Preparation of Low-Phenylalanine Macro Peptides and Estimation of its Phenylalanine Content by Fluorometric Technique

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Abstract: The aims of the study were to prepare macro peptides low in phenylalanine (Phe) from non-conventional raw materials, and to demonstrate the feasibility of using the fluorometric technique to measure the diminution of their Phe content. Aqueous solution of flours of legumes, and amaranth panicles were used to elaborate the concentrates by using isoelectric precipitation. These protein concentrates, and a whey solution were incubated with proteolytic enzymes to hydrolyze the peptide link at the aromatic amino acids, and then these macro peptides were filtrated through activated charcoal, in order to reduce its phenylalanine concentration. The Phe concentration, of the each prepared macro peptides, was analyzed by using fluorometric technique, and it was later validated by using HPLC. The crude protein contents in the concentrates have varied from 90% in the protein isolate from lentils, 76% in those from the frijol white, and 44% in those from amaranth panicles. Protein concentrates, and whey were hydrolyzed by using the following enzymes: pepsin from the pig gastric mucosa, protease from *Aspergillus oryzae*, and protease type XIV from *Streptomyces griseus*. It was determined that the enzymes with the better hydrolysis capacity, were the proteases from *S. griseus* and *A. oryzae*. The macro peptides with non-linked phe were filtered through activated charcoal. Reductions of Phe of up to 99% in the second and third filtrate were observed and this reduction was corroborated by using HPLC technique. It was also established the higher sensitivity of the fluorometric method to detect Phe, than the HPLC technique.

Keywords: Flours, *L-phenylalanine*, PKU food, dietary supplementation.

INTRODUCTION

In developing countries, foods for people with nutritional special regimes; such as consumers with phenylketonuria (PKU), are usually imported. As a consequence, they are non-traditional, in short supply and expensive; and its availability depends on factors, such as: governmental importing policies, availability of foreign currency, among others. The use of national products in the preparation of these foods would be of great impact because this will decrease the dependence factors, mentioned above. Some of this protein sources are whey and grains (legumes and amaranth). Legumes, especially *Phaseolus* genus are the main source of protein for a large group of the population [1]. Proteins from these grains and whey contain a high level of Phenylalanine (Phe), therefore the elimination of Phe in them is needed, before their use in the production of food for PKU consumers.

This procedure is feasible through combined methodologies [2-4]. They are performed throughout several steps; such as: First, production of the protein concentrate by precipitation, using the isoelectric point technique. Second, the aromatic amino acids reduction; especially Phe, is reached by using enzymes that will break those bounds, linking these amino acids. Protease from *Streptomyces griseus* is a mixture of at least three proteolytic activities including an extracellular serine protease. Protease, from *Aspergillus oryzae*, contains both endo-protease and exo-peptidase activities [5]. Pepsin is an endo-peptidase, which hydrolyzes internal peptide bonds of the protein. Its action is uppermost effective on peptide bonds close to aromatic amino acids (phenylalanine, tyrosine) [6]. Studies have shown that *S. griseus* and *S. oryzae* proteases are the most effective proteases with a much higher protein recovery and degree of hydrolysis than the tested papain [7]. Finally, the reduction of Phe content in the protein hydrolyzed can be done by using filtering treatment with activated charcoal.

The content of Phe in the hydrolized protein is relatively low, so the procedure to detect it has to be of high sensitivity (low detection limit). These methods include enzymatic colorimetric, fluorometric, HPLC, and mass spectrometric [8]. The amino acids automatic HPLC analyzer is the most used [9]. These techniques

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are time consuming, expensive, and require the use of solvents. In order to control the manufacturing process in the food industry, it is necessary a rapid and accurate method to determine the Phe in the food. The fluorescent ultra micro essay UMTEST PKU (SUMA) used in the detection of Phe in blood is based on the reaction of phenylalanine present in the blood sample with ninhydrin [8, 10, 11]. The amount of Phe in the blood is measured by the quantity of released fluorescence. This method could be used in order to measure the presence of the Phe in the liquid hydrolyzed protein, proving the fact of the elimination of Phe in free or low–Phe hydrolizate.

The objectives of the research were the preparation of macro peptides Phe-free from amaranth, lentils, white beans, and whey powders. In order to obtain the macro peptides Phe-free, each one of the proteins were precipitated by using isoelectrical point. This process was followed by an enzymatic hydrolysis, and later by a filtration through activated charcoal column. And finally, the technique of fluorometric was used to verify the elimination of Phe from the filtrate.

