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Removal of Phenylalanine from Protein Hydrolysates Prepared with Rice

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Abstract: Aiming the preparation of low-phenylalanine protein hydrolysates from rice, 2 pancreatins were used for the hydrolytic process and the activated carbon was used as adsorbent. Second derivative spectrophotometry was used to evaluate the efficiency of Phenylalanine (Phe) removal. Also, the peptide profiles of the protein hydrolysates were analysed. Initially, rice proteins were extracted by an alkaline process, varying the pH (9.0, 10.0 or 12.0), the stirring time (15 or 60 min) and the velocity of centrifugation (106, 425 or 2,660×g). Then, 12 protein hydrolysates were prepared using 2 different Enzyme: Substrate (E:S) ratios (1 and 2%) and Rice Protein Extract (REP) concentrations (1-3 g/100 mL). The protein content of extracts changed from 19.63-77.92% and the highest extraction yield (84.74%) was obtained at pH 12.0, after stirring for 60 min and centrifuging at 425×g. The Phe removal was similar for both pancreatins, varying from 72-100% and giving final Phe contents from 0.28-95 mg/100 g of product. For pancreatin 1, the best results were obtained with REP concentration of 3% and E:S of 2%, while for pancreatin 2 REP concentrations as well as E:S of 1 and 2% produced the smallest Phe content. The peptide profile obtained with pancreatin 2 was more advantageous than pancreatin 1, since it produced much smaller large peptide and little higher oligopeptide contents.

Key words: Protein hydrolysates, rice, pancreatin, activated carbon, phenylalanine

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important foodstuffs for people in many countries, including Brazil, where it is consumed daily. The production of rice in Brazil was of 11.5 million tons in 2006/2007 (IBGE, 2006). Although, the protein content of rice is the smallest among the cereals, the nutritional value of its proteins is greater (around 7.3%) than other grains, due to their higher digestibility and lysine content, which is the limitant aminoacid of cereals (Juliano, 1993).

Phenylketonuria (PKU) is a metabolic disease associated with the metabolism disorder of Phenylalanine (Phe), in which the oxidation of this amino acid is impaired due to the deficiency of the hydroxylase phenylalanine enzyme (Moszczynski and Idziak, 1993). Untreated patients show serious mental retardation and their expectation of life is drastically reduced (Lopez-Bajonero *et al*., 1991; Shimamura *et al*., 1999; Mira and Marquez, 2000). The nutritional therapy for PKU is based on limitation of protein ingestion, reducing Phe supply to the minimum and promoting the normal growth of patients with other nutrients (Mahan and Stump, 1998; Dutra-De-Oliveira, 1998).

Cereals are among foodstuffs whose ingestion by phenylketonurics must be controlled (Aguiar, 2002). This explain the necessity of removing Phe from rice aiming its free use in the diet of these patients, especially in Brazil, where it is one of the main component of the daily diet.

Most of the methods used for Phe removal from proteins are based on the principle that a sufficient amount of Phe is exposed or liberated by enzymatic hydrolysis and then, removed by gel filtration, adsorption by activated carbon or resins (Lopez-Bajonero *et al*., 1991; Outinen *et al*., 1996). Our group has been removing Phe from milk and whey proteins, using activated carbon or a resin as adsorbents (Lopes *et al*., 2005; Delvivo *et al*., 2006; Soares *et al*., 2006). In case of cereals, proteins must be previously extracted. Also, different enzymes have been used in our laboratory for hydrolyzing varied protein

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sources (Morato *et al*., 2000; Carreira *et al*., 2004; Lopes *et al*., 2005; Soares *et al*., 2007). However, in this research the 2 pancreatins were tested for the first time.

In order to evaluate the efficiency of Phe removal, its amount must be determined either in the protein source or in their hydrolysates, after having used an appropriate adsorption method. Several techniques to quantify Phe among other amino acids are available in the literature, including the ion-exchange chromatography using the amino acid analyzer (Kan and Shipe, 1981) reverse high performance liquid chromatography (Badoud and Pratz, 1984; Bidlingmeyer *et al*., 1984; Carisano, 1985; Vendrell and Avéles, 1986; Alaiz *et al*., 1992) hydrophylic interaction chromatography (Alpert, 1990) and Spectrophotometric methods of Second order (SDS) (Brandts and Kaplan, 1973; Matsushima *et al*., 1975; O'Haver, 1979; Silvestre *et al*., 1993; Rojas *et al*., 1998).

