Enhancement of Antioxidant Capacities of Salmon Viscera Waste as an Animal Feed Additive: Effects of Continuous Process With Acid/Alkali Swelling, Enzymatic Hydrolysis, and Glycation

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Abstract

Salmon viscera, a protein-rich by-product of salmon processing, offers significant potential as a valuable ingredient in companion animal feed. This study aimed to enhance the antioxidant capacities of salmon viscera hydrolysates through a systematic process involving acid/alkali swelling, enzymatic hydrolysis, and glycation. The viscera were washed and swollen with distilled water (pH 5.75), acid (pH 3.18), or alkali solution (pH 8.17), followed by hydrolysis using Alcalase at 55 °C for 1 h. Glycation was subsequently performed with 1 % (w/v) D-glucose, 1 % D-xylose, or a combination of 0.5 % D-glucose and 0.5 % D-xylose at 95 °C for 1 h. The acid swelling (39.16 %) and alkali swelling (39.27 %) resulted in a significantly higher degree of hydrolysis than the control (38.16 %) and increased the release of free amino acids from 419.7 mg.100 mL^{-1} (control) to 512.8 mg.100 mL^{-1} (acid treatment) and 533.3 mg.100 mL^{-1} (alkali treatment), respectively ($P < 0.05$). Notably, alkali swelling combined with glycation, particularly with xylose, significantly enhanced the antioxidant activities of the hydrolysates. However, certain combinations of swelling and glycation resulted in diminished antioxidant activity, potentially due to the variability in glycation progress. These findings suggest that optimised combinations of acid/alkali swelling and glycation, particularly using alkali swelling and xylose, can effectively enhance the antioxidant properties of salmon viscera hydrolysates, making them promising additives for companion animal feed.

Keywords: collagen swelling, Maillard reaction, pet food, protein hydrolysate, salmon by-products

Introduction

Salmon farming has emerged as the fastest-growing sector in the aquaculture industry, with salmonid production projected to reach 5 million metric tons by 2050 (Albrektsen et al., 2022). According to the Food and Agriculture Organization (FAO), global salmon consumption was approximately 2.6 million tons in 2022, and the salmon market was valued at $14.87 billion in 2021 (FAO, 2022). In salmon fillet processing, by-products such as heads, frames, fins, skin, and viscera account for nearly 50 % of the whole fish (Stevens et al., 2018). Among these, salmon viscera, which include digestive tracts and internal organs, are valuable by-products (Opheim et al., 2015). Given their high protein content (8 – 9 %), salmon viscera have been used as a nutritionally rich resource for producing animal-grade hydrolysates and fish meal, commonly used in animal feed (Wu et al., 2011; Opheim et al., 2015). Moreover, protein hydrolysates derived from salmon viscera have been identified as a promising marine protein source, capable of enhancing the growth performance of chicken broilers (Opheim et al., 2016). Therefore, the effective utilisation of salmon by-products presents a crucial industrial challenge, both in terms of sustainable management and value-added production.

According to the National Pet Owners Survey conducted by the American Pet Products Association, the U.S. pet industry has experienced substantial growth, with total expenditures amounting to USD136.8 billion in 2022 (APPA, 2023). This expansion has driven the development of diverse pet products

designed to enhance the health and lifespan of companion animals (de Robillard et al., 2022). In contrast to livestock, which has a controlled lifespan for economic purposes, companion animals are expected to live extended, healthy lives, free from disease. Salmon, recognised for its palatability and nutritional profile, has emerged as a key ingredient in premium pet food. However, the elevated cost of salmon-based products may hinder their accessibility for consumers. As a result, modern pet owners increasingly emphasise both the nutritional and functional attributes of pet food, highlighting the necessity for innovative ingredients and advanced processing technologies to enhance the functional properties of animal feed additives. The identification and optimisation of novel ingredients are critical for developing effective, cost-efficient feed additives in the expanding pet food market.

Protein hydrolysis of fish viscera is a widely implemented industrial process designed to enhance nutrient absorption in livestock by improving protein digestibility (Opheim et al., 2016). Traditional hydrolysis methods include chemical hydrolysis, involving pH adjustment, and enzymatic hydrolysis, which is favoured in industrial settings due to its mild operating conditions and precise peptide bond cleavage (Villamil et al., 2017). Optimising hydrolysis efficiency is particularly critical, as it directly impacts protein recovery rates and the generation of antioxidative low molecular weight peptides (Foh et al., 2010). Structurally, fish viscera are encapsulated by internal membranes primarily composed of collagen, a triple-helical protein complex with substantial resistance to enzymatic degradation (Zhang et al., 2013; Espinales et al., 2023). Collagen, a polymer electrolyte, exhibits pH-dependent swelling behaviour, with the degree of swelling increasing in both acidic and alkaline environments that deviate from its isoelectric point (Ding et al., 2022). Given these properties, it can be expected that acid/alkali swelling of salmon viscera could enhance the enzymatic hydrolysis process by weakening the structural integrity of collagen, thereby facilitating more efficient protein breakdown.

The Maillard reaction, sometimes referred to as glycation in biological concepts, is a non-enzymatic reaction that forms covalent bonds between free amino acids, peptides, or proteins and reducing sugars, significantly affecting the nutritional, sensory, and functional properties of food and feed (Tamanna et al., 2015). Studies investigating the antioxidant properties of protein hydrolysates derived from fishery by-products have demonstrated that glycation can enhance these properties by improving radical scavenging activity and reducing power (Zeng et al., 2012; Korczek et al., 2020). Furthermore, glycation has been shown to effectively mitigate undesirable fishy odours and off-flavours in protein hydrolysates from salmon by-products, thereby enhancing their flavour profile (Kouakou et al., 2014). These findings suggest that glycation is a viable processing technique not only for enhancing antioxidant activity but also for improving the sensory characteristics of protein hydrolysates.

