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Protein Extraction and Preparation of Protein Hydrolysates from Rice with Low Phenylalanine Content

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Abstract: Aiming the introduction of rice in the phenylketonurics diet, the protein extraction and phenylalanine (Phe) removal processes were studied. For protein extraction, an enzymatic method was used and for Phe removal, a papain and an Activated Carbon (AC) were used. The influence of protein:AC ratio, type and way of using AC was tested. The efficiency of Phe removal was evaluated by second derivative spectrophotometry. The results showed that the condition which gave the highest protein extraction yield (63.4%) was that using a sample concentration of 1:10 (w/v) at a temperature of 50°C, as well as an enzyme:substrate ratio of 10:100 at a reaction time of 5 h. Activated carbon was efficient for removing Phe, leading to values above 70% for most of the samples and the best result (94.1% of Phe removal) was found for a protein:AC ratio of 1:88, using simultaneously three types of AC (20×50, 12×25, 6×12 mesh), which led to a final Phe content of 82.5 mg kg^{-1} of hydrolysate.

Key words: Rice, proteins, enzymes, activated carbon, phenylalanine

INTRODUCTION

Rice is the number one food crop in the world. It is also nutritious and hypoallergenic, which makes rice products desirable food ingredients (Shih and Daigle, 2000). Rice protein is valuable because it has unique hypoallergenic properties and ranks high in nutritive quality (rich in the essential amino acid lysine) among the cereal proteins (Ju *et al*., 2001). Among occidental countries, Brazil occupies the first position in rice production and this cereal plays an important role in the diet of Brazilian people (Barata, 2005).

Phenylketonuria (PKU) is a metabolic disease associated with the metabolism disorder of phenylalanine (Phe) and its nutritional therapy is based on limitation of protein ingestion, reducing Phe supply to the minimum (Wasserstein *et al*., 2006). The introduction of rice in the diet of phenylketonurics is restricting since it is part of food having controlled use for PKU (Aguiar, 2002). Therefore, the development of rice with low Phe content is of great interest.

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As showed before by our group, the first stage in the development of a low-Phe cereal involves enzymatic protein extraction and hydrolysis, followed by Phe removal, using an adsorbent support, such as activated carbon (Capobiango *et al*., 2007).

The evaluation of the efficiency of Phe removal is achieved by quantifying this amino acid in the protein source and in their hydrolysates. Our group has already successfully used the second derivative spectrophotometry for this purpose in different foods (Soares *et al*., 2006; Delvivo *et al*., 2006; Lopes *et al*., 2008).

The present study represents an important step in the process for obtaining low-Phe rice since its goal was the optimization of the enzymatic protein extraction and hydrolysis, using varied reaction conditions. Also, the optimization of Phe removal was studied, using activated carbon as adsorbent.

MATERIALS AND METHODS

A commercial rice flour was kindly furnished by Ferla-L. Ferenczi (São Paulo, SP, Brasil). A protease from *B. licheniformis* (EC 3.4.21.14) was kindly furnished by Prozyn (São Paulo, SP, Brazil). A papain (EC 3.4.22.2) was kindly furnished by AB Enzymes of Brazil (Barueri, SP, Brazil). L-pheylalanine, L-tyrosine and L-tryptophan were purchased from Sigma (St. Louis, MO, USA). Activated carbon (granulated, n. 119, 20×50, 12×25, 6×12 mesh) was purchased from Carbomafra S.A (Curitiba, PR, Brazil).

This research project was conducted from January to December, 2008, in the Laboratório de Bromatologia/Pesquisa da Faculdade de Farmácia-Universidade Federal de Minas Gerais.

Determination of the Chemical Composition of Rice Flour

The contents of moisture, protein, lipid, minerals and dietary fiber were determined according to the Association of Official Agricultural Chemists methods (AOAC, 1995). The carbohydrates were calculated by difference. The conversion factor of nitrogen to protein was 5.95 (Nielsen, 1998).

