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Research Article

Effect of the water-to-rainbow trout by-product ratio on autolysis, oxidation, and antioxidant activity of protein hydrolysates

Reyhaneh Mirzaei Emamzadeh[1], Mehdi Nikoo[1*], Hassan Ahmadi Gavlighi[2]

[1]*Department of Pathobiology and Quality Control, Artemia and Aquaculture Research Institute, Urmia University, Urmia, 57179-44514, Iran;* [2]*Department of Food Science and Technology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran*

Abstract

The effects of water-to-rainbow trout by-product ratio on hydrolysis, oxidation, and antioxidant activity of protein hydrolysates were investigated. Di- and tri-peptides were the dominant fractions, followed by free amino acids (FAA) and small oligopeptides. At a 100% water-to-by-product ratio, hydrolysates obtained after 1 h exhibited peptide profiles comparable to those produced at 0% water. In contrast, after 3 h, hydrolysates prepared with 0% water contained higher amounts of FAA and small peptides. Across all samples, DPPH radical-scavenging activity remained high (> 70%). The highest metal-chelating capacity was observed for the 50% water sample at 3 h ($p < 0.05$). However, increasing the water content to 75% and 100% resulted in a significant decrease in chelating activity ($p < 0.05$). The highest ferric reducing antioxidant power (FRAP) values were recorded with 0% water at 1 h ($p < 0.05$), whereas increasing the water content to 100% lowered FRAP. Similarly, the lowest thiobarbituric acid reactive substances formation was found at a 50% water addition ($p < 0.05$). Eicosapentaenoic acid and docosahexaenoic acid accounted for 0.23–0.43% and 0.97–2.19% of the total fatty acids, respectively. However, the differences among treatments were not significant. Overall, the results showed that peptide profiles, oxidation status, and antioxidant activity are influenced by the water content during autolysis.

Keywords: Autolysis, By-products, Oxidation, Protein hydrolysates, Water content.

Introduction

Rainbow trout (*Oncorhynchus mykiss*) is a widely cultured aquaculture species. According to the Food and Agriculture Organization (FAO), global production of rainbow trout reached 808,000 ton in 2023 (FAO, 2025). By-product of rainbow trout is largely discarded as waste (Vázquez et al., 2019; Stevens et al., 2018). Nevertheless, hydrolysis of rainbow trout by-products using different commercial proteases yields protein hydrolysates and peptides with antioxidant activity (Ketnawa et al., 2018; Nguyen et al., 2017). Trypsin and myofibril-bound serine proteinase (MBSP; 8–23 kDa) have been identified as the principal proteases in the pyloric ceca of rainbow trout. These enzymes exhibited high activity at 55 °C and pH 7.0 and retained more than 87% of their initial activity after 1 h of incubation at 40 °C (Andevari et al., 2019). Autolysis of rainbow

* **Correspondence:** M. Nikoo (m.nikoo@urmia.ac.ir).
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trout by-products indicated that a treatment of 1 h at 40 °C was sufficient to generate hydrolysates enriched in small peptides. In contrast, gradually increasing the temperature or performing autolysis at higher fixed temperatures reduced enzymatic activity and was accompanied by a higher accumulation of protein carbonyls. Notably, trypsin activity remained comparatively high at lower temperatures (Nikoo et al., 2021). Similarly, hydrolysis of defatted *Sardinella sindensis* mince, particularly in the presence of an antioxidant, could attenuate lipid oxidation as hydrolysis time increased (Sarteshnizi et al., 2019). Collectively, these findings suggest that the functional properties and safety of protein hydrolysates intended for food applications or animal feed are, to a considerable extent, determined by lipid oxidation occurring during the hydrolysis of seafood by-products.

During hydrolysis, water and substrates are mixed at a predetermined ratio prior to enzyme addition, followed by adjustment to the optimal pH and temperature specific to each proteolytic enzyme (Wubshet et al., 2019). Therefore, the appropriate water content is a critical factor affecting the reaction rate and, the structural and biological properties of the resulting peptides. At higher water contents, a more extensive water layer may form around the enzyme surface, which can hinder diffusion of substrates to the enzyme active sites and thereby limit enzymatic turnover (Kuepethkaew et al., 2017). Moreover, high water ratio compared with by-product weight will necessitate additional evaporation step prior to spray drying, which can increase energy consumption (Petrova et al., 2018). Given that by-product contain ~ 70% moisture (Malcorps et al., 2021), some water is also released from the by-product during hydrolysis, which further increase water volume and the drying energy consumption. It is not known to what extent the water content can be reduced during autolysis of rainbow trout by-product without affecting autolysis efficiency, oxidation, and the antioxidant activity of protein hydrolysates. The aim of this study was to investigate the effect of water content on the production and properties of protein hydrolysates and lipid oxidation during autolysis of rainbow trout by-products.

Materials and Methods

Preparation of rainbow trout by-products

Fresh rainbow trout processing by-products (viscera + heads + frames) were obtained from a fish market in Urmia (January 2022) and transported to the Fisheries Processing Laboratory of the Artemia and Aquaculture Research Institute within 20 min in an ice-box.

Autolysis

Ground by-products were mixed with distilled water at ratios of 0%, 25%, 50%, 75%, and 100% and homogenized for 2 min using a homogenizer (Heidolph DIAX900; Heidolph Instruments GmbH, Schwabach, Germany). Autolysis was carried out according to Cao et al. (2009) at 40 °C and an initial pH of 7.1 for 1, 2, and 3 h in a water bath (Model SD15, Tehran, Iran). During autolysis, the mixtures were continuously stirred using a mechanical stirrer (FTDS-11; Sci Finetech Co., Seoul, South Korea). Samples were collected after 1, 2, and 3 h. After passing through a mesh cloth for initial filtration, samples were centrifuged (Z 326 K; Hermle Labortechnik GmbH, Wehingen, Germany) at 6000 × g and 4 °C for 10 min. The resulting supernatants were dried using a freeze-dryer (type 102042, Christ, Osterode, Germany) for 24 h.

Molecular weight distribution of peptides

The molecular weight distribution of peptides was determined using a TSK gel 2000 SWXL (300 ×7.8 mm) column connected to Waters 600 high-performance liquid chromatography (HPLC) (Waters Co., Milford, MA, USA) with a 2487 UV detector at 215 nm. Cytochrome C (12,384 Da), bacitracin (1,423 Da), the tetrapeptide GGYR (451 Da), and the tripeptide GGG (189 Da) were used as molecular weight standards (Nikoo et al., 2021).

