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Study of Brazilian Beans: Protein Extraction and Hydrolysis Followed by Phenylalanine Removal

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ABSTRACT

Beans occupy an important place in the diet of Brazilian people but their use by phenylketonurics is forbidden. In this way, this study aimed at reducing Phenylalanine (Phe) content from protein beans using activated carbon as Phe adsorbent. The effect of some parameters was evaluated, such as initial pH, enzyme: substrate ratio, temperature, type of protease and protein: activated carbon ratio. The efficiency of Phe removal was assessed by second derivative spectrophotometry. The results showed that the percentage of Phe removal varied from 25.4 to 87.9% and the final Phe content from 433.7 to 2679.8 mg Phe 100 g^{-1} hydrolysate. The action of a protease from *Papaya carica* gave rise to the highest percentage of Phe removal (81.5%) and the best values for enzyme: substrate ratio, protein:activated carbon ratio and temperature were 10:100, 1:88 and 50°C, respectively. The pH of 8.4 was chosen as the best one, since no adjustment was needed for initiating the hydrolytic reaction. The conditions used in this work were appropriate for obtaining low-Phe beans.

Key words: Phenylalanine, pH, enzyme: substrate ratio, temperature, protein: AC ratio

INTRODUCTION

Beans are a good source of proteins (20 to 40%) and occupies an important place in the diet of Brazilian people (Tharanathan and Mahadevamma, 2003; De Almeida Costa *et al*., 2006). However, considering that the phenylalanine (Phe) content of food proteins is around 5% and that the Brazilian legislation establishes a maximum limit of 100 mg Phe 100 g^{-1} product in the diet of phenylketonurics (Brazil, 2010), a reduction of Phe content of beans is required to be incorporated in the diet of these patients.

In fact, phenylketonuria is a metabolic disease associated with the metabolism disorder of phenylalanine and its nutritional therapy is based on limitation of protein ingestion, reducing Phe supply to the minimum and promoting the normal growth of patients with other nutrients (Malloy-Diniz *et al*., 2004; Hamman *et al*., 2005; Monteiro and Candido, 2006; Wasserstein *et al*., 2006). Untreated patients show serious mental retardation and their expectation of life is drastically reduced (Bajonero *et al*., 1991; Shimamura *et al*., 1999; Mira and Marquez, 2000).

The method for removing Phe from leguminous plants involves initially two enzyme assisted processes, i.e., protein extraction and hydrolysis. In fact, the first stage in the development of low-Phe legumes is the protein extraction and some studies already used an enzymatic method for extracting proteins from corn (Capobiango *et al*., 2007), wheat flour (Carreira *et al*., 2008) and rice flour (Silvestre *et al*., 2009a). Then, proteins are hydrolyzed in order to expose Phe residues and our group has been employing different proteases and reaction conditions for hydrolyzing proteins from varied sources (Silvestre *et al*., 2009a; Silva *et al*., 2010; Souza *et al*., 2010).

Finally, Phe removal is achieved using adsorbent supports. Among them we have already tested a resin (Delvivo *et al*., 2006) and the Activated Carbon (AC) (Lopes *et al*., 2008; Silva *et al*., 2007). In this study, we used a protease from *Bacillus licheniformis* for extracting proteins from beans, six proteases from different sources for the hydrolytic stage and the activated carbon to remove Phe.

The evaluation of the efficiency of Phe removal is achieved by quantifying this amino acid in the protein source and in their hydrolysates after AC treatment. In present study, we used the second derivative spectrophotometry for this purpose, as we have already done in previous studies with different foods (Lopes *et al*., 2007; Soares *et al*., 2007; Carreira *et al*., 2008).

Present study represents therefore, an important step for obtaining low-Phe beans, where the effect of some parameters, such as initial pH, enzyme: substrate ratio, temperature, type of protease and protein:AC ratio was evaluated.

