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Enzymatic predigestion of amaranth proteins

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Abstract
Partial protein hydrolysis is an issue of interest in the development of ingredients for the production of special foods. The amaranth grain, which is known for its nutritional properties, allows obtaining two main fractions by using differential milling: a hyperproteic flour with approximately 40% proteins with a complete amino-acids profile, and semolina with approximately 90% starch. The mixture of both products creates raw materials suitable for the elaboration of special foods. The enzymatic protein hydrolysis assays were performed on an equal-part mixture of amaranth hyperproteic flour and amaranth semolina, using commercial proteinases Alcalase™ and Flavourzyme™. Hydrolysates were obtained with hydrolysis degrees ranging from 30 to 58%. The molecular weights of the obtained peptides and polypeptides were between 0.5 and 240 kDa. The solubility for the 2-7 pH range is not lower than 75% and the amino-acids profile is not different to that of the raw material.

1. Introduction

Different pathologies and/or dietary dysfunctions make it necessary to consume foods that are known as specials foods, some of which are formulated with predigested proteins and may also contain carbohydrates, which in turn may or may not be predigested. Supplements are also considered special foods (Clemente, 1999a; Pedrón-Giner *et al.,* 2011) and are consumed by sportsmen and women, elderly people and individuals that have no specific pathologies but require a special contribution to their regular diet.

According to the degree of protein hydrolysis, special foods are classified in: polymeric, oligomeric and elemental. Oligomeric foods mainly contain protein hydrolysates in peptide state, in addition to dextrins and maltodextrins that are formed by the hydrolysis of starch, as well as a low amount of vegetable fats, and they have practically displaced elemental foods because in the latter, the fractions resulting from protein hydrolysis have an unacceptable flavor and high osmolarity (Clemente, 1999a; Pedrón-Giner *et al*., 2011); (Nielsen, 1997) On the other hand, low-molecular-weight peptides produced by a controlled protein hydrolysis are more effective than native proteins in the absorption process of the gastrointestinal system. (Villanueva *et al*., 1999), (Nielsen, 1997), (Damodaran, 1997), (Schwenke, 1997)

As source of proteins, raw materials of animal and/or vegetable origin are used, and although the nutritional quality of the animal origin materials is greater, nowadays such materials are being questioned because of the unpleasant flavors and odors that appear during extensive hydrolysis (Clemente, 1999a; Pedrón-Giner *et al*., 2011). In addition, formulas with animal protein hydrolysates with high or low

hydrolysis degrees do not ensure lack of allergenicity, but only hypo-allergenicity (Nielsen, 1997), (Salcedo-Chávez, 2002) On the other hand, there is a high degree of concern regarding the presence of sporadic viral sources in animal meats, as well as residues or metabolites of chemical and/or pharmacological substances (Clemente, 1999a; Pedrón-Giner *et al*., 2011). Therefore, the use of vegetable origin raw materials has gained importance because they can provide proteins and also carbohydrates; in the case of cereals, usually as starch. On the other hand, although having a high nutritional value, soybean is currently questioned because it has undergone genetic modifications.

The amaranth grain (*Amaranthus spp*.) shows a high potential to be used in food formulations. The average protein content is 19% (dry basis), with an amino-acids balance that is considered as one of the best amongst vegetable origin proteins (Segura-Nieto *et al*., 1994). According to Bressani & Garcia Vela (1990), proteins in the amaranth grain are distributed as follows: 40% of globulins and albumins, 2% of prolamins and 40% of glutelins. The lysine content (5.5 g/100 g protein) of the amaranth grain must be noted. According to several authors, leucine is the first limiting amino-acid.

Differential milling of the whole amaranth grain, developed in CIDTA with physical methods and only by a dry process (Tosi *et al.*, 2000), allows obtaining three different composition fractions: a hyperproteic flour with a protein content of approximately 39.6% and the largest amount of lipids; a semolina fraction with 89.0% starch and a fraction rich in fibers (23.2%). By mixing these fractions it is possible to prepare substrates with different contents of proteins, carbohydrates and lipids on which different hydrolysis processes can be carried out.