MATERIALS AND METHODS

Materials

The raw materials used were: Whey powder (**W**), lentil (*Lens culinaris*), white beans (*Phaseolus vulgaris*) and amaranth panicle flours. Lentil, white beans and whey (dehydrated) were acquired at the local market. The amaranth panicles were gathered from the crops of *Amaranthus dubius,* which are growing at Miranda State, Venezuela.

Enzymes

Pepsin from porcine gastric mucosa (powder, 800-2500 units/mg protein) EC232-629-3, protease from *Aspergillus oryzae* P-6110 Sigma, EC 232-752-2 ≥500 units/g, and protease type XIV bacterial from *Streptomyces griseus* (5.9 units/mg solid) P5147.

Methods

Flours Preparation and Analysis

Flours from beans, lentils and amaranth panicles were prepared. Each materials was individually milled in a hammer mill (Fitz Mill Comminuting Machine, Model D, The Fitzpatrick Company; Inc., Chicago, USA). The milled flours were sieved using a sieve equivalent to 60 *mesh* (0. 5 mm; Tyler standard). Flours were coded (L=lentil beans; B=white beans, and A=amaranth panicles) and packaged in plastic hermetic containers, and later stored at room temperature for further analysis and use.

The moisture and crude protein of flour were determined by using official methods [12].

Elaboration of the Protein Concentrates

To obtain the concentrates, two combined procedures were used [13, 14]. Three batches of 200 g of flour of each material (W, L, B, A) were dissolved in 400 mL of water, and the pH was adjusted to reach a value of 11 using a solution of NaOH (0.1 N) and shaking it for 60 min. The suspension was stored at 4 °C overnight, and centrifuged at 0.6 x g for 10 min. The pH of the supernatant was adjusted until it reached 5.4 by adding solution of HCl (0.1 N). This supernatant was left overnight at 4 °C to precipitate the proteins. An adjustment for the protein precipitation was needed for the amaranth panicle flour; as follows: The pH of the supernatant was set at 4, with HCl 0.1 N, instead of 5.4. Finally, for all of them, the suspension was centrifuged at 0.6 x g for 10 min and the precipitate was neutralized with solution of NaOH (0.1N). Neutralized precipitated solutions were coded (PL=lentil beans; PB=white beans and PA=amaranth panicles)

Enzymatic Reduction of the Phe by Using Two ways

1. Laboratory Assay

The enzymatic reductions of Phe were achieved following combined procedures [3, 15]. 500 mL of each concentrated were heated at 90 °C for 10 min, and then cooled at room temperature. Samples were dropped in a coded piece of absorbent filter paper (S&S 903) to quantify the initial content of Phe in the concentrated. These pieces of paper were left to dry. The pH in the leftover concentrated protein was adjusted to reach 2 by using a 0.1N of HCl solution. 2.5 g of pepsin from porcine gastric mucosa was added to the solution which was later homogenized and incubated at 37°C during 2 h. Samples were taken each hour from this solution and dropped in a coded piece of absorbent filter paper. Each piece of paper was left to dry. The pH of the leftover solution was adjusted to reach 6.5 with 0.1N of NaOH solution and 2.5g of protease from *Aspergillus oryzae* or protease type XIV bacterial from *Streptomyces griseus* was added. The solution was homogenized and incubated at 40°C during 5h, taking sample each hour for Phe

quantification. Finally to inactivate the enzymatic reaction (hydrolysis) was the solution was heated at 90°C. The solution was cooled at room temperature and was filtered three times through an activated charcoal column. Samples were taken on each one of the filtrate. The filtrate was dried by using lyofilization (2mBar; -48 to -50°C; Freeze dry system of Labconco freezone 4.5)