Enzymatic Extraction of Proteins

Initially, the sample was mixed with water in three different concentrations (1:3, 1:5 and 1:10, w/v) and a quantity of sodium benzoate was added to obtain a final concentration of 0.1 g 100 mL^{-1}. After the adjustment of pH to 10.5 with a 3 mol L^{-1} sodium hydroxide solution, the mixture was set on an oil bath at 50°C, under stirring. The protease from *Bacillus licheniformis* was added at two E:S ratios (5:100 and 10:100) and the protein extraction was performed for 5 h. Then, the mixture was centrifuged at 1,700 x g for 15 min, at 25°C and between each centrifugation, the residue was washed with water (Capobiango *et al*., 2007). Finally, the residue was separated from the supernatant, freeze-dried (Freezone freeze-dryer, 77500 model, Labconco, Kansas City, USA) and its protein content was determined (AOAC, 1995).

The Extraction Yield (EY) was calculated using the Eq. 1.

$$EY = \frac{\left[(A\times B)-(C\times D)\right]}{(A\times B)}\times 100 \quad (1)$$

Where:

A = Protein content of rice flour

B = Amount of rice flour used for protein extraction

C = Weight of residue obtained in the protein extraction
D = Protein content of residue

Preparation of Protein Hydrolysate

The enzymatic hydrolysates were prepared from Rice Protein Extract (RPE) solution using papain. The pH was measured (7.4), the solution was set on an oil bath at 60°C, under stirring and the enzyme was added in such a concentration to attain an enzyme:substrate ratio (E:S) of 4:100. After 5 h of hydrolysis, the reaction was stopped by heating in a water-bath at 80°C for 20 min (Soares *et al*., 2006). Finally, the hydrolysate was freeze-dried.

Removal of Phenylalanine from Protein Hydrolysate

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, the hydrolysate was passed through the syringe under pression (compressor Diapump, Fanem, 089-A model, number BE11778, São Paulo, SP, Brazil).

Evaluation of the Efficiency of Phe Removal

For evaluating the efficiency of Phe removal, its content in rice flour and in its hydrolysate 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 to 280 nm. Second derivative spectra were drawn (Cecil spectrophotometer, CE2041 model, Buck Scientific, England) and the area of a negative peak was used to calculate the amount of Phe in the samples, employing a standard curve. In case of protein hydrolysate, this same procedure was employed after the treatment with activated carbon. A software GRAMS-UV (Galactic Industries Corporation, Salem, NH, USA) was used to draw the second derivative spectra. For the standard curve, stock solutions of Phe (6.05×10^{-4} mol L^{-1}), Tyr (5.52×10^{-4} mol L^{-1}) and Trp (4.90×10^{-4} mol L^{-1}) were prepared in the same buffer solutions cited above. Then, 10 mL of each solution were mixed and successive dilutions of this mixture were made to have Phe concentrations in a range from 0.13 to 1.01×10^{-4} mol L^{-1}. Spectra of these diluted solutions were recorded from 250 to 280 nm the area of third negative peak of Phe spectra were plotted in function of its concentration.

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

$$\text{Pheremovel}\,(\%) = \frac{\left[A-(B\times C/D)\right]}{A}\times 100 \qquad (2)$$

Where:
A = Phe content of rice flour
B = Phe content of protein hydrolysate, after AC treatment
C = Protein content of rice flour
D = Protein content of protein hydrolysate

Evaluation of the Effect of Some Parameters on Phe Removal

As shown in Table 1, the following parameters and values were tested: protein:AC ratio (1:88, 1:44 and 1:22) and type as well as way of using AC employing the same quantity of three different meshes (type A, n° 119, 20×50 mesh; type B, 12×25 mesh; type C, 6×12 mesh),

Table 1: Conditions employed for preparing rice protein hydrolysates and for removing phenylalanine

	Enzymatic hydrolysis		Phe removal	
Hydrolysates	Protease	E:S	Protein:AC Ratio	AC type
01	Papain	4:100	1:88	A
02	Papain	4:100	1:88	B
03	Papain	4:100	1:88	C
04	Papain	4:100	1:88	A e B
05	Papain	4:100	1:88	A e C
06	Papain	4:100	1:88	B e C
07	Papain	4:100	1:88	A, B e C
08	Papain	4:100	1:44	A, B e C
09	Papain	4:100	1:22	A, B e

E:S: Enzyme:substrate ratio; AC: Activated carbon; AC types: Type A: 20×50 mesh, Type B: 12×25 mesh, Type C: 6×12 mesh

in the same column, for the protein:AC ratio of 1:88. The way of using AC refers to the use of the three types of AC isolated (A, B or C) or in two kinds of associations. First, using simultaneously two of them, that is, A and B; A and C; B and C; second, using the three types together (A, B and C). For the column containing more than one AC type, the C one was placed on the top followed by B type and the A type in the bottom. In all cases the total amount of AC was of 2 g.