Protein hydrolysis processes that use acids or bases are no longer in use because they can cause the decomposition of certain essential aromatic amino-acids, give way to isomerization processes and cause the formation of toxic compounds. Due to high protein selectivity, enzymatic protein hydrolysis can be performed in a controlled manner and in moderate conditions of temperature and pH, and the appearance of degradation products is infrequent (Guadix *et al*, 2000). On the other hand, it is necessary to consider that during the hydrolysis processes of food proteins, as the hydrolysis advances, the resulting products are substrates for the following fragmentations to peptides of lower molecular weight. This is how several hydrolysis processes take place, creating a competition between the original protein and the peptides resulting from hydrolysis (Adler-Nissen, 1986). Commercial exoproteases and endopeptidases were used in the protein hydrolysis processes studied in the present work, the latter are essential to avoid the intense bitter flavor related with the hydrophobicity of high-molecular-weight peptides (Maehashi & Huang, 2009). According to Clemente *et al.*, (1999a; 1999b), the use of Alcalase™ and Flavourzyme™ sequentially in the chickpea flour hydrolysis allows obtaining a hydrolysate with a hydrolysis degree of 34% and an antigenic activity above 90%.

The present work shows the results obtained for the determination of operating conditions of protein hydrolysis processes by an enzymatic pathway using Alcalase™ and Flavourzyme™ on a substrate formulated with an equal-part mixture of amaranth hyperproteic flour and amaranth semolina.

2. Materials and Methods

2.1. Materials

An equal-part mixture (w/w) of amaranth hyperproteic flour and amaranth semolina (*A. cruentus*, 2009 crop), crushed in a cold mill to pass through a 50 ASTM mesh sieve, was used as substrate. The compositions of the hyperproteic meal and the semolina that correspond to two fractions of the amaranth grain differential milling (Tosi *et al*., 2000) and the equal-part mixture, which is the substrate to be hydrolyzed, are detailed in Table 1. Table 2 includes the amino-acids composition of the analyzed mixture and the hydrolysis products and the compositions recommended by FAO (FAO-WHO, 1991).

Table 1. *Percent composition of hyperproteic meal (HM) and semolina (S) produced by differential milling of whole amaranth grain and the substrate to be hydrolyzed*

Parameter	**Differential Milling**		**Substrate to be hydrolyzed 50% HM - 50% S**
	Hyperproteic meal	**Semolina**	
Moisture	8.0	8.4	8.2
Protein	34.6	5.7	20.2
Ashes	7.0	1.0	4.0
Hexane solubles	23.7	2.3	13.0
Carbohydrates (*)	26.7	82.6	54.7
Fibers	17.0	0.6	8.8
Starch	9.7	82.0	45.9

(*) by difference

Table 2. *Amino-acids content in the hydrolysis substrate (50% of amaranth hyperproteic flour and 50% amaranth semolina) and in hydrolysates*

Amino-acid	Amino-acids content (g/100 g protein)		
	Amaranth hyperproteic meal + semolina	Hydrolysate	FAO
Isoleucine	3.6 ± 0.1	2.3 ± 0.1	2.8
Leucine	5.6 ± 0.4	4.0 ± 0.3	6.6
Lysine	6.7 ± 0.1	5.0 ± 0.1	5.8
Methionine	1.9 ± 0.2	1.1 ± 0.1	2.5
Phenylalanine	4.0 ± 0.2	3.3 ± 0.1	-
Tyrosine	3.4 ± 0.2	2.6 ± 0.2	6.3
Threonine	3.8 ± 0.2	3.1 ± 0.2	3.4
Valine	4.2 ± 0.2	5.0 ± 0.3	3.5
Alanine	3.6 ± 0.1	2.4 ± 0.2	-
Arginine	6.7 ± 0.2	4.8 ± 0.2	-
Aspartic acid	7.9 ± 0.1	6.9 ± 0.2	-
Glutamic acid	15.7 ± 0.5	14.2 ± 0.3	-
Glycine	7.7 ± 0.1	5.9 ± 0.1	-
Histidine	2.5 ± 0.1	1.9 ± 0.1	1.9
Proline	3.9 ± 0.4	2.0 ± 0.2	-
Serine	6.7 ± 0.2	6.0 ± 0.3	-
Tryptophan	1.2 ± 0.1	1.0 ± 0.2	-
Cystine	2.0 ± 0.2	1.6 ± 0.2	-

From preliminary assays, enzymes Alcalase™ and Flavourzyme™ 500 MG (Novozymes, Bagsvaerd, Denmark) were selected to perform protein hydrolysis. Alcalase™ is an endoproteinase produced by the *Bacillus lichenisformis*, while Flavourzyme™ is an enzymatic complex of fungal endo- and exopeptidases. All the enzymes used were food grade.