Color parameters of protein hydrolysates

Color parameters of the samples were determined using a colorimeter (Color Flex; Virginia, USA) by measuring L^* (lightness), a^* (redness/greenness), and b^* (yellowness/blueness). Prior to analysis, the instrument was calibrated using standard black and white tiles (L^* = 92.24, a^* = −1.31, b^* = 1.18). All measurements were performed in triplicates.

Lipid oxidation

Lipid oxidation was assessed using the thiobarbituric acid (TBA) reactive substance method. Briefly, 500 µL of the reaction solution was mixed with 2.5 mL of TBA reagent (0.375% TBA, 15% trichloroacetic acid (TCA), and 0.25 M hydrochloric acid) and heated for 10 min at approximately 95 °C. The mixture was then cooled to 4 °C and centrifuged (Z 326 K, Hermle Labortechnik GmbH, Wehingen, Germany) for 8 min at 4 °C and 4000 × g. The intensity of the pink chromophore in the supernatant was measured at 532 nm (Buege & Aust, 1978). Malondialdehyde (MDA) content was expressed in mg/kg by constructing a standard curve using 1,1,3,3-tetramethoxypropane (0–6 ppm).

Fatty acid composition

Fatty acid composition was determined according to Miquel and Browse (1992). Analyses were performed using a gas chromatograph (Agilent Technologies 7890A; Santa Clara, CA, USA) equipped with a DB225MS column. Briefly, 1 g of sample was mixed with 1 mL of extraction solution (2.5% sulfuric acid in 98% methanol; 1:40 v/v) and incubated at 80 °C for 1 h. Then, 500 µL of hexane and 1.5 mL of 0.5% sodium chloride solution were added to extract fatty acid methyl esters. The mixture was centrifuged at 4000 rpm for 10 min, and the upper phase containing hexane and methyl esters was collected. Fatty acid levels were calculated based on the relative percentage of the total peak area, with the sum of all peaks set to 100%. The polyene index (PI): ((C20:5 + C22:6)/C16:0) was calculated as a measure of polyunsaturated fatty acid (PUFA) damage (Šimat et al., 2020).

Antioxidant activity

DPPH radical scavenging activity

Antioxidant activity was evaluated using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Intarasirisawat et al., 2013). Protein hydrolysates (5 mg/mL) were added at different concentrations to 1 mL of ethanolic DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (0.1 mM) and mixed for 10 s. The samples were then incubated in the dark for 30 min, after which the absorbance was measured at 517 nm using a spectrophotometer. The percentage of free-radical inhibition was calculated using the following equation:

DPPH RSA (%) = Abs. control – Abs. sample / Abs. control × 100

Metal chelating activity

Iron-chelating activity was determined according to Intarasirisawat et al. (2013). Briefly, 300 µL of the sample (5 mg/mL) was mixed with 35.5 µL of $FeCl_2$ and incubated for 3 min. Subsequently, 5 mM ferrozine (4,4'-(3-(pyridin-2-yl)-1,2,4-triazine-5,6-diyl) dibenzenesulfonic acid) was added, and the mixture was incubated at room temperature for 20 min. Absorbance was measured at 562 nm, and iron-chelating activity was calculated and expressed as a percentage.

Ferric-reducing antioxidant power (FRAP)

FRAP was determined according to Benzie and Strain (1996). Briefly, 150 µL of the peptide solution (5 mg/mL) was mixed with 2850 µL of FRAP reagent (containing 5 mL of 20 mM $FeCl_3$, 5 mL of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and 50 mL of 300 mM acetate buffer, pH 3.6). The mixture was incubated at 37 °C for 30 min, and absorbance was measured at 593 nm. Antioxidant activity was expressed as micromoles of Trolox equivalents, based on a Trolox standard curve (25–150 µM).

Statistical analysis

Statistical analyses were performed using SPSS software (version 16; SPSS Inc., Chicago, IL, USA). Data were analyzed by two-way analysis of variance (ANOVA), and mean comparisons were conducted using Duncan's multiple range test. Statistical significance was considered at a 95% confidence level ($P \leq 0.05$). Results are reported as mean ± standard deviation (SD) based on three independent replicates.

Results and Discussion

Molecular weight distribution of peptides

SEC-HPLC analysis showed that peptides of varying sizes were generated following the autolysis of by-products. A representative chromatogram illustrating the molecular weight distribution of peptides obtained using a 0% water ratio and a 1-h hydrolysis

time is presented in **Figure 1A**. In this sample, di- and tri-peptides (180–500 Da) were the dominant components, accounting for approximately 30% of the total peptides, followed by free amino acids (approximately 25%). Peptides with molecular weights in the range of 500–1000 Da (small oligopeptides) represented approximately 22% of the total peptide fraction. The present findings are consistent with previous results from this laboratory, which reported that di- and tri-peptides (51.6 – 58.4%) and free amino acids (27.3 – 32.9%) were the predominant peptide fractions in rainbow trout by-product protein hydrolysates (Nikoo et al., 2021). Protein hydrolysates prepared from trout heads and trimmings using Alcalase and papain contained a higher percentage of peptides with molecular weights above 1 kDa (52 – 58%) (Vázquez et al., 2019). It has been reported that the type of by-product and the protease applied has significant effect on the hydrolysis extent as seen in mackerel (*Scomber scombrus*) and sardine (*Sardina pilchardus*) by-products (Fuentes et al., 2024). A comparison of different water-to-by-product ratios showed significant differences in the peptide profiles among the 15 samples (**Fig. 1B**).