The reliability of SDS for quantifying Phe in proteins has been reported by some authors, especially between 250 and 270 nm, since parameters such as pH and the addition of other elements are controlled (Brandts and Kaplan, 1973; Ichikawa and Terada, 1979; O'Haver, 1979; Cahill and Padera, 1980; Grant and Bhattacharyya, 1985; Rojas *et al*., 1998).

Our group has been testing the SDS for several purposes. Thus, we used successfully this technique for determining the hydrolysis degree of protein hydrolysates (Silvestre *et al*., 1993) for evaluating the encapsulation rate of protein hydrolysates (Barbosa *et al*., 2004) as well as for estimating the Phe removal of hydrolysates from milk and whey proteins (Lopes *et al*., 2005; Soares *et al*., 2006) whey (Delvivo *et al*., 2006) and corn flour (Capobiango *et al*., 2006).

Over the past years, some authors have shown that preparations rich in oligopeptides, especially di and tripeptides, from partially hydrolysed proteins could be utilized more efficiently and have a higher nutritive value than the intact protein and an equivalent mixture of free aminoacids (Hara *et al*., 1984; Keohane *et al*., 1985; Grimble *et al*., 1986; González-Tello *et al*., 1994; Boza *et al*., 2000). Thus, the nutritional value of protein hydrolysates depends, among other factors, on the peptide chain length and therefore, it is important to characterize the peptide profiles of these preparations.

For characterizing the peptide profiles of protein hydrolysates, we developed a technique that showed to be efficient for fractionating peptides according to their size, especially those bellow 1000 Da (Silvestre *et al*., 1994b). Since then, we have been using this method for analysing several protein hydrolysates prepared in our laboratory (Morato *et al*., 2000; Barbosa *et al*., 2004; Carreira *et al*., 2004; Lopes *et al*., 2005; Barbosa *et al*., 2004; Soares *et al*., 2007).

The aim of the present research was to remove Phe from rice proteins, using 2 pancreatins for the hydrolytic process and the activated carbon as adsorbent. Also, the peptide profiles of the protein hydrolysates were analysed.

MATERIALS AND METHODS

L-pheylalanine, L-tyrosine, L-tryptophan, one of the pancreatin used (p-1500, from porcine pancreas, Activity = 11,7 U mL^{-1}, where, one unit of activity is defined as the amount of enzyme that liberates 1 μg of tyrosine in 1 min at 37°C) were purchased from Sigma (St. Louis, MO, USA) and will be called in the present work pancreatin 1. The other pancreatin (Corolase PP, from porcine pancreas, Activity = 18,9 U mL^{-1}, where, one unit of activity is defined as the amount of enzyme that liberates 1 μg of tyrosine in one min at 37°C) was kindly furnished by AB Enzymes of Brazil (Barueri, SP, Brazil) and will be called in the present work pancreatin 2. Activated carbon (granulated, 119, 20-50 meshes) was purchased form Carbomafra S.A. (Curitiba, PR, Brazil). Polished rice grain, type 1, was purchased in the market of our city (Belo Horizonte, MG, Brazil).

The HPLC system consisted of one pump (HP 1100 Series), an UV-VIS detector, coupled to a computer (HPchemstation HP1100, Germany). A poly (2-hydroxy-ethylaspartamide)-silica (PHEA) column, 250×9.4 mm, 5 23 μm, 200 Å pore size (PolylC, Columbia, MD), was used for HPLC. For HPLC, water was purified by passage through a Milli-Q water purification system (Aries-Vaponics, EUA). All solvents used for the HPLC were carefully degassed by sonication for 10 min before use.

The spectrophotometer used was CECIL, CE2041 model, Buck Scientific, England with a software GRAMS-UV (Galactic Industries Corporation, Salem, NH, EUA).

Determination of the chemical composition of the rice: Initially, the rice grains were ground in a mill (Marconi TE 020, n. 870348, Piracicaba, SP, Brazil). The contents of moisture, protein, lipid and total ash were determined according to the Association of Official Agricultural Chemists methods (AOAC, 1995). The conversion factor of nitrogen to protein was 5.95 (Juliano, 1993). The carbohydrate content was calculated by difference.

