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ANTIOXIDANT ACTIVITY OF CHACHAFRUTO (*Erythrina edulis*) SEED HYDROLYSATES OBTAINED BY SEQUENTIAL ENZYMATIC HYDROLYSIS

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ABSTRACT

The current study evaluates the effect of sequential hydrolysis on degree of hydrolysis and antioxidant activity of Chachafruto (*Erythrina edulis*) storage proteins hydrolysates, which is an endemic South American legume. Besides, sequential enzymatic hydrolysis of Chachafruto, storage proteins was studied to determine the type of protein and combination of proteases that would produce hydrolysates with the highest *in vitro* antioxidant activity. In order to obtain each of the storage proteins of Chachafruto separately, sequential extraction was carried out. A 500 mL reactor with a magnetic stirrer at 960 rpm was used, which were controlled by means of an automatic titrator. The reaction was monitored through the degree of hydrolysis, calculated by the pH-stat method. First, each enzyme as single enzyme was used in hydrolysis process, then Flavourzyme followed by Alcalase 2.4L® and Neutrase followed by Alcalase 2.4L® were used to produce hydrolysates from albumins, globulins and glutelins from Chachafruto. Antioxidant activity was evaluated in each hydrolysate by oxygen radical antioxidant capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2' -azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) methods. The results showed that Chachafruto seeds flour have above 20% of protein, which contain 46.36%, 11.39%, 42.01% and 0.23%, of albumins, globulins, glutelins, and prolamins, respectively. Besides, it is possible to increase the degree of hydrolysis and antioxidant activity by sequentially using Flavourzyme-Alcalase and Neutrase-Alcalase, compared to hydrolysis with a single enzyme, for all storage protein evaluated. Among the storage proteins from Chachafruto, the albumins hydrolyzed showed the highest antioxidants. Both, Neutrase-Alcalase and Flavourzyme-Alcalase hydrolysates showed antioxidant activity, being Flavourzyme-Alcalase combination which showing the higher antioxidant activity. In conclusion, Chachafruto seed can produce a flour with high protein contained, which storage protein are potential source for functionals compound with antioxidant activity, when are subjected to enzymatic hydrolysis by Alcalase, Flavourzyme and Neutrase enzymes, either as single and sequential enzymatic hydrolysis process.

Key words: Degree of hydrolysis, bioactive peptides, storage protein, *in vitro* activity

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INTRODUCTION

There is a global trend in the search for new protein sources driven by consumers' interest in new nutrients, reducing the consumption of animal-based proteins, and functional compounds [1]. These functional compounds are known to prevent or reduce non-communicable diseases which are causing disability and mortality worldwide, such as neurodegenerative, cardiovascular, cancer and diabetes diseases [1]. These diseases often share common etiological characteristics such as oxidative stress, hypertension, inflammation and metabolic disorders [1].

Bioactive compounds of protein origin play an important role in the treatment of these pathologies or diseases. Vegetable proteins found in foods such as barley and maize are a source of compounds with antioxidant activity [2]. Among vegetable products, underutilized plant products can contribute to the generation of bioactive compounds of protein origin. In this context, Chachafruto (*Erythrina edulis*), an endemic South American legume, has a protein content ranging from 12% to 18%, whose quality is similar to that of eggs and superior to other legumes. It is used in Colombia mainly as shadow tree and animal feed , with few uses in human nutrition and as medicinal plant, but more recently, it has been reported as a source of antioxidant compounds [3]. However, this plant has not been used as a source of antioxidant compounds in industrial level yet, for that reason, it is necessary improving the antioxidant activity of its hydrolysates, to increase the possibility of use of it at this level.

One of the strategies for obtaining bioactive compounds from proteins is enzymatic hydrolysis, which can release small isolated protein fragments that are encrypted in the original chain and once released, can provide protective functions against non-communicable diseases [4]. These peptides can exhibit antioxidant, antihypertensive, antimicrobial, antithrombotic, anticancer, metal chelating or anticoagulant properties, among others [4]. The antioxidant properties of peptides are related to their composition, structure and hydrophobicity. The amino acids Tyr, Trp, Met, Lys and Cys are examples of amino acids that exert this activity. Amino acids with aromatic residues can donate protons of electrons to deficient radicals [4].

A recent classification of bioactive compounds has emerged, distinguishing between mono and multifunctional peptides, based on whether they possess one or multiple associated biological activities. In the latter group, peptides derived from Chachafruto (Erythrina edulis) can be found, to which Palma-Albino *et al.* [3] attributed antioxidant, antihypertensive, antidiabetic activities (through *in vitro* analysis), and neuroprotective activity through analysis in SH-SY5Y cell lines in 2021.

One of the most important aspects in the development of an enzymatic hydrolysis reaction lies in achieving high degrees of hydrolysis (DH), as this parameter

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quantitatively describes the progress of the reaction. Thus, a high value indicates that hydrolysis has occurred to a significant extent and is associated with a greater likelihood of obtaining bioactive peptides, given that these peptides usually have molecular weights below 6 kDa [4].