MATERIALS AND METHODS

This study was conducted in March 2008 until December 2010 in University of Minas Gerais (UFMG, Belo Horizonte, Brazil).

Materials: The Brazilian beans (*Phaseolus vulgaris*) were purchased at a market of Belo Horizonte (Minas Gerais, Brazil). Three packages of the same lot of carioca beans type 1 were used (Pink, Belo Horizonte, MG, Brazil). The proteases from *Bacillus licheniformis* (Protemax® 580 L) and from *Bacillus subtilis* (Protemax® N200) were kindly furnished by Prozyn (Sao Paulo, SP, Brazil). Another protease from *Bacillus subtilis,* (Corolase® 7089), one from *Aspergillus sojae* (Corolase® LAP), one from *Bacillus stearothermophilus* (Corolase® TS) and one from vegetal origin (*Papaya carica*-Corolase® L10) were kindly furnished by AB Enzymes of Brazil (Barueri, SP, Brazil). Activated carbon (granulated, n. 119, 20×50 mesh, 12×25 mesh, 6×12 mesh) was purchased from Carbomafra S.A. (Curitiba, PR, Brazil). L-phenylalanine, L-tyrosine and L-tryptophan were purchased from Sigma (St. Louis, MO, USA).

Methods

Determination of the chemical composition of beans: Initially, the beans were ground in a mill (Marconi TE 020, 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 (Greenfield and Southgate, 1992). The carbohydrate content was calculated by difference.

Enzymatic extraction of proteins: In this step, the method used was the one previously described by our group for extracting proteins from rice flour (Silvestre *et al*., 2009a).

Table 1: Hydrolytic conditions employed for preparing protein hydrolysates and for removing phenylalanine

	Enzymatic hydrolysis				Phe removal
Hydrolysates	Proteases	pH	E:S	Temperature (°C)	Protein:AC ratio
H1	*B. licheniformis*	8.4	4:100	50	1:88
H2	*B. stearothermophilus*	8.4	4:100	50	1:88
H3	*B. subtilis* (Prozyn)	8.4	4:100	50	1:88
H4	*B. subtilis* (AB enzymes)	8.4	4:100	50	1:88
H5	*Aspergillus sojae*	8.4	4:100	50	1:88
H6	*Papaya carica*	8.4	4:100	50	1:88
H7	*Papaya carica*	8.4	4:100	50	1:44
H8	*Papaya carica*	8.4	4:100	50	1:16
H9	*Papaya carica*	8.4	4:100	25	1:88
H10	*Papaya carica*	8.0	4:100	50	1:88
H11	*Papaya carica*	9.0	4:100	50	1:88
H12	*Papaya carica*	11.0	4:100	50	1:88
H13	*Papaya carica*	8.4	5:100	50	1:88
H14	*Papaya carica*	8.4	7:100	50	1:88
H15	*Papaya carica*	8.4	10:100	50	1:88

E:S: Enzyme:substrate ratio; AC: Activated carbon; Phe: Phenylalanine

Preparation of protein hydrolysates: Fifteen hydrolysates were prepared by varying the following parameters: enzyme type, pH, enzyme:substrate ratio and temperature (Table 1). Initially, a volume of 40 mL of protein extract was placed in a flask and the pH was measured (8.4). For some samples, the pH was adjusted for 8.0, 9.0 and 11.0. Then, the protein extract was set on an oil bath under stirring until the desired temperature was reached. After stabilizing the temperature, the enzyme was added in a sufficient quantity to achieve the E:S ratio desired (Table 1). At the end of the reaction, the process was stopped by heating in a water bath at 80°C for 20 min.

Removal of phenylalanine from protein hydrolysates: The removal of Phe from protein hydrolysates using activated carbon was described before by our group (Silvestre *et al*., 2009a).

Evaluation of the efficiency of Phe removal: The evaluation of the efficiency of Phe removal was performed by measuring the free Phe in beans and in its hydrolysates after AC treatment, using the Second Derivative Spectrophotometry (SDS), as described before by our group (Silvestre *et al*., 2009a).