Given the industrial and biochemical background mentioned above, we hypothesised that the continuous process involving acid/alkali swelling, enzymatic hydrolysis, and glycation could enhance the antioxidant capacities of protein hydrolysates derived from salmon viscera waste. Therefore, this study aimed to investigate the effects of acid/alkali swelling and glycation with xylose and/or glucose on the hydrolysis characteristics and antioxidant capacities of protein hydrolysates from salmon viscera.

Materials and Methods

Ethical approval

This study used salmon viscera sourced from a local salmon fillet processing facility that imports and processes frozen salmon and hence no ethical approval was required for this research.

Raw material

Salmon viscera were sourced from a local salmon fillet processing facility that imports and processes frozen salmon (*Salmo salar*) from Chile. The viscera, including all intact organs, were manually removed during the gutting process. On the same day, approximately 10 kg of viscera were randomly collected from a visceral collection container. The collected viscera were sealed in a plastic bag, refrigerated, transported to the laboratory, and stored at 4 °C for 24 h.

Procedure of sample preparation

Acid/alkali swelling

The salmon viscera were thoroughly washed multiple times with running tap water to remove residual digestive matter and prevent spoilage due to endogenous enzyme activation. The cleaned viscera were then weighed and divided into three groups (500 g each). Each group was individually immersed in an equal volume of one of three swelling solutions at different pH levels: an acid solution (2 % (v/v) apple vinegar, pH 3.18), deionised and distilled water (DDW, pH 5.75), and an alkali solution (2 % (w/v) sodium bicarbonate, pH 8.17). The total acidity of the apple vinegar was 6–7 %. The reagents for pH adjustment were selected based on their safety for human and animal consumption. The acid/alkali swelling was conducted at 4 ºC for 18 h. Following the procedure, the viscera were drained and rinsed several times with running water to remove any residual swelling solution.

Enzymatic hydrolysis

Each group of pre-treated salmon viscera was enzymatically hydrolysed using Alcalase 2.4 L

(declared activity of 2.4 AU.kg^{-1}, density of 1.18 g.mL^{-1}, Novozymes, Bagsværd, Denmark), with enzyme activity standardised to 0.1 AU.g^{-1} protein (Ovissipour et al., 2008). The pH of distilled water was adjusted to 8.0 using 1 M NaOH, and the swelled salmon viscera was blended at a 1:1 ratio. Enzymatic hydrolysis was conducted in a constant-temperature water bath (JSIB-22T, JS Research Inc., Gongju, Korea) set to 55 ºC for 1 h, followed by heating at 85 ºC for 20 min to inactivate the enzyme. The hydrolysate was cooled to room temperature for approximately 1 h and then filtered through a test sieve (500 µm mesh, line thickness 315 µm, 885705, Chung Gye Sanggong Sa, Seoul, Korea) to remove the insoluble matter. The filtrate (salmon viscera raw hydrolysate) was used to determine the degree of hydrolysis and for free amino acid content and was stored at 4 ºC until further glycation processing.

Glycation process

In each treatment group, the salmon viscera raw hydrolysate was divided into five equal aliquots (200 mL each) for further glycation process: 1) unheated control (raw salmon viscera hydrolysate), 2) only heated salmon viscera hydrolysate without reducing sugar, 3) salmon viscera hydrolysate heated with 1 % (w/v) D-glucose (Glu, purity of 98.0 %, Samchun Chemical, Seoul, Korea), 4) salmon viscera hydrolysate heated with 1 % (w/v) D-xylose (Xyl, purity of 98.5 %, Samchun Chemical, Seoul, Korea), and 5) salmon viscera hydrolysate heated with a combination of 0.5 % (w/v) Glu and 0.5 % (w/v) Xyl. The heating process for glycation was carried out in a constant-temperature water bath set at 95 °C for 1 h (Zhang et al., 2020). After heating, the reaction mixture was cooled, and the browning index was determined. The remaining samples were stored at -70 °C and subsequently lyophilised using a freeze dryer (80 × 10^{-3} Torr pressure, PVTFD10R, Ilshin Lab, Daejeon, Korea). The freeze-dried samples were used for further antioxidant capacity analysis.

Analysis of salmon viscera hydrolysate

pH changes during enzymatic hydrolysis

The pH change during enzymatic hydrolysis was monitored using an electric pH meter (Orion star A211; Thermo Fisher Scientific, USA). The pH meter was calibrated at room temperature using pH standard buffers (pH 4.01, 7.00, and 10.01).

Degree of hydrolysis

The degree of hydrolysis (DH) was measured in triplicate by the *o*- phthalaldehyde (OPA) method (Jung et al., 2015). The OPA reagent was prepared as follows: 7.62 g di-sodium tetraborate decahydrate and 200 mg sodium-dodecyl-sulphate were dissolved in 150 mL deionised water. One hundred and sixty mg of *o*-phtahldialdehyde was dissolved in 4 mL of 99 % ethanol, and 176 mg dithiothreitol was added to the solution by rinsing with deionised water. The solution was adjusted to 200 mL with deionised water. The OD_{sample} value was obtained by measuring the absorbance at 340 nm after reacting 400 µL of salmon viscera hydrolysate with 3 mL of OPA reagent for 2 min. The OD_{total} value was measured by additional acid hydrolysis at 120 °C for 12 h in a dry oven by adding 10 mL of 6 N hydrochloric acid (HCl) to the 1 mL of salmon viscera hydrolysate sample, and the absorbance was measured by reacting with the 3 mL of OPA reagent for 2 min. The degree of hydrolysis was calculated as follows; Degree of hydrolysis (%) = (OD_{sample} - OD_{blank}) / (OD_{total} - OD_{blank}) × 100.