During enzymatic hydrolysis of proteins, there is a gradual decrease in the reaction rate, which has been attributed primarily to several factors: (i) a decrease in the concentration of peptide bonds susceptible to hydrolysis by proteases, (ii) possible enzyme inhibition caused by hydrolysis products as substrates, (iii) thermal denaturation of the enzyme, and (iv) release of short peptides and free amino acids that cause enzyme inhibition. From this perspective, one possibility to increase the final DH in the hydrolysis reaction would be to use multiple enzymes sequentially so that, when the first enzyme is reaching its performance limit, another enzyme is introduced that uses new cleavage sites or is not inhibited by the reaction products in the same way as the first enzyme, thereby revitalizing the reaction [4].

The type of enzyme used in the reaction is one of the factors that most affect the activity of the hydrolysates and the quantity of peptides produced. Proteases are classified as endo- or exopeptidases based on the cleavage site in the protein of origin, depending on whether the cleavage occurs at the ends or inside the protein chain, respectively. They can also be differentiated according to the functional group in their active site, such as serine, aspartate, cysteine, or metalloproteases. Proteases are further classified as alkaline, acidic, or neutral based on their optimal pH [4].

Some of the proteases that have been used to hydrolyze plant proteins are Alcalase®, Flavourzyme®, Neutrase®, and the sequential combination of some of them. Flavourzyme® is a mixture of enzymes with endo- and exopeptidase activities, produced by fermentation of Aspergillus oryzae, which has broad specificity to produce small-sized peptides and free amino acids. On the other hand, endopeptidases such as Alcalase® and Neutrase® cleave peptide bonds within the polypeptide chain, releasing small and medium-sized peptides [5]. Alcalase is a commercial serine endopeptidase produced by fermentation with Bacillus licheniformis [6] and has already been used for the production of antioxidant peptides from Chachafruto [7]. Combinations of Neutrase-Alcalase and Flavourzyme-Alcalase, have been used in sequential enzymatic hydrolysis of several vegetable substrates with positive results [2], but these combinations have not been used in hydrolysis of storage proteins of Chachafruto (*Erythrina edulis*) yet [2].

Enzymatic hydrolysis of complex substrates such as plant products poses a challenge because they consist of different proteins, making it difficult to identify from which proteins the peptides with the highest activity come. Therefore, separating the

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proteins in some way before hydrolysis would allow for the identification of which protein can deliver the highest quantity and quality of peptides for a specific biological activity. In this regard, the biological activity of enzymatic hydrolysates from the albumin, globulin, and glutelin fractions of E. edulis has been evaluated, thus finding that the most prominent ones are those from albumin [3].

So far, studies on the biological activity of Chachafruto hydrolysates have mainly focused on antioxidant activity from single enzyme hydrolysis [7], and very few studies have evaluated effects of sequential enzymatic hydrolysis. This study focused on sequentially studying enzymatic hydrolysis with different proteases of the storage proteins of Chachafruto (*Erythrina edulis*) to determine the type of protein and the combination of proteases that produce the hydrolysate with the highest antioxidant activity.

MATERIALS AND METHODS

Plant material

Fruits of Chachafruto (*Erythrina edulis*) were collected in the rural area of the Boquerón neighborhood in the municipality of Ibagué, Tolima. These are legumes up to 35 centimeters long, dark green in color and contain up to 6 large kidney-shaped seeds inside. Once collected, the plant material was transported to the laboratory under low light and humidity conditions. The seeds and pods were separated, followed by the separation of the seed coat and the rest of the seed. Once the seeds were cleaned, they were cut into smaller slices and dried in an oven at a temperature between 40 and 45°C for 48 hours.

Obtaining and characterization of Chachafruto (*Erythrina edulis*) seed flour

The dried seeds were ground using a Thomas Wiley electric grinder (Thomas Scientific, USA). The flour obtained was sieved through a 200 µm mesh to achieve a smaller particle size. The material was ground further using a Moen GXP650 food processor (Moen, USA). Subsequently, the flour was sieved through a 10 µm mesh and stored at 20°C in airtight containers for further use.

Reagents

The reagents 2,2'-Azino-bis (3-ethylbenzenothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), Crocin, 2,2'-azobis(2-metilpropionamidine) dichlorhydrate (AAPH), fluorescein sodium salt, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Alcalase 2.4 L, Flavourzyme®, and Neutrase 0.8L® were purchased from Novo Nordisk Co (Bagsvaerd, Denmark). The substrate hippyl-histidyl-leucine (HHL) and the enzyme ACE (EC 3.4.15.1, 5.1 U/mg), both for *in vitro* and ex vivo tests, Captopril, fenilefrina, Carbacol, KCl, NaOH, HCl and L-NAME,

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were purchased from SIGMA-ALDRICH® (St. Louis, MO, USA). Other chemicals used were analytical grade (Merck, USA).

Fractionation of Chachafruto protein (*Erythrina edulis)*

In order to obtain each of the storage proteins of Chachafruto (*Erythrina edulis*) separately, sequential extraction was carried out with a 1:10 ratio (flour:solvent) (g:mL) for all extractions, and two extractions were performed for each fraction following the methodology proposed by Guerra *et al.* [8], with some modifications. The solvents used for each kind of storage protein were:

Albumins: Distilled water

Globulins: 5% NaCl and buffer (0.001M K_2HPO_4 , 0.01M EDTA), in relation 60:40 (mL:mL), to pH 7,5.

Gluetlins: NaOH 0.1 N.

Prolamines: 70% ethanol in water.