2. Pilot Assay

In this case, the whey powder was treated with both; pepsin from porcine gastric mucosa, and protease from *Aspergillus oryzae* following combined procedures [3, 4]. 25L of a 5% aqueous solution of whey (1,250 g whey/25L distilled water) was heated at 90°C during 10 min, and cooled a 37°C and its pH was adjusted at 2 with 0.1N of HCl solution. 12.5 g of pepsin from porcine gastric mucosa (1:100, enzyme: sustrate) was added to the whey aqueous solution and incubated at 37°C during 2h in a bioreactor BioFlo 4500®, with a peristáltica Easy-Load® II, master flex® L/S® model 77200-50) shaking it at 75 rpm. Samples were taken for Phe analysis as was described as mentioned above. Afterward, the pH was adjusted to reach 6.5 using a solution of 0.1N of NaOH, and 25g of protease from *Aspergillus oryzae* was added. The solution was homogenized and incubated in the bioreactor at 40°C during 5h. Samples were taken each hour for Phe determination. Finally, the enzymatic inactivation (hydrolysis) was reached by heating the solution to 90°C. The solution was cooled at room temperature and 187.5 g of activated charcoal was added and mixed. The mix was filtered three times through a Buchner funnel with filter paper Whatman Nº 1. This filtrate was dried by using a spray dryer atomizer model F-11BAA06, with 170 °C of inlet temperature and outlet 90-100 °C. The powder was packed in polyethylene bag, then placed inside of plastic container and kept refrigerated at 5±1°C for further analysis.

Quantification of Phenylalanine by Fluorometric Test Kit (FTK) and its Validation with Chromatography Techniques

The FTK quantification was achieved following combined procedures [8, 10, 11]. In the assay procedure 3mm in diameter with the dried sample spot discs of calibrators, control and samples were punched out into each well of the elution microplates, and incubated with 70 µL of ethanol 70% (v/v) in a humid chamber for 30 min at room temperature. Ten micro liters of eluate were transferred to white opaque polystyrene ultra-micro plates containing 10 µL of reaction mixture, pH 5.8 containing 20 mmol/L ninhydrin, 8.3 mmol/L L-leucyl-L-alanine, and 31 mmol/lL sodium azide, in 0.2 mol/l disodium succinate buffer solution. Plates were incubated at 60 °C for 1 h in a humid chamber. A fluorescent complex was obtained by adding 10 µL of copper reagent (1.2mmol/L copper sulfate, 75.5mmol/L potassium sodium tartrate, and 31 mmol/L sodium azide) to the reaction ultra-micro plate. 5–15 min later the fluorescence was measured automatically in the fluorometer–photometer reader. Reading values in fluorescence units (FU) were directly transferred to the computer after measurement. The automatic validation and interpretation of the results were done using software developed specially for the UMTEST PKU. Samples with Phe concentrations above the cut-off value were marked and printed as elevated [8].

The Phe levels obtained by FTK were compared with those obtained by TLC with duration of three days [16, 17]. Amino acid profiles of the whey, and amaranth panicle macro peptide were performed by HPLC [18].

RESULTS AND DISCUSSION

Moisture, Dry Material, Crude Protein and Phe Content of Flours, Concentrate and Macro Peptides

Flours contain a relatively high protein content in general; lentil having the highest one and whey the lowest (Table **1**).

Preparation of Protein Concentrate

As can be seen, in Table **1** the crude protein content of the concentrates varied from 44 to 90% with a phenylalanine content of 0.86; 0.84; 0.56 and 3.07 mg/100g dry matter (DM) in PL, PA, PB and PW, respectively. The hydrolysis of the whey was done with 5% of whey aqueous, because the whey powder was a protein concentrate in itself,

As it can also be seen in Table **1**, the Phe content in the filtrate or macro peptide of each sample has been reduce notably to the point of reaching a value of cero in the macro peptide obtained from the legumes.

The assay using the fluorometric kit was more précised and accurate to detect the Phe concentration in food than the HPLC assay. This fluorometric method, which has been reported to be very précised for blood Phe analysis, is also very accurate and sensitive for food research as it has been proved in this study. This

Table 1: Moisture, Dry Material, Crude Protein and Phe Content of Flours, Concentrate and Macro Peptides from Legumes, Amaranth Panicle and Whey