Free amino acid content

The free amino acid content of salmon viscera hydrolysates was analysed in triplicate after pre-treatment with trichloroacetic acid (TCA). A 16 % TCA solution (v/v) was added to an equal volume of salmon viscera protein hydrolysate and heated in a constant-temperature water bath preheated to 100 °C for 15 min. After cooling for 30 min, the supernatant obtained after centrifugation at 2,000 ×g for 15 min was filtered using a 0.45 µm syringe filter (DISMIC-25CS, Advantec MFS Inc., Tokyo, Japan) and diluted ten-fold. For free amino acid analysis, an amino acid analyser (L-8900 Amino Acid Analyzer, Hitachi, Tokyo, Japan) was used. Twenty microliters of each sample were injected into the amino acid analyser equipped with a Hitachi AAA PH column (#2622 PH column, 4.6 mm I.D. × 60 mm, Hitachi, Japan). The column temperature was set to 57 °C, with a flow rate of 0.4 mL.min^{-1}. The absorbance of the sample was measured at 570 nm, or at 440 nm for proline specifically. The limit of detection of amino acids was at least 0.1 µg.mL^{-1}, and the limit of quantification was below 0.3 µg.mL^{-1}. The R^2 values of calibration curves were over 0.98, respectively.

Analysis of glycated salmon viscera hydrolysate

Browning index

The browning index of the glycated salmon viscera hydrolysate was performed in triplicate by the method of Habinshuti et al. (2022). The samples were diluted with distilled water (ten-fold) and centrifuged at 3,000 ×g for 10 min (4 °C). The absorbance of the supernatant was read at 420 nm.

2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity

ABTS radical scavenging activity was performed in triplicate by the method of Binsan et al. (2008). Fresh $ABTS^{+}$ reagent was prepared by mixing 7.4 mM ABTS and 2.6 mM potassium persulphate in a ratio of 1:1 (v/v) in a dark room at room temperature for 12 h, and then the prepared $ABTS^{+}$ reagent was mixed with methanol in a ratio of 1:15 (v/v). The raw hydrolysate (150 µL) was

reacted with 2.85 mL of the fresh $ABTS^{+}$ reagent in a darkroom at room temperature for 2 h. The absorbance of the reactant was read at 734 nm, and ABTS radical scavenging activity was expressed as µmol trolox equivalent (TE).mL^{-1}.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined in triplicate using the method of You et al. (2010). Six hundred microliters of 5 mM 1,10-phenanthroline solution, 600 µL of 5 mM $FeSO_4$ solution, 600 µL of 15 mM ethylenediaminetetraacetic acid (EDTA) solution, and 400 µL of 0.2 M sodium phosphate buffer (pH 7.4) were mixed. The sample (600 µL) was reacted with 800 µL of 0.01 % hydrogen peroxide (H_2O_2) and 2.2 mL of the mixed reagent at 37 °C for 1 h. The absorbance of the reactant was measured at 560 nm. OD_blank was measured by adding 800 µL of distilled water instead of hydrogen peroxide, and OD control was measured by adding 600 µL of distilled water instead of the sample. Hydroxyl radical scavenging activity was calculated by the following equation: Hydroxyl radical scavenging activity (%) = $(OD_{treatment} - OD_{control}) / (OD_{blank} - OD_{control}) \times 100$.

Reducing power

Reducing power was measured in triplicate using the method of Wu et al. (2003). Two millilitres of 0.2 M sodium phosphate buffer (pH 6.6) and 2 mL of 1 % potassium ferricyanide were reacted with 2 mL of the sample at 50 °C for 20 min. Then, 2 mL of 10 % TCA was added and the mixture was centrifuged at 1,500 ×g for 20 min. Two millilitres of the supernatant were mixed with 2 mL of distilled water and reacted with 0.4 mL of 0.1 % ferric chloride at room temperature for 10 min. The absorbance of the reactant was measured at 700 nm.

Inhibition of lipid peroxidation

The inhibition of lipid peroxidation induced by iron was performed in triplicate by the method of Gu et al. (2009). The LLS solution was prepared by dissolving lecithin (10 $mg.mL^{-1}$) in 0.01 M sodium phosphate buffer solution (pH 7.4). Fifteen grams of TCA, 0.37 g 2-thiobarbituric acid (TBA), and 2 mL of HCl were dissolved in distilled water and finally adjusted to 100 mL with distilled water (TCA-TBA-HCl solution). One millilitre of LLS solution, 1 mL of 0.4 mM ascorbic acid, 1 mL of 0.4 mM ferric chloride ($FeCl_3$), and 1 mL of sample were mixed and heated in the constant-temperature water bath at 37 °C for 60 min. After heating, 2 mL of TCA-TBA-HCl solution was added, heated again in the water bath at 100 °C for 15 min, and then cooled in ice water for 20 min. The cooled sample was centrifuged at 750 ×g for 10 min (4 °C), and the absorbance of the supernatant was read at 532 nm (OD_{Sample}). Blank was measured by adding 1 mL of distilled water instead of the sample (OD_{Blank}). The lipid peroxidation inhibition rate was calculated by the following equation: Inhibition percentage (%) = $(OD_{Blank} - OD_{Sample}) / OD_{Blank} \times 100$.