Each solution was shaken for 90 min at 20°C, then centrifuged using a refrigerated centrifuge Hermle-Z32HK (Hermle Labortechnik, Germany), at 10,000 × g for 15 minutes at 4°C. The supernatant in each fraction was adjusted to the isoelectric point with 0.1 N HCl. Once each protein was precipitated, they were resuspended and neutralized to pH 7.0, with 0.1 N NaOH. Subsequently, a dialysis process was carried out for 48 hours with water changes every 8 hours. After this process, each of the protein concentrates was lyophilized at -50°C using a Buchi Lyovapor (Buchi Labortechnik AG, Switzerland) [8].

Enzymatic hydrolysis

The isolated water-soluble proteins (Albumins, Globulins and Glutelins) were subjected to enzymatic hydrolysis processes, in substrate concentration according to table 1, in order to achieve the highest degree of hydrolysis (DH) and maximum antioxidant activity [9]. First, each enzyme as single enzyme was used in hydrolysis process, then Flavourzyme followed by Alcalase 2.4L® and Neutrase followed by Alcalase 2.4L® were used to produce hydrolysates from albumins, globulins and glutelins from Chachafruto (*Erythrina edulis*).

The purpose of using enzymes in the first phase (Flavourzyme, Neutrase) was to cleave sites that were initially inaccessible to Alcalase 2.4L but could be exposed to its action once the native protein was partially hydrolyzed. The conditions used for each hydrolysis stage are shown in Table 1, regardless of whether the enzyme acts alone or in sequential combination. During hydrolysis with individual enzymes, the times were set to 120 minutes for all enzymes, but in the processes with sequential enzymes, the times for Neutrase and Flavourzyme were increased to 135 minutes while maintaining the Alcalase time at 120 minutes.

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At the end of the process, the enzymes were inactivated by placing the system in bain-marie, TSGP05 Model 0.1 ◦C precision (Thermo Scientific, USA) at 85°C for 15 minutes. Subsequently, dialysis was performed for 48 hours with water changes every 6 hours to remove salts present in the medium and prevent interference in subsequent determinations.

Reaction system and hydrolysis control mode

In all cases, a 500 mL reactor with a magnetic stirrer at 960 rpm was used, which were controlled by means of a combined glass electrode connected to an automatic titrator Titrando 842 (Metrohm, Switzerland). The reaction was monitored through the DH, expressed as the ratio between the number of hydrolyzed peptide bonds (h) and the number of total peptide bonds in the native protein per unit of weight (ht). The DH was calculated with equation (1), using the pH-stat method [13].

$$DH\% = \frac{BN_B}{M_p \alpha h_{Tot}} * 100 \qquad (1)$$

Where B is the consumed volume of the base in L, MP is the mass of the protein in kg, NB is the concentration of the base, and α is the degree of dissociation of the amino groups released during the reaction. Here a ht of 7.5 Eq/Kg was employed, which was calculated by the o-phthalaldehyde (OPA) method, while α and pK were calculated with equations (2) and (3), respectively [13].

$$\alpha = \frac{10^{pH-pK}}{(1 + 10^{pH-pK})} \qquad (2)$$

$$pK = 7.8 + \frac{298 - T}{298 * T} * 2400 \qquad (3)$$

***In vitro* Antioxidant Activity**

The oxygen radical antioxidant capacity (ORAC) method was carried out according to that proposed by Gómez *et al.* [9], with some modifications. For that method, fluorescein (FL) (Oakville, Ontario, Canada) was used as the "fluorescent probe." A mixture of 150 µL of FL (1 µM) and 25 µL of the samples were pre-incubated for 30 min at 37°C. Then, 25 µL of an 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) solution in potassium dihidrogenofosfato/di-Sodio hidrogenofosfato (PBS) (250 mM) was added. Fluorescence intensity was measured every 2 min during 120 min (λexc = 485 nm and λem = 520 nm), and the area under curve was calculated. The Trolox calibration curve was prepared for a concentration range of 0-200 µM, and the results were reported as µmol TE/g of protein [9].

For the 2,2-Difenil-1-Picrilhidrazilo (DPPH) method, the methodology described by Braca *et al.* [10], was followed with some modifications. One milliliter of the hydrolysate solution (0-1 mg/mL) was mixed at different concentrations (300, 500,

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700 y 900 mg/L) with 4 mL of 0.15 mM DPPH solution (in 95% ethanol). The mixture was vigorously shaken and then incubated in the dark at 20°C for 30 minutes. The absorbance of the resulting solution was measured at 517 nm using a UV-Vis microplate reader (Multiskan® GO Thermo Scientific). Ethanol and Trolox (Oakville, Ontario, Canada) were used as control and standard samples, respectively. The radical scavenging capacity of the samples was measured as a decrease in the absorbance of the DPPH radical, and it was calculated using Equation 4 [10]:

$$\%\ \mathrm{DPPH} = \frac{A_c - A_M}{A_c} x100 \quad (4)$$

Where: % DPPH is the DPPH radical scavenging activity expressed as a percentage
AC: is the Control of absorbance
AM: Is absorbance of the reaction mixture

