regulating advanced glycosylation end products of Maillard reaction. Food Chem 404:134541. https://doi.org/10.1016/j.foodchem.2022.134541
Khan S, Rehman A, Shah H, Aadil RM, Ali A, Shehzad Q, Ashraf W, Yang F, Karim A, Khaliq A, Xia W (2022) Fish protein and its derivatives: The novel applications, bioactivities, and their functional significance in food products. Food Rev Int 38:1607–1634. https://doi.org/10.1080/87559129.2020.1828452
Kittiphattanabawon P, Benjakul S, Visessanguan W, Shahidi F (2012) Gelatin hydrolysate from blacktip shark skin prepared using papaya latex enzyme: Antioxidant activity and its potential in model systems. Food Chem 135:1118–1126. https://doi.org/10.1016/j.foodchem.2012.05.080
Kuzan A (2021) Toxicity of advanced glycation end products (Review). Biomed Reports 14:46. https://doi.org/10.3892/br.2021.1422
Laouicha S, Senator A, Kherbache A, Bouriche H (2020) Total phenolic contents and antioxidant properties of algerian *Arbutus unedo L*. Extracts. J Drug Deliv Ther 10:159–168. https://doi.org/10.22270/jddt.v10i3-s.4182
Lertittikul W (2005) Porcine plasma protein-sugar maillard reaction products (MRPs): some factors affecting antioxidant activity and their application. Prince of Songkla university
Liu B, Li N, Chen F, Zhang J, Sun X, Xu L, Fang F (2022a) Review on the release mechanism and debittering technology of bitter peptides from protein hydrolysates. Compr Rev Food Sci Food Saf 21:5153–5170. https://doi.org/10.1111/1541-4337.13050
Liu Q, Li J, Kong B, Jia N, Li P (2014) Antioxidant capacity of maillard reaction products formed by a porcine plasma protein hydrolysate-sugar model system as related to chemical characteristics. Food Sci Biotechnol 23:33–41. https://doi.org/10.1007/s10068-014-0005-8
Liu S, Sun H, Ma G, Zhang T, Wang L, Pei H, Li X, Gao L (2022b) Insights into flavor and key influencing factors of Maillard reaction products: A recent update. Front Nutr 9. https://doi.org/10.3389/fnut.2022.973677
Mittal A, Singh A, Aluko RE, Benjakul S (2021a) Pacific white shrimp (*Litopenaeus vannamei*) shell chitosan and the conjugate with epigallocatechin gallate: Antioxidative and antimicrobial activities. J Food Biochem 45. https://doi.org/10.1111/jfbc.13569
Mittal A, Singh A, Benjakul S (2021b) The impact of chitosan film or chitosan/chitosan–epigallocatechin gallate conjugate composite film on the quality changes of Asian sea bass (*Lates calcarifer*) slices stored in air or under vacuum packaging. Int J Food Sci Technol 56:6025–6038. https://doi.org/10.1111/ijfs.15203
Nalinanon S, Benjakul S, Kishimura H, Shahidi F (2011) Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. Food Chem 124:1354–1362. https://doi.org/10.1016/j.foodchem.2010.07.089
Nikoo M, Regenstein JM, Yasemi M (2023) Protein hydrolysates from fishery processing by-products: production, characteristics, food applications, and challenges. Foods 12:4470. https://doi.org/10.3390/foods12244470
Nilsuwan K, Chantakun K, Chotphruethipong L, Benjakul S (2021) Development of hydrolysis and defatting processes for production of lowered fishy odor hydrolyzed collagen from fatty skin of sockeye salmon (*Oncorhynchus nerka*). Foods 10:2257. https://doi.org/10.3390/foods10102257
Nooshkam M, Varidi M, Bashash M (2019) The Maillard reaction products as food-born antioxidant and antibrowning agents in model and real food systems. Food Chem 275:644–660. https://doi.org/10.1016/j.foodchem.2018.09.083
Patil U, Saetang J, Zhang B, Benjakul S (2022) Use of tuna visceral pepsin in combination with trypsin as digestion aid: enhanced protein hydrolysis and bioavailability. Foods 12:125. https://doi.org/10.3390/foods12010125
Ribeiro PVM, Tavares JF, Costa MA, Mattar JB, Alfenas RC (2019) Effect of reducing dietary advanced glycation end products on obesity-associated complications: a systematic review. Nutr Rev 77:725–734. https://doi.org/10.1093/nutrit/nuz034
Ruiz-Roca B, Navarro MP, Seiquer I (2008) Antioxidant properties and metal chelating activity of glucose-lysine heated mixtures: relationships with mineral absorption across Caco-2 cell monolayers. J Agric Food Chem 56:9056–9063. https://doi.org/10.1021/jf801718h
Shakoor A, Zhang C, Xie J, Yang X (2022) Maillard reaction chemistry in formation of critical intermediates and flavour compounds and their antioxidant properties. Food Chem 393:133416. https://doi.org/10.1016/j.foodchem.2022.133416
Sharma K, Nilsuwan K, Hong H, Fan X, Benjakul S (2024) Debittering of salmon frame protein hydrolysate and plastein using maillard reaction as affected by types of sugar. Int J Food Sci Technol 59:1560–1571. https://doi.org/10.1111/ijfs.16904
Sharma K, Nilsuwan K, Ma L, Benjakul S (2023) Effect of liposomal encapsulation and ultrasonication on debittering of protein hydrolysate and plastein from salmon frame. Foods 12:761. https://doi.org/10.3390/foods12040761
Sharma K, Nilsuwan K, Zhang B, Hong H, Benjakul S (2022) Protein hydrolysate from salmon frame debittered by plastein reaction: amino acid composition, characteristics and antioxidant activities. Int J Food Sci Technol. https://doi.org/10.1111/ijfs.16183
Singh P, Kumar R, Sabapathy SN, Bawa AS (2008) Functional and edible uses of soy protein products. Compr Rev Food Sci Food Saf