Statistical Analysis

All experiments were replicated three times and all measurements were carried out in triplicate. 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 analysis of variance was performed 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 (Gomes, 2000).

RESULTS AND DISCUSSION

Chemical Composition of Rice Flour

The results of the analysis of some components of rice flour are shown in Table 2. In general, the values found here are close to those of the furnisher and the literature. Some differences among the data may be associated to some factors such as the analytical method used, the climatic and soil conditions, the injuries of the grain and the presence of pests. Moreover, the protein content of grains may be influenced by the germination degree (Gonçalves *et al*., 2003).

Efficiency of Protein Extraction

As shown in Table 3, the Sample Concentration (SC) and the E:S ratio affected the Extraction Yield (EY) and the highest value (63.4%) was obtained for the extract (number 6) which employed a SC of 1:10 and an E:S ratio of 10:100.

Generally, the incomplete extraction of proteins from foodstuffs is probably due to the interference of the matrix, since proteins may interact with other sample constituents, such as lipids and carbohydrates, reducing its solubility (Fischer *et al*., 2001). Also, according to Agboola *et al*. (2005), in case of cereals the starch is the main contaminant of protein extract, reducing the extraction yield.

The results obtained here are into the range of most of the works found in the literature for rice or its by-products. Thus, Euber *et al*. (1991) used several proteases for extracting

Table 2: Chemical composition of rice flour

References	Moisture	Protein	Lipids	Total Ash	Carbohydrates
	----------	----------	(g kg^{-1})	----------	----------
Values found[1]	9.27	6.61[3]	0.82	0.42	82.88
Ferla[2]	9.60	6.96[4]	0.90	0.60	81.94
Hagenimana *et al.* (2006)	12.40	6.71[4]	0.41	0.39	80.10
Murthy *et al.* (2007)	11.00	7.50[4]	0.80	0.70	80.00
Ilo *et al.* (1999)	-	8.13[5]	0.77	0.47	-
USDA (1989)	11.89	6.05[6]	1.42	0.61	80.13
USP (2005)	11.60	6.90[4]	1.10	0.70	79.70

[1]Values found in the present work. [2]Values from raw matter label. [3]Conversion factor from nitrogem to protein = 5.95. [4]No mention about the conversion factor from nitrogem to protein. [5]Conversion factor from nitrogem to protein = 6.25. [6]Conversion factor from nitrogem to protein = 5.85

Table 3: Effect of sample concentration and of the enzyme:substrate ratio on protein extraction yield

Protein extracts	Sample concentration (w/v)	E:S ratio	Extraction yield (%)
1	1:3	5:100	43.7[f]
2	1:3	10:100	47.9[e]
3	1:5	5:100	53.6[d]
4	1:5	10:100	60.3[b]
5	1:10	5:100	57.8[c]
6	1:10	10:100	63.4[a]

E:S (Enzyme:substrate ratio). The values represent the means of triple repetition. Different letters are significantly different ($p<0.05$)

proteins from rice and reported an yield changing from 17.7 to 75%. The extraction of proteins from rice bran, by the action of varied enzymes (amylases and some proteases), after a physical treatment of the sample (ultra-sound, ultra-turrax, high pressure), produce lower yields changing from 56.2 to 66.6% (Tang *et al.*, 2002). Using a protease from *Aspergillus* sp. associated with different carbohydrases, it was possible to extract from 25.94 to 57.89% of proteins from rice bran (Ansharullah and Chesterman, 1997).

Our group tested the same enzyme used here for extracting proteins from a commercial corn flour and the EY for this cereal was higher than that of rice, since the values changed from 71.5 to 86.8% (Capobiango *et al.*, 2007). An explanation for these results may be associated to the fact that rice proteins show disulfide and noncovalent bonds inside the polypeptide chains and between these chains and other compounds such as lipids and carbohydrates, which reduce the protein solubility (Fang *et al.*, 1992).