Statistical analysis

All data were expressed as mean ± standard error (S.E.). Data from all measured variables with three independent replicates were analysed using the general linear model (GLM) procedure in SPSS 18.0 software (SPSS Inc., Chicago, USA). All trait models were used to determine the antioxidant capacities of the glycated salmon viscera hydrolysates, considering the pre-treatment effect, glycation treatment effect, and their interaction as fixed effects, and independent batch as a random effect. For variables with statistically significant effects at a 5 % critical value, Duncan's multiple range test was used to determine the significance of the differences between treatments ($P < 0.05$).

Results

Effects of acid/alkali swelling on hydrolysis characteristics

The pH changes during Alcalase-mediated enzymatic hydrolysis of the swelled salmon viscera were monitored over 60 min (Table 1). Previous studies have shown that pH control methods, such as the pH-stat adjustment and free-fall pH methods, do not significantly impact the hydrolysis characteristics of salmon frame protein (Valencia et al., 2021). Therefore, the free-fall pH method was adopted in this study due to its operational convenience. A rapid decline in pH was observed during the initial 20 min of enzymatic hydrolysis across all pre-treatment groups. Both distilled water and acid swelling treatments resulted in similar final pH values (6.28 and 6.24, respectively), whereas the alkali swelling maintained a relatively higher final pH of 6.94.

The degree of hydrolysis (DH) was significantly influenced by the acid/alkali swelling (Table 1). The DH of salmon viscera reported in earlier studies, using various enzymes such as Protamex, papain, and bromelain, ranged from 35.0 % to 50 % (Opheim et al., 2015). Consistent with these findings, our results demonstrated a DH range of 38.16 % to 39.27 %. Notably, both acid and alkali swelling significantly increased the DH compared to the distilled water swelling control ($P < 0.05$), and no significant difference was observed between the acid and alkali swelling treatments. The yields of salmon hydrolysates, excluding insoluble residues, were 67.2 % for alkali swelling treatment, 65.8 % for acid swelling treatment, and 64.8 % for distilled water treatment, aligning with the DH results (data not shown).

The free amino acid content of swelled and Alcalase-hydrolysed salmon viscera hydrolysates is presented in Table 2. A total of eight essential amino acids and nine non-essential amino acids were detected. Alkali swelling significantly enhanced the total free amino

Table 1. pH changes during Alcalase-mediated hydrolysis for 60 min and the degree of hydrolysis (DH) of acid/alkali swelled salmon viscera.

Time	Distilled water swelling	Acid swelling	Alkali swelling
Initial (controlled pH)	8.00	8.00	8.00
5 min	6.44	6.37	7.01
10 min	6.35	6.31	6.91
15 min	6.29	6.26	6.87
20 min	6.27	6.24	6.87
30 min	6.26	6.25	6.88
40 min	6.28	6.24	6.89
50 min	6.27	6.24	6.92
60 min	6.28	6.24	6.94
Degree of hydrolysis (%)	38.16 ± 0.11^{b}	39.16 ± 0.28^{a}	39.27 ± 0.12^{a}

Mean ± standard error (n = 3).
The swelling conditions for salmon viscera waste were as follows; acid solution (pH 3.18), deionised and distilled water (pH 5.75), and alkali solution (pH 8.17).
The Alcalase-mediated hydrolysis (0.1 AU.g protein^{-1}) was performed at 55 °C for 1 h.
^{a,b}Means with the same letter within a row are not significantly different ($P > 0.05$).

Table 2. Free amino acid content of swelled and Alcalase-hydrolysed salmon viscera.

Amino acids (mg.100 mL^{-1})	Distilled water swelling	Acid swelling	Alkali swelling
Total amino acid	419.7 ± 7.5^{c}	512.8 ± 28.7^{ab}	533.3 ± 38.1^{a}
Essential amino acid			
Phe	35.1 ± 2.2^{b}	41.5 ± 2.7^{ab}	47.4 ± 2.0^{a}
Lys	25.6 ± 1.4^{b}	25.4 ± 1.8^{b}	35.5 ± 1.4^{a}
Met	25.0 ± 0.5^{b}	25.7 ± 1.6^{ab}	30.6 ± 2.1^{a}
Thr	18.5 ± 0.7^{b}	20.1 ± 1.3^{b}	30.4 ± 1.5^{a}
Val	22.7 ± 1.3^{b}	25.8 ± 2.7^{b}	37.7 ± 1.4^{a}
Ile	24.8 ± 2.3^{b}	28.4 ± 3.1^{b}	40.4 ± 2.7^{a}
Leu	44.7 ± 2.6^{b}	51.6 ± 4.4^{b}	60.8 ± 2.8^{a}
Non-essential amino acid			
Asp	29.2 ± 0.2^{b}	29.4 ± 1.4^{b}	40.7 ± 1.6^{a}
Tau	18.9 ± 0.9^{b}	21.6 ± 0.7^{b}	27.3 ± 1.4^{a}
Ser	15.8 ± 0.9^{b}	18.1 ± 0.8^{b}	28.9 ± 1.0^{a}
Glu	51.9 ± 0.2^{b}	55.1 ± 3.2^{b}	71.8 ± 2.4^{a}
Gly	9.9 ± 0.7^{b}	11.3 ± 0.6^{b}	17.5 ± 0.6^{a}
Ala	23.0 ± 1.0^{b}	25.3 ± 1.4^{b}	35.8 ± 1.3^{a}
Tyr	37.8 ± 4.1^{b}	65.9 ± 8.2^{a}	83.2 ± 6.4^{a}
Orn	14.3 ± 4.8	7.2 ± 0.4	8.6 ± 0.4
His	7.5 ± 0.6^{b}	8.7 ± 0.1^{b}	12.6 ± 0.5^{a}
Cys	5.8 ± 0.2^{b}	6.0 ± 0.5^{b}	9.5 ± 0.3^{a}
Ans	2.5 ± 0.0^{b}	12.9 ± 0.6^{a}	13.8 ± 0.7^{a}
Car	–$^{1)}$	1.0 ± 0.2^{a}	1.0 ± 0.3^{a}
Arg	2.1 ± 0.1^{c}	23.8 ± 1.1^{b}	33.8 ± 1.0^{a}
Pro	0.1 ± 0.2^{c}	8.1 ± 0.4^{a}	4.5 ± 0.0^{b}