Raw material	Moisture (%)	Dry material (%)	Crude Protein (%)	Phe (g/100g DM) HPLC	Phe (g/100g DM) Fluorometric
Lentil flour	9.5 ± 0.1	90.5±0.1	23.2 ± 0.1	1,4[1]	ND
Lentil concentrate (PL)*	NA	5.0±0.2	90.0	ND	0.86
Lentil macro peptide*	NA	4.4±0.3	78.2±0.5	ND	0.03
Amaranth flour	7.8±0.5	92.2±0.5	18.4±0.5	0.76[1]	ND
Amaranth concentrate (PA)*	NA	5.0±0.0	44.0	ND	0.84
Amaranth macro peptide	4.4±0.3	95.6±0.00	6.5±0.2	0.0	0.00
White bean flour	11.3±0.5	88.7±0.5	15.3±1.2	0.91[1]	ND
White bean concentrate PB)*	NA	5.0±0.0	76.0	ND	0.56
White bean macro peptide	10.8±0.4	89.2±0.4	47.01±1.03	ND	0.00
Whey powder (PW)	5.1±0.16	94.9±0.1	14.5± 0.19	0.32	3.07
Whey macro peptide	5.0±0.00	95.0±0.0	10.9±0.06	0.01	0.42

Values are the means of three determinations (n = 3). Different letters in the same row indicate statistically significant differences. ($p \leq 0.05$). [1]FAO, 1981: ND= Not determined.

method only works for the amino acid Phe because the fluorescence is developed when this amino acid is treated with ninhydrin and a peptide. The other amino acids, which are normally found in plasma and protein hydrolyzates, yield no fluorescence [19]. The reaction of the Phe in the sample with ninhydrin, in optimal conditions of pH and temperature, is forming a little fluorescent complex. Adding copper ions amplifies the fluorescence, increasing its intensity with a previous addition of L-Leucyl-L-alanine to the reaction mixture. The emitted fluorescence intensity is proportional to the concentration of Phe in the mix [8,10].

Lentil Hydrolyzed and Macro Peptide

Figure **1**, shows hydrolysis rates of the three enzymes studied on the PL solution. The Figure **1** also shows that the enzyme pepsin is the one that releases less Phe. By contrast, the enzymes proteases from *A. griseus* or *oryzae,* released much more amino acid Phe. Protease breaks up the proteins by hydrolyzing the peptide links.

Both proteases used to hydrolyze the proteins in all the samples have shown an optimal performance, which is seen by the increase of values close to 5 to

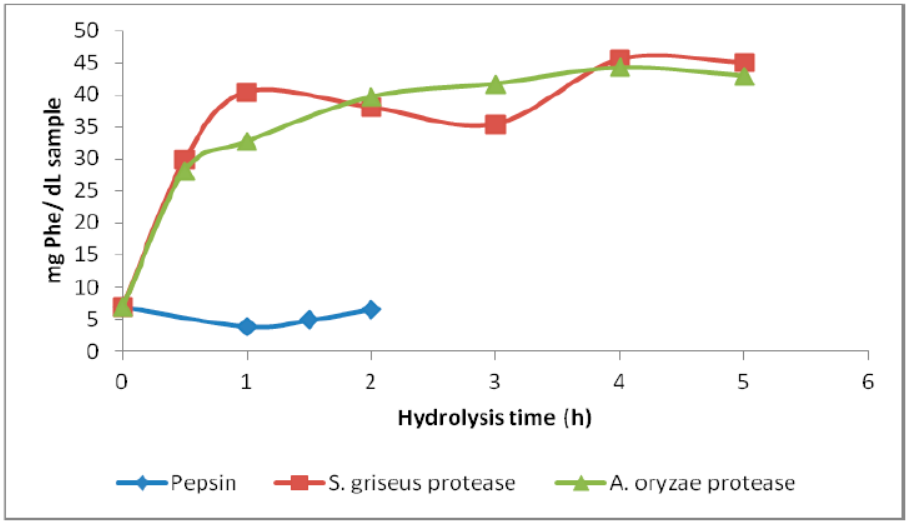

Figure 1: Progress curves for the reaction of enzymes: pepsin, and proteases from *S. griseus* and *A. oryzae* on a 5% aqueous solution of lentil flour.