The way SC and E:S ratio affected protein extraction may be also evaluated in Table 3. Thus, comparing the three extracts using an E:S of 5:100, 1 (SC = 1:3) with 3 (SC = 1:5) and with 5 (SC = 1:10), as well as the other three sing an E:S of 10:100, 2 (SC = 1:3) with 4 (SC = 1:5) and with 6 (SC = 1:10), we can observe that the higher the dilution of the sample the greater the EY, since the values changed from 43.7 to 53.6 and to 57.8% for the first case and from 47.9 to 60.3 and to 63.4% for the second, respectively. This result could be explained by the higher mobility of the enzyme in a more diluted medium, making easier its contact with protein molecules. No work was found in the literature concerning the effect of the SC on the protein extraction of rice or other cereal.

The comparison of the results obtained for the extracts 1 with 2 (SC of 1:3), 3 with 4 (SC of 1:5) and 5 with 6 (SC of 1:10) shows the advantage of an E:S of 10:100 for all cases, since the results for this E:S value were higher than those using an E:S of 5:100, that is, the EY values changed from 43.7 to 47.9%, 53.6 to 60.3% and from 57.8 to 63.4%, respectively. In fact, the use of higher amount of enzyme is expected to promote stronger protein hydrolysis and, consequently, higher extraction yield.

Euber *et al.* (1991) also described the same effect since using a pancreatin in a concentration of 2% for extracting proteins from rice gave rise to an EY of 75.5% compared to 71.2% obtained with an enzyme concentration of 1%.

Efficiency of Phenylalanine Removal

The standard curve presented a good correlation coefficient, as shown in Fig. 1. The data in Table 4 show that activated carbon was efficient to remove Phe from rice protein hydrolysates prepared with pancreatin. For all hydrolysates, the removal changed from 25.7 to 94.1% and the final Phe content from 82.5 to 1,038.4 mg kg^{-1} of product. The Phe content of rice flour was 283.1 mg kg^{-1}. In case of some hydrolysates where the Phe removal was low, these results could be probably associated to fact that the hydrolytic conditions were not sufficient to cleave some bonds between proteins and other components, such as starch, reducing its solubility and therefore hindering some sites in their molecules for the enzyme attack and exposition of Phe.

It is worth stating that a certain amount of Phe in the product is desirable, since this amino acid is an essential one and its presence in the diet is important for the normal growing process of children (Hendriksz and Walter, 2004; Lara *et al*., 2005).

No work was found in the literature concerning the removal of Phe from rice flour proteins and the only study with another cereal was that one of our group. Thus, we obtained Phe removal from 68.63 to 97.55% using a corn flour as protein source (Capobiango *et al*., 2007).

Some studies of our laboratory and of other researchers applied this process for milk proteins. Thus, our group had previously used the AC for removing Phe from skim milk and whey and obtained values ranging from 93.6 to 99.0%, from 75 to 99%, respectively. Among

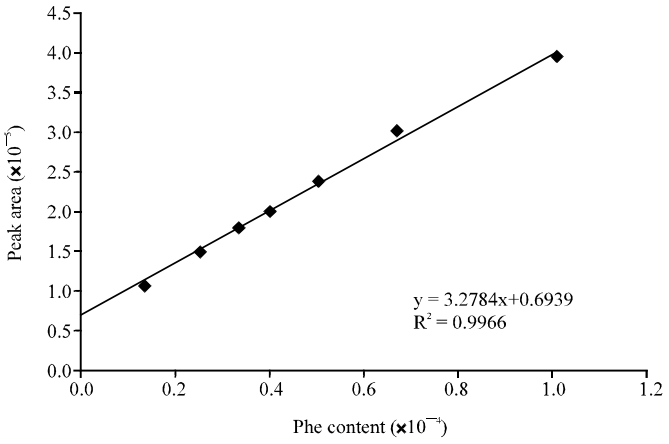

Fig. 1: Standard curve of Phe

Table 4: Phenylalanine removal and final Phenylalanine contents of protein hydrolysates

Hydrolysates	Phe removal (%)	Final Phe content (mg Phe kg^{-1} of hydrolysate)
01	82.6^{c}	243.2^{g}
02	73.0^{f}	377.3^{d}
03	25.7^{i}	1,038.4^{a}
04	86.1^{b}	194.3^{h}
05	76.8^{e}	324.2^{e}
06	63.8^{g}	505.9^{c}
07	94.1^{a}	82.5^{i}
08	78.4^{d}	301.9^{f}
09	44.0^{h}	782.6^{b}