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 factorial analysis was used to evaluate the effect of some parameters over the Phe removal. 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 beans: The results of the analysis of some components of beans are shown in Table 2. In general, the values found here are close to those found in the literature. Some differences among the data may be associated to some factors such as the cultivar, the analytical method, the climatic and soil conditions, the injuries of the beans and the presence of pests. Moreover, the protein content of beans may be influenced by the germination degree (Goncalves *et al.*, 2003).

It can be observed in Table 2 that the values found for proteins (23.7%), lipids (1.7%), Ashes (3.4%) and carbohydrates (71.2%) were very close to those reported by Antunes *et al.* (1995) (23.4, 1.4, 4.2 and 71.0%, respectively) and found in the Brazilian Table of Food Composition (TACO, 2006) (23.2, 1.5, 4.1 and 71.2%, respectively). Considering that in both works the same cultivar was used, these differences may be due to effects of environmental factors and growing conditions, because according to Sgarbieri (1996), the chemical composition of beans depends on the cultivation conditions and the cultivar. The values obtained in the present work are also similar to the ones of another Brazilian Table of Food Composition (TBCA, 2008) (21.1, 1.5, 4.1 and 73.3%, respectively). Some observed differences could be justified by the fact that the latter represents not only the cultivar carioca, but values of several types of *Phaseolus vulgaris*.

Efficiency of phenylalanine removal: The results obtained for Phe removal from different protein hydrolysates of beans are shown in Table 3. The Phe content in beans was of 1527.45 mg Phe 100 g^{-1} of beans. As can be seen in this table, the use of activated carbon was efficient for removing Phe from protein hydrolysates of beans obtained by the action of proteases from different sources, since the percentage of Phe removal varied from 25.4 to 87.9% and the final Phe content from 433.7 to 2679.8 mg Phe 100 g^{-1} hydrolysate. Thus, the hydrolysate with the lowest Phe content could be partly used for the development of modified beans with reduced Phe content and therefore, be inserted in the diet of phenylketonurics.

It should be noted that phenylalanine is an essential amino acid and therefore, must be obtained through feeding. In this way, a certain amount of this compound must be present in the products for phenylketonurics to ensure protein synthesis and normal growth. Moreover, the operational conditions necessary to achieve approximately 100% of Phe removal, as shown before by our group (Soares *et al.*, 2006), would increase the costs for scaling up the process.

Some works reported the Phe removal from several foods using AC, such as skimmed milk (93.6 to 99%) (Lopes *et al.*, 2005; Soares *et al.*, 2006), whey (75 to 99%) (Delvivo *et al.*, 2006; Silva *et al.*, 2007), wheat flour (37.4 to 66.3%) (Carreira *et al.*, 2008), rice grains (85 to 100%) (Lopes *et al.*, 2008), corn meal (68.63 to 97.55%) (Capobiango *et al.*, 2007), rice flour (25.7 to 94.1%)

Table 2: Chemical composition of the cultivar of beans used in the present work and of other ones

	Cultivars			
Nutrients (% of dry matter)	Carioca[1]	Carioca[2]	Carioca[3]	Varied cultivars[4]
Proteins	23.7	23.4	23.2	21.1
Lipids	1.7	1.4	1.5	1.5
Ashes	3.4	4.2	4.1	4.1
Carbohydrates	71.2	71.0	71.2	73.3

[1]Cultivar used in the present work. [2] Antunes *et al.* (1995). [3]Brazilian Table of Food Composition-TACO (2006). [4]Brazilian Table of Food Composition-TBCA (2008): average values for different cultivars