Mean ± standard error (n = 3).
The swelling conditions for salmon viscera waste were as follows; acid solution (pH 3.18), deionised and distilled water (pH 5.75), and alkali solution (pH 8.17).
The Alcalase-mediated hydrolysis (0.1 AU.g protein^{-1}) was performed at 55 °C for 1 h.
$^{1)}$Less than detectable limits.
$^{a-c}$Means with the same letter within a row are not significantly different ($P > 0.05$).

acid content in the hydrolysates compared to distilled water and acid swelling treatments. Apart from ornithine and proline, all detected amino acids were present in higher concentrations in the alkali swelled hydrolysates than in those treated with distilled water ($P < 0.05$). The ornithine content in the distilled water treatment (control) was higher than that in the acid and alkali swelling treatments; however, the difference was not significant. These findings show that alkali swelling markedly alters the hydrolysis characteristics of salmon viscera, leading to increased DH and elevated free amino acid content.

Alkali swelling resulted in the highest levels of essential amino acids such as Phe, Lys, Met, Thr, Val, Ile, and Leu, indicating its superior role in releasing bioactive peptides and free amino acids. The increase in leucine (60.8 mg.100 mL^{-1}) and isoleucine (40.4 mg.100 mL^{-1}) after alkali swelling is particularly noteworthy, as these branched-chain amino acids (BCAAs) are crucial for muscle metabolism and animal nutrition (Zhang et al., 2017). Lysine content also significantly increased with alkali swelling (35.5 mg.100 mL^{-1}), which is important for protein synthesis and feed formulation (Liao et al., 2015). Thus, enhanced amino acid release through alkali swelling could improve the nutritional profile of hydrolysates, making them valuable for pet food formulations and functional feed additives.

Effects of acid/alkali swelling on glycation efficiency

The browning index of swelled, Alcalase-hydrolysed, and glycated salmon viscera hydrolysates is depicted in Figure 1. The browning index is a quantitative indicator of the intensity of enzymatic and non-enzymatic browning reactions, including glycation (Kouakou et al., 2014). A significant interaction between swelling and glycation effects was observed. The browning index of unheated control (raw hydrolysates) was slightly lower in acid and alkali swelling treatments compared to the distilled water treatment ($P < 0.05$). Glycation with xylose (1 % Xyl or 0.5 % Xyl + 0.5 % Glu) significantly increased the browning index compared to the raw hydrolysate ($P < 0.05$). The highest browning index was observed for salmon viscera hydrolysates subjected to acid/alkali swelling followed by glycation with xylose ($P < 0.05$). The glycation process, particularly with xylose, likely led to the formation of advanced glycation end-products, which are characterised by their chromophore groups with maximum absorbance at 420 nm (Chung et al., 2012). The lower browning index observed in glucose-glycated samples suggests that the glycation reaction may have been less efficient under the specific chemical conditions (pH, temperature, and time) used in this study.

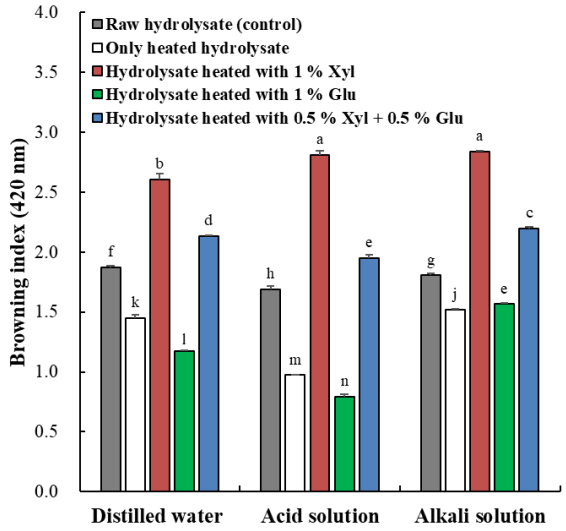

Fig. 1. Browning index of swelled, Alcalase-hydrolysed, and glycated salmon viscera. The swelling conditions for salmon viscera waste were as follows; acid solution (pH 3.18), deionised and distilled water (pH 5.75), and alkali solution (pH 8.17). The Alcalase-mediated hydrolysis (0.1 AU.g protein^{-1}) was performed at 55 °C for 1 h. Except for raw hydrolysate, all treatments were heated to induce glycation at 95 ° C for 1 h. Xyl, D-xylose; Glu, D-glucose. Error bars represent each standard error (S.E., n = 3). a-n Means sharing with the same letters are not significantly different ($P < 0.05$).