For the determination of antioxidant activity by 2,2' -azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), the methodology described by Vasconcelos *et al.* [11], was followed. The ABTS+ radical was obtained by mixing ABTS (7 mM) and potassium persulfate (final concentration of 2.45 mM). This mixture was left to stand for 16 hours at 20°C, and then it was diluted with ethanol to achieve an absorbance of 0.7 ± 0.02 at 734 nm. The sample was prepared by mixing 3.43 mL of adjusted ABTS+ solution with 70 µL of the hydrolysates at different concentrations (final concentration of 0.2 at 200 µg/mL), and the absorbance values were recorded after 6 minutes of reaction. TROLOX was used as a standard (0.1-1 µg/mL), and Equation (5) was used for its respective calculation [11]:

$$\%\ \mathrm{ABTS} = \frac{A_{ABTS} - A_{6\ min}}{A_{abts}} x100 \quad (5)$$

Where: %ABTS is the percentage of ABTS radical stabilizing activity.
AABTS is the absorbance of ABTS+ before adding the sample.
A6min is the absorbance of the reaction mixture at 6 minutes.

Experimental plan

All experiments were performed in triplicate. For antioxidants analysis, mean comparison analysis and analysis of variance (ANOVA) were applied, and Tukey's multiple range tests were used to determine significant differences between samples using Statgraphics Centurion software. The groups of mean comparison were formed with each antioxidant type of assay (ABTS, DPPH and ORAC).

RESULTS AND DISCUSSION

Chemical analysis of flour

The chachafruto seed flour obtained after cleaning and grinding the product, underwent a bromatological analysis, which is presented in Table 2. It can be

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observed that the protein content (20.7% bs) is in the same order of magnitude as reported by Palma-Albino *et al.* [3] (18.6 %), Intiquilla *et al.* [7] (18.6%), Guerra-Almonacid *et al.* [8] (18.5%), and Córdova [12] (17.13%).

The total ash content is associated with the total mineral content present in the sample; and in this case, it is in the same order of magnitude as those obtained by Palma-Albino *et al.* [3], Córdova [12], Villafuerte and Enriquez [13], and Castañeda [14], who reported values of 3.95%, 5.84%, 4.8% and 5.32%, respectively. Regarding the minerals present in Erythrina edulis flour, Miraya [15] reports values for phosphorus, potassium, calcium and magnesium (0.30%, 1.90%, 0.24%, and 0.15%, respectively) that, in some cases, differ from those obtained in this study, where calcium and phosphorus are slightly lower but magnesium has a much higher value. Differences in bromatological results may be due to various factors such as the extraction method, moisture content of the flour, or agronomic conditions of the crop [16].

Protein concentrate extraction

The extraction of storage proteins from Chachafruto flour was carried out sequentially, first obtaining albumins, followed by globulins, then glutelins, and finally prolamins, resulting in protein contents of 46.36%, 11.39%, 42.01% and 0.23%, respectively. The final extraction yield of total proteins was 64.33%. In the case of the obtained prolamins extract, the protein content was too low to be considered for further studies.

The values reported in this study differ from those of Arango-Bedoya *et al.* [16], who reported values for sequentially extracted chachafruto reserve proteins as follows: glutelins 39.49%, albumins 34.23%, globulins 26.24% and prolamins 0.029%. The possible reason for the differences with this study may lie in the fact that a double extraction is performed at each step.

Enzymatic hydrolysis

Figures 1, 2 and 3 show the results of individual enzymatic hydrolysis with Alcalase (A), Flavourzyme (F) and Neutrase (N), respectively, for the fractions of protein concentrates obtained in the previous section. It can be observed that Alcalase is the enzyme that achieves the highest degrees of hydrolysis (DH) in each of the fractions, followed by Neutrase, and finally Flavourzyme.

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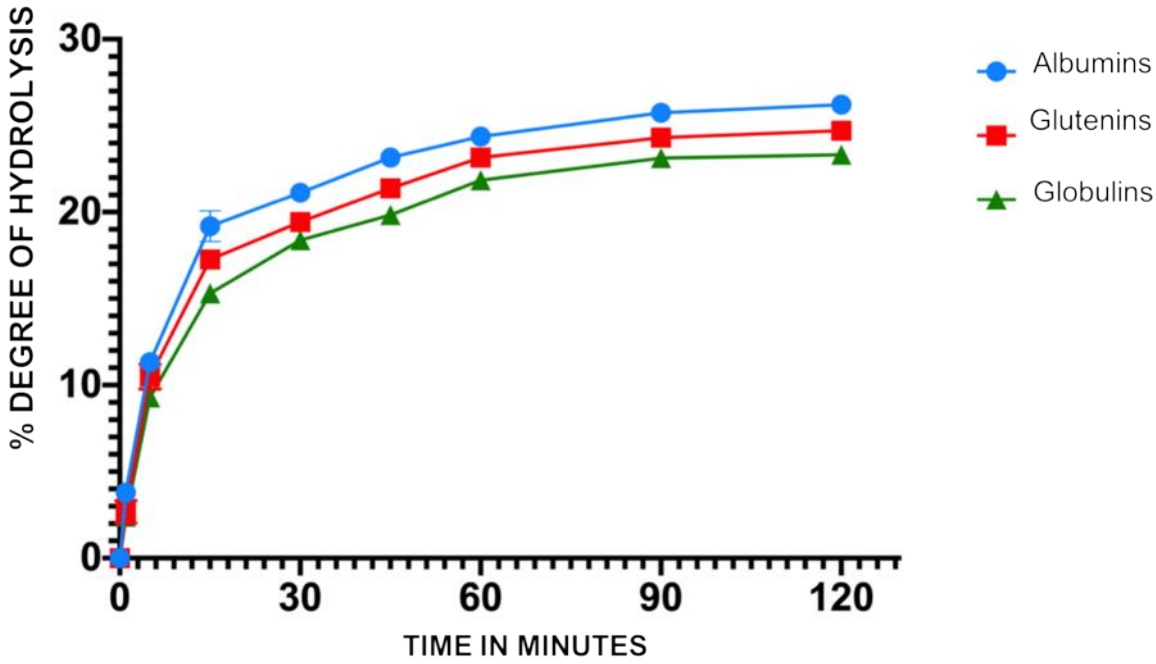

