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ANTIOXIDANT ACTIVITY OF PEPTIDES OBTAINED BY ENZYMATIC HYDROLYSIS FROM PROTEINS OF AMARANTH (*Amaranthus hypochondriacus* L.) STUBBLE

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ABSTRACT

This research work is focused on the enzymatic hydrolysis of amaranth (*Amaranthus hypochondriacus*) stubble to obtain peptides and demonstrate their antioxidant activity. To this aime, we analyzed extracts of leaves and stems of this plant species. Amaranth stubble was dried and grounded to 0.1 mm particle size and treated by enzymatic digestion using Flavourzyme® or Alcalase®. The hydrolysis degree was 16.31 % and 12.64 %, for each digestion, respectively. The peptides obtained showed antioxidant activity in all the range of molecular size from < 1 kDa to >10 kDa. Our findings indicate that amaranth stubble is an available material that can be used to obtain peptides with antioxidant activity.

Keywords *Amaranthus*, antioxidant activity, peptides.

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INTRODUCTION

Antioxidants are essential for neutralization of free radicals and for remedying reactive oxygen species (ROS) damage on cells (Admassu *et al*., 2017). Interest in discovering novel effective-nontoxic-natural compounds with antioxidative activity has substantially increased in recent years. Peptides also have excellent potential as antioxidant additives in foods because they can inactivate pro-oxidative species (Montoya-Rodríguez *et al*., 2015). Understanding the relationship between peptide composition and antioxidant activity could lead to the development of new class of effective, multifunctional, generally recognized as safe (GRAS) antioxidants that could be used in many food applications (Saito *et al*., 2003).

Protein-rich foods are expensive and scarce in most countries. Therefore, many research groups have focused on the search for proteins from unconventional sources, such as those of plant origin (Montesano *et al*., 2020, Balandrán-Quintana *et al*., 2019). One of the unconventional protein sources is the amaranth (*Amaranthus* *hypochondriacus* or *Amaranthus cruentus*), plant fraction (APF), which consists of the stem and leaves after the amaranth grain harvest (Ngugi *et al*., 2017, Soriano-Santos *et al*., 1992). The APF is considered as an agro-industrial waste, and farmers use it as feed for livestock (Manyelo *et al*., 2020). The APF could be exploited as a source of peptides with biological activity including their antioxidant capacity. Indeed, proteins obtained from amaranth have a high content of bioactive properties, including antioxidant and anticancer capacity. Such proteins, however, must be hydrolyzed to release bioactive peptides (Montoya-Rodriguez *et al*., 2015).

Leaves and stems of amaranth are a good source of protein, that have not been fully explored and exploited. Since peptides obtained by enzymatic hydrolysis of protein from amaranth waste (*Amaranthus hypochondriacus* L.) could have antioxidant activity, the objective of this work was to determine the antioxidant activity of the peptide fractions obtained by mono-enzymatic hydrolysis from the proteins of APF.

MATERIALS AND METHODS

All salts were from J.T. Baker and enzymes from Sigma-Aldrich, except where otherwise noted. Amaranth (*Amaranthus hypochondriacus* L.) plants were collected in Tulyehualco in Mexico City (19° 15′ 13″ NL, 99° 00′ WL, 2247 m altitude), drying at 65 °C., ground in industrial blender (Waring laboratory, Torrington, CT, USA) and screened to particle size of 1 mm. The ground samples were defatted with acetone (5 mL g^{-1}) while being stirred for 16 h, 3 times. The residual acetone was eliminated in gas extraction hood and stored for further extraction of the proteins. The flour was called flour from amaranth plant fraction (FAPF).

Protein extraction were performed according to Hoover *et al*. (1991) with some modifications: Defatted FAPF was re-suspended in water (6:1 v:w), the pH was adjusted to 11 with 1 N NaOH, the suspension was agitated for 1 h at 400 rpm and filtered using two sieves (80 and 100 mesh). The bagasse was eliminated. The filtrated was collected and allowed to stand for 30 min at room temperature to settle down the solids. The supernatant decanted was adjusted to pH 4.5 with 1 N HCl, and centrifuged at 1,317 × g for 12 min at 4 °C (Megapure Thermocentrifuge, Thermo Scientific; Waltham, MA, USA). The bottom was resuspended in 1 L glass bottle, lyophilized at -47 °C and 13 × 10^{-3} mbar (Labconco FreeZone 4.5 Liter Freeze Dry System, Kansas City, MO, USA), and stored at -20 °C, until use. This fraction was called protein concentrate (PC).