Table 3: Phe removal and Phe contents of samples

Samples	Phe removal (%)	Phe content (mg Phe 100 g^{-1} of sample)
Beans	-	1527.45
H1	77.9^{cd}	795.30
H2	70.3^{e}	1067.30
H3	69.6^{e}	1092.80
H4	60.8^{f}	1409.40
H5	73.5^{de}	951.90
H6	81.5^{b}	665.50
H7	60.2^{f}	1429.20
H8	25.4^{g}	2679.80
H9	69.4^{e}	1099.30
H10	75.6^{cd}	878.30
H11	80.1^{bc}	716.60
H12	73.0^{de}	971.70
H13	82.7^{b}	623.50
H14	81.5^{b}	649.00
H15	87.9^{a}	433.70

Phe: Phenylalanine. Phe contents of hydrolysates: 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

(Silvestre *et al*., 2009b), whey protein concentrate (55.6 to 81.3%) (Silva *et al*., 2010) and whole milk (32.7 to 75.9%) (Souza *et al*., 2010).

Other authors reported the use of AC for Phe removal from casein. Thus, Bajonero *et al*. (1991) removed 92% of Phe from protein hydrolysates of sodium caseinate obtained by the action of a protease of *Aspergillus oryzae* followed by a papain. Employing a system of three enzymes (chymotrypsin, carboxypeptidase A and leucine aminopeptidase) (Moszczynski and Idziac, 1993) removed 89.5% of Phe from casein hydrolysates. No updated work has been found in the literature.

Effect of some parameters on phenylalanine removal: Some of the parameters which influence Phe removal were analyzed taking into account the reduction of costs for scaling up the process. Thus, it was considered that a lower E:S ratio is associated with the use of a smaller amount of enzyme required to hydrolyze proteins; a lower temperature is associated to a smaller energy consumption and a smaller amount of activated carbon (higher protein: AC ratio) means lower costs, since AC is the most expensive material used in the process.

Effect of enzyme type: The evaluation of the use of different proteases on Phe removal was carried out by comparing the results of the hydrolysates from H1 to H6. It can be observed in Fig. 1 that the hydrolysate H6, obtained by the action of a protease from *Papaya carica* showed the highest percentage of Phe removal (81.5%) and therefore, the lowest level of final Phe content (665.5 mg Phe 100 g^{-1} of hydrolysate).

This performance of a papain could be explained, at least in part, by the fact that it has a broad range of optima values of action and therefore, the conditions of pH and temperature used for the preparation of protein hydrolysates (pH 8.4 and 50°C) are within this range (pH from 3 to 9 and temperature from 50 to 70°C), contrarily to what happened in relation to the other enzymes for which the pH and the temperature were different from their optima values, as shown in Table 4. Thus, H6 obtained with papain showed the highest Phe removal (81.5%), followed by H1

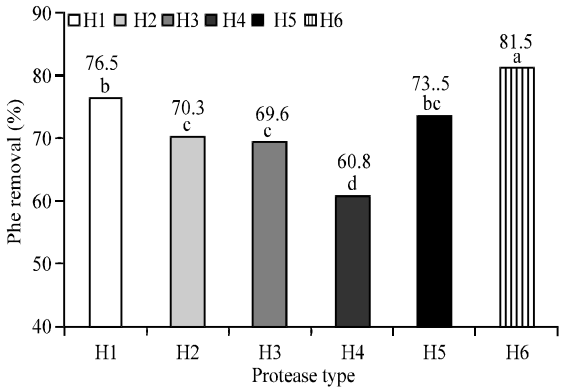

Fig. 1: Effect of enzyme type on Phe removal from protein hydrolysates of beans. Different letters are significantly different ($p<0.05$) for different hydrolysate. H1 = *B. licheniformis*; H2 = *B. stearothermophilus*; H3 = *B. subtilis* (Prozyn); H4 = *B. subtilis* (AB enzymes); H5 = *Aspergillus sojae*; H6 = *Papaya carica*