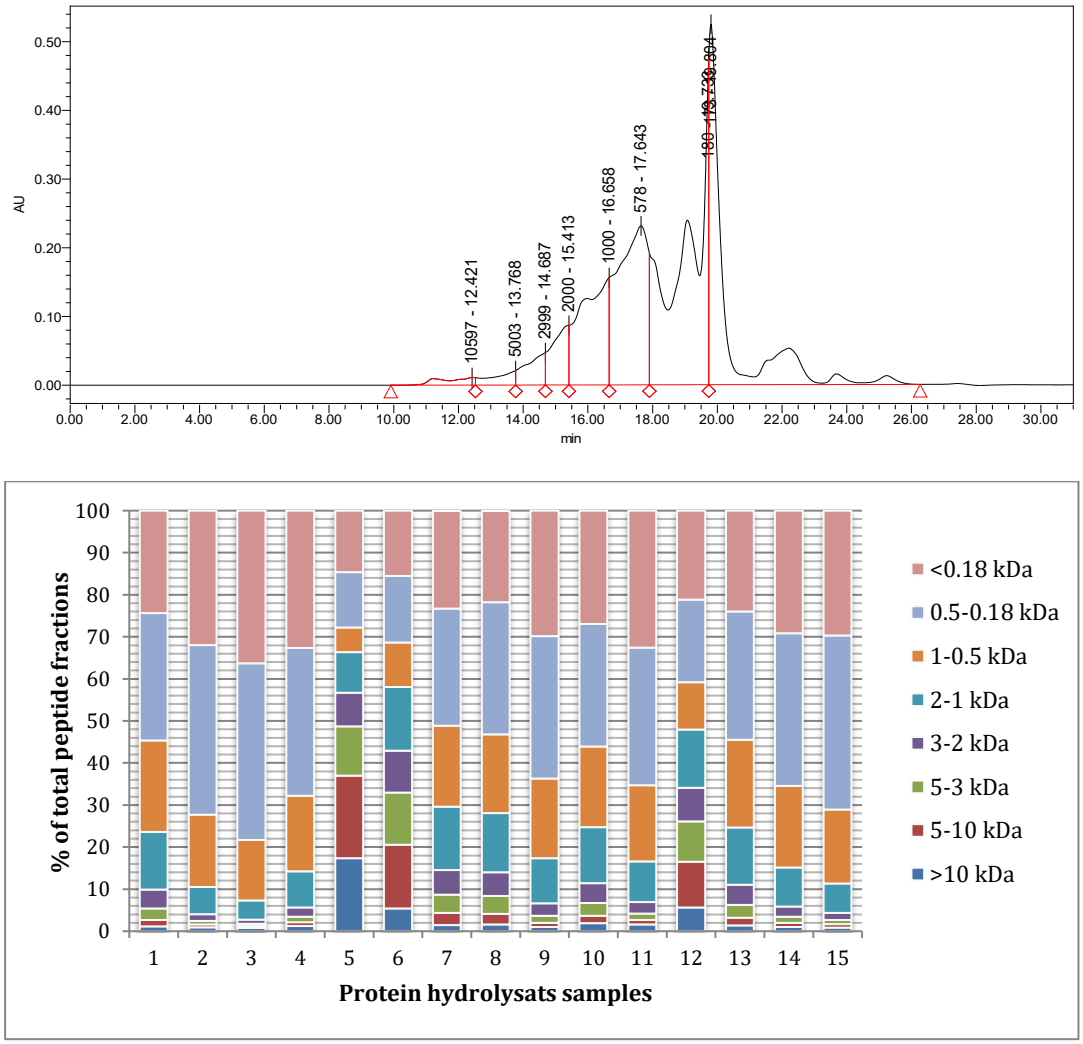

Figure 1. Chromatogram of the molecular weight distribution of peptides at a water ratio of zero and a duration of 1 h (ranging from < 180 Da to > 10 kDa) (**A**, above), and the percentages of various peptide fractions in the protein hydrolysates under different water-to-waste ratios and autolysis times (**B**, below).

Increasing the water ratio to 50% reduced the proportions of dipeptides, tripeptides, and FAA, whereas the fraction with a MW of 500-1000 Da remained largely unchanged. In contrast, the percentage of peptides within the 1 – 10 kDa range was higher than that observed under the 0% water condition. At a 100% water ratio, the peptide profile of the hydrolysate after 1 h was comparable to that obtained at 0% water for the same duration. However, when the 3-h samples were compared, dipeptides, tripeptides, and FAA were present at higher levels under the 0% water condition. This discrepancy may be related to the enhanced accessibility of endogenous enzymes to the by-product proteins in the absence of added water.

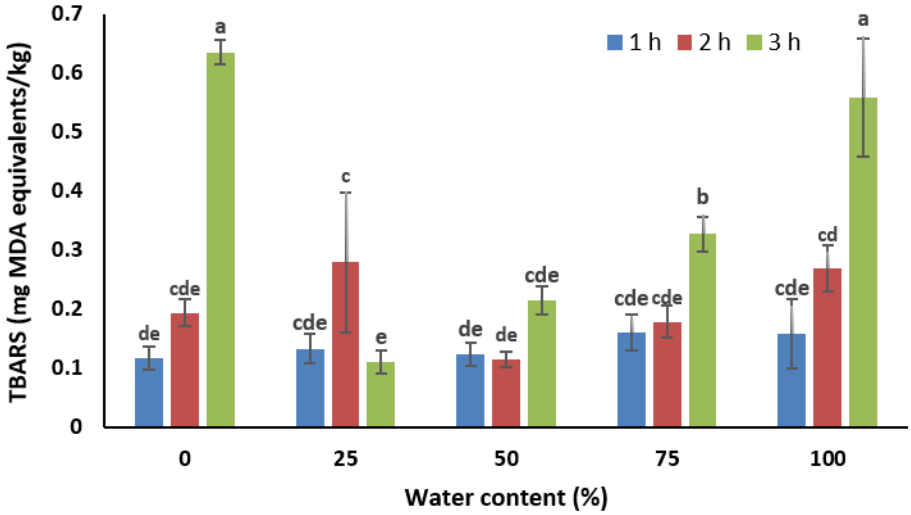

Figure 2. Formation of TBARS in rainbow trout by-product protein hydrolysates in relation to the water-to-waste ratio and autolysis time