Figure 1: Enzymatic hydrolysis of Chachafruto (Erythrina edulis) proteins with Alcalase

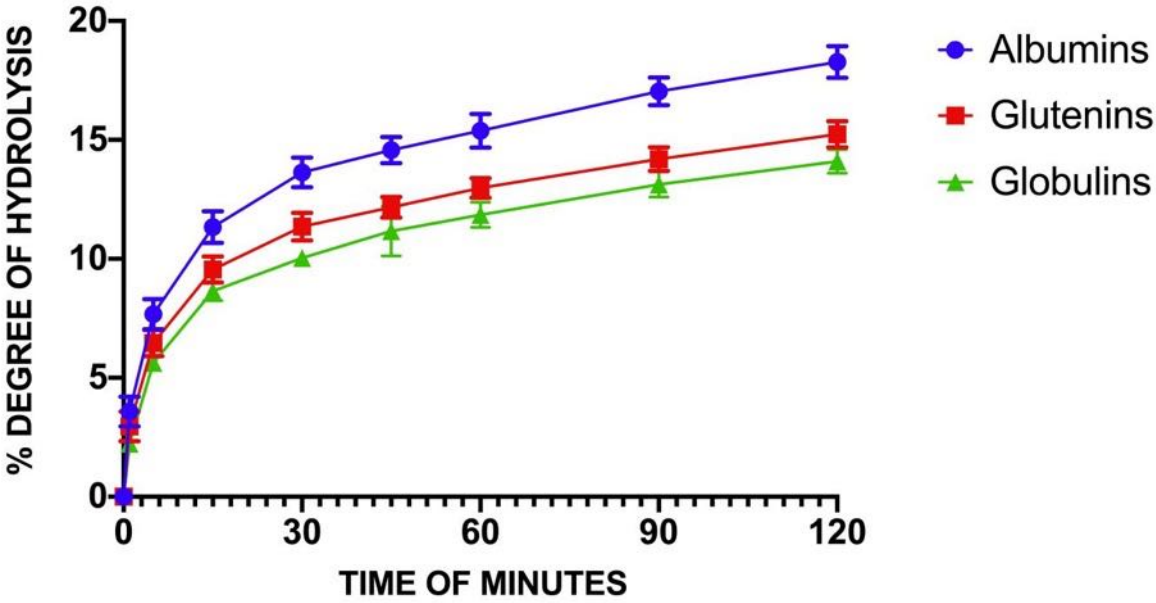

Figure 2: Enzymatic hydrolysis of Chachafruto (Erythrina edulis) proteins with Neutrase

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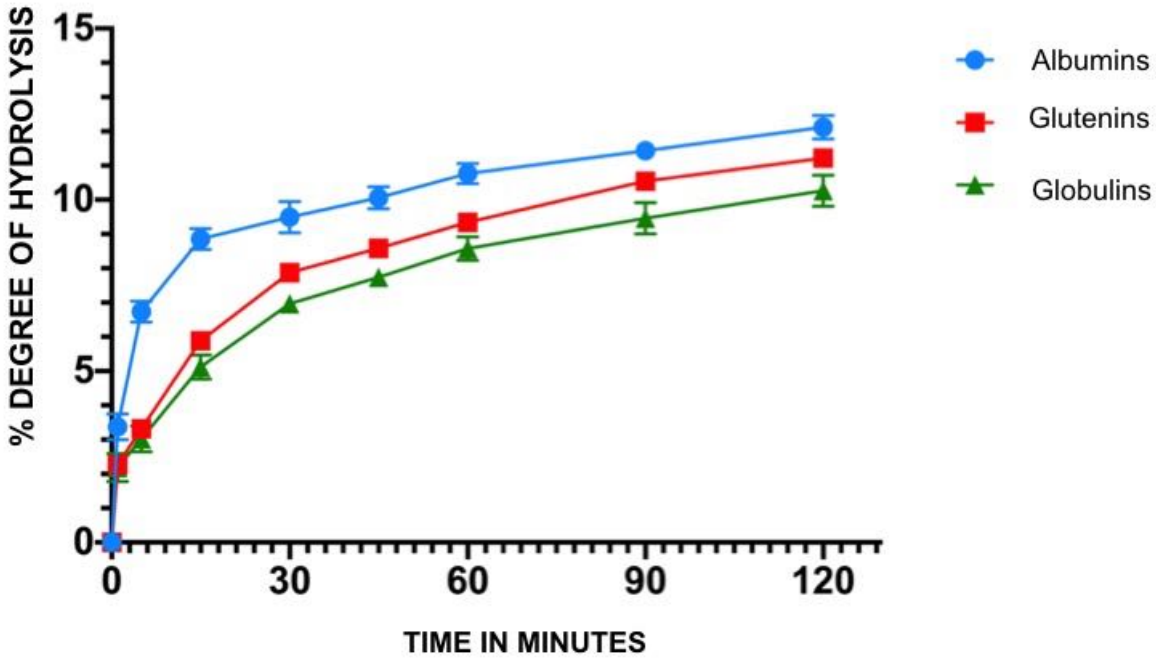