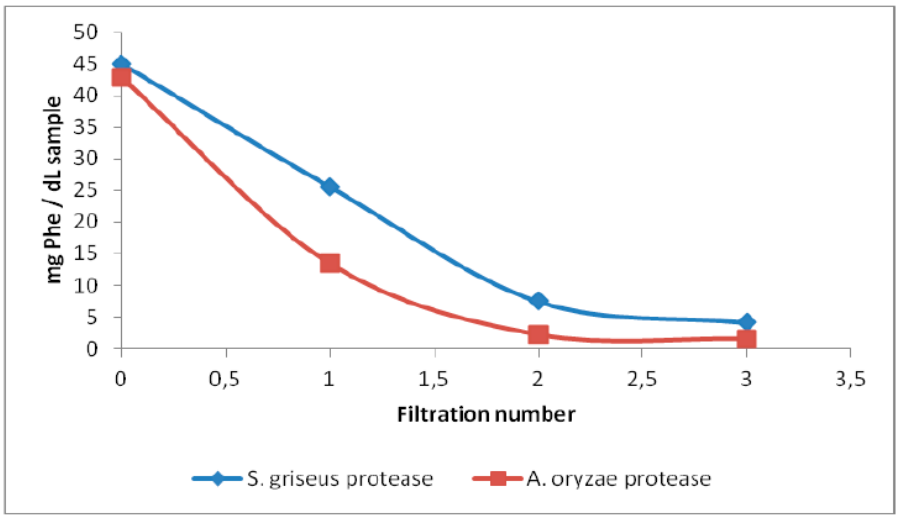

Figure 2: Phe reduction by filtration through activated charcoal column of the lentil macro peptide.

reach 45 mg Phe/dL of Phenylalanine in the solutions. It has been postulated that although all serine-proteases have the same catalytic mechanism of action, their substrate specificity is very different [20]. However, in this study; both enzymes (bacterial and fungus) have shown the same higher hydrolysis behavior.

Figure **2**, shows the effect of the activated charcoal on the reduction of the Phe content in the whey protein concentrate (PL). The Figure **2** also shows that the reduction of Phe is achieved during the second filtration time, resulting in a reduction of approximately 45 to 1.54 mg Phe/dL sample or 0.86 to 0.03 (g/100g DM).

Amaranth Panicle Hydrolyzed and Macro Peptide

When assaying the two enzymes mentioned above, under the same reaction condition of a 5% amaranth panicle flour aqueous solution, the same results reported for the lentil flour were found (Figure **3**). Figure **3** also shows that the pepsin enzyme has low activity when used by itself.

Analysis of the previous results, encourage the use of the protease from *A. oryzae*, for the hydrolysis of white beans and whey flour (5% aqueous solutions), following the procedure outlined above.

Figure **4** is showing the decreasing of the content of Phe, after three times of filtering through activated charcoal. In Figure **5** it can be seen the validation of the fluorometric techniques. In the Figure it is denoted the Phe spot of the CA sample that are not present in the CN and the HP replicates. Data of the HPLC analysis of this sample (Table **2**) is showing that the Phe content is cero. This result is also a validation of the fluorometric results observed in Figure **5**.

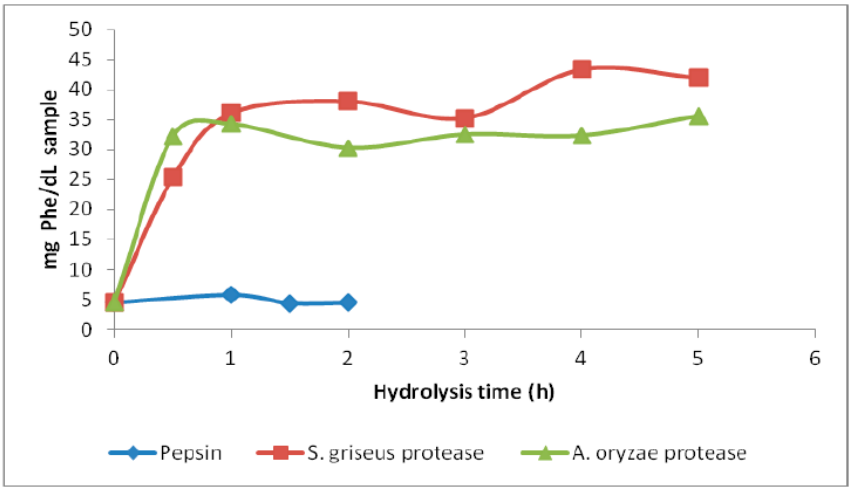

Figure 3: Progress curves for the reaction of enzymes: pepsin, and proteases from *S. griseus* and *A. oryzae* on a 5% aqueous solution of amaranth panicles flour.