Lipid oxidation

TBARS formation in the protein hydrolysate was significantly lower at 1-h of autolysis than at the other time points, although it increased progressively (p < 0.05) with autolysis time (**Fig. 2**). Oxidation levels at 3 h were higher across all water ratios, except for the 25% treatment, in which TBARS reached its maximum after 2 h and then decreased. This decrease might be attributed to the specific properties of the enzymatic reaction medium at this water ratio, compared with other treatments that resulted in the highest lipid oxidation and secondary TBA-reactive products after 2 h. However, the precise mechanism could be elucidated by measuring volatile compounds, such as hexanal and fatty acid content. At 3 h, TBARS values increased significantly compared with 2 h, especially for the 0% and 100% water ratios (p < 0.05), whereas they decreased slightly for the 50% and 75% water treatments. Notably, the 50% water treatment consistently produced the lowest TBARS levels across all time points relative to the other samples (p < 0.05). Marine oils are rich in long-chain unsaturated fatty acids, which are highly susceptible to oxidative degradation (Jacobsen et al., 2008). TBARS in shrimp hepatopancreas were reported to increase significantly when autolysis was performed at a constant temperature of 60 °C for an extended period (150 min) (Senphan & Benjakul, 2012). An increase in TBARS reflects an enhanced breakdown of hydroperoxides and the subsequent formation of secondary lipid oxidation products (Medina et al., 2009). Accordingly, in rainbow trout processing by-products, the prolonged hydrolysis time likely promoted hydroperoxide decomposition and accelerated the generation of secondary lipid oxidation products during autolysis, particularly with 0% and 100% water ratio. It has been stated that hydrolysis with zero added water is more suitable for by-products and raw materials with high water content and lower proportions of bone/shells, due to better homogenization and mixing during the

reaction (Hopkins et al., 2025). In previous studies on hake (*Merluccius merluccius*) by-catch, a solid content of 50% combined with a hydrolysis time of 2 h was identified as the optimal hydrolysis condition, as it ensured enhanced functional properties and antioxidant activity (Iñarra et al., 2023). Similarly, in this study, water ratio of 50% and autolysis time of 2 h led to the lowest TBARS.

Table 1. Fatty acid composition of rainbow trout by-product oil in relation to water ratio and autolysis time