Table 4: Optima and used pH and temperature values of the enzymes

		pH		Temperature (°C)	
Hydrolysates	Enzymes	Used	Optimum*	Used	Optimum*
H1	*B. liccheniformis*	8.4	9.5	50	60
H2	*B. stearothermophilus*	8.4	8.0	50	80
H3	*B. subtilis* (Prozyn)	8.4	7.0 to 7.5	50	55
H4	*B. subtilis* (AB enzymes)	8.4	7.0	50	55
H5	*Aspergillus sojae*	8.4	9.0	50	70
H6	*Papaya carica*	8.4	3.0 to 9.0	50	50 to 70

* Values furnished by Prozyn in 2005 and AB Enzymes in 2001, 2002 and 2003

(*B. liccheniformis*), H2 (*B. stearothermophilus*-70.3%), H3 (*B. subtilis* from Prozyn-69.6%), H5 (*Aspergillus sojae*-73.5%) for which no significant difference was found and finally, H4 (*B. subtilis* from AB Enzymes-60.8%).

The effect of the enzyme type on Phe removal from protein hydrolysates was also evaluated. Thus, using a pepsin and a papain to prepare hydrolysates of powder milk, no significant difference ($p \leq 0.05$) was found between these two treatments in terms of Phe removal (97.1 and 97.6%, respectively) (Soares *et al.*, 2006). In another study, a pancreatin and a papain were immobilized in two supports for hydrolyzing whey proteins and it was shown that when the support was activated carbon, the action of the former enzyme was more advantageous since it led to a higher Phe removal (95 and 89%, respectively). Contrarily, using alumina as support, the action of papain removed more Phe than pancreatin (97 and 92%, respectively) (Silva *et al.*, 2007). In a study where different proteases (one of *Bacillus stearothermophilus*, two of *Bacillus subtilis*, one of *Aspergillus sojae*, a pancreatin and a papain) were used in the preparation of protein hydrolysates of rice flour, the highest Phe removal was achieved with papain, reaching 94.1% (Silvestre *et al.*, 2009b). Eight enzymes (papain, pancreatin, proteases from *B. stearothermophilus*, *A. sojae*, *A. oryzae*, *B. amyloquefaciens* and two from *B. subtilis)* were used by our group for preparing WPC hydrolysates and a pancreatin was the most efficient having led to a Phe removal of 81.3% (Silva *et al.*, 2010).

Effect of protein:activated carbon ratio: The effect of the protein: AC ratio on Phe removal was evaluated by comparing the data of hydrolysates H6 (1:88), H7 (1:44) and H8 (1:16) in Table 3. It can be observed that the beneficial effect of using a higher ratio (lower amount of AC) was not obtained, as the percentages of Phe removal were of 82, 60 and 25% for ratios of 1:88, 1:44 and 1:16, respectively.

The activated carbon is the adsorbent responsible for Phe removal. Therefore, there is an explanation for obtaining smaller Phe removal with the use of higher protein:AC ratio. Probably, the reduction of the amount of AC inside the column produces a smaller surface of contact between the adsorbent and the amino acid leading to a rapid saturation of AC which reduces its capacity of retaining Phe. This phenomenon gives rise to protein hydrolysates with higher Phe content.

Five works have been carried out aiming at verifying the influence of protein:AC ratio on Phe removal from protein hydrolysates obtained from different protein sources. In three of them, no beneficial effect was observed with the use of a larger protein: AC ratio. Thus, three values for this ratio were tested (1:118, 1:90 and 1:60) but no significant difference was detected among the results, whose average was of 97% for Phe removal from protein hydrolysates of skimmed milk (Soares *et al*., 2006). The use of protein: AC ratios of 1:88, 1:44 and 1:22 led to Phe removal from corn flour hydrolysates of 84.0%, 62.4% and 54.1%, respectively (Capobiango *et al*., 2007). In case of protein hydrolysates from rice flour, the ratios of 1:88, 1:44 and 1:22 gave rise to Phe removal of 94.1, 78.4 and 44.0%, respectively (Silvestre *et al*., 2009b).