Sodium dodecyl sulfate, 2,4,6-trinitrobenzenesulfonic acid (TNBS), DL-alpha-amino-n-butyric acid, protein standards for electrophoresis and molecular weight markers, acrylamide, N,N′-methylenebisacrylamide, diethyl (ethoxymethylene) malonate and Coomassie brilliant blue G-250 were provided by Sigma Chemical Co. (St. Louis, MO, USA), D-Salt™ dextran desalting columns (5K MWCO) were provided by Pierce Biotechnology Inc. (Rockford, IL, USA). The rest of the reagents, of analytical quality, were provided by Merck (Darmstad, Germany).

2.2. Methods

2.2.1. Experimental Design

By applying both enzymes sequentially, in accordance with their mode of action on proteins, each experiment was performed in two stages, because the pH needs to be adjusted to the optimal values of each enzyme used. The time of protein hydrolysis was considered as the independent variable with values of 20, 60, 120, and 180 minutes, the water/substrate relationship was constant and equal to 10 w/w. The amount of enzyme units/per gram of protein relationship was adopted by means of preliminary assays (see ahead). The following were selected as results: nitrogen recovery percentage, hydrolysis degree, protein solubility, polypeptides profile by SDS-PAGE, molecular weights profile by exclusion chromatography, amount of nitrogen found when forming peptides with two or more peptide bonds, and the amino-acids profile.

2.2.2. Determination of Enzymatic Activity

The enzymatic activities of the selected enzymes were determined in accordance with the supplier's protocols. To determine the best concentrations of Alcalase™ and Flavourzyme™, preliminary assays were performed at different concentrations with a maximum time of hydrolysis of 120 minutes. Preliminary assays were performed with the methodology described in the following item: *Protein hydrolysis process.*

From the obtained preliminary results (see Results) the following relationship was adopted: Alcalase™ 60 AU/kg protein/Flavourzyme™ 50 LAPU/kg protein.

2.2.3. Protein Hydrolysis Process

One hundred grams of substrate were suspended in 1000 mL water at pH 7, corresponding to the action of the Alcalase™ enzyme, under constant agitation and in a thermostatic bath at 60 ºC. The hydrolysis (time 0) starts with the addition of the Alcalase™ enzyme; after two-thirds of the total time of the trial elapsed, pH was adjusted to 6.5, temperature was set at 50ºC and Flavourzyme™ MG 500 enzyme was added. Owing to the fact that the optimum pH interval of Alcalase™ ranges between 6.5 and 8, it was considered that this enzyme acts throughout the total time of the assay. The values for the temperature and pH were controlled continuously, maintaining the indicated values throughout the assay; the pH was adjusted by adding solutions of NaOH or H_3PO_4.

To stop the hydrolysis process, once the time of the assay was completed, enzymes were deactivated maintaining agitation, lowering the pH to 4 and increasing the temperature to 95 ºC during 10 minutes. After deactivation was completed, pH was adjusted to 7 and agitation was maintained until the temperature reached 35 ºC.

Hydrolysates were separated from the remaining solids by centrifugation at 4000 × *g* for 40 minutes; solids were

washed three times with 30 mL distilled water and centrifuged each time in the aforementioned conditions; all liquid fractions were collected in a 200-mL volumetric flask and distilled water was added to reach the calibration mark.

2.2.4. Content of Hydrolyzed Protein (Recovered Nitrogen)

The total hydrolyzed protein content was determined by micro-Kjeldahl, method Aa 5-38 (AOAC, 1990) on an aliquot of hydrolysate using a nitrogen-to-protein conversion factor of 6.25 to convert the determined nitrogen. The result was expressed as the percentage of protein content in the hydrolysate with regard to the protein content in the substrate.

2.3. Hydrolysis Degree

The hydrolysis degree is defined as the percentage of hydrolyzed peptide bonds and was calculated by the determination of free amino groups in the hydrolysates and its comparison to the total of free amino groups determined by total hydrolysis of a 10 mg sample of the substrate, with 4 mL of 6N HCI, during 24 hours at 110 ºC. For the determination of the free amino groups, the technique proposed by Adler-Nissen (1979) was used, with 2,4,6-trinitrobenzenesulfonic acid as reagent.