Crude protein in the PC sample was determined using the Kjeldahl method (method 933.05) of the A.O.A.C. (2000), using a conversion factor (fN) of 5.85 for amaranth grain (Scilingo *et al*., 2002). The yield of protein was calculated with the eq. 1.

$$\text{Yield (\%)} = (\text{protein in PC (mg)})/(\text{Total protein in FAPF (mg)})\times 100 \qquad 1)$$

Protein concentrate hydrolysis was performed with Alcalase® from *Bacillus licheniformis* or Flavourzyme® from *Aspergillus oryzae* according to Hamada (2000), with a slight modification. The enzymatic reaction for Alcalase® was performed in an agitated jacketed reactor at 50 °C, the pH was adjusted by using 50 mM Tris-HCl buffer pH 8.0 and kept constant by automatic titration by 0.1 N NaOH. For Flavourzyme® the reaction was performed in phosphate buffer pH 7.0. In both cases the reaction was stopped by heating the reaction mixture for 20 min at 82 °C. In each reaction mixture aliquots were taken during hydrolysis (0, 15, 30, 60, and 90 min). The hydrolysate was centrifuged at 10 000 ×g for 20 min at 4 °C (Thermo Scientific Megapure Thermocentrifuge) and 0.02 % sodium azide, stored at -20 °C until use. The product obtained was stored at room temperature to determine the degree of hydrolysis through the orthophenylphthaldehyde method (OPA) (Nielsen *et al*., 2001). To determine the percentage of the degree of hydrolysis a calibration curve with L-serine was used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the procedure previously described by Laemmli (1970). The supernatants of both enzymatic hydrolysates of the PC were fractionated by ultrafiltration according to *Cho et al*. (2004). Ultrafiltration units (Stirred Ultrafiltration Cell, Models 8200 and 8050, Millipore; Burlington, MA, USA) equipped with magnetic stirrer were used to prevent sedimentation. Four membranes with different molecular weight cuts (MWCO) were used: 10 kDa; 5 kDa; 3 kDa and 1 kDa.

Protein was determined in the peptide fractions by Bradford method (Bradford, 1976) with 10 μL of the sample, following the manufacturer´s instructions, with a BSA (1 mg mL^{-1}) calibration curve.

The antioxidant activity from Ultrafiltration fractions were determined with 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich), according to Pukalskas *et al*. (2002). The ABTS in PBS to obtain an absorbance of 0.800 ± 0.030 at 734 nm and used Trolox as standard (0.5 - 2.5 mM) to estimate the Trolox equivalent antioxidant capacity (TEAC).

The experimental design was completely random in a factorial arrangement with two factors (enzyme and peptide fraction): enzyme with 2 levels; and peptide fractions with five levels (>10, 5-10, 3-5, 1-3, <1 kDa) for each enzymatic treatment. The multiple comparisons between means were carried out with SAS. ver 9.1 (2003).

RESULTS AND DISCUSSION

Protein concentration in peptides obtained by enzymatic hydrolysis of APF was determined in each (Table 1). The results showed that the concentration of protein was 14.4 ±1.2 % in FAPF and 13.7±0.3 % in the PC. Similar results were obtained by López-Mejía *et al*. (2014), who reported 15.49 %.

The variation in protein content reported was due to differences in physiological states, origin of the crop or different parts of the plant. Alfaro *et al.* (1987), performed protein analysis on amaranth plants at 25, 40 and 60 days, respectively, and found that the longer the period after plant emergence, the lower the protein concentration. Those findings are in full agreement with our results, since samples were obtained after the harvest of the seeds. There is a large amount of protein with the potential to produce bioactive peptides instead of being considered agricultural waste.

Table 1. Protein concentration in different samples of raw materials and plant developmental stages (age) of *Amaranthus* spp. plants.

Raw material	Protein concentration (%)		Reference
	Flour	Concentrate fraction	
Leaves	25.3	51.4	Metri-Ojeda *et al*. (2019)
Leaves	25.25		Ngugi *et al*. (2017)
Seeds	17.4	40.01 (364 mg·g^{-1})	Pospišil *et al*. (2006)
Stubble	15.49		López-Mejía *et al*. (2014)
Plants:after seedling			
25 days	29.5		Alfaro *et al*. (1987)
40 days	22.7		
60 days	14.4		
Stubble	14.4±1.2	13.7±0.3	This work

The electrophoretic profile of the PC of the stubbles were determined (data not shown). There were four main protein bands in PC (MW: 66.2, 35, 31.0 and 15 kDa). It has been reported that amaranth grain contains a higher proportion of albumin, followed by globulins and glutelins (Soriano *et al*., 1992; Barba de la Rosa *et al*., 1992). Other investigations reported that the albumin present in the amaranth grain corresponds to protein fractions of PM of 45, 36, 26, 22 and 17 kDa (Segura-Nieto *et al*., 1992; Silva-Sánchez *et al*., 2004).