Extraction of rice proteins: The method of Connor *et al*. (1976), based on the alkaline extraction was used for extracting proteins from rice. Thus, 10 g of ground rice was mixed with 5 vol of water, the pH was adjusted to 9,

10 or 12, with dilute sodium hydroxide (3.0 mol L^{-1}). The mixture was stirred for 15 or 60 min. The protein concentrate was separated from the residue by centrifugation twice at 106, 425 or 2,660×g for 10 min. Between each centrifugation, the residue was washed with water. Both fractions (the residue and the supernatant, which was called Rice Protein Extract-RPE) were neutralized by adding diluted hydrochloric acid and dried by lyophilization. The supernatant (protein extract) was used for preparing protein hydrolysates. The parameters used for protein extraction are shown in Table 1.

The protein content of the RPE was determined by Kjeldhal method (AOAC, 1995) and the Extraction Yield (EY) was calculated using the Eq. 1.

$$EY\ (\%) = \frac{\text{RPE protien}\,(\%) \times \text{RPE mass}}{\text{Rice protien}\,(\%) \times \text{Rice mass}} \qquad (1)$$

Where:

RPE protein (%) = Protein content of RPE, in g/100 g.
RPE mass (g) = Amount of RPE obtained after lyophilization, in g.
Rice protein (%) = Protein content of rice grain, in g/100 g.
Rice mass (g) = Amount of rice grain used in the protein extraction, in g.

Preparation of rice hydrolysates: Twelve hydrolysates were prepared from RPE solutions (concentrations from 1-3 g/100 mL), using the 2 pancreatins, according to the procedure used before by our goup (Delvivo *et al*., 2006). The RPE used was the one that produced the highest extraction yield. First, a quantity of sodium benzoate was added to obtain a final concentration of 0.1 g/100 mL. Then, the pH was adjusted to 9.0 with 3.0 mol L^{-1} NaOH solution and remained constant until the end of the hydrolytic process. The solutions were heated in a water-bath at 25°C and the enzymes were added in such a concentration to attain the desired enzyme:substrate ratios (1 and 2%). After 5 h of hydrolysis, the reactions were stopped by the reduction of pH to 3.0 by adding phosphoric acid and the samples were, then, freeze-dried (Labconco, Freezone model, n. 77500, Kansas City, MI, USA). The other parameters of hydrolysis are listed in Table 2.

Removal of phenylalanine from rice hydrolysates: The removal of Phe from protein hydrolysates using activated carbon was described before by our group (Soares *et al*., 2006). Briefly, the activated carbon was previously hydrated for 10 min and placed inside a disposable syringe of 20 mL containing a filter of nylon and wool glass, manufactured in our laboratory. Then, 10mL of a hydrolysate solution (80 mg/100 mL) was added to the column and the eluate was collected and filtered through qualitative research.

Table 1: Parameters and Results of protein extraction from rice

Rice protein extract	pH	Stirring time (min)	Velocity of centrifugation (× g)	Protein content (g 100^{-1}g)	Extraction yield (%)
RPE 1	9.0	15	2660	69.58	61.3^{d}
RPE 2	10.0	15	2660	63.37	58.7^{d}
RPE 3	10.0	60	2660	62.83	58.2^{d}
RPE 4	12.0	15	2660	59.77	65.0^{c}
RPE 5	12.0	60	2660	60.33	78.3^{b}
RPE 6	12.0	60	106	19.63	35.9^{e}
RPE 7	12.0	60	425	77.92	84.7^{a}

RPE = Rice Protein Extract; The values represent the means of triple repetitions. Different letters are significantly different ($p<0.05$)

Table 2: Hydrolytic conditions employed for preparing rice protein hydrolysates

Hydrolysates			
Pancreatin 1	Pancreatin 2	RPE concentration (g 100^{-1} mL)	E:S (%)
H1	H7	1	1
H2	H8	1	2
H3	H9	2	1
H4	H10	2	2
H5	H11	3	1
H6	H12	3	2

E:S = Enzyme:Substrate ratio, RPE = Rice Protein Extract; Pancreatin 1: From Sigma; Pancreatin 2: From AB enzymes

Evaluating the efficiency of Phe removal: For evaluating the efficiency of Phe removal, its content in rice and in its hydrolysates was estimated by Second Derivative Spectrophotometry (SDS), as described before by our group (Lopes *et al*., 2005). Briefly, the samples were hydrolysed (5.7 mol L^{-1} HCl, 110°C, 24 h) and their absorbance measured from 250-280 nm. Second derivative spectra were drawn (CECIL spectrophotometer, CE2041 model, Buck Scientific, England) and the area of a negative peaks were used to calculate the amount of Phe in the samples, employing a standard curve. In case of protein hydrolysates, this same procedure was employed after the treatment with activated carbon. A software GRAMS-UV (Galactic Industries Corporation, Salem, NH, EUA) was used to draw the second derivative spectra.