Solubility Percentage of proteins from the hydrolysate, which are soluble at a specific pH

Aliquots were taken from the hydrolysates, the pH was adjusted with HCI to the selected values 2.0; 3.0; 4.0; 4.5; 5.5; 6.5; and 7.0, it was maintained for 30 minutes and the solids were separated by centrifugation at 4000 × *g* for 30 minutes; the protein content of the supernatant was determined using the micro-Kjeldahl method. A nitrogen-to-protein conversion factor of 6.25 was used (Villanueva *et al*., 1999). Solubility was expressed as a percentage and was defined as the relationship between the protein that keeps soluble at a specific pH and the total protein content of the hydrolysate.

2.4. Polypeptides Profile by SDS-PAGE

SDS-PAGE electrophoresis for molecular weights between 14.2 and 66 kDa was performed according to the Laemmli's method (1970). The gel of 100 g/L SDS, consisted of a polyacryilamide resolution gel of 0.12 g/g (pH 8.8) and a stacking gel of 0.10 g/g (pH 6.8). The lengths of the resolution and stacking gels were 50 and 15 mm, respectively. A BioRad model 1653301 equipment (Bio Rad Laboratories Inc., Hercules, CA, USA) was used. After electrophoresis, the gels were stained by immersion in a solution of 2.5 g/L Coomassie Brilliant Blue G-250, 0.45 mL/mL methanol and 0.09 mL/mL acetic acid. As reference, Sigma (St. Louis, USA) molecular weight markers were used, ranging from 14.2 to 66 kDa.

For molecular weights between 3.48 and 10.6 kDa, the method proposed by Swank & Munkres (1971) was used, and a Sigma molecular weight marker for molecular weights between 2.5 and 17 kDa.

A solution obtained from the substrate only treated with water was used as control, at the same conditions of temperature, pH and time described in the experimental design section.

2.5. Molecular Weights Profile by Exclusion Chromatography

Samples were passed through D-Salt™ dextran desalting columns (5K MWCO) (Pierce Biotechnology Inc. Rockford, IL, USA) to eliminate non-protein compounds. A Shimadzu LC 10AS high-pressure liquid chromatograph, with a Phenomenex BioSep SEC-S 3000 (length: 300 mm; ID: 7.8 mm) column was used, with a Shimadzu SPD UV-Vis 10A detector. The injected volume was 20 µL eluted with 20 mM phosphate buffer, 0.5 M sodium chloride pH 8.3, at a flow rate of 0.6 mL/min; measuring at 214 nm. Calibration was done with blue dextran (2000 kDa), catalase (240 kDa), α-amylase (200 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa) and riboflavin (0.5 kDa).

2.6. Amino-Acids Profile

Amino-acids profile was determined by high-pressure liquid chromatography after acid hydrolysis and derivatization with diethyl (ethoxymethylene) malonate according to the technique proposed by Alaiz *et al*., (1992) and Villanueva *et al.*, (1999). Methionine, tryptophan, cystine and cysteine were not determined with this method (see ahead).

A Shimadzu LC 10AS HPLC chromatograph was used, with a Supelco C18 reversed phase column (length: 300 mm, ID: 7.8 mm), at 25 ºC, with a binary gradient system, and a Shimadzu SPD UV-Vis 10A detector; reading was performed at 484 nm. The elutant used was a 25 mM solution of sodium acetate with 0.02% of sodium azide (pH 6.0) (A) and acetonitrile (B), and flow was set to 0.9 mL/min as follows: between 0.0-3.0 min, linear gradient from A/B (91:9) to A/B (86:14); between 3.0-13.0 min, elution with A/B (86:14); between 13.0-30.0 min, linear gradient from A/B (86:14) to A/B (69:31); between 30.0-35.0 min, elution with A/B (69:31).

Methionine content was determined by the Tonkovié & Hadžija method (1977), while tryptophan, cystine and cysteine contents were determined with the methods proposed by Villegas *et al.*, (1984).

All assays and determinations of the results were performed in triplicate.

