ANTIOXIDANT CAPACITY OF PEPTIDES DERIVED FROM THE ENZYMATIC HYDROLYSIS OF RAINBOW TROUT (*Oncorhynchus mykiss*) SKIN: EFFECT OF PASTEURIZATION AND ULTRASONICATION PRE-TREATMENT

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ABSTRACT

Rainbow trout skin and viscera are considered non-commercial by-products with high protein content. This research aimed to determine the incidence of pasteurization and ultrasonication during the obtaining of antioxidant peptides by the hydrolysis of trout skin proteins with Alcalase and Flavourzyme. Solutions of freeze-dried skin were prepared (20 % w/v), and two pretreatments were performed: pasteurization (90 ºC for 10 min) and a combination of pasteurization and ultrasonication (40 kHz x 15 min). Hydrolysis was for 8 hours with Alcalase (pH 9, 55°C) and Flavourzyme (pH: 7, 50°C) sampling every 2 hours. The degree of hydrolysis was determined by TNBS and SDS-PAGE. Antioxidant activity was determined by DPPH and FRAP. The ultrasonication does not affect the release of peptides with antioxidant capacity. In addition, greater antioxidant capacity was determined in hydrolysates with Flavourzyme. Results would allow establishing the conditions for the optimized obtaining of this type of peptide fractions.

Keywords: Enzymatic hydrolysis; antioxidant activity; skin trout proteins; bioactive peptides, pre-treatment.

INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is characterized by its ability to adapt to different habitats by modifying its nutritional content [1]. However, the increase in the production and commercialization of this species has generated a greater amount of waste such as heads, bones, skin, and viscera [2]. These wastes have a high protein content, so they are used in the generation of bioactive peptides, which are of interest to the food, pharmaceutical, or cosmetic industries [3]. One method to obtain these peptides is through enzymatic hydrolysis [4]. Currently, in search of increasing the yield and biological activity of these peptides, the use of enzymes has been combined with the application of new technologies [5]. One of these technologies is ultrasonication which, through cavitation, shear, shock waves, and heat, causes physical and chemical changes that affect the protein, increasing the degree of hydrolysis and improving the antioxidant capacity of the peptides [6]. In the case of pasteurization, this heat treatment causes a breakdown of the protein, facilitating the enzymatic reaction and resulting in a greater number of peptides with antioxidant activity [7], [8]. That is why the objective was to know the effect of pasteurization and ultrasonication as pretreatments in enzymatic hydrolysis on the antioxidant activity of rainbow trout skin peptides.

EXPERIMENTAL SECTION

On a farm in the state of Hidalgo, Mexico, 15 fresh rainbow trout were acquired. The skin was separated, cleaned, and cut into 2 cm pieces to be frozen and freeze-dried. With freeze-dried rainbow trout skin, a 1:4 (w/v) suspension was prepared with deionized water, and it was used to prepare a 20% solution with 0.1 M Tris-HCl buffer. Subsequently, the solutions were divided into two groups: pasteurization (90°C x 10 min) as pretreatment and pasteurization plus ultrasonication (40 kHz x 15 min). The suspensions were hydrolyzed with Alcalase (pH 9, 55°C) and Flavourzyme (pH: 7, 50°C) for 0, 2, 4, 6 and 8 hours.

Free amino groups were measured using the trinitrobenzene sulfonic acid (TNBS) method [9]. 250 µL of sample, 2 ml of phosphate buffer, and 2 mL of 0.1% TNBS solution were mixed. It was incubated for 1 h at 50°C, and the reaction was stopped with 4 ml of 0.1 N HCl. The absorbance was measured at 340 nm. To determine the content, a standard curve was performed with glycine. The results were expressed as g/L of free amino groups (NH^{2-}).

The separation of the peptides was carried out by Tris-Tricine polyacrylamide gel electrophoresis following the methodology of Schägger and von Jagow [10], with modifications by González-Olivares et al.,[11]. Separation and concentration gels were made with 15% and 4% acrylamide respectively, from a 40% Bio-Rad Acrylamine solution (19:1 ratio of acrylamide: bisacrylamide with 5% crosslinker) in Tris-HCl buffer. In an electrophoresis chamber, the gels were placed in Tris-Tricine-SDS-HCl buffer (pH: 8.25), and the samples were injected together with the standard. The electrophoretic run was run at 95V for 6 hours. Gels were stained with Comassie blue G-250 (Bio-Rad) and analyzed by Gel-Doc software.