Then, the efficiency of Phe removal was calculated according to Eq. 2:

$$\text{Phe removal}\,(\%) = \frac{\text{Initial amount of Phe} - \text{Final amount of Phe}}{\text{Initial amount of Phe}} \times 100 \qquad (2)$$

Where:

Initial amount of Phe = Amount of Phe in whey (mol L^{-1}).

Final amount of Phe = Amount of Phe in hydrolysates (mol L^{-1}) treated by activated carbon or by the resin.

Characterization of peptide profiles of rice hydrolysates: This characterization was performed in 2 stages: fractionation of the peptides, according to their size and their quantification. The fractionation of rice hydrolysates was carried out by Size-Exclusion HPLC (SE-HPLC) on a PHEA column, according to the method developed by our group (Silvestre *et al*., 1994a, b), using 0.05 mol L^{-1} formic acid as the mobile phase at a flow rate of 0.5 mL min^{-1}. Twenty microliters of 0.4% hydrolysate solutions were injected on the column. Peptides were detected at 3 wavelengths: 230, 280 and 300 nm. The fractions were separated according to the elution time: F1, from 13.2-18.2 min (large peptides with more than 7 amino acid residues); F2, from 18.2-21.7 min (medium peptides, with 4-7 amino acid residues); F3, from 21.7-22.7 min (di-and tripeptides) and F4, from 22.7-32 min (free amino acids).

The rapid method of Correct Fraction Area (CFA) developed by our group (Silvestre *et al*., 1994a,b) was used for quantifying peptides and free amino acids in SE-HPLC fractions of whey hydrolysates. The samples were fractionated and the CFA values calculated with aid of a standard curve, prepared by using rice as the substrate. Briefly, 5 rice standard hydrolysates (2 using trypsin and 3 using pancreatin) were prepared and then fractionated in 4 fractions by SE-HPLC, as described above. The 4 fractions were collected and submitted to an amino acid analysis. The calculation of CFA was performed using the formulas described by Silvestre *et al*. (1994b). A standard curve was drawn correlating the CFA with the amino acid contents of the fractions. In order to find the amino acid contents of the rice hydrolysates prepared by using the 2 pancreatins, their CFA were taken to this curve.

Statistical analysis: All experiments were replicated 3 times and all measurements were carried out in triplicate. Differences between means of areas were evaluated by Analysis of Variance (ANOVA) and Duncan test (Pimentel-Gomes, 2000). Differences were considered to be significant at $p<0.05$ throughout this study. The least square method was used to fit the standard curve and the adequacy of the linear model ($y = ax+b$) was tested at $p<0.05$. The factorial analysis was used to evaluate peptide and free amino acid contents of chromatographic fractions. The analysis of variance was performed for each condition, in order to investigate the presence of significant effects among treatments ($p<0.05$) and in these cases the Duncan test was applied to establish the differences among the means (Pimentel-Gomes, 2000).

RESULTS AND DISCUSSION

Chemical composition of rice: The results of the analyisis of some components of rice in Table 3 show that the values found for proteins and carbohydrates are close to those of USDA (2006) (United States Department of Agriculture), USP (2005, University of Sao Paulo, Brazil) and of Juliano (1993). The ash content is closer to the value of USDA (2006) and that of lipids is closer to the value of USP. The differences observed, especially for lipid and ash contents, are expected and may be associated to some factors such as climatic and soil conditions, method of analysis, injuries of the grain and the presence of pests.

Efficiency of protein extraction from rice: The data in Table 3 shows that the alkaline method used here was efficient for removing proteins from rice grains. The protein content of extracts changed from 19.63-77.92% and the highest extraction yield (84.74%) was obtained at pH 12.0, after stirring for 60 min and centrifuging at 425×g.