Fatty acids	0			25			50			75			100		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
C14:0	0.96±0.028^{b}	0.94±0.014^{b}	0.93±0.014^{b}	1.14±0.00^{ab}	1.08±0.007^{ab}	1.09±0.042^{ab}	1.12±0.021^{ab}	1.07±0.07^{b}	1.18±0.134^{ab}	1.39±0.212^{a}	1.06±0.134^{ab}	1.06±0.021^{b}	1.09±0.028^{ab}	1.08±0.049^{ab}	1.04±0.00^{b}
C14:1n5	0.18±0.014^{de}	0.17±0.004^{e}	0.17±0.004^{e}	0.21±0.00^{abc}	0.20±0.002^{abc}	0.20±0.002^{abc}	0.21±0.006^{abc}	0.20±0.006^{abc}	0.22±0.03^{abc}	0.26±0.03^{a}	0.23±0.02^{ab}	0.22±0.03^{abc}	0.21±0.004^{abc}	0.21±0.009^{abc}	0.21±0.002^{abc}
C16:0	15.36±0.36^{g}	15.04±0.05^{g}	15.11±0.02^{g}	17.48±5.00^{fg}	16.68±0.07^{g}	16.59±5.01^{g}	17.03±0.17^{g}	16.40±0.35^{g}	16.92±0.86^{g}	17.50±2.23^{g}	16.84±0.68^{g}	16.28±0.041^{g}	16.22±0.08^{g}	16.31±0.41^{g}	16.07±0.23^{g}
C16:1n7	2.65±0.02^{c}	2.52±0.00^{c}	2.52±0.00^{c}	3.18±0.00^{b}	2.99±0.05^{c}	3.02±0.05^{c}	3.07±0.07^{b}	2.98±0.07^{c}	3.11±0.19^{b}	3.18±0.28^{b}	2.92±0.17^{c}	2.78±0.01^{c}	2.94±0.04^{c}	1.96±1.49^{c}	2.90±0.02^{c}
C18:0	5.02±0.18^{gf}	4.98±0.02^{gf}	5.05±0.01^{gf}	6.82±0.00^{de}	5.57±0.07^{gfe}	5.59±0.05^{gfe}	5.90±0.29^{ef}	5.77±0.02^{fe}	5.68±0.12^{fe}	4.66±0.65^{g}	5.42±0.21^{gfd}	5.51±0.04^{gfe}	5.32±0.04^{gfed}	5.38±0.005^{gfed}	5.41±0.07^{gfed}
C18:1n9	28.89±1.51^{abc}	29.74±0.67^{a}	29.32±0.61^{ab}	27.65±0.00^{abc}	26.17±1.04^{bcd}	25.56±0.08^{cd}	25.05±1.26^{cd}	27.26±1.12^{abcd}	26.38±0.18^{bcd}	23.51±2.44^{d}	25.56±0.65^{bcd}	25.56±0.85^{bcd}	25.44±0.12^{bcd}	25.63±0.51^{bcd}	25.98±0.03^{bcd}
C18:1n7	1.33±1.16^{a}	1.09±0.73^{a}	2.07±0.95^{a}	1.32±0.00^{a}	1.21±0.02^{a}	1.02±0.69^{a}	2.50±1.52^{a}	0.67±0.57^{a}	1.27±0.06^{a}	1.46±0.70^{a}	1.24±0.43^{a}	0.49±0.29^{a}	1.63±0.16^{a}	1.63±0.07^{a}	1.56±0.003^{a}
C18:2n6Cis	31.84±0.87^{ab}	32.16±0.009^{ab}	32.11±0.16^{ab}	34.72±0.00^{a}	32.80±0.05^{ab}	32.65±0.06^{ab}	31.63±0.14^{ab}	31.49±0.05^{ab}	31.33±0.10^{ab}	29.80±3.37^{b}	32.70±0.18^{ab}	33.05±0.34^{ab}	33.09±0.14^{ab}	32.89±0.17^{ab}	32.87±0.44^{ab}
C18:3n3	3.40±0.07^{a}	3.41±0.007^{a}	3.35±0.03^{ab}	3.42±0.00^{a}	3.25±0.007^{ab}	3.21±0.005^{ab}	3.02±0.01^{ab}	3.03±0.001^{ab}	3.04±0.01^{ab}	1.63±1.57^{b}	3.14±0.003^{ab}	3.24±0.08^{ab}	3.69±0.56^{a}	3.23±0.002^{ab}	3.24±0.0007^{ab}
C20:0	0.25±0.01^{a}	0.24±0.002^{a}	0.11±0.16^{a}	0.24±0.00^{a}	0.23±0.001^{a}	0.23±0.002^{a}	0.11±0.16^{a}	0.23±0.005^{a}	0.22±0.01^{a}	0.16±0.03^{a}	0.21±0.009^{a}	0.23±0.003^{a}	0.11±0.16^{a}	0.22±0.01^{a}	0.11±0.16^{a}
C20:1n9	0.84±0.01^{a}	0.83±0.008^{ab}	0.83±0.007^{ab}	0.82±0.00^{ab}	0.79±0.007^{abc}	0.77±0.01^{abc}	0.75±0.01^{abc}	0.74±0.02^{abc}	0.70±0.03^{c}	0.55±0.04^{d}	0.75±0.04^{bc}	0.79±0.02^{abc}	0.74±0.04^{abc}	0.78±0.03^{abc}	0.80±0.009^{abc}
C20:2n6	1.45±0.01^{abc}	1.46±0.003^{abc}	1.45±0.01^{abc}	1.57±0.00^{ab}	1.50±0.002^{ab}	1.48±0.007^{ab}	1.33±0.04^{bc}	1.34±0.01^{bc}	1.28±0.07^{c}	0.98±0.12^{d}	1.31±0.07^{bc}	1.40±0.02^{abc}	1.35±0.01^{bc}	1.36±0.04^{bc}	1.39±0.01^{abc}
C20:4n6	0.78±0.007^{b}	0.77±0.004^{b}	0.74±0.01^{b}	0.84±0.00^{b}	0.82±0.001^{b}	0.80±0.009^{b}	1.01±0.07^{ab}	1.02±0.02^{ab}	0.98±0.05^{ab}	0.84±0.0.03^{b}	0.98±0.11^{ab}	1.04±0.05^{ab}	1.55v0.61^{a}	1.03±0.03^{ab}	1.08±0.004^{ab}
C20:3n3	0.10±0.02^{c}	0.10±0.01^{c}	0.11±0.00^{b}	0.11±0.00^{b}	0.14±0.03^{b}	0.130.007^{b}	0.08±0.04^{b}	0.19±0.04^{b}	0.11±0.01^{c}	0.07±0.01^{b}	0.11±0.0002^{a}	0.12±0.004^{b}	0.19±0.03^{a}	0.09±0.002^{a}	0.12±0.01^{b}
C20:5n3 (EPA)	0.3±0.01^{c}	0.36±0.02^{c}	0.34±0.00^{c}	0.33±0.00^{b}	0.31±0.002^{c}	0.30±0.01^{b}	0.43±0.18^{b}	0.32±0.0007^{b}	0.30±0.04^{c}	0.23±0.002^{b}	0.33±0.006^{c}	0.37±0.01^{c}	0.36±0.05^{c}	0.32±0.02^{c}	0.33±0.01^{c}
C22:0	0.13±0.01^{c}	0.16±0.008^{c}	0.12±0.00^{c}	0.17±0.00^{b}	0.12±0.04^{c}	0.17±0.04^{b}	0.14±0.03^{b}	0.20±0.005^{a}	0.18±0.07^{b}	0.08±0.007^{b}	0.20±0.11^{a}	0.15±0.005^{b}	0.15±0.05^{b}	0.13±0.05^{b}	0.15±0.03^{b}
C22:1n9	0.11±0.01^{d}	0.47±0.54^{a}	0.10±0.02^{d}	0.10±0.08^{d}	0.09±0.02^{d}	0.07±0.007^{d}	0.19±0.11^{b}	0.09±0.012^{d}	0.07±0.007^{d}	0.08±0.04^{d}	0.08±0.01^{d}	0.13±0.06^{c}	0.11±0.02^{d}	0.10±0.004^{d}	0.10±0.007^{d}
C22:6n3 (DHA)	2.19±0.07^{c}	2.17±0.01^{c}	2.08±0.01^{c}	2.07±0.00^{c}	1.93±0.0007^{c}	1.94±0.01^{c}	1.94±0.007^{c}	2.15±0.11^{c}	1.91±0.44^{c}	0.97±0.01^{d}	1.82±0.32^{c}	2.14±0.09^{c}	1.76±0.16^{c}	1.84±0.20^{c}	1.94±0.07^{c}
∑SFA	21.74±0.60^{gc}	21.38±0.08^{g}	21.33±0.11^{g}	19.05±0.0^{gf}	23.72±0.20^{ge}	23.61±0.05^{ge}	24.33±0.32^{e}	23.69±0.40^{ge}	24.21±0.79^{e}	23.80±3.11^{ab}	23.84±0.48^{ge}	23.27±0.10^{ghc}	22.90±0.34^{ghc}	23.15±0.50^{ghc}	22.80±0.11^{ghc}
∑MUFA	34.03±0.42^{ab}	34.46±0.03^{a}	35.03±0.37^{a}	33.37±0.00^{abc}	31.54±0.00^{cd}	31.68±0.56^{cd}	31.66±0.01^{bcd}	32.00±0.43^{bcd}	31.77±0.11^{bcd}	29.07±3.45^{d}	30.80±0.92^{dc}	29.94±1.13^{dc}	31.13±0.00^{dbc}	31.33±0.37^{dbc}	31.57±0.07^{dc}
∑PUFA	38.70±0.62^{c}	40.46±0.40^{abc}	38.99±1.56^{dc}	43.43±0.0^{a}	40.78±0.42^{abc}	40.54±0.77^{abc}	39.48±0.41^{bc}	39.58±0.15^{bc}	38.97±0.73^{bc}	34.57±1.89^{d}	40.43±0.33^{abc}	41.39±0.61^{abc}	41.90±1.09^{ab}	40.80±0.13^{abc}	41.00±0.37^{abc}
∑n-3	6.07±0.13^{b}	6.04±0.04^{b}	5.88±0.02^{c}	5.93±0.00^{b}	5.62±0.02^{d}	5.57±0.02^{d}	5.47±0.14^{d}	5.69±0.06^{d}	5.35±0.50^{d}	2.91±1.57^{e}	5.41±0.31^{d}	5.87±0.19^{c}	5.89±0.33^{c}	5.49±0.23^{d}	5.62±0.07^{d}
∑n-6	34.07±0.90^{ab}	34.39±001^{ab}	34.30±0.19^{ab}	37.13±0.00^{a}	35.12±0.07^{ab}	34.94±0.05^{ab}	33.98±0.26^{ab}	33.59±0.24^{ab}	33.85±0.09^{ab}	31.62±3.48^{b}	34.99±0.007^{ab}	35.48±0.41^{ab}	35.98±0.74^{a}	35.27±0.09^{ab}	35.34±0.45^{ab}
∑n-3/n-6	0.17±0.00^{c}	0.17±0.001^{c}	0.17±0.001^{c}	0.15±0.00^{ab}	0.15±0.0007^{ab}	0.15±0.00^{ab}	0.15±0.009^{ab}	0.16±0.009^{ab}	0.16±0.001^{c}	0.15±0.014^{ab}	0.09±0.062^{ab}	0.15±0.009^{ab}	0.16±0.002^{a}	0.16±0.005^{a}	0.15±0.007^{ab}	0.15±0.00^{ab}
∑n-6/n-3	5.60±0.02^{a}	5.69±0.03^{a}	5.79±0.10^{a}	5.82±0.05^{a}	6.23±0.03^{a}	6.26±0.01^{a}	6.20±0.11^{a}	5.94±0.05^{a}	6.30±0.55^{a}	13.08±8.27^{a}	6.47±0.37^{a}	6.04±0.12^{a}	6.11±0.22^{a}	6.42±0.28^{a}	6.28±0.16^{a}
Total FA	96.33±0.53^{ab}	95.81±0.70^{ab}	96.59±0.08^{a}	95.27±0.0^{ab}	95.96±0.14^{ab}	95.85±0.43^{ab}	95.90±0.11^{ab}	95.28±0.18^{ab}	94.96±0.15^{ab}	87.45±8.47^{b}	95.08±0.78^{ab}	94.62±0.40^{ab}	95.96±0.72^{ab}	95.29±0.00^{ab}	95.39±0.56^{ab}
PI	0.16±0.006^{g}	0.16±0.001^{g}	0.15±0.00^{g}	0.13±0.00^{g}	0.13±0.00^{g}	0.13±0.00^{g}	0.13±0.01^{g}	0.15±0.01^{g}	0.13±0.03^{g}	0.06±0.07^{g}	0.12±0.02^{g}	0.15±0.01^{g}	0.12±0.01^{g}	0.13±0.01^{g}	0.14±0.01^{g}
EPH + DHA	2.58±0.08^{b}	2.53±0.007^{b}	2.42±0.02^{b}	2.41±0.00^{b}	2.24±0.0007^{b}	2.25±0.02^{b}	2.38±0.17^{b}	2.47±0.11^{b}	2.21±0.49^{b}	1.21±0.01^{b}	2.16±0.32^{b}	2.52±0.10^{b}	2.02±0.25^{b}	2.17±0.22^{b}	2.27±0.09^{b}