The degree of hydrolysis (DH) was determined from the mono-enzymatic hydrolysis with Alcalasa or Flavourzyme® of the PC of the stubbles. The results obtained are shown in Figure 1. For Flavourzyme® it was 16.31 % and for Alcalasa 12.64 %.

During the first 30 min of reaction the DH with both enzymes increases fast and at a constant rate. The hydrolysis using Alcalase® between 30 and 120 min reached its maximum value and there was no significant difference between the DH obtained in that time interval. On the other hand, with Flavourzyme® there was an increase in the DH of proteins from 30 to 120 min. However, the maximum degree of hydrolysis remained constant from 60 min.

The DH obtained of PC from the stubble with both enzymes were like those reported for other mono-enzymatic systems with vegetable proteins, Sun (2011) reviewed the enzymatic hydrolysis of soy proteins founding that DH was usually between 1 % and 39.5 %. In other studies, it has been reported that hydrolysates with a DH >10 % originate peptides with physiological activities such as antihypertensive, antioxidant among others. Therefore, it is very likely that the peptide fractions obtained from the amaranth stubble protein hydrolysates have antioxidant activity.

The antioxidant activity of the five peptide fractions obtained after ultrafiltration of the hydrolyzate obtained with Flavourzyme® or Alcalase® is showed in the Figure 2.

It was observed that all the peptide fractions had antioxidant activity. Fractions >10, 3-5 and 1-3 kDa showed significant differences among them, while fractions of 5-10 and <1 kDa showed that there were no significant differences between them regarding the

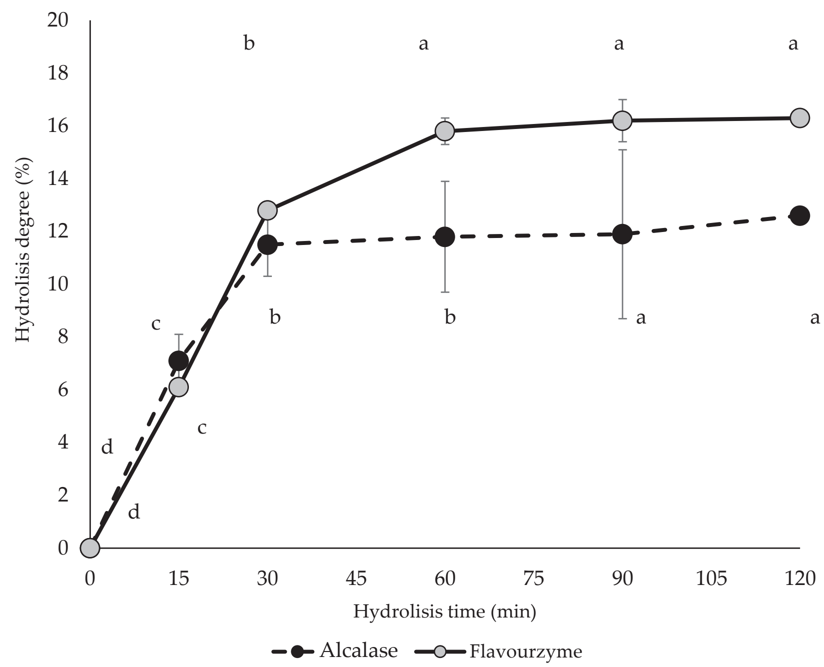

Figure 1. Kinetics of the degree of hydrolysis of PC concentrate with Alcalase® and Flavourzyme® obtained from amaranth (*Amaranthus hypochondriacus* L.) stubble. Different letters on the same curve indicate significant differences between treatments ($p \leq 0.05$).

