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Effect of Enzyme Type, Mode of Enzyme Action and Temperature on the Obtention of Low Phenylalanine Hydrolysates from Wheat Flour

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Abstract: With the aim of obtaining wheat flour with low phenylalanine (Phe) content, protein extracts were prepared using an enzymatic method with a protease from *Bacillus licheniformis*. Then, the protein extracts were hydrolyzed by the action of one commercial enzyme (pancreatin or Panc) and a crude enzymatic extract obtained from pineapple peel (CE). The effect of some parameters was evaluated, such as enzyme type, mode (isolated or successive association), order of enzyme action and temperature (30, 35, 40, 50 and 70°C). The Activated Carbon (AC) was used as adsorbent and the efficiency of Phe removal was evaluated by second derivative spectrophotometry, measuring Phe content in wheat flour and in its hydrolysates after AC treatment. The results showed that the use of CE followed by Panc at 50°C was the most advantageous condition, leading to a Phe removal of 66.3% and a final Phe content of 522.4 mg kg^{-1} of hydrolysates.

Key words: Wheat flour, protein hydrolysates, association of enzymes, phenylalanine, removal

INTRODUCTION

Taking in consideration three points i.e., 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 wheat in the diet of phenylketonurics is restrict since it is part of food having controlled use for these patients (Kanufre *et al*., 2001); this cereal plays an important role in the diet of Brazilian people; one can conclude that the development of wheat flour with low Phe content is of great interest.

The first stage in the development of low-Phe wheat flour is the protein extraction followed by enzymatic hydrolysis to expose or release Phe residues. Then, an adsorbent support such as activated carbon is used for removing these residues. Our group has already employed all these steps for obtaining low-Phe corn (Capobiango *et al*., 2007) and rice (Lopes *et al*., 2008).

Aiming at the reduction of process costs, since commercial proteases must be imported and therefore show high price, we prepared in the laboratory a crude enzymatic extract (CE) from an industrial waste (pineapple peel) and used it isolated and in association with a commercial pancreatin (Panc).

In order to evaluate the efficiency of Phe removal, its amount must be determined either in the protein source or in its hydrolysates, after having used an appropriate adsorption method. Our group has already successfully used the second derivative spectrophotometry for this purpose in different foods (Delvivo *et al*., 2006; Soares *et al*., 2006; Lopes *et al*., 2008).

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Having as final goal the preparation of wheat flour with low-Phe content, this work involved the study of protein hydrolysis using these two enzymes (CE and Panc), evaluating the effect of mode (isolated and successive association), order of enzyme action as well as temperature.

MATERIALS AND METHODS

This study was conducted of August, 2007-2008.

Materials

A commercial wheat flour was purchased in the market of our city (Belo Horizonte, MG, Brazil). A protease from *B. licheniformis* (Protemax® 580 L) was purchased from Prozyn (São Paulo, SP, Brazil). A pancreatin from porcine pancreas (Corolase PP) was purchased from AB Enzymes of Brazil (Barueri, SP, Brazil). L-phenylalanine, L-tyrosine and L-tryptophan were purchased from Sigma (St. Louis, MO, USA). Activated carbon (granulated, n. 119, 20×50 mesh, 12×25 mesh, 6×12 mesh) was purchased from Carbomafra SA (Curitiba, PR, Brazil). The crude enzymatic extract was prepared in the laboratory from pineapple peel *(Ananas comosus)*. The spectrophotometer used was CECIL, CE2041 model, Buck Scientific, England with a software GRAMS-UV (Galactic Industries Corporation, Salem, NH, USA). The freeze dryer was from Labconco (77500 model, Kansas City, MI, USA) and the stirrer from Fisatom (São Paulo, SP, Brazil). The cutter with maximum capacity of 3 kg was from Sire (model Super cutter, São Paulo, SP, Brazil). The vacuum pump was from Fanem (089-A model, number BE11778, São Paulo, SP, Brazil).

Methods

Determination of the Chemical Composition of Wheat Flour

The contents of moisture, protein, lipid, minerals and dietary fiber were determined according to the Association of Official Agricultural Chemists methods (Brasil, 2005). The carbohydrates were calculated by difference. The conversion factor of nitrogen to protein was 5.70 (Nielsen, 1998).