3. Results

Figure 1 shows that the higher hydrolysis degrees are obtained with the Alcalase™ 60 AU/kg protein and Flavourzyme™ 35 LAPU/kg protein relationship; but it was considered convenient to increase the Flavourzyme™ concentration to 50 LAPU/kg protein to avoid the presence of bitter flavors.

3.1. Degree of Protein Hydrolysis

Figure 1 shows the hydrolysis degrees in terms of the hydrolysis times for the relationship Alcalase™ 60 AU/kg protein/Flavourzyme™ 50 LAPU/kg protein. A 49% degree of hydrolysis was achieved in 60 minutes, and then slowly increased up to 54% after 180 minutes.

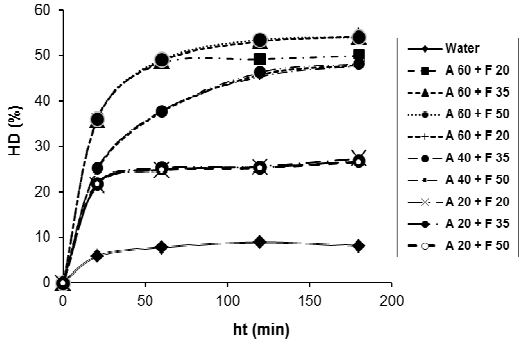


Figure 1. *Hydrolysis degree (HD) as a function of time of hydrolysis (ht)*

Protein hydrolysis was also verified when the treatment was carried out only with water (Bressani & Garcia Vela (1990); Gorinstein *et al*., 1991); hydrolysis degree values at the abovementioned times were 7.8 and 8.1. It was estimated that endogenous proteases were responsible for these hydrolytic phenomena.

3.2. Content of Hydrolyzed Protein (Recovered Nitrogen)

Figure 2 (RN vs HD) shows that the amount of hydrolyzed protein by enzymatic pathway increased rapidly in the first 40 minutes of the hydrolysis process until it reached values close to 85.5 after 180 minutes.

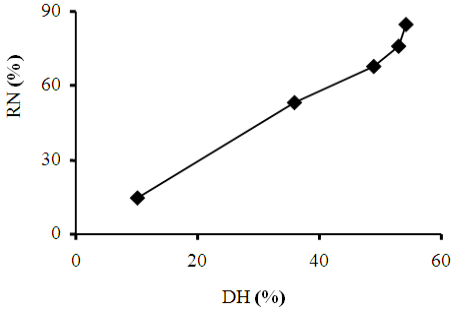


Figure 2. *Percentage of recovered nitrogen (RN) as a function of hydrolysis degree (HD)*

Solubilization of the proteins treated only with water also occurred, caused by the hydrolytic action of the endogenous proteases or the presence of amaranth proteins that solubilized at pH 7.

The electrophoretic analysis (Figure 5) and the molecular weights profile (Figure 6) of the hydrolysates showed that proteins extracted with water have higher molecular weight structures than the ones present in the hydrolysates with Alcalase™ and Flavourzyme™. This owes to the fact that amaranth contains water-soluble albumins, known as 2MPRS, which are rich in methionine and lysine.

3.3. Solubility

Figure 3 shows that the solubility of the hydrolysates does not undergo significant changes in the total pH range of the assays, ranging between 79.7 and 92.7%. On the other hand, the solubility of the original proteins changed strongly as a function of pH, decreasing up to a pH of 5.5, where they exhibit the lower solubility. From that pH value, solubility starts to increase, reaching the higher solubility at pH 7 (for the interval of the tested pH).