In two other studies, the beneficial effect of using lower amount of AC was observed. Thus, working with four enzymatic hydrolysates of corn flour obtained by pancreatin action, following different hydrolytic conditions and using protein: AC ratios of 1:88.5, 1:16 and 1:8, the result obtained with a 1:8 ratio (86.1%) was similar to those with 1:88.5 (85.4%) (Capobiango *et al*., 2007). Working with protein hydrolysates of whole milk, it was noted the advantage of using a smaller amount of AC, since the 1:22 ratio gave rise to a higher Phe removal (75.9%) than 1:44 (39.7%) and 1:88 (45.4%) (Souza *et al*., 2010).

Effect of the reaction temperature: Aiming at the evaluation of this parameter on Phe removal, the results of the hydrolysates H9 (25°C) and H6 (50°C) were compared. It can be observed in Table 3 that the advantage of using a lower reaction temperature was not found, since the Phe removal at 50°C (81.5%) was higher than at 25°C (69.4%).

The greater efficiency of Phe removal after hydrolysis at 50°C may be related to a higher enzyme activity of *Papaya carica* at this temperature, which could be explained by the fact that this temperature is within the optimum range of action of this enzyme, i.e., from 50 to 70°C, according to its furnisher. Thus, the use of a temperature value inside this range favors the interaction between the enzyme and the substrate leading to a higher Phe exposure and release and consequently, to an increased Phe removal by the activated carbon.

Moreover, it is known that the temperature may affect the three-dimensional structure of proteins and that the activity of an enzyme is directly associated with its conformation. In this way, the temperature may have some effect on enzyme activity (Lehninger *et al*., 2006). In the case of the present study, the use of a temperature of 50°C may have caused a change in the conformation of the protein structure which favored the interaction between the enzyme and the substrate (proteins), leading to an increased exposure or release of Phe and consequently, to an increased Phe removal by the activated carbon.

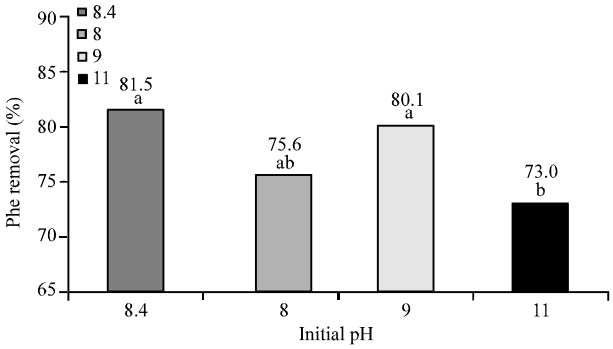

Fig. 2: Effect of initial pH of hydrolytic reaction on Phe removal from protein hydrolysates of beans. Different letters are significantly different ($p<0.05$) for different hydrolysates

Two studies showed the advantage of using lower temperature. Thus, the hydrolysis of whey at 25°C produced higher Phe removal than at 50°C when some reaction conditions were used, such as the use of an E:S of 0.1:100 associated to the ultrafiltration of the hydrolysates (88 and 86%, respectively) as well as an E:S of 1:100 with no ultrafiltration (9 and 94%, respectively) (Delvivo *et al*., 2006). Among different reaction conditions tested for hydrolyzing proteins from whole milk using a protease of *B. subtilis*, the use a temperature of 30°C showed to be more advantageous than 50°C, when the E:S was of 1:100 and the reaction time of 1:30 h, leading to a Phe removal of 38.2 and 32.7%, respectively (Souza *et al*., 2010).