Enzymatic Extraction of Proteins

Initially, the sample was mixed with water (1:3 w/v) and the pH was adjusted to 9.5 with a 3 mol L^{-1} NaOH solution. Then, the mixture was set on an oil bath at 40°C, under stirring. The protease from *Bacillus licheniformis* was added at an enzyme:substrate (E:S) ratio of 10:100 and the protein extraction was performed for 2 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. Finally, the residue was separated from the supernatant, dried in an oven at 65°C and its protein content was determined.

Preparation and Characterization of Enzymatic Extract from Pineapple Peel

Initially, the pineapple samples were washed and their peel removed, processed in a cutter and filtrated through a gauze. Then, they were centrifuged at 6,000×g for 15 min, at 4°C and a volume of 10 mL of a solution containing 4.0×10^{-3} mol L^{-1} of EDTA and 10^{-2} mol L^{-1} of cystein was added to the supernatant. Finally, this pineapple crude enzymatic extract (CE) was frozen.

The characterization of this extract included the determination of optimum values for pH, temperature and time, which was performed according to the methods described by Dias *et al*. (2008), with some modifications related to the substract (use of hemoglobin instead of casein) and buffer solutions (buffer phosphate instead of tris(hydroxymethyl)aminomethane -TRIS).

Preparation of Protein Hydrolysates

Eight enzymatic hydrolysates were prepared from Wheat Flour Protein Extract (WFPE) solution varying some parameters, such as type of enzyme (Panc or CE), mode of enzyme action (isolated or

Table 1: Hydrolytic conditions employed for preparing protein hydrolysates

Hydrolysates	Proteases	Mode of action	Temperature (°C)
H1	CE	Isolated	70
H2	CE	Isolated	70
H3	Panc	Isolated	50
H4	CE+Panc	Successive association	70/50
H5	Panc+CE	Successive association	50/70
H6	CE+Panc	Successive association	35
H7	CE+Panc	Successive association	40
H8	CE+Panc	Successive association	50
H9	CE+Panc	Successive association	70

CE: Crude enzymatic extract obtained from pineapple peel. Panc: Pancreatin

in association), order of enzyme action (CE+Panc, Panc+CE) and temperature (30, 35, 40, 50 and 70°C) (Table 1).

Initially, the pH of 50 mL of the protein extract was adjusted to the desirable value with a 3 mol L^{-1} NaOH solution or with a 0.1 mol L^{-1} HCl solution. In the isolated action of Panc, its optimum pH 7.0 was tested, while two values were used for CE, i.e., its optimum pH 6.0 as well as the optimum of commercial bromelin (pH 8.0). In case of the successive association of these two enzymes, only the optima values for each enzyme were tested.

After the adjustment of pH, the WFPE was set on an oil bath under stirring, varying the temperature values of 35, 40, 50 and 70°C. Then, CE was added in such a concentration to attain an E:S ratio of 10:100 and let act for 1 h 30 min followed by the Panc that was added at an E:S ratio of 4:100, for 3 h 30 min. After 5 h of hydrolysis, the reaction was stopped by heating in a water-bath at 90°C for 20 min and the hydrolysates were freeze-dried. Their protein and phenylalanine contents were determined by micro-Kjeldahl (Brasil, 2005) and second derivative spectrophotometry (Lopes *et al*., 2008), respectively.

Removal of Phenylalanine from Protein Hydrolysates

The removal of Phe from protein hydrolysates using Activated Carbon (AC) was described before by our group (Soares *et al*., 2006). Briefly, 2 g of activated carbon were previously hydrated for 10 min and placed inside a disposable syringe of 20 mL containing a filter of nylon and wool glass, manufactured in this laboratory. Then, a volume of the hydrolysate was passed through the syringe under pression (compressor Diapump, Fanem, 089-A model, number BE11778, São Paulo, SP, Brazil) in order to obtain a protein:AC ratio of 1:88.5.

Evaluation of the Efficiency of Phe Removal

For evaluating the efficiency of Phe removal, its content in wheat flour and in protein hydrolysates was estimated by Second Derivative Spectrophotometry (SDS), as described before by our group (Lopes *et al*., 2008). 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, CE 2041 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.