The antioxidant activity of the hydrolysates was measured by the DPPH and FRAP methods following the methodology proposed by Ramírez-Godínez et al.,[12]. For the determination by DPPH, 100 µL of hydrolysate, with 2.9 mL of DPPH reagent (0.1 mM 2.2-diphenyl-1-picrylhydrazyl in methanol) was placed in tubes. After 50 min the absorbance was measured at 515 nm. A standard curve was performed with Trolox. The results were expressed as IC_{50}. For the FRAP methodology, 250 µL of the hydrolysates together with 1 ml of FRAP reagent (300 acetate buffer pH 3.6, 10mM TPTZ solution, and 20 $FeCl_3$ $6H_2O$ solution) were added to 10 mL with deionized water. They were incubated at 37°C for 15 min and the absorbance was measured at 593 nm. A curve was performed with $FeSO^{4-}$ $7H_2O$ in 40 mM HCl. The results were expressed as mg equivalents of Fe^{2+}.

The protein concentration in the supernatants of the rainbow trout skin suspensions had a significant difference before pasteurization and after pasteurization (Table 1). In the case of the suspension at pH 7, it was 1.84 to 1.99 g/L and in the suspension at pH 9, it was 1.67 to 1.83 g/L, before and after pasteurization respectively. The higher concentration of proteins in pasteurized suspensions is because exerted by heating and a pH higher than 6 [7], [13]. Due to these results, pasteurization pretreatment was applied to all samples. Likewise, the enzymatic hydrolyses were applied according to the optimal pH of each of the enzymes used (Alcalase pH 9 and Flavourzyme pH 7). Four different suspensions were obtained to hydrolyze according to the pretreatments and enzymes used (PA, pasteurized sample hydrolyzed with Alcalase; PUA, pasteurized and ultrasonicated sample hydrolyzed with Alcalase; PF, pasteurized sample hydrolyzed with Flavourzyme and PUF to the pasteurized and ultrasonicated hydrolyzed sample with Flavourzyme).

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Table 1. Effect of pasteurization on the protein content of rainbow trout skin suspension after centrifuge*

Treatment	pH	Protein Supernatant mg/L	Protein Sediment %
No treatment	7	1848.69 ± 25.96	17.34
	9	1673.99 ± 10.57	16.39
Pasteurization	7	1990.26 ± 13.91	17.00 ± 0.28
	9	1832.63 ± 38.37	14.47 ± 0.40

*20% (w/v) suspension of rainbow trout paste with 0.1M Tris-HCl buffer
The protein content of the supernatant and sediment were determined with the Bradford and Kjeldahl methods, respectively.

According to the results obtained in the analysis by the TNBS technique, differences were observed in the concentration of free amino groups (Table 2). The Alcalase enzyme caused a higher degree of hydrolysis even immediately after adding the enzyme (PA: 5.97 g/L NH^{2-}, PUA: 5.00 g/L NH^{2-}). Subsequently, a decrease in the concentration of free amino groups was observed. In contrast, those hydrolyzed with Flavourzyme presented lower concentrations of these groups at the initial time (PF: 1.40 g/L NH^{2-}, PUF: 0.85 g/L NH^{2-}). However, the release of free amino groups increased after 2 hours of hydrolysis (PF: >7.51 g/L NH^{2-} and PUF: >6.16 g/L NH^{2-}). In general, a greater release of amino groups was observed in the hydrolysates at 6 h regardless of the enzyme used. This effect on the release of amino groups is related to the specificity of the enzyme used. It is known that during the first hours of hydrolysis, Alcalase generates a greater number of cleavages to the right of the peptide bonds with glutamic acid. This amino acid is mainly present in the α1 subunit of trout skin collagen [14], [15].

Table 2. Effect of sonication and enzyme on the degree of hydrolysis of pasteurized rainbow trout skin.