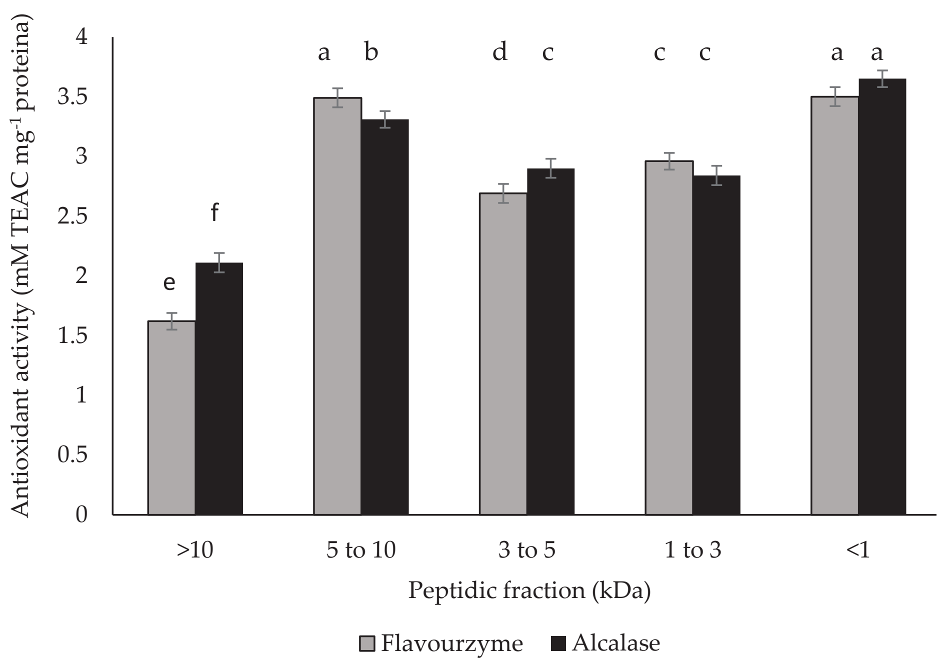

Figure 2. Antioxidant activity of the five peptide fractions of the hydrolysate from amaranth (*Amaranthus hypochondriacus* L.) stubbles with Flavourzyme® and Alcalase®. Different letters indicate significant differences ($p \leq 0.05$).

enzymatic conversion of both Flavourzyme® and Alcalase®. Moreover, these fractions also were the fractions with the highest antioxidant activity. Fractions >10, 5-10, 3-5 and 1-3 kDa between enzymes showed significant differences, while between fractions <1 kDa with the two enzymes and the 5-10 kDa fraction with Flavourzyme® there were no significant differences. On the other hand, the 3-5 kDa fraction with Alcalase® and the 1-3 kDa fraction with Flavourzyme® showed no significant difference between them. Fractions 5-10 and <1 kDa were the ones that obtained the highest antioxidant activity regardless of the enzyme.

The study confirmed that bioactive peptide fractions can be released from amaranth stubbles by Flavourzyme® or Alcalase®-mediated digestion. Amaranth proteins are a good source of raw material to produce peptides with antioxidant properties. The peptide fractions showing antioxidant activity are a mixture of peptides.

Table 2 shows a comparison between the results obtained in this work and values reported by other authors, in referred to peptides with antioxidant activity, obtained

Table 2. Peptides with antioxidant activity, obtained from proteins from plant sources using different enzymes.

Source	Enzyme	Degree of Hydrolysis (%)	Antioxidant activity assay	Fractions	Amount of protein	Reference
Amaranth Stubble	Flavourzyme®	16.31	ABTS (mM TEAC g^{-1})	<1 kDa	3.50	This work
	Alcalase®	12.64		<1 kDa	3.65	
Beans	Alcalase® -Flavourzyme®	43.01	ABTS (mM TEAC g^{-1})	<1 kDa	888.43	Ruiz-Ruiz *et al*., 2011
	Pancreatic pepsin	26.15		<1 kDa	1985.50	
Sweet Potatoe	Alcalase®	---	Fe^{2+} chelating capacity	< 3 kDa	82.27	Zhang *et al*., (2014)
			Radical hydroxil (HO·)	< 3 kDa	59.74	
Soy	Flavourzyme®	18.8	FRAP (mM Ascorbic acid equivalent)	>50 kDa	1.009	Moure *et al*., (2006)
		36.6	Reduction power (mM) Ascorbic acid equivalent	>50 kDa	0.4×10^{-3}	
		63.4	Hydroxyl radical (OH^-)	30 & 50 kDa	69.75	
		42.1	ABTS (mM Trolox eq.)	>50 kDa	6.64	
			ABTS (IC_{50}) (mg mL^{-1})		0.811	
			Chelating metal (IC_{50})		0.766	
			Reducing Power		0.274	
	Papain		DPPH (IC_{50}) (mg mL^{-1})		1.48	
			ABTS (IC_{50}) (mg mL^{-1})		0.892	
			Chelating metal (IC_{50})		0.801	
			Reducing Power		0.29	

from proteins from plant sources using different enzymes. The focus of this research was to isolate and identified peptides with antioxidant activity from amaranth after enzymatic digestion.

CONCLUSIONS

Amaranth stubble is a viable source for obtaining proteins that can release bioactive peptides antioxidant by –enzymatic commercial mediated digestion. The peptide fractions produced by Flavourzyme® or Alcalase® had antioxidant activity, and the higher activity was observed with <1 kDa and 5-10 kDa fractions, regardless of the enzyme used.

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