Figure 3: Enzymatic hydrolysis of Chachafruto (Erythrina edulis) proteins with Flavourzyme

With all three enzymes studied, it is observed that albumins achieve the highest DH, followed by glutelins and globulins, indicating that, in that same order, they are the proteins with the highest probability of producing low molecular weight peptides, which have been associated with a greater likelihood of being bioactive peptides [17]. Similar results were observed in the hydrolysis of tea proteins, which showed how antioxidant activity increased with DH [18].

The increasing in DH is important because, high DH implies peptides with low molecular weights, which in turn have direct relationship with higher antioxidant activity. In that sense, wheat bran hydrolysates fractions with molecular weights <1 kDa exhibited the highest antioxidant activity compared to higher molecular weight fractions [19]. Similarly, soy hydrolysates with molecular weight <3 kDa showed greater antioxidant activity measured by the DPPH method. They also reduced the production of reactive oxygen species and lipid peroxidation and stimulated the production of antioxidant enzymes in Caco-2 cells [17]. Hydrolysates of yam bean seeds with molecular weights <1 kDa obtained by using membranes exhibited higher antioxidant capacities than fractions of the same hydrolysate with higher molecular weight [20].

In different studies, it has been reported that Alcalase hydrolysates outperform those of other enzymes in terms of antioxidant activity. This is the case in the hydrolysis of soy proteins with Alcalase and Neutrase [17]; hydrolysis of a by-product milk

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production with Alcalase and Flavourzyme; hydrolysis of sweet potato protein with Alcalase, Neutrase, Protamex, and Flavourzyme; hydrolysis of corn gluten with Alcalase and Protamex; hydrolysis of corn flour comparing five enzymes, including Alcalase; hydrolysis of defatted corn flour with Alcalase, papain, Neutrase, and Flavourzyme; hydrolysis of rice bran with Neutrase, Flavourzyme, and Alcalase; barley glutelin hydrolysates with Flavourzyme and Alcalase; hydrolysis of rapeseed protein with Alcalase, pepsin+pancreatin, Flavourzyme, and thermolysin; hydrolysis of coconut protein with Alcalase, Neutrase, bromelain, and papain; hydrolysis of plum bone protein extract with Alcalase, thermolysin, Flavourzyme, and protease P; hydrolysis of cherry bone with seeds containing significant amounts of protein with Flavourzyme, Alcalase, and thermolysin; hydrolysis of olive seed protein with Alcalase, thermolysin, Neutrase, Flavourzyme, and PTN; Camellia oleifera hydrolysates with Alcalase, Flavourzyme, trypsin, Neutrase, and papain; flaxseed protein hydrolysates with papain, trypsin, pancreatin, Alcalase, and Flavourzyme; wild almond hydrolysis with pepsin, trypsin, chymotrypsin, Alcalase, and Flavourzyme [2]. The same way hydrolysis of Erythrina edulis concentrate with Alcalase, Neutrase, and Flavourzyme [7]; hydrolysis of brown teff with Protamax, Flavourzyme and Alcalase; hydrolysis of fennel seeds with different proteases, including Alcalase; and hydrolysis of spent brewery grain with Alcalase and other enzymes [2].

It is important to note that all figures (1-3) have the typical shape of hydrolysis curves, with a quick increase in DH at first minutes, then DH decreases continuously until the final, which variability is in range of acceptability with value less than 10%, in all cases. This behaviour has been observed in several protein sources and it has been attributed to among other reasons, denaturation of the enzyme. Some authors have proved the enzymatic inactivation, adding a second enzyme dose to system of reaction [22]. However, if in the second dose the same enzyme is used, inhibition by hydrolysis products could persist. For that reason in this study sequential enzymatic hydrolysis was proposed, but in this case with different enzymes to avoid the phenomenon of inhibition by hydrolysis products.

Sequential Enzymatic Hydrolysis

Based on the results in Figures (1-3), Alcalase was selected to be used in the second stage of sequential hydrolysis, because by starting with a greater number of peptides, it would have the highest possibility of driving the reaction to a higher degree of hydrolysis and, therefore, a greater presence of smaller peptides.