The efficiency of Phe removal was calculated according to Eq. 1:

$$\text{Phe removal (\%)} = \frac{\left[A - (BxC/D)\right]}{A} \times 100 \quad (1)$$

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

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 (Pimentel-Gomes, 2000).

RESULTS AND DISCUSSION

Chemical Composition of Wheat Flour

The results of the analysis of some components of wheat 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).

Hydrolytic Parameters

The optima values found for pH, temperature and time, corresponding to the maxima values of specific activity of pineapple crude enzymatic extract were 6.0, 70°C and 1 h 30 min, respectively. For the commercial pancreatin, the data of the furnisher were pH 7.0, temperature of 50°C and 5 h.

Efficiency of Phenylalanine Removal

The data in Table 3 show that Phe removal changed from 37.4 to 66.3% and the final Phe content from 522.4 to 973.4 mg kg^{-1} of hydrolysate. The Phe content in wheat flour was 354 mg kg^{-1}.

Although the level of Phe removal was not so high, for the reconstitution of wheat flour to be introduced in the phenylketonurics diet, the hydrolysate with the lowest Phe content (hydrolysate 8) should be added to the residue (starch) separated at the moment of the protein extraction, in such amount to have a final Phe content bellow 0.1 mg kg^{-1} which is the limit allowed by Brazilian Legislation for containing this kind of patient (Capobiango *et al*., 2007).

Table 2: Chemical composition of wheat flour

Nutrients	Values found[1]	TACO[2]	FAO[3]	Label[4]
Moisture	13.68	13.0	9.4	-
Protein	8.57	9.8	10.6	10
Lipid	1.29	1.4	1.8	1
Minerals	0.75	0.8	1.1	-
Dietary fiber	2.40	2.3	2.1	2.0
Carbohydrates	86.96	75.0	7.2	76

[1]Values found in the present study, in g kg^{-1} of product. [2]TACO (2006). [3]FAO (2008). [4]Label of the commercial wheat flour used in the present study

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 study was found in the literature concerning Phe removal from wheat proteins, but our group was able to remove almost all this amino acid from proteins of other cereals. Thus, we obtained Phe removal varying from 85 to 100%, from 68.6 to 97.6% and from 25.7 to 94.1%, using rice grains (Lopes *et al*., 2008), corn flour (Capobiango *et al*., 2007) and rice flour (Vieira *et al*., 2008), respectively, as proteins sources.

Effect of Some Hydrolytic Parameters

Type of Enzyme and Isolated Enzymatic Action

These parameters can be evaluated by comparing the hydrolysates prepared with CE (H1 and H2) and the one with Panc (H3), separately.

As shown in Table 3 between the two hydrolysates obtained with CE, the one prepared at pH 8.0 (H2) produced higher Phe removal (41.9%) than the one at pH 6.0 (H1-37.4%), although the last value corresponds to CE's optimum pH of action.

On the other hand, there was no significant difference between the results obtained with CE at pH 8.0 (H2) and with Panc (H3), leading to values of 41.9 and 42.5% of Phe removal and final Phe contents of 904.2 and 893.9 mg kg^{-1} of hydrolysate, respectively. Therefore, the use of CE at pH 8.0, associated with Panc was chosen to study the effect of the other hydrolytic parameters on Phe removal.

No study was found in the literature concerning the effect of the type of enzyme on Phe removal of wheat flour. Therefore, the results here were compared with those of other protein sources using different enzymes from the present study. In most of these studies, the removal of Phe was superior to the one achieved in the present study.

In earlier studies obtained by our group, Soares *et al*. (2006) also evaluated the effect of the type of enzyme on Phe removal by AC, from protein hydrolysates of milk powder. Thus, when using papain (BIOBRÁS, Montes Claros, Brazil) and pepsin (Sigma-Aldrich St.Louis, U.S.A.), no significant differences were found in the percentage of Phe removal which were 97.6 and 97.1%, respectively. However, when working with commercial rice flour protein hydrolysates, Vieira *et al*. (2008) observed that the use of papain produced a higher percentage of Phe removal (94.1%) compared with the *B. stearothermophilus* protease (66.1%).

Another researcher achieved a Phe removal of only 36%, using the enzyme Rhozyme 62 for the enzymatic casein hydrolysates preparation, a lower percentage than the one obtained with CE in this study (average of 39.64%) (Cogan *et al*., 1981).