Effects of swelling and glycation on antioxidant activities of salmon viscera hydrolysate

Significant interactions between swelling and glycation effects were observed across all antioxidant assays. The ABTS radical scavenging activity of swelled, Alcalase-hydrolysed, and glycated salmon viscera hydrolysates is shown in Figure 2. Previous research on rainbow trout visceral hydrolysates reported ABTS radical scavenging activity of 1,469

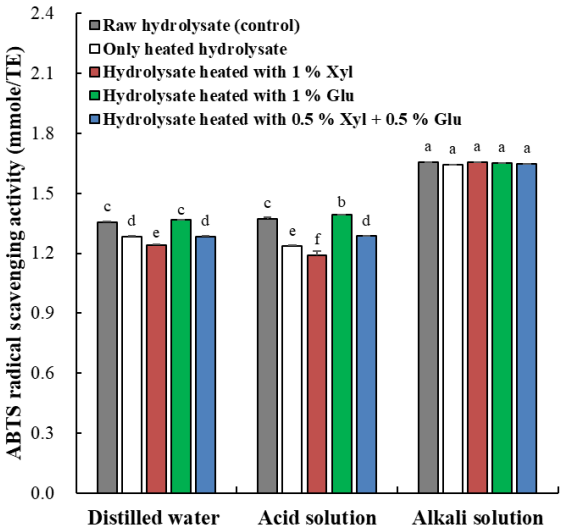

Fig. 2. ABTS radical scavenging activity of swelled, Alcalase-hydrolysed, and glycated salmon viscera. The swelling conditions for salmon viscera waste were as follows; acid solution (pH 3.18), deionised and distilled water (pH 5.75), and alkali solution (pH 8.17). The Alcalase-mediated hydrolysis (0.1 AU.g protein^{-1}) was performed at 55 °C for 1 h. Except for raw hydrolysate, all treatments were heated to induce glycation at 95 °C for 1 h. Xyl, D-xylose; Glu, D-glucose. Error bars represent each standard error (S.E., n = 3). a-f Means sharing with the same letters are not significantly different ($P < 0.05$).

μmol TE.g^{-1}, which is comparable to the results obtained in this study (1,192.2-1,656.0 μmol TE.g^{-1}) (Vásquez et al., 2022). Alkali swelling treatment consistently resulted in higher ABTS radical scavenging activity compared to other treatments, regardless of glycation ($P < 0.05$). However, glycation did not significantly alter the ABTS radical scavenging activity of alkali swelled hydrolysates ($P > 0.05$), indicating that glycation had minimal impact on the radical scavenging capacity in these samples.

The hydroxyl radical scavenging activity is shown in Figure 3. In the raw hydrolysates, acid swelling treatment resulted in significantly higher hydroxyl radical scavenging activity compared to distilled water or alkali swelling treatments. However, glycation led to obvious changes in hydroxyl radical scavenging activity depending on the swelling conditions. Glycation improved scavenging activity in the distilled water and alkali swelling treatments, while a reduction in activity was observed following acid swelling treatment.

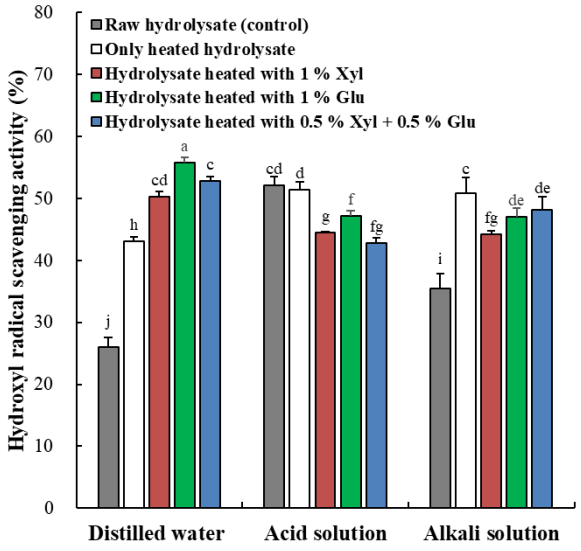

Fig. 3. Hydroxyl radical scavenging activity of swelled, Alcalase-hydrolysed, and glycated salmon viscera. The swelling conditions for salmon viscera waste were as follows; acid solution (pH 3.18), deionised and distilled water (pH 5.75), and alkali solution (pH 8.17). The Alcalase-mediated hydrolysis (0.1 AU.g protein^{-1}) was performed at 55 °C for 1 h. Except for raw hydrolysate, all treatments were heated to induce glycation at 95 °C for 1 h. Xyl, D-xylose; Glu, D-glucose. Error bars represent each standard error (S.E., n = 3). a-j Means sharing with the same letters are not significantly different ($P < 0.05$).