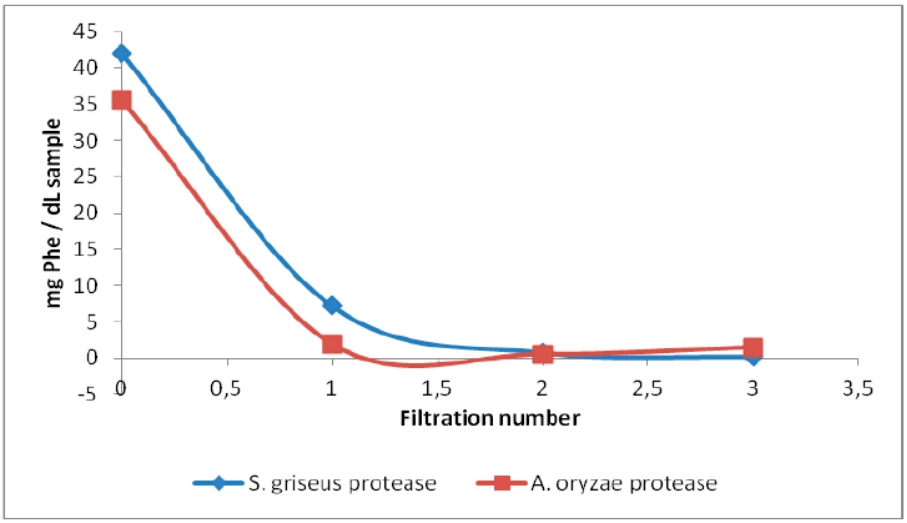

Figure 4: Phe reduction by filtration through activated charcoal column of the amaranth panicles macro peptide.

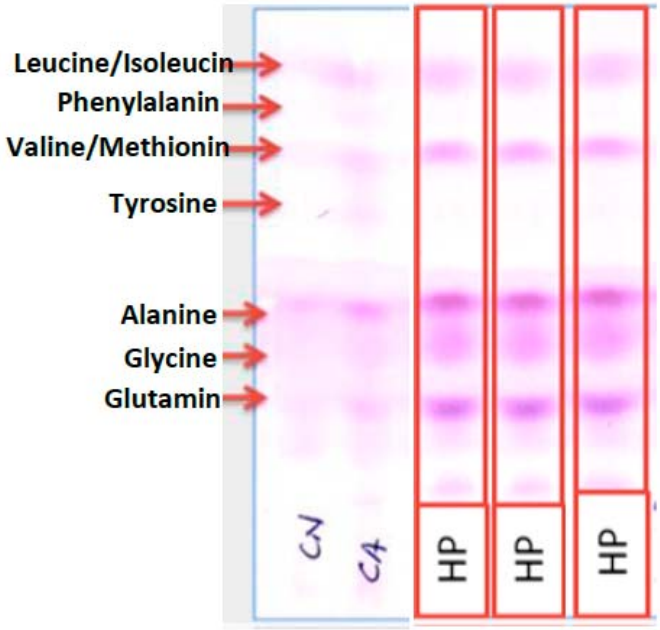

Figure 5: Thin layer chromatography analyses of the amaranth panicles macro peptide (HP) filtrated three times through a charcoal column. CN: Normal blood control; CA: Atypical blood control laboratory prepared with high level of the amino acids.

White Beans Hydrolyzed and Macro Peptide

Figure **6** is showing the combined hydrolytic action of the enzymes pepsin and protease from *Aspergillum oryzae* in the 5% aqueous solution of white bean flour.

The initial amount of Phenylalanine detected was 2.40 mg Phe/ dL sample, showing an increase at the end of the hydrolysis of 28.14 mg Phe/dL of sample or 0.05 to reach 0.56 g Phe/100g db (see Table **1**). Similar results were reported in foodstuff, and milk [3, 15].

Figure **7** is showing the decreasing of the content of Phe, after three times of filtering through activated charcoal. After completing the three filtrates, the percentage of Phenylalanine removed, was calculated using the formula [21]:

$$\%\ Phe\ removed = \frac{\text{Initial } Phe \text{ content - final } Phe \text{ content}}{Phe \text{ initial content}} = 98.94$$

By using this formula, a high value of the amino acid reduction was obtained with this methodology. Soarez *et al.* (2006) [15] got a lower Phe content (92%) from casein hydrolysates.