No report was found in the literature concerning the protein content of extracts from rice grain. Some works presented some data from rice bran. Thus, Connor *et al*. (1976) obtained protein concentrates with 23-31% of protein, values near only to that found here for RPE 6. However, the results of Lindsay *et al*. (1977) for the protein concentrates reached some values (19.44- 54.00% of protein) which were closer to those obtained here for other extracts (4 and 5).

Regarding the Extraction Yield (EY), around 65% of proteins were extracted from rice bran, at pH 9.0 with 15 min stirring, according to Lindsay *et al*. (1977) and this result is similar to that of RPE 1 (61.32%), which employed the same conditions. Tang *et al*. (2002) reported EY varying from 56.2 -66.6%, using an enzymatic extraction of proteins from bran rice, values that are in the same range of those obtained here.

The data in Table 1 may also be used to evaluate the effect of different parameters on the protein extraction. Thus, the effect of pH on the protein extraction involves the comparison of 2 groups. First, using 15 min of stirring: RPE 1 with RPE 2 with RPE 4 and second, using

Table 3: Chemical composition of rice

Nutrients	Values found[1]	USDA[2]	USP[3]	JULIANO[4]
	(g 100^{-1} g)			
Moisture g (%)	12.52	11.62	12.33	-
Protein g (%)	8.05	7.13	6.73	6.3-7.1
Lipids g (%)	1.05	0.66	0.89	0.3-0.5
Total ash g (%)	0.65	0.64	0.48	0.3-0.8
Total sugars g (%)	77.73	79.95	79.57	77.0-89

[1]: Values found in the present work, [2]: USDA Nutrient database for standard reference (2006), [3]: USP:Food Compposition Table of University of São Pulo, Brazil (2005), [4]: Juliano (1993)

60 min of stirring: RPE 3 with RPE 5 (Table 1). In the former, no difference was shown between pH 9.0 (61.3%) and 10.0 (58.7%), but the EY at pH 12.0 (65.0%) was greater than pH 10.0 (58.7%). In the second group, also the EY was higher at pH 12.0 (78.3%) than at pH 10.0 (58.2%). These results agree with the studies of Ju *et al.* (2001), showing that 98% of glutelins, the larger protein fraction of rice, are extracted at pH 11.8 and at pH below 10.0 the solubility of this fraction are drastically reduced.

Regarding the stirring time, no effect was observed at pH 10, since no difference was shown between 15 min (RPE 2 = 58.7%) and 60 min (RPE 3 = 58.2%). However, at pH 12 and 60 min (RPE 4 = 78.3%) of stirring gave higher EY than 15 min (RPE 5 = 65.0%).

Also, the velocity of centrifugation affected the EY, as can be seen in Table 3, comparing among them, the values of RPE 5 with RPE 6 and with RPE 7. Thus, the EY was much greater at 425×g (84.7%) than at 106×g (35.9%). However, it was smaller at 2660×g (78.3%) than at 425×g. A possible explanation for this result could be associated to the precipitation of the starch at this velocity which could have carried part of the protein.

Spectra and standard curve of phenylalanine: The absorbance and SDS spectra of Phe, in a mixture of aromatic amino acids and of hydrolysate H5, in pH 6.0, are shown in Fig 1. In case of Phe (Fig. 1B), we can see 4 negative peaks, indicated by letters a, b, c and d, situated within the range of 250-280 nm with maxima at 253, 258, 263, 268 and 273 nm and minima at 257, 262, 267 and 272 nm.

The SDS spectrum of H6 is close to that of Phe, with negative peaks situated in almost the same wavelengths. The likeness among the spectra of standard amino acids and proteins had previously been described by Ichikawa and Terada (1977) working with several native and denaturated proteins. The same result was previously achieved in our laboratory using papain for hydrolysing casein (Barbosa *et al.*, 2004) and skim milk (Soares *et al.*, 2004; Lopes *et al.*, 2005).

Concerning the standard curve of Phe, its linear regression, employing the area of negative peak c, was highly significant ($p<0.01$) and the correlation coefficient and the curve equation were $y = 3.0077x + 0.7587$ and $R^2 = 0.9576$. This result is in agreement with others in the literature (Ichikawa and Terada, 1977; Zhao *et al.*, 1996) and also with previous studies carried out in our laboratory (Barbosa *et al.*, 2004; Soares *et al.*, 2004; Lopes *et al.*, 2005), since in all these works a linearity for the standard curve of Phe, in presence of Tyr and Trp in several concentrations, was shown.