Hydrolysis time (h)	Hydrolysis degree Free amino groups [NH_2^-] (g/L) PA	PF	PUA	PUF
0	$5.97 \pm 0.02^{Ae*}$	1.40 ± 0.02^{Ce}	5.00 ± 0.01^{Be}	0.85 ± 0.02^{De}
2	6.47 ± 0.01^{Cd}	7.51 ± 0.03^{Ad}	$7.25 \pm 0.07^{Bc*}$	6.16 ± 0.07^{Dd}
4	8.51 ± 0.02^{Dc}	10.14 ± 0.02^{Ab}	9.34 ± 0.07^{Bb}	8.69 ± 0.03^{Cc}
6	9.59 ± 0.01^{Ca}	10.65 ± 0.01^{Aa}	10.64 ± 0.01^{Aa}	10.17 ± 0.01^{Ba}
8	8.76 ± 0.01^{Bb}	7.79 ± 0.03^{Cc}	6.25 ± 0.01^{Dd}	8.99 ± 0.07^{Ab}

Results expressed as mean of three repetitions ± standard deviation. [a-e] In columns indicate significant difference between the hydrolysis time. [A-D] Between rows, indicate significant difference between treatments.

In the case of Flavourzyme, being endo- and exopeptidase, it generates a higher degree of hydrolysis compared to other enzymes such as Alcalase, papain, pepsin, or trypsin, as has been reported in hydrolysis of sturgeon skin [16], [17]. Regarding the effect of ultrasonication as pretreatment, an increase in the concentration of free amino groups was observed only in those hydrolyzed with Alcalase. In the other treatments, the change in concentration was without any effect. It has been reported that the degradation of collagen during ultrasonication could cause the aggregation of compact proteins resistant to the hydrolysis of some enzymes, which results in a new conformation of the structures [18], [19].

After peptide separation by SDS-PAGE, the gels were analyzed and the molecular weights of the fractions obtained were determined (Figure 1). In the electropherograms, high molecular weight fractions were observed at time 0 which correspond to unhydrolyzed trout skin proteins (collagen). This high molecular weight protein represents 60% of the rainbow trout skin composition, mainly type I collagen, which has two chains α1 and α2 [20], [21]. Using SDS-PAGE electrophoresis in native collagen, the α chains that make it up have been identified with weights between 111 to 117 kDa; cross-linked β and γ components with weights similar to 200 kDa can also be identified with less intensity [20], [22]. It has also been identified that in partial hydrolysis of collagen peptides with weights of 50 to 90 kDa are found, while more extensive hydrolysis generates peptides with weights less than 40 kDa [23].

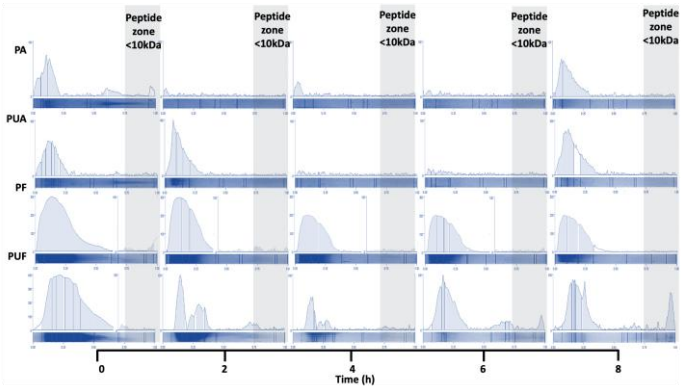

Figure 1. Electropherograms of peptide separation in hydrolyzed rainbow trout skin. PA, pasteurized sample hydrolyzed with Alcalase; PUA, pasteurized and ultrasonicated sample hydrolyzed with Alcalase; PF, pasteurized sample hydrolyzed with Flavourzyme and PUF to the pasteurized and ultrasonicated hydrolyzed sample with Flavourzyme.

However, in the hydrolysates with Alcalase (PA and PUA) peptide fractions with molecular weight greater than 25 kD were present. In contrast, in those hydrolyzed with Flavourzyme (PF and PUF), the highest concentration of peptides was those with molecular weights less than 10 kDa. It is known that the ability to produce lower molecular weight peptides by Flavourzyme corresponds to its endo- and exo-peptidase capacity, which could be involved in a higher degree of hydrolysis and, consequently, greater production of lower molecular weight peptides [24].