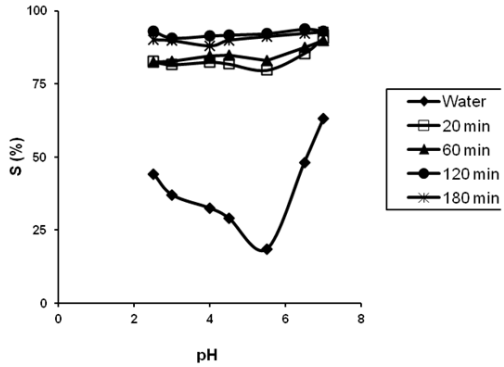
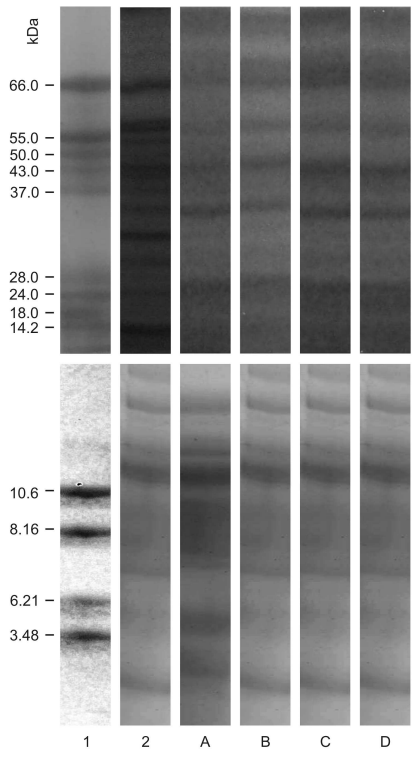


Figure 3. *Solubility as a function of the pH of the substrate (mixture of hyperproteic flour and semolina) and in the hydrolysates as a function of time*

3.4. Polypeptides Profile by SDS-PAGE



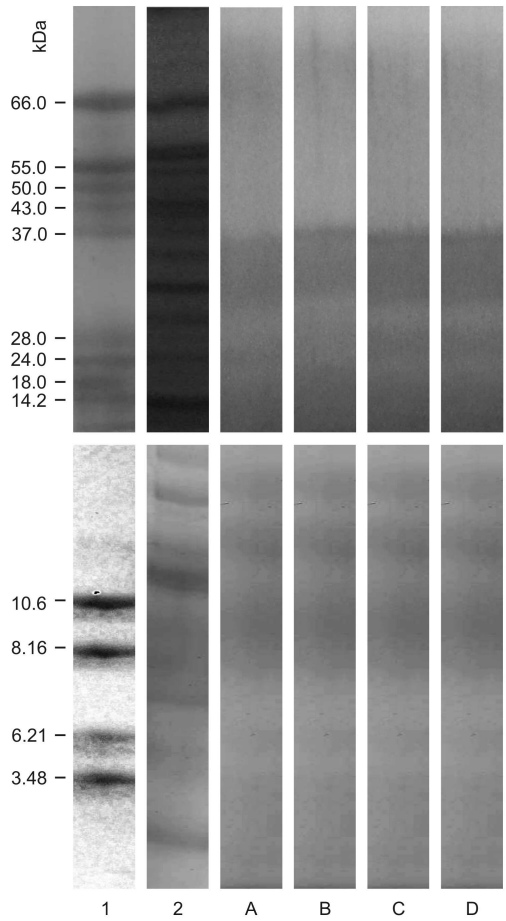
1 Standards; 2 Water; A 20 min; B 60 min; C 120 min; D 180 min

Figure 4. *SDS-PAGE profile of hydrolysates obtained with water.*

The polypeptides profiles obtained by SDS-PAGE from the hydrolysates obtained only with water (Figure 4)

showed that the intensity of the bands decreases as the time of hydrolysis increases. As a function of the time of hydrolysis only with water, it was possible to observe protein bands between 14.2 and 32 kDa after 60 minutes and protein bands between 14.2 and 20 kDa after 120 minutes, and only bands below 14.2 kDa could be observed after 180 minutes.

The profiles of hydrolysates obtained with Alcalase™ and Flavourzyme™ (Figure 5) showed that after 20 minutes the enzymes ruptured the easily accessible bonds, not only in the solubilized proteins but also in those that remained in solid state. The proteins that partially maintain their structures correspond to the 14.2 – 32 kDa bands which are not observable after 120 minutes of hydrolysis because the action of enzymes deepens, leaving bands between 14.2 and 20 kDa. Bands between 14.2 and 55 kDa can be observed after 180 minutes of hydrolysis.



1 Standards; 2 Water; A 20 min; B 60 min; C 120 min; D 180 min

Figure 5. *SDS-PAGE profile of hydrolysates obtained with Alcalase™ and Flavourzyme™*

The presence of polypeptides between 20 and 55 kDa can be explained considering the fact that in the first stages of hydrolysis the complex tertiary structures are modified in such a way that they allow the enzymes to rupture peptide bonds previously inaccessible due to steric impediment, allowing the separation of heavy fractions in the original protein chain. This was in agreement with the results obtained by Konishi *et al.* (1991), related with the resistance of the albumin-2 fraction to some proteases.