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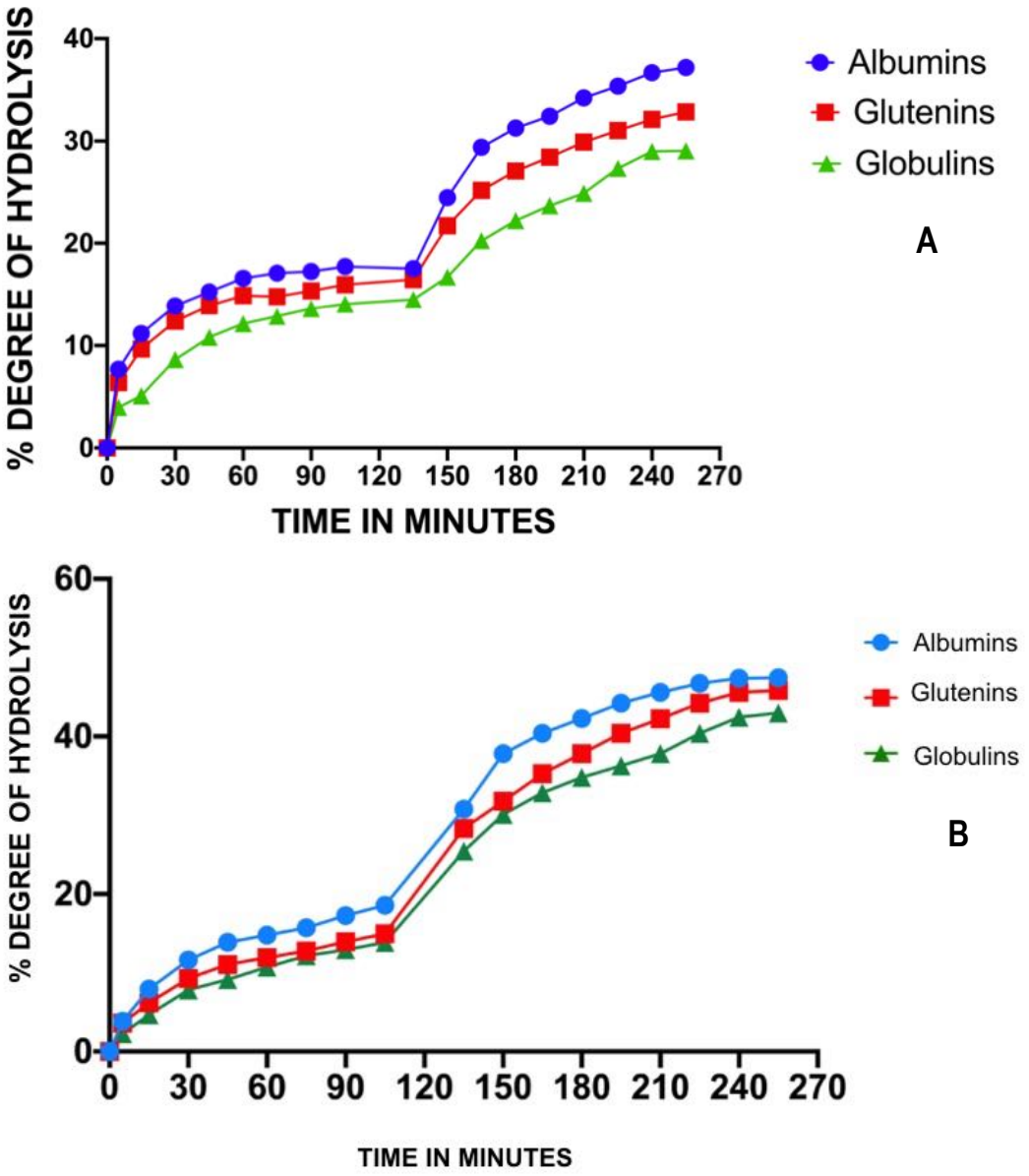

Figure 4: Enzymatic hydrolysis of Chachafruto (*Erythrina edulis*) with sequential process A) Flavourzyme-Alcalase B) Neutrase-Alcalase

Figure 4 presents the enzymatic hydrolysis curves performed by sequential application of Flavourzyme-Alcalase (Figure 4a) and Neutrase-Alcalase (Figure 4b). It can be observed that, when applying sequential hydrolysis to the reaction system, the degree of hydrolysis (DH) obtained by each enzyme is very similar to when it was applied individually. Additionally, Alcalase adds a DH value that is almost equal to what it obtained when used as the single enzyme, resulting in a DH that is practically the sum of the DH values obtained by each enzyme individually. This indicates that the cleavage sites of each enzyme are different, and therefore, the hydrolysis performed by the first enzyme does not diminish the DH obtained with the

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second enzyme. For this reason, the combination of enzymes allows for an increased possibility of obtaining smaller peptides, which, as mentioned before, produce hydrolysates potentially more bioactive.

In the same vein, it is clear that the hydrolysates obtained with Neutrase-Alcalase (NA) reach higher DH values than those of Flavourzyme-Alcalase (FA). In the latter case, Alcalase starts from a lower DH and reaches lower DH values than in the first case. These results require the analysis of biological activity for both processes to determine if the combination with higher DH corresponds to the one with the highest *in vitro* biological activity.

In vitro Antioxidant Activity

Table 3 shows the results of the antioxidant activity of the hydrolysates from Chachafruto proteins obtained through sequential hydrolysis. It can be observed that the albumin hydrolysates achieve the highest activities in both enzyme combinations, followed by the glutelin hydrolysates, and finally the globulin hydrolysates. This is consistent with the fact that higher DH leads to greater biological activity in the hydrolysates [4]. However, it should be noted that the FA hydrolysates, despite having lower DH values than the NA hydrolysates, exhibit similar or even better antioxidant activity values in many cases.