The antioxidant activity of the hydrolysates by the DPPH method was expressed as IC_{50} (Table 3). It was observed that the antioxidant capacity of the PF hydrolysates (8.20–1.43 µg/mL) was greater than that of the PA hydrolysates (21.33–1.79 µg/mL), and it was at 6 h when the highest antioxidant activity was found (PA: 2.47 and PF: 1.43) (Table 3). In contrast, the FRAP results (Table 3) demonstrated greater antioxidant activity at 8 hours in PA and PF samples, while in PUF it was at 6 hours. Furthermore, no relationship was observed between the degree of hydrolysis or enzyme.

Table 3. Antioxidant activity of hydrolysates with Alcalase and Flavourzyme from pasteurized and ultrasonicated pasteurized rainbow trout skin.

Hydrolysis time (h)	PA	PF	PUA	PUF
	DPPH (IC_{50} µg/mL)			
0	21.33 ± 0.46^{Ba}	8.20 ± 0.06^{Da}	19.73 ± 0.40^{Ca}	36.12 ± 1.00^{Aa}
2	4.81 ± 0.04^{Cc}	2.69 ± 0.03^{Db}	9.00 ± 0.06^{Bb}	10.68 ± 0.19^{Ab}
4	5.61 ± 0.06^{Cb}	2.14 ± 0.02^{Dc}	8.40 ± 0.10^{Ac}	7.67 ± 0.12^{Bc}
6	2.47 ± 0.04^{Cd}	1.43 ± 0.01^{De}	7.07 ± 0.04^{Ad}	4.05 ± 0.07^{Bd}
8	1.79 ± 0.02^{Ce}	1.74 ± 0.00^{Cd}	8.05 ± 0.08^{Bc}	9.66 ± 0.13^{Ab}
	FRAP (mg eq. Fe^{2+}/100 ml)			
0	1.20 ± 0.09^{Ba}	1.20 ± 0.03^{Ba}	1.22 ± 0.02^{Aa}	1.20 ± 0.03^{Da}
2	1.19 ± 0.13^{Bb}	1.60 ± 0.01^{Aa}	0.77 ± 0.03^{Cc}	0.83 ± 0.03^{Ec}
4	1.10 ± 0.01^{Bb}	1.13 ± 0.01^{Bb}	0.92 ± 0.02^{Bc}	1.72 ± 0.02^{Ba}
6	1.58 ± 0.03^{Ab}	1.06 ± 0.16^{Bc}	0.81 ± 0.03^{Cd}	2.03 ± 0.04^{Aa}
8	1.73 ± 0.02^{Aa}	1.75 ± 0.01^{Aa}	0.99 ± 0.01^{Bc}	1.59 ± 0.06^{Cb}

Results expressed as mean of three repetitions ± standard deviation. [a-e] In columns indicate a significant difference between the hydrolysis time. [A-D] Between rows indicate significant differences between treatments.

Ultrasonication did not favor the antioxidant activity in the hydrolysates with Alcalase or Flavourzyme. Previously Yaghoubzadeh et al.[25], reported a similar behavior of the antioxidant activity in rainbow trout skin hydrolysates, in which the hydrolysates with Flavourzyme presented greater inhibitory power by the DPPH method than those obtained with Alcalase. Also, when comparing the values obtained in this work, trout skin hydrolysates achieve greater antioxidant activity by DPPH, compared to what was reported for stingray skin gelatin hydrolysates, which presented an IC_{50} value of 1.98. mg/mL [26].

The antioxidant activity in peptides has been related to the presence of amino acids with nucleophilic side chains with sulfur (taurine, cysteine, and methionine) or with aromatic side chains (tryptophan, tyrosine, and phenylalanine), amino acids that are present in skin proteins of fish [27].

For the enzymatic hydrolysis of rainbow trout skin, the factors that allowed obtaining better characteristics such as higher soluble protein content, a higher degree of hydrolysis, low molecular weight peptides (<10 kDa), and higher antioxidant activity were pasteurization, hydrolysis by 6 hours and the use of flavourzyme. While ultrasonication as pretreatment did not improve the characteristics of the hydrolysates. These results could be the beginning of the optimization of processes for obtaining peptides with antioxidant activity by enzymatic hydrolysis of trout skin and waste byproducts.

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