Table 2: Amino Acids Profile (g/100g of Dry Basis) of Amaranth Panicles and Whey Macro Peptides Filtrated Three Times Through a Charcoal Column

Aminoacids	Amaranth panicles	Whey
Asparagine (Asn)	2.63	0.72
Gluthamine (Gln)	3.16	1.16
Serine (Ser)	1.45	0.77
Threonine (Thr)	0.87	0.70
Glycinae(Gly)	1.48	0.17
Alanine (Ala)	0.00	0.00
Arginine (Arg)	3.55	1.80
Proline (Pro)	0.62	0.37
Valine (Val)*	0.66	0.40
Methyonine (Met)	0.00	0.25
Isoleucine (Ile)	0.00	0.39
Leucine (Leu)	0.71	0.66
Triptófano (Trp)	0.00	0.00
Phenylalanina (Phe)	0.00	0.01
Cysteine (Cys)	1.82	0.57
Lysine (Lys)	0.83	0.51
Histidine (His)	4.54	0.18
Tyrosine (Tyr)	0.15	0.11

White bean hydrolyzed and macro peptide.

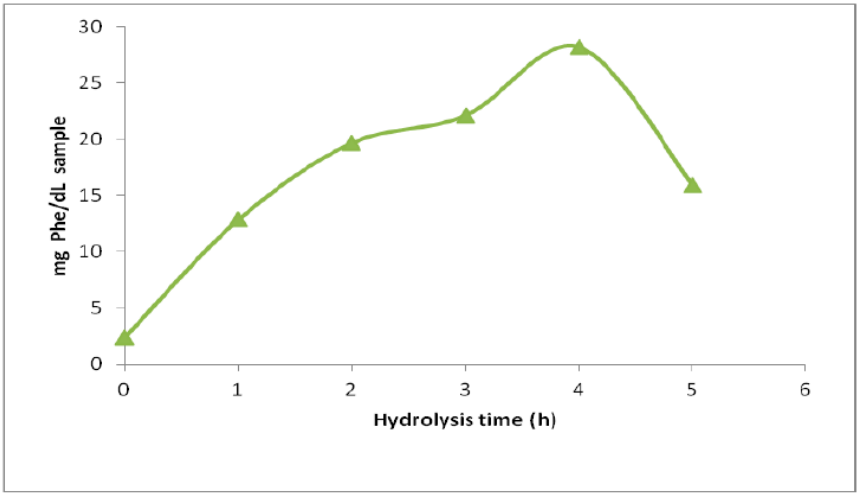

Figure 6: Progress curves for the reaction of proteases from *A. oryzae* on a 5% aqueous solution of white beans flour.

On the other hand, Moszczynski and Idziac (1993) [22] were able to remove 95% of Phe of casein hydrolysates even though they used more severe conditions: hydrolysis during 72h and further treatment with activated carbon by 5.5 h.

Whey Hydrolyzed and Macro Peptide

Figure **8**, is showing the combined hydrolytic action of the enzymes pepsin and protease from *Aspergillum oryzae,* in the 5% whey powder aqueous solution. As it was mentioned before this procedure was assayed at a pilot level. As can be seen, the enzyme reaction tendency was similar to previous products. However, the whey powder showed to have more Phe at the initial stage. The Figure **9** also shows similar tendencies to previous assays. The whey macro peptide was also analyzed by HPLC (Table **2**) and it was found a reduction from 0.36 g/100g DM in the dry whey to 0.01 g/100g DM in the macro peptide.

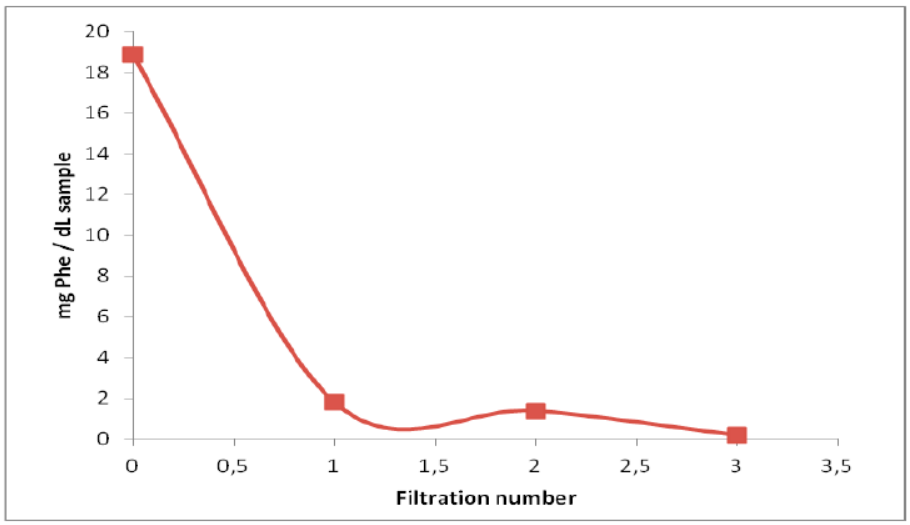

Figure 7: Phe reduction by filtration through activated charcoal column of the white bean macro peptide.