7:14–28. https://doi.org/10.1111/j.1541-4337.2007.00025.x

Sivaraman B, Shakila RJ, Jeyasekaran G, Sukumar D, Manimaran U, Sumathi G (2016) Antioxidant activities of squid protein hydrolysates prepared with papain using response surface methodology. Food Sci Biotechnol 25:665–672. https://doi.org/10.1007/s10068-016-0117-4

Tagrida M, Benjakul S (2020) Ethanolic extract of Betel (*Piper betle L.*) and Chaphlu (*Piper sarmentosum Roxb*.) dechlorophyllized using sedimentation process: Production, characteristics and antioxidant activities. J Food Biochem 44. https://doi.org/10.1111/jfbc.13508

Vate N, Benjakul S (2013) Antioxidative activity of melanin-free ink from splendid squid (*Loligo formosana*). Int Aquat Res 5:9. https://doi.org/10.1186/2008-6970-5-9

Vhangani LN, Van Wyk J (2016) Antioxidant activity of Maillard reaction products (MRPs) in a lipid-rich model system. Food Chem 208:301–308. https://doi.org/10.1016/j.foodchem.2016.03.100

Wei Q, Liu T, Sun DW (2018) Advanced glycation end-products (AGEs) in foods and their detecting techniques and methods: A review. Trends Food Sci Technol 82:32–45. https://doi.org/10.1016/j.tifs.2018.09.020

Xu Z, Bai H, Ma X, Wu Y, Wu Z, Yang A, Mao W, Li X, Chen H (2023) Cytological evaluation by Caco-2 and KU812 of non-allergenic peptides from simulated digestion of infant formula in vitro. Food Sci Hum Wellness 12:817–824. https://doi.org/10.1016/j.fshw.2022.09.020

Zhang Q, Tong X, Qi B, wang Z, Li Y, Sui X, Jiang L (2018) Changes in antioxidant activity of Alcalase-hydrolyzed soybean hydrolysate under simulated gastrointestinal digestion and transepithelial transport. J Funct Foods 42:298–305. https://doi.org/10.1016/j.jff.2018.01.017

Ziegler H (2007) Flavourings Production, composition, applications, regulations, Second edi. Wiley-VCH, Aufsess, Germany