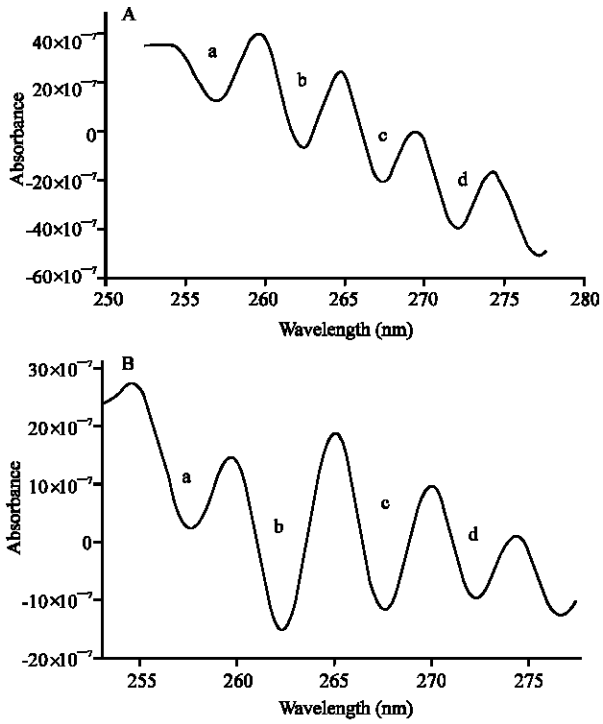

Fig. 1: Second derivative spectra. (A) Phe at pH 6.0 in the presence of Tyr and Trp. (B) Hydrolysate H6 at pH 6.0

Table 4: Phe removal and final Phe contents of rice protein hydrolysatesv

Hydrolysates	Final Phe content* (mg Phe/100g of hydrolysate)	Removal percentage
H1 (Panc 1)	95.00^a	72
H2 (Panc 1)	78.35^b	77
H3 (Panc 1)	63.53^d	82
H4 (Panc 1)	8.12^f	98
H5 (Panc 1)	32.03^e	91
H6 (Panc 1)	0.28^k	100
H7 (Panc 2)	1.57^i	100
H8 (Panc 2)	2.77^h	99
H9 (Panc 2)	5.26^g	98
H10 (Panc 2)	0.39^j	100
H11 (Panc 2)	68.05^c	80
H12 (Panc 2)	68.34^c	80

*: Final Phe content = Phe content after treatment with activated carbon. Panc 1 = pancreatin 1, from Sigma; Panc 2 = pancreatin 2, form AB enzymes. The values represent the means of triple repetitions. Different letters are significantly different ($p<0.05$)

Efficiency of phenylalanine removal: The data in Table 4 show that activated carbon was efficient to remove Phe from rice protein hydrolysates prepared with the two pancreatins.

The Phe content of rice grain was 344.8 mg/100 g. Although the activity of pancreatin 2 is higher than that on of pancreatin 1, no great differences were observed among the results showed for both enzymes, since Phe removal changed from 72% to almost 100% for pancreatin 1 and from 80% to almost 100% for pancreatin 2. For all

hydrolysates, the final Phe content changed from 0.28-95 mg/100 g of product and was inferior to the maximum value established by the Brazilian Legislation for products intended for phenylketonurics, that is 0.1 g/100 g (Brasil, 2002). Although pancreatin 2 has a lower activity, the final Phe content was a little smaller with pancreatin 1 (0.39 and 0.28 mg Phe/100 g, respectively). It is worth stating that the method we used for determining enzyme activity is based on the measurement of tyrosine liberated, under controlled conditions, by the hydrolysis of casein (Çalýk *et al*., 2002) and it seems that there is not a direct correlation between this parameter and the exposure of Phe as well as its further removal by activated carbon.

Thus, all these hydrolysates could be added to the starch (residue of protein extraction) in order to reconstitute the original rice grain and be used in the phnylketonurics diet. This new rice would contain a protein amount close to the original one, with the advantage of having low amount of Phe as well as proteins in the hydrolysed form, which are nutritionally superior to the intact ones found in rice or another food.