The reducing power of the glycated salmon viscera hydrolysates is presented in Figure 4. Acid and alkali swelling treatments exhibited significantly higher reducing power than distilled water swelling treatment, irrespective of glycation. The highest reducing power was observed in alkali swelled raw hydrolysates ($P < 0.05$). After glycation, the reducing power of alkali swelled hydrolysates decreased significantly, while a slight increase was noted in the acid or distilled water

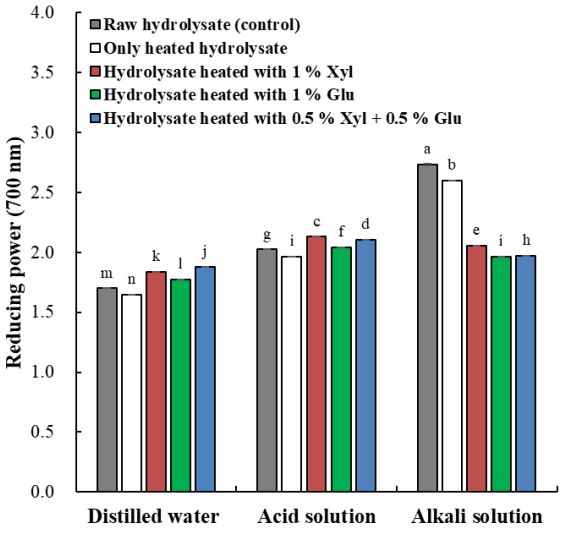

Fig. 4. Reducing power of swelled, Alcalase-hydrolysed, and glycated salmon viscera. The swelling conditions for salmon viscera waste were as follows; acid solution (pH 3.18), deionised and distilled water (pH 5.75), and alkali solution (pH 8.17). The Alcalase-mediated hydrolysis (0.1 AU.g protein^{-1}) was performed at 55 °C for 1 h. Except for raw hydrolysate, all treatments were heated to induce glycation at 95 °C for 1 h. Xyl, D-xylose; Glu, D-glucose. Error bars represent each standard error (S.E., n = 3). a-n Means sharing with the same letters are not significantly different ($P < 0.05$).

swelled counterparts ($P < 0.05$).

The inhibition of lipid peroxidation induced by iron, expressed as the inhibition percentage, is shown in Figure 5. The inhibition percentage of glycated salmon

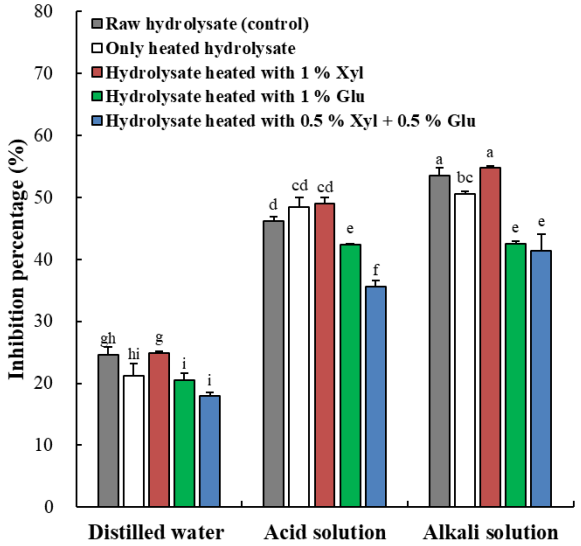

Fig. 5. Inhibition percentage of lipid peroxidation induced by iron of swelled, Alcalase-hydrolysed, and glycated salmon viscera. The swelling conditions for salmon viscera waste were as follows; acid solution (pH 3.18), deionised and distilled water (pH 5.75), and alkali solution (pH 8.17). The Alcalase-mediated hydrolysis (0.1 AU.g protein^{-1}) was performed at 55 °C for 1 h. Except for raw hydrolysate, all treatments were heated to induce glycation at 95 °C for 1 h. Xyl, D-xylose; Glu, D-glucose. Error bars represent each standard error (S.E., n = 3). a-i Means sharing with the same letters are not significantly different ($P < 0.05$).

viscera hydrolysates ranged from 24.6 % to 53.6 %, similar to that reported for protein hydrolysates from sardinella viscera (33.9 %) (Bougatef et al., 2010). Acid and alkali swelling treatments led to significantly higher inhibition percentages compared to distilled water swelling treatment. However, glycation with glucose (1 % Glu or 0.5 % Xyl + 0.5 % Glu) reduced the inhibition percentage across all swelling methods, indicating a potential adverse effect of glycation on the lipid peroxidation inhibitory capacity of the salmon viscera hydrolysates. These results show that antioxidant properties of salmon viscera hydrolysate could be improved only through a specific combination of swelling and glycation processes.

Discussion

Effects of acid/alkali swelling on hydrolysis characteristics

Enzymatic hydrolysis, which releases free amino acids and peptides, typically leads to an increase in hydrogen ion concentration, causing a decrease in pH (Foh et al., 2010). Similar biochemical changes were observed in this study during Alcalase-mediated hydrolysis (Table 1). However, the decline in pH was less pronounced in the alkali swelled samples, likely due to the presence of residual hydroxide ions, despite several washings. Additionally, acid and alkali swelling treatments significantly enhanced the DH of salmon viscera hydrolysates. Generally, adjusting the pH away from the isoelectric point of fish proteins, which is around pH 5.2–5.5, is an effective strategy to improve protein solubility (Kim et al., 2003; Ramakrishnan et al., 2023). Acidic or alkaline conditions can increase protein solubility, thereby improving the DH of protein hydrolysates (Tan et al., 2015; Prihanto et al., 2019). These changes are particularly relevant for collagen, whose isoelectric point is near neutral pH (7.4) (Achilli et al., 2010). Our findings demonstrate that both acid and alkali swelling can effectively enhance the efficacy of Alcalase-mediated hydrolysis in salmon viscera.