*Different lowercase letters in the same row indicates significant difference among treatments at $p < 0.05$.

Fatty acid composition

Across the by-products oils produced under different hydrolysis conditions, polyunsaturated fatty acids (34 – 43%) constituted the largest fraction, followed by monounsaturated fatty acids (29–34%) and saturated fatty acids (19–24%; **Table 1**). Among the individual fatty acids, linoleic acid (C18:2n6-cis), vaccenic acid (C18:1n9), and palmitic acid (C16:0) were the most abundant. These findings are consistent with those reported by Nikoo et al. (2021) regarding changes in fatty acid composition as a function of hydrolysis time and temperature in rainbow trout by-product. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) accounted for 0.23–0.43% and 0.97 – 2.19% of the total fatty acids, respectively. However, the differences among treatments were not significant. Overall, the combined EPA and DHA fractions accounted for no more than 2.5% of the total fatty acids. In contrast, oil recovered from the waste of farmed sea bass and sea bream contained approximately 12% EPA and DHA, which is substantially higher than that observed in rainbow trout by-product. Moreover, higher EPA and DHA levels have been reported for oils obtained from marine fish by-products, such as bluefin tuna (31%) and sardines (30%) (Šimat et al., 2020).

The polyene index is commonly used as an indicator of polyunsaturated fatty acid oxidation and the formation of secondary lipid oxidation products (Šimat et al., 2020). A comparison of water-to-by-product ratios showed that the lowest polyene index was recorded at a 75% water level after 3 h of hydrolysis, whereas the highest value occurred at a 0% water level after 2 h (**Fig. 3**). At the 1-h time point, across all ratios, the minimum value was observed at 75% water and the maximum at 0% water. Likewise, at the 2-h time point, the lowest index corresponded to the 75% water treatment, while the highest was again associated with the 0% water condition.

Oil obtained from Atlantic salmon processing by-products, particularly oil recovered from viscera using alcalase-assisted hydrolysis, has been shown to undergo oxidation (Liu et al., 2021). In oils derived from farmed sea bass and sea bream by-products, which contain lower levels of EPA + DHA, the polyene index was lower than that reported for oils from sardine and tuna by-products and cod liver oil, all of which are richer in EPA + DHA (Šimat et al., 2020). Notably, the polyene index of the rainbow trout by-product oil was lower than that of the aforementioned species' oils.

Antioxidant activity of protein hydrolysates

The antioxidant activity of protein hydrolysates obtained from rainbow trout processing by-products was evaluated in relation to the water-to-substrate ratio and hydrolysis time, using DPPH radical scavenging, ferrous-ion chelating ability, and ferric reducing power assays (**Fig. 3**). All hydrolysates showed high DPPH radical scavenging activity, exceeding 70%. These results suggest that autolysis promotes the cleavage of peptide bonds, generating smaller peptides with inherent antioxidant properties. At a 0% water ratio, the highest antioxidant activity was observed at hydrolysis times of 1 and 3 h, with a slight decline at 2 h. At water ratios of 25% and 50%, the free-radical scavenging activity was comparable at the 1- and 2-h time points. Across all samples, the lowest antioxidant activity was recorded for the 75% and 100% water treatments at hydrolysis times of 1 and 2 h. In a previous study, Nikoo et al. (2021) reported that the DPPH radical scavenging activity of protein hydrolysate produced from trout processing by-product was highest at 40 °C with a 1-h autolysis period. This was followed by samples generated using a gradual temperature increase at 1 h and those produced at a constant 40 °C at 2 h, all of which also exhibited high antioxidant activity. DPPH is a stable free radical commonly employed to assess the free-radical scavenging and antioxidant activities of protein hydrolysates obtained from food sources, including seafood. The DPPH radical-scavenging activity reflects the ability of antioxidants to reduce radical species (Quan and Benjakul, 2019). Consequently, the protein hydrolysate obtained from rainbow trout processing by-products, particularly at a 0% water ratio, exhibited a high proton-donating capacity relative to the other treatments. Under industrial and pilot-scale production conditions, this property may enhance radical-scavenging activity while reducing water consumption in the reactor.