Similar level of Phe removal was previously obtained by our group (75-99%), using activated carbon for removing Phe from enzymatic hydrolysates of skim milk (Lopes *et al*., 2005; Soares *et al*., 2004) and of whey (Delvivo *et al*., 2006).

No work was found in the literature concerning the removal of Phe from rice protein hydrolysates. Instead, other protein sources were reported, using activated carbon as adsorbent. Thus, Kitagawa *et al*. (1987), after hydrolysing whey proteins with actinase, in pH 6.5 at 37 °C, treated these preparations with activated carbon and removed 97% of Phe. However, the conditions for the treatment with this adsorbent were not mentioned. Lopez-Bajonero *et al*. (1991) reduced 92% the level of Phe from hydrolysates of skim milk or sodium caseinate obtained by the action of papain and a protease from *Aspergillus orysae*. Using a mixture of 3 enzymes (chymotrypsin, carboxypeptidase A and leucine aminopeptidase). Moszczynski and Idziak (1993) removed 95% of Phe from casein hydrolysates. However, these authors employed more severe conditions than those used by our group, that is, a very long time for hydrolysis (72 h) and for the treatment with activated carbon (5.5 h).

Effect of hydrolytic parameters on the phenylalanine removal: In fact, the action of enzymatic hydrolysis on Phe removal is very complex, as can be observed in Table 4. Thus, for pancreatin 1 the effect of the substrate (RPE) concentration must be evaluated by comparing 2 groups of hydrolysates (Table 2); first (E:S = 1%): H1 (1%), H3 (2%) and H5 (3%); and second (E:S = 2%): H2 (1%), H4 (2%) and H6 (3%). The results in Table 4 show that for both groups, the RPE concentration of 3% was the best one since the higher the concentration the higher was the Phe removal, that is, the lesser was the final Phe content. In case of pancreatin 2, the opposite was observed when the E:S was of 1% and the RPE concentration of 1% gave the smallest final Phe content (comparison among H7, H9 and H11). However, the RPE concentration of 2% produced the best result, when the E:S used was of 2% (comparison among H8, H10 and H12). Therefore, all the three substrate conentrations tested can lead to good results, depending on the other hydrolytic parameters used, especially, type of enzyme and E: S.

The action of another hydrolytic parameter that can be evaluated is the E:S ratio. One can observe in Table 4 that, for pancreatin 1 in all possible cases of comparison (H1 with H2-RPE concentration of 1%; H3 with H4-RPE concentration of 2%; H5 with H6-RPE concentration of 3%) the E:S of 2% produced the smallest final Phe content. However, for pancreatin 2, the results were more diverse. For RPE concentration of 1%, the smallest final Phe content was obtained with E:S of 1% while for RPE concentration of 2%, the smallest final Phe content was obtained with E:S of 2% and for RPE concentration of 3% the E:S had no effect on Phe removal.

No report was found in the literature concerning the effect of hydrolytic parameters on Phe removal. However, our group has been working on this subject, especially related to the effect of E:S ratio. Thus, depending on the substrate and hydrolytic conditions employed the use of a higher E:S was either beneficial (Delvivo *et al*., 2006) or harmful (Lopes *et al*., 2005; Delvivo *et al*., 2006). Also, in some cases no effect of E:S ratio was shown (Delvivo *et al*., 2006; Soares *et al*., 2006).

Although, it is theoretically expected that using higher E:S ratios will produce greater Phe removal due to its more intense release, all these results show that in practice this procedure is much more complex and involves the concurrent effect of some parameters such as temperature, type and concentration of substrate and enzyme.

Peptide profiles of protein hydrolysates: The protein hydrolysates were resolved in four fractions. A typical chromatographic pattern is shown in Fig. 2. The SE-HPLC technique used here showed to be efficient in fractionating protein hydrolysates, especially peptides of molecular mass lower than 1,000 Da, as previously reported by our group (Silvestre *et al*., 1994a; Morato *et al*., 2000; Barbosa *et al*., 2004; Carreira *et al*., 2004; Lopes *et al*., 2005; Soares *et al*., 2007).