The amino acid composition of protein hydrolysates is a key determinant of their nutraceutical and functional properties. It is well-established that the generation of certain free amino acids can significantly enhance the antioxidant capacities of protein hydrolysates (Chalamaiah et al., 2012). For instance, hydrophobic amino acids such as leucine, isoleucine, alanine, valine, and glycine play critical roles as proton donors or radical scavengers (Zamora-Sillero et al., 2018). Aromatic amino acids like phenylalanine, tyrosine, and histidine can donate electrons to free radicals, exhibiting high radical scavenging activity (Amiri et al., 2013). Sulphur-containing amino acids such as cysteine and taurine contribute to reducing oxidative stress by scavenging reactive oxygen species (ROS) (Kim et al., 2020). Furthermore, dipeptides like carnosine and anserine, which were most abundant in alkali swelled salmon viscera hydrolysate (Table 2), are known for their antioxidant properties in muscle tissue (Wu et al., 2003). The increased levels of lysine and arginine in alkali swelled hydrolysates could also facilitate the formation of glycated products during glycation (Liu et al., 2020). Thus, the observed increase in specific free amino acids following alkali swelling treatment likely contributes to the enhanced antioxidant capacities of salmon viscera hydrolysates.

Effects of acid/alkali swelling on glycation efficiency

The formation of glycated products in sugar-amino acid model systems has been extensively studied, with the glycation process being influenced by factors such as the concentrations of reducing sugars and amino acids, heating conditions (time and temperature), and the chemical environment (water activity and pH) (Kitts et al., 2012; Xu et al., 2017). According to Ajandouz and Pulgserver (1999), high pH values (between pH 6 and 12) can enhance glycation by accelerating the deprotonation of α-amino groups in amino acids. Additionally, Laroque et al. (2008) reported that xylose, due to its higher reactivity compared to glucose, could induce a faster glycation rate of amino acids. These factors likely explain why acid/alkali swelling treatment followed by glycation with xylose provided optimal conditions for producing glycated products in salmon viscera hydrolysates.

Effects of swelling and glycation on antioxidant activities of salmon viscera hydrolysate

In this study, acid and alkali swelling treatments positively influenced the antioxidant activities of salmon viscera hydrolysates. As previously mentioned, alkali swelling treatment resulted in significantly higher ABTS radical scavenging activity and reducing power compared to distilled water pre-treatment. Additionally, both acid and alkali swelled hydrolysates exhibited high inhibition percentages in lipid peroxidation induced by iron (Fig. 5). The enhanced ABTS radical scavenging activity and reducing power observed in the alkali swelled samples are likely due to the electron-donating properties of certain free amino acids (e.g., phenylalanine, cysteine, tyrosine, leucine) and antioxidant peptides (Kumar et al., 2011; Yu et al., 2012; Qin et al., 2020). The increased recovery of free amino acids through swelling process appears to be a important factor contributing to the enhanced antioxidant activity in salmon viscera waste.

Contrary to expectations, glycation did not generally enhance the antioxidant activity of swelled salmon viscera hydrolysates, with the exception of hydroxyl radical scavenging activity (Figs. 2–5). Specifically, the ABTS radical scavenging activity of acid and distilled water pre-treated hydrolysates decreased after glycation with reducing sugars (Fig. 2). Additionally, glycation with reducing sugars reduced the hydroxyl radical scavenging activity and reducing power of acid and alkali swelled hydrolysates (Figs. 3 and 4).

Previous studies have similarly reported unchanged or reduced antioxidant capacities following glycation. For example, Oh et al. (2013) found no change in the ABTS radical scavenging activity of milk protein hydrolysates after glycation. Nie et al. (2017) observed that the hydroxyl radical scavenging activity of chicken bone hydrolysates varied depending on peptide concentration and molecular weight, with some higher concentrations of glycated hydrolysates showing no scavenging activity. Chen et al. (2019) also noted a decrease in the reducing power of fish gelatine hydrolysates after glycation. The glycation process, which consists of three stages, produces various Maillard reaction products (MRPs) with differing antioxidant properties at each stage (Cui et al., 2021). Therefore, the progression of glycation likely plays a crucial role in determining the antioxidant capacities of glycated protein hydrolysates.

The results of this study suggest that while acid/alkali swelling treatment can improve the degree of hydrolysis of salmon viscera, leading to enhanced antioxidant activity due to the release of free amino acids, the effects of glycation on antioxidant activity are less straightforward. Despite supporting evidence from previous studies, glycation either diminished or had no significant impact on antioxidant activity under certain conditions, limiting definitive conclusions. It is likely that the observed results were influenced by the varying progress of glycation, which was affected by pre-treatment conditions and the presence of reducing sugars.

Conclusion

This study industrially designed a continuous process involving acid/alkali swelling, Alcalase-mediated hydrolysis, and glycation to enhance the antioxidant activity of salmon viscera hydrolysates. The acid/alkali swelling significantly improved the efficiency of enzymatic hydrolysis, leading to an increased release of free amino acids. Notably, significant interactions between the swelling conditions and glycation with different reducing sugars (glucose and/or xylose) were observed, particularly with xylose, which suggests an optimal strategy for enhancing the antioxidant properties of salmon viscera hydrolysates. However, it is important to note that antioxidant activity varied depending on the specific combination of swelling and glycation, potentially due to differences in the glycation process. From the perspective of the pet food industry, the enhanced antioxidant properties of hydrolysates produced through this process may contribute to improving feed quality and extending shelf life, offering both economic and nutritional advantages. Consequently, by promoting the sustainable utilisation of fishery processing by-products, our approaches align with global efforts toward waste valorisation and circular economy practices.

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