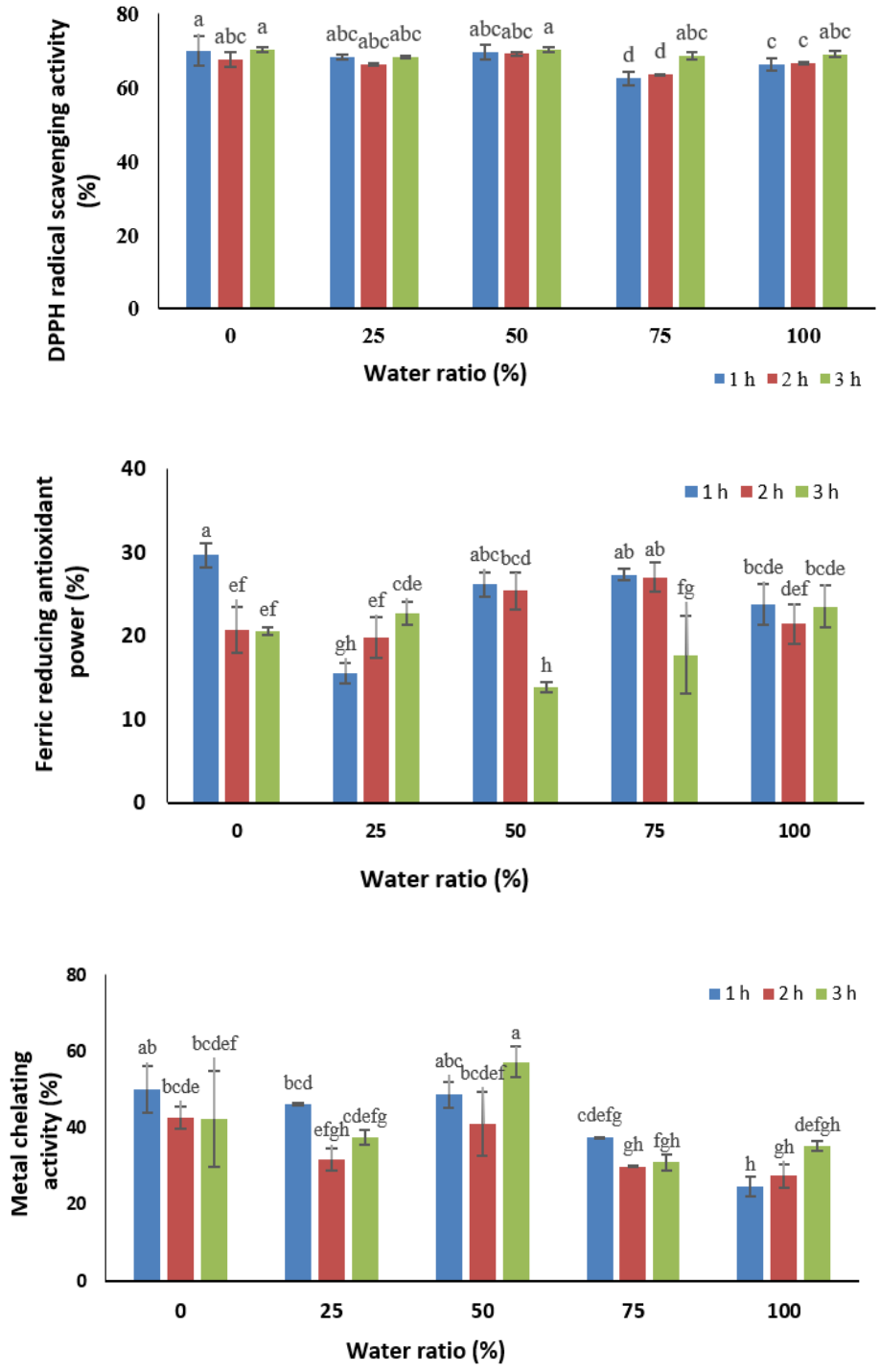

Figure 3. Antioxidant activity of rainbow trout by-product protein hydrolysates in relation to the water-to-waste ratio and autolysis time.

The metal-chelating activity ranged from 31% to 50% across water ratios of 0% to 50%. The protein hydrolysate prepared with 50% water and a 3-h reaction time exhibited the highest chelating activity (57%; $p < 0.05$). However, increasing the water content to 75% and 100% resulted in reduced antioxidant activity ($p < 0.05$). Among the samples, that processed at 40 °C for 1 h showed lower chelating activity compared to the other samples, with activity increasing as the autolysis time was extended (Nikoo

et al., 2021). In general, autolysis yields small peptides that increase the number of free amino and carboxyl groups capable of binding to and chelating metal ions. The results demonstrated that autolysis influences the antioxidant activity of peptides derived from rainbow trout processing by-products through various mechanisms.