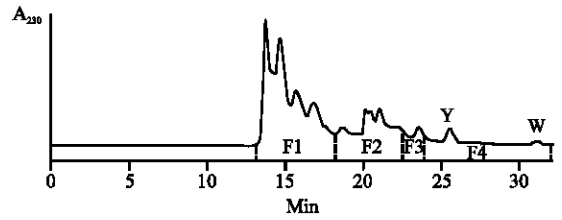

Fig. 2: Chormatographic profile of a rice protein hydrolysate. (pH 1,9, 37°C, 5h) at 230nm. F1: Large peptides (>7 aminoacid residues). F2: Medium peptides (4-7 aminoacid residues); F3: Di and tripeptides; F4: Free amino acids. Y = Tyrosine peak, W = Tryptophan peak

In order to choose the hydrolysate which showed the best peptide profile from the nutritional point of view, the statements of some authors must be considered. According to Frenhani and Burini (1999), during the metabolism of proteins, the first stage of their hydrolysis leads to the formation of oligopeptides containing 2-6 aminoacid residues and free aminoacids. Then, these peptides are broken to di- and tripeptides. Finally, the proteins are absorbed in the form of di- and tripeptides as well as of free aminoacids, where the absorption of the formers is quicker. González-Tello *et al.* (1994) also reported the advantage of the di-and tripetides over the free aminoacids, concerning the velocity of absorption.

For each enzyme, the characterization of peptide profiles was performed for the hydrolysates that showed the smallest final Phe contents. Comparing the 2 hydrolysates prepared with pancreatin 1 (H4 with H6), the data in Table 5 shows that the best peptide profile was obtained by H4, which presented the highest oligopeptide and the lowest free aminoacid contents. Its only disadvantage was its little higher large peptide content. These results also show the advantage of using a RPE concentration of 2% over 3%, for pancreatin 1.

In case of pancreatin 2, the peptide profile of H7 was better than that of H10, since it showed higher oligopetide and lower large peptide and aminoacid contents. These results also show the advantage of using a RPE concentration of 1% over 2% and of E:S of 1% over 2%, for pancreatin 2.

Comparing the best results obtained by both enzymes, H4 for pancreatin 1 and H7 for pancreatin 2, one can conclude that the use of the second enzyme was a little more advantageous from the nutritional point of view, since it produced much smaller large peptide and little higher oligopeptide contents. Its only inconvenient is related to its much higher amount of free aminoacids. Similarly to what was stated above for Phe removal, although pancreatin 2 has a lower activity, its use gave rise to a better peptide profile. Thus, a higher release of tyr by the hydrolysis of casein is not necessarily associated to a better peptide distribution.

Table 5: Peptide and free aminoacid contents of chromatographic fractions of rice protein hydrolysates

Chromatographic fractions	Hydrolysates			
	H4-Panc1	H6-Panc 1	H7-Panc 2	H10-Panc 2
F1	$31.5^{b/1}$	$24.6^{b/2}$	$15.2^{d/4}$	$20.2^{c/3}$
F2	$47.1^{a/1}$	$29.0^{a/4}$	$33.7^{a/3}$	$36.9^{a/2}$
F3	$15.7^{c/32}$	$22.2^{c/2}$	$31.6^{b/1}$	$20.6^{c/2}$
F4	$5.7^{d/4}$	$24.2^{b/1}$	$19.5^{c/3}$	$22.2^{b/2}$
F2+F3	62.8^{b}	51.2^{d}	65.3^{a}	57.5^{c}

The values are in % of nmmols of the four fractions. The values represent the means of triple repetition. Different letters are significantly different ($p<0.05$) for different fractions of the same hydrolysate. Different numbers are significantly different ($p<0.05$) for the same fraction of different hydrolysates

No report was found in the literature concerning the peptide profile of rice protein hydrolysates.

CONCLUSION

The alkaline method used here extracted around 85% of proteins from rice grain, at pH 12.0, after stirring for 60 min and centrifuging at 2000 rpm. Both pancreatins tested were able to produce rice protein hydrolysates containing low Phe contents, since the removal of this aminoacid by activated carbon and the final amount of Phe reached 100% and 0.28 mg/100 g, respectively. The best protein extract concentration and E:S ratio were, respectively, of 3 and 2% for pancreatin 1, while for pancreatin 2 the values of 1 and 2% for both parameters gave the smallest Phe contents. The use of pancreatin 2 produced more advantageous pepetide profiles than pancreatin 1, giving rise to much smaller large peptide and higher oligopeptide contents.

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