Table 3: Phe removal and final Phe contents of protein hydrolysates

Hydrolysates	Phe removal (%)	Final Phe content (mg kg^{-1} of hydrolysate)
H1	37.4^{e}	973.5^{a}
H2	41.9^{d}	904.2^{b}
H3	42.5^{d}	893.9^{b}
H4	66.4^{a}	562.0^{d}
H5	49.9^{c}	779.0^{c}
H6	61.5^{b}	598.2^{f}
H7	57.5^{c}	661.0^{e}
H8	66.3^{a}	522.4^{g}
H9	56.9^{c}	$669.7^{d,e}$

Final Phe content: Phenylalanine 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

Successive Association

The data obtained for successive action of CE and Panc, can be used to evaluate the order of enzyme addition. Thus, comparing the results obtained with hydrolysates 4 (CE+Panc) with 5 (Panc+CE), it can be observed in Table 3 that the action of CE before Panc (H4) was more advantageous than in case where this order was inverted (H5), since a higher Phe removal was achieved (66.41%) giving rise to a lower Phe content (562.0 mg kg^{-1} of hydrolysate).

No data concerning the effect of the successive combination of enzymes on the Phe removal from wheat flour or other cereals was found in the literature.

The results obtained here may be compared with the ones previously described by our group, using milk powder and different enzymes than those used here.

Thus, Lopes *et al*. (2007) used the successive association of an *Aspergillus oryzae* protease with a papain, at E:S ratio of 1:100 and 2:100, during 1 h and 4 h, respectively, in the milk powder preparation of protein hydrolysates. In this study, the percentage of Phe removal obtained was 99%. Soares *et al*. (2006) compared the effect of two successive associations, pepsin/*Aspergillus oryzae* protease and papain/*Aspergillus oryzae* protease and observed a higher efficiency (98% removal) in the second combination compared with 94% achieved in the first one. For both studies, the final Phe contents of the products were much higher than those obtained here using the successive association CE and pancreatin.

Isolated×Association Action

The effect of these two types of enzymatic treatment can be evaluated by comparing the hydrolysates that showed the highest Phe removal in isolated (H2) and in association actions (H4). The results in Table 3 show the advantage of the second treatment over the first one, giving rise to 66.4% and 41.9% of Phe removal, respectively.

Arai *et al*. (1986), using the Pronase E isolately and in three successive associations (one with papain, another with quimiotripsin and one third with pepsin) in the whey protein hydrolysates preparation, reported percentages Phe removal of 99, 98.8, 98.9 and 99.2%, respectively, being superior to the results gotten in the present study. However, this study has not found significant differences in the final Phe content of the hydrolysates attained by pronase isolated action and the ones with pronase in successive association with other enzymes.

Temperature

One can evaluate the effect of the temperature on Phe removal by comparing the results obtained for hydrolysates 6, 7, 8 and 9 (35, 40, 50 and 70°C, respectively). It can be observed in Table 3 that the temperature affected the Phe removal and the highest value (66.3%) was reached at 50°C, followed by 35°C (61.6%). The lowest values were obtained with the temperatures of 40°C (57.5%) and 70°C (57%), with no significant difference between them.

The highest Phe removal achieved using 50°C could be explained, at least in part, by the fact that this value corresponds to the optimum temperature for Panc action. On the other hand, the same was not observed at 70°C, although this is the CE's optima temperature. An explanation for this result may be associated to the fact that even though the use of higher temperatures increases the yield of the enzymatic reactions, it can also lead to enzyme inactivation, impairing the hydrolysis process (Fennema, 1996).

In a earlier study of four group, Lopes *et al*. (2007), working with milk hydrolysates, reported higher Phe removal (98%) at the temperature of 50°C than at 25°C (94%), as in the present study. This result was explained by the greatest Phe exposition achieved by a stronger hydrolysis degree at 50°C.

CONCLUSION

The isolated use of a crude enzymatic extract obtained from pineapple peel (CE) and a commercial pancreatin (Panc) produced low Phe removal (41.9 and 42.5%, respectively). However, the successive association of these enzymes, using CE followed by Panc, both at 50°C, was able to remove up to 66.4% of this amino acid, which corresponds to a content of 522.4 mg kg^{-1} of hydrolysate.

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