Table 2. Color parameters of rainbow trout by-product protein hydrolysates in relation to water ratio and autolysis time

Autolysis time (h)	Water content (% of by-products weight)	L^*	a^*	b^*
1	0	71.17 ± 0.59^{d}	4.22 ± 0.98^{g}	35.13 ± 1.01^{b}
	25	65.45 ± 0.33^{fg}	7.94 ± 0.39^{c}	31.05 ± 0.73^{e}
	50	76.25 ± 0.87^{b}	4.09 ± 0.25^{g}	31.10 ± 1.20^{e}
	75	76.30 ± 1.30^{b}	2.98 ± 0.62^{h}	25.43 ± 0.93^{f}
	100	68.19 ± 0.09^{e}	6.40 ± 0.22^{de}	33.92 ± 0.44^{bcd}
2	0	63.38 ± 0.45^{hi}	6.51 ± 0.13^{de}	37.45 ± 0.24^{a}
	25	67.22 ± 1.93^{ef}	9.57 ± 0.16^{a}	30.37 ± 1.44^{e}
	50	78.33 ± 0.93^{a}	1.71 ± 0.43^{i}	25.59 ± 0.45^{f}
	75	65.85 ± 0.05^{g}	6.68 ± 0.19^{d}	32.98 ± 1.41^{d}
	100	67.93 ± 1.38^{e}	5.86 ± 0.48^{def}	34.74 ± 0.52^{bc}
3	0	62.09 ± 0.58^{i}	9.31 ± 0.77^{ab}	34.06 ± 0.72^{bcd}
	25	67.06 ± 1.43^{ef}	8.00 ± 0.89^{c}	34.47 ± 0.99^{cd}
	50	72.97 ± 0.55^{c}	5.23 ± 0.34^{f}	30.98 ± 0.47^{e}
	75	64.29 ± 0.06^{gh}	8.64 ± 0.04^{bc}	34.87 ± 0.05^{bc}
	100	68.70 ± 0.20^{e}	5.72 ± 0.37^{ef}	31.53 ± 0.92^{e}

*Different lowercase letters in the same column indicates significant difference among treatments at $p < 0.05$

Different water ratios and autolysis durations significantly affected the FRAP activity of the protein hydrolysate samples. At water levels of 0%, 50%, and 75%, FRAP values decreased significantly as hydrolysis time increased. Conversely, at a 25% water ratio, increasing the hydrolysis time led to an increase in antioxidant activity ($p < 0.05$). The highest FRAP value was observed at 0% water content and a hydrolysis time of 1 hour ($p < 0.05$). Increasing the water content to 100% of the waste weight resulted in reduced antioxidant activity ($p < 0.05$). A comparison of the different water ratios indicated that, based on FRAP activity, it is possible to reduce water usage relative to the weight of the by-product. Furthermore, shorter hydrolysis times resulted in the highest FRAP values across the three water ratios (0%, 50%, and 75%; $p < 0.05$). The high content of small-sized peptides in the protein hydrolysate facilitates greater electron donation to free radicals, thereby terminating chain reactions (Nikoo et al., 2021).

Color parameters of protein hydrolysates

The impact of autolysis at different water-to-by-product ratios on the color parameters of the protein hydrolysate was evaluated (**Table 2**). During the first hour of hydrolysis, the hydrolysate samples prepared at 50% and 75% water ratios exhibited higher whiteness values than the other treatments. Thereafter, the highest whiteness was recorded for the 0% water ratio ($p < 0.05$). At the 0% water ratio, whiteness declined significantly with increasing autolysis time ($p < 0.05$), whereas no significant change was detected at the 100% water ratio ($p < 0.05$). Under the 0% water ratio, redness increased from 1 to 3 h, reaching a maximum at 3 h ($p < 0.05$). Notably, the sample obtained after 2 h of autolysis at a 50% water ratio showed the lowest redness value ($p < 0.05$). At a zero-water ratio, yellowness values were higher during autolysis, whereas increasing the water ratio reduced yellowness ($p < 0.05$). At a 50% water ratio, yellowness was lower at 2 and 3 h relative to the other treatments; however, it increased significantly when the water ratio was raised to 75% and 100% ($p <0.05$). The oxidation of the protein hydrolysate was attributed to heme proteins and pro-oxidants present in the fillet. Fish processing by-products are typically less uniform than fillets and may contain high levels of heme pigments, such as hemoglobin. Prior studies have reported that hemoglobin can promote lipid and protein oxidation in seafood products (Richards &

Hultin, 2002; Wu et al., 2021; Larsson & Undeland, 2010). In addition, it has been shown that hemoglobin, in combination with rainbow trout abdominal fat, can accelerate oxidative reactions during the hydrolysis of by-products (Nikoo et al., 2021).

Pires et al. (2015) reported higher yellowness and redness values in protein hydrolysates prepared from carp by-products. They attributed these findings to the greater amount of haem proteins released during alkaline solubilization. In the present study, oxidation was more pronounced at a 0% water ratio, particularly after 3 h of autolysis. This may be related to the closer interaction between pro-oxidants and oxidation-sensitive substrates, which could accelerate pigment formation and darkening of the resulting protein hydrolysate. Sardine (*Sardinella aurita*) protein hydrolysate prepared from pre-treated muscle, characterized by lower hemoglobin content, showed a lighter color than that obtained from untreated muscle (Sarteshnizi et al., 2019). After 3 h of autolysis, the hydrolysates produced at a 50% water ratio exhibited the highest lightness and the lowest redness and yellowness values. Overall, the results confirmed that the water percentage in the reactor significantly affected the color characteristics of the protein hydrolysate derived from trout processing by-products.

Conclusion

In this study, the effect of the water-to-waste ratio on the hydrolysis of rainbow trout by-products was investigated. Based on the TBARS formation in the resulting protein hydrolysates, a hydrolysis time of 1 h was identified as optimal across all tested water-to-by-product ratios. Moreover, considering DPPH free-radical scavenging and ferric-ion reducing activities, the optimal conditions were a 0% water ratio and a 1-h hydrolysis time. Finally, with respect to iron-ion chelating activity, the highest performance was achieved at 50% water ratio and 3-h hydrolysis duration. In the industrial production of protein hydrolysates from aquaculture by-product, increasing the water-to-waste ratio elevates processing costs, particularly those related to drying or evaporating excess water. Therefore, reducing the water ratio can improve operational efficiency during the drying step. The findings of this study indicate, first, the feasibility of producing small, water-soluble peptides without adding water during hydrolysis. Specifically, over a hydrolysis period of 1–3 h at a constant temperature, 70–90% of the generated peptides exhibited molecular weights below 1,000 Da. This ratio was influenced by the percentage increase in water. Despite the potential for peptide production, oxidative reactions varied across the different water ratios.

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Conflicts of interest

The authors declare that they have no conflict of interest

Disclaimer

AI tool (avalai.ir) has been used for language editing to improve the readability and clarity of sentences. Unless this, no AI-based tools were used to generate or alter research data in this work.

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