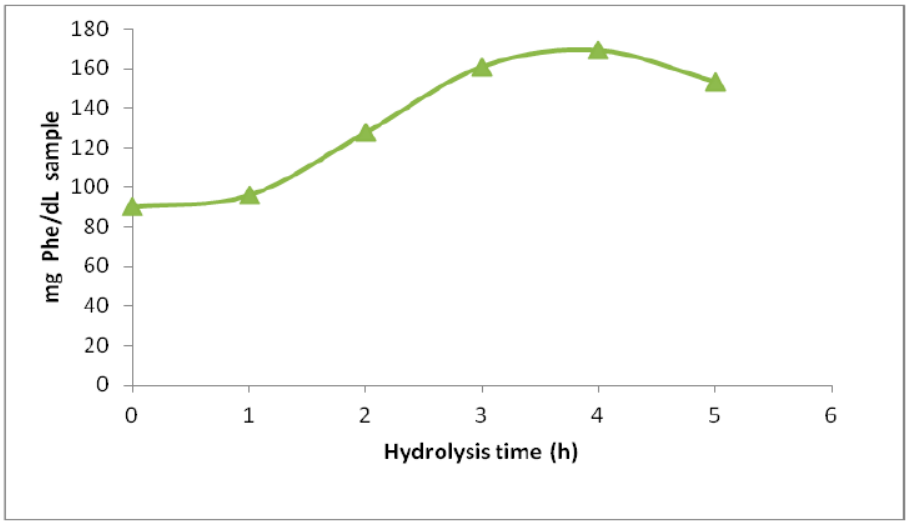

Figure 8: Progress curves for the reaction of proteases from *A. oryzae* on a 5% aqueous solution of whey flour.

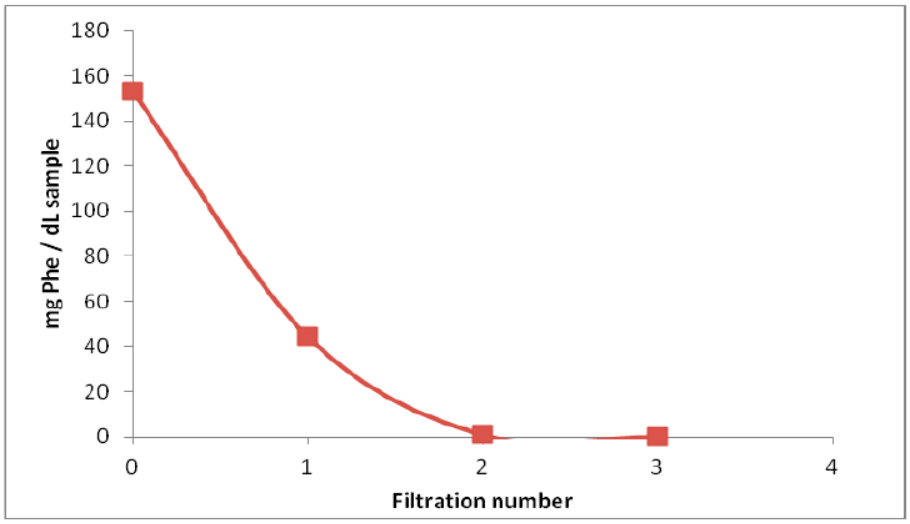

Figure 9: Phe reduction by filtration through activated charcoal column of the whey macro peptide.

CONCLUSION

There is a great feasibility for the high-efficiency protein concentrate production using precipitation with isoelectric point. In addition, it was determined that the enzymes from *Aspergillum oryzae,* and *Streptomyces griseus* have the highest hydrolysis effect in the 5% solution of each concentrate. It was also demonstrated, as well, the effective use of activated charcoal packed in a column, in order to reduce almost a 99% the Phenylalanine. And finally, it can be concluded that the more sensitive determination of Phe was by using the fluorometry method, as compared with HPLC technique.

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