Final Phe content: Phe content after treatment with activated carbon. The values represent the means of triple repetition. Different letters are significantly different ($p<0.05$) for different hydrolysates

the other authors, Kitagawa *et al.* (1987), after hydrolyzing whey proteins with actinase, at 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. Bajonero *et al.* (1991) reduced92% 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 three enzymes (chymotrypsin, carboxypeptidase A and leucine aminopeptidase), Moszczynski and Idziac (1993) removed 95% of Phe from casein hydrolysates. However, these researchers employed more severe conditions than those used by our group, that is, a very long time for hydrolysis (72 h). Three of the hydrolysates (1, 4 and 7) prepared in the present study showed Phe removal percentages that are close to those cited above, while two of them (3 and 9) were as low as those reported by Cogan *et al.* (1981) since, they obtained only 36% of Phe removal after the hydrolysis of casein using a protease from *Aspergillus niger*.

Effect of Some Parameters on Phenylalanine Removal

The parameters studied here are protein: AC ratio as well as type and way of using AC. For evaluating the effect of the protein:AC ratio one must compare the hydrolysates 7 (protein:AC = 1:88), 8 (protein:AC = 1:44) and 9 (protein:AC = 1:22) in the Table 4. As expected, the use of the lowest ratio (1:88-the highest amount of AC) produced the highest Phe removal (94.1%), followed by 1:44 (78.4%) and 1:22 (44.0%).

Considering that no study from other researchers was found in the literature concerning the effect of protein:carbon ratio on Phe, the results obtained here were compared with those previously described by our group. Thus, some skim milk hydrolysates were prepared by the action of three proteolitic enzymes (papain, pepsin and a protease from *Aspergillus oryzae*) and, contrarily to the present work, no significant differences were observed for the values obtained using three protein:AC ratios (1:60, 1:90 and 1:118). However, the average of Phe removal was of 97%, which was close to the result obtained here for hydrolysate 7 (Soares *et al.*, 2006). In another study, a pancreatin was used for preparing corn flour hydrolysates and the same effect obtained here for protein:AC ratio was observed, that is, the lowest value (1:88.5) led to the highest Phe removal (84%), followed by 1:16 (62.4%) and 1:8 (54.1%) (Capobiango *et al.*, 2007).

The effect of AC type can be evaluated by comparing the results obtained for the hydrolysates 1, 2 and 3 (Table 4), which showed that the higher the meshes the higher Phe removal, since the values were 82.6% for type A, 73.0% for type B and 25.7% for type C. This result could be explained by the fact that smaller particle size provides a larger surface and a longer time of contact between AC and the sample. No study was found in the literature concerning the study of the effect of this parameter on Phe removal.

The data in Table 4 allows evaluating also the effect of way of using AC. One can observe that for the association of two types of AC (hydrolysates 4, 5 and 6), the use of type A with B (hydrolysate 4) was the most advantageous leading to the highest Phe removal (86.1%, hydrolysate 4), followed by type A with C (76.8%, hydrolysate 5) and, finally, type B with C (63.8%, hydrolysate 6). The results also show that the association of three types of AC (94.1%, hydrolysate 7) was the most efficient way of using AC, since it produced higher Phe removal than the association of two types (86.1%, hydrolysate 4) as well as than the isolated use of one type of AC (82.6%, type A, hydrolysate 1) which was the most disadvantageous procedure. In a previous study of our group, the same was observed since the use of the association of these three types of AC led to the highest Phe removal from protein hydrolysates of corn flour (Capobiango *et al.*, 2007). However, contrarily to the

present study, no significant difference was observed between the use of type A alone or in association with type B.

CONCLUSION

The enzymatic method used here (protease from *Bacillus licheniformis,* sample concentration of 1:10, 50°C, E:S ratio of 10:100, reaction time of 5 h) was efficient for extracting proteins from rice flour leading to an yield of 63.4%. The highest level of Phe removal (94.1%) was obtained using the association of three types of AC (20×50, 12×25, 6×12 mesh) and a protein:AC ratio of 1:88.

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