Hydrolysis allows recognizing four polypeptide groups, one with a molecular weight between 55 and 64 kDa, and a second group between 33 and 37 kDa, such groups correspond to the alpha subunit of amarantin, a storage protein which is similar to the legumin in leguminous seeds; amarantin has a molecular weight above 300 kDa (Martinez *et al.*, 1997). The third group contains polypeptides between 18 and 25 kDa, which correspond to the beta subunit of amarantin and the fourth group has molecular weights between 14.2 and 18 kDa.

In the present work, only three (between 33 and 37; 18 and 25; and 14.2 and 18 kDa) of the four groups mentioned are present in all assays as products of hydrolysis. The second group (that corresponds to the beta subunit of amarantin) and the third one were described by Juan *et al.* (2007). The disappearance of the 32 kDa band may be due to native grain enzymes or to microbial flora contamination of the grain.

3.5. Molecular Weights Profile by Exclusion Chromatography

The initial degree of hydrolysis, at time zero, was estimated from molecular weights of soluble proteins in the elution buffer after 180 minutes of extraction. The molecular weights of the extracted proteins reached 2000 kDa. Nevertheless, there are some fractions with molecular weights around 200 kDa. It was considered that such fractions were produced by the action of endogenous enzymes in the amaranth grain (Figure 6).

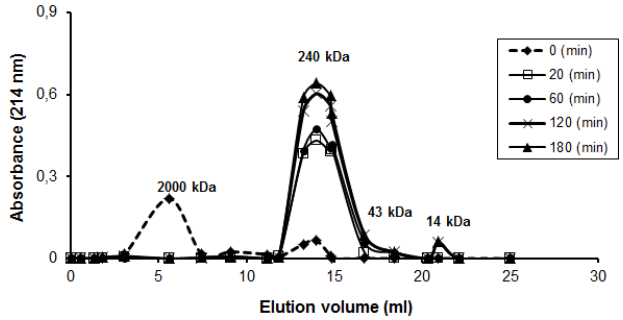


Figure 6. *Molecular weights profiles of proteins as a function of time of hydrolysis.*

A reduction of molecular weights was observed in the proteins of the hydrolysates with regard to the ones that correspond to time zero. The proteins with different molecular weights in the hydrolysates depend on the time of hydrolysis. A significant increase in the amount of proteins with molecular weights between 240 and 200 kDa was observed in a hydrolysis time of 20 minutes. This value continued increasing for hydrolysis times of 60, 120 and 180 minutes. Peptides ranging between 43 – 29 kDa and 14 – 0.5 kDa were found in hydrolysis times of 120 and 180 minutes. No differences were verified between the profiles corresponding to hydrolysis times of 120 and 180 minutes.

3.6. Amino-Acids Profile

The composition of amino-acids in hydrolysates obtained after 180 minutes of hydrolysis is described in Table 2. Amino-acids produced by hydrolysis with Alcalase™ and Flavourzyme™ are not substantially different than the composition of the substrate, and this indicates that there is no amino-acids loss due to the action of the hydrolysis process.

4. Discussion

The controlled hydrolysis of proteins contained in the mixtures of amaranth hyperproteic flour and amaranth semolina can result in several peptide fractions. With the commercial proteases tested, the best effect was obtained when both enzymes were used sequentially; the effect of Alcalase™ enzyme acting independently was in second place. It was verified that controlled hydrolysis using several commercial enzymes allows obtaining hydrolysis products that contain different peptides with molecular weights ranging between 240 – 200; 49 – 43; 43 – 29 and 29 – 0.5 kDa. Hydrolysis processes using Alcalase™ and Flavourzyme™ allow obtaining products with solubility values between 0.75 and 0.9 g of nitrogen/g of total nitrogen for tested pH ranges between 2 and 7. The amino-acids profile obtained with hydrolysis showed a slight modification with regard to the original profile of the substrate.

There was no verification of starch hydrolysis attributable to endogenous enzymes present in the hyperproteic meal and semolina.

The obtained results show that in a first approach it is possible to conduct partial hydrolysis of amaranth proteins to obtain ingredients which can be used in the formulation of foods for special diets.

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