Effect of initial pH: For evaluating the effect of initial pH of hydrolysis on Phe removal from protein hydrolysates of beans the results of the samples H6 (pH 8.4), H10 (pH 8.0), H11 (pH 9.0) and H12 (pH 11.0) may be compared in Fig. 2. One can note that no significant difference was found among the results obtained at pH 8.4, 8.0 and 9.0. However, the percentage of Phe removed at pH 11.0 was lower. These results could be explained because the first three pH values are within while the last one is out of the range of optimum pH of activity of the enzyme (protease from *Papaya carica*-pH from 3.0 to 9.0). In this way, considering that no adjustment should be made for working at pH 8.4, this value was chosen as the best one.

Effect of enzyme:substrate ratio: Having as a purpose to assess the effect of the enzyme: substrate ratio on Phe removal, the results of four samples were compared: H6 (E:S = 4:100), H13 (E:S = 5:100), H14 (E:S = 7:100) and H15 (E:S = 10:100). As can be seen in Fig. 3, the advantage of using a lower E:S ratio (smaller amount of enzyme) was not observed, since the highest Phe removal was achieved with an E:S ratio of 10:100. However, the use of the smallest E:S ratio (4:100) could be beneficial because no significant difference was found among the percentage of Phe removal using this value and the two other higher ones (5:100 and 7:100).

In some studies the effect of E:S ratio on Phe removal from protein hydrolysates for different protein sources and enzymes was evaluated. Thus, using corn flour as raw material and a pancretin in various hydrolytic conditions, in some cases it was shown the advantage of using a lower E:S ratio (1:100 and 2:100), which led to higher Phe removal (86.7 and 79.0%, respectively) (Capobiango *et al*., 2007). When the protein source was the whey, the action of a papain in different reaction conditions showed in some cases the advantage of using lower E:S ratio, comparing 0.01:100 with 0.1:100 (98 and 93% of Phe removal, respectively) and 0.1:100 with 1:100

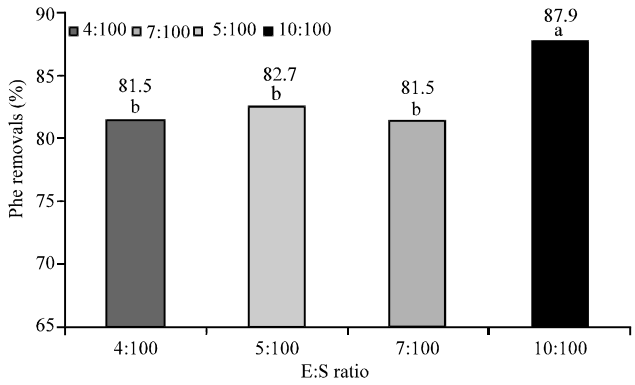

Fig. 3: Effect of enzyme:substrate ratio on Phe removal from protein hydrolysates of beans. Different letters are significantly different ($p<0.05$) for different hydrolysates

(88 and 86%, respectively) (Lopes *et al*., 2005). In case where papain was immobilized on activated carbon for hydrolyzing whey, this same beneficial effect was observed, because the E:S of 1:100 removed 95% of Phe while this percentage was 89% with an E:S of 2:100 (Silva *et al*., 2007). The use of a pancreatin for hydrolyzing WPC in a E:S ratio of 1:100 was more beneficial than 2:100, because more Phe was removed (81 and 63%, respectively) (Silva *et al*., 2010).

These results demonstrate that in spite of the theoretical expectation that the use of a higher E:S ratio leads to a greater degree of hydrolysis and consequently to a higher Phe exposure and removal, in practice this procedure is far more complex than expected and depends on other factors, such as type of substrate and enzyme, pH, length and temperature of the hydrolytic reaction.

CONCLUSION

The use of a protease from *Bacillus licheniformis* for extracting proteins from Brazilian beans and of several proteases for the hydrolytic stage, followed by the treatment with AC as adsorbent, showed to be a successful process to remove Phe. The best result was found when employing papain in an E:S ratio of 10:100; a protein:AC a ratio of 1:88; an initial pH of 8.4 and a temperature of 50°C, leading to 87.93% of Phe removal.

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