The results of this study are higher than those from Intiquilla *et al.* [7], who worked with Neutrase, Alcalase and Flavourzyme as single enzymes in hydrolysis of *Erythrina edulis* seed protein. Similarly, the results are higher than results from Correa *et al.* [23], who evaluated antioxidant capacity of enzymatic hydrolysates of protein from *Erythrina edulis* seeds with gastric and gastrointestinal digestion and their ultrafiltrates. That indicates the important effect of hydrolysis single storage protein and the sequential enzymatic process, which are the most importants novelties in this study.

On the other hand, the results of this study are lower than those of Palma-Albino [3] in the sequential hydrolysis of Chachafruto (*Erythrina edulis*) with pepsin, pancreatin and Alcalase. However, it must be considered that they used three enzymes, while in the present study, two enzymes were used. On the other hand, these results are in line with previous studies where antioxidant peptides were obtained by enzymatic hydrolysis of plant substrates using Alcalase. Examples include the production of antioxidant peptides by hydrolyzing proteins from wheat bran [19], yam bean seeds [20], soybean [17], peas, rapeseed, tea seeds [18], *Lupinus mutabilis, Jatropha curcas,* maize, amaranth and its seeds, chickpea, rice, glutinous rice, peas, lead tree, fenugreek, sorghum, walnut, hemp, cocoa almonds, sweet potato, Chinese chestnut, peanut, Chachafruto, Chungkukjang, among others [2]. Some vegetable

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waste has also been hydrolyzed with Alcalase to produce antioxidant hydrolysates, such as asparagus waste, tomato seeds, plum stones, among others [2].

These results demonstrate that albumin hydrolysates from Chachafruto (*Erythrina edulis*) seed flour, obtained by sequential enzymatic hydrolysis, offer an opportunity as a source of compound antioxidants with potential for food preservation or health applications in prevention or reduction of diseases associated with oxidative stress, as neurodegenerative, cardiovascular or cancer diseases [1]. That is due to direct relationship that has been observed between the antioxidant and anticancer activity of different compounds, including several types of peptides [9]. This is the case of peptides obtained by enzymatic hydrolysis from red tilapia viscera (*Oeraochomys spp*) [24], oyster (*Saccostrea cucullata*) [25], blood clam (*Tegillarca granosa*) muscle [26], eel (*monopterus sp*) protein [27] and pacific white shrimp (*Litopenaeus vannamei*) [28]. Besides, also is due to relationship observed between antioxidant activity and other activities as anti hypertensive [29] and anti-diabetic [30].

CONCLUSION AND RECOMMENDATIONS FOR DEVELOPMENT

Based on the results of this study, it can be concluded that enzymatic hydrolysates of Chachafruto seed storage protein can increase their degree of hydrolysis when subjected to sequential enzymatic hydrolysis compared to hydrolysis with a single enzyme. Hydrolysates obtained by sequential hydrolysis with Neutrase-Alcalase have a higher degree of hydrolysis than those obtained with Flavourzyme-Alcalase. Chachafruto seed can produce a flour with high protein contained, which storage protein can be hydrolyzed to produce functionals compound with antioxidant activity, using Alcalase, Flavourzyme and Neutrase enzymes. However, Flavourzyme-Alcalase exhibit higher antioxidant activitiy than Neutrase-Alcalase hydrolysates or each enzyme individually.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1: Conditions used in each stage of enzymatic hydrolysis

				Substrate Concentration (S)		
Enzyme	Temperature	pH	E/S	Albumins	Globulins	Glutelins
Alcalase	50°C	8.0	5%	4.4	3.85	4.60
Flavourzyme	45°C	7.5	5%	4.4	3.85	4.60
Neutrase	45°C	7.5	5%	4.4	3.85	4.60

Table 2: Phisicochemical analysis of Chachafruto seeds flour (*Erythrina edulis*)

Parameter	Units	Average	SD	CV
Ashes	%	5,6	0,306	0,054
Protein	%	20,7	1,440	0,070
Ethereal extract	%	0,8	0,208	0,250
Fiber	%	4,8	0,289	0,060
Calcium	%	0,1	0,020	0,154
Magnesium	%	0,0	0,012	0,247
Sodium	mg/Kg	160,1	9,311	0,058
Potassium	%	2,1	0,419	0,203
Iron	mg/Kg	114,2	4,718	0,041
Cupper	mg/Kg	10,3	0,451	0,044
Manganese	mg/Kg	10,6	0,900	0,085
Zinc	mg/Kg	37,2	3,317	0,089
Boron	mg/Kg	17,2	4,350	0,253
Phosphorus	%	0,2	0,021	0,098
Sulfur	%	0,2	0,045	0,260

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Table 3: Antioxidant activity of hydrolyzed of Chachafruto (*Erythrina edulis*) obtained by sequential hydrolysis (mM Trolox/g)

Hydrolyzed	ABTS	DPPH	ORAC
Glutelins FA	624,36[b]	576,36[a]	857,30[d]
Albumins FA	785,14[c]	664,55[a]	1284,00[f]
Globulins FA	536,19[a]	540,00[a]	813,10[b]
Glutelins NA	608,23[b]	560,00[a]	577,10[a]
Albumins NA	767,67[c]	630,91[a]	1033,00[e]
Globulins NA	520,91[a]	550,91[a]	832,23[c]
Ascorbic acid (control +)	1011,25[d]	970,80[b]	1510,11[g]
H_2O (Control -)	0	0	0

Different superscript indicate statistically significant differences

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