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Preparation of Low-phenylalanine Whey Hydrolysates, Using Papain and Pancreatin Immobilized on Activated Carbon and Alumina

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Abstract: This study involves the preparation of whey hydrolysates with low phenylalanine (Phe) content aiming the treatment of phenylketonuria. For hydrolysing the proteins, two enzymes were used, papain and pancreatin, in an immobilized form, on Activated Carbon (AC) and alumina (AL) and three enzyme: substrate ratios (E:S) were tested for each enzyme. Activated carbon was used to remove Phe from hydrolysates. The second order spectrophotometry was used to evaluate the efficiency of Phe removal as well as the losses of tyrosine (Tyr) and tryptophan (Trp). The results showed that the activated carbon was efficient to remove Phe from whey hydrolysates and the values changed from 84-97%. Tyr and Trp losses varied from 45-70 and from 63-78%, respectively, depending on the enzyme and the immobilization support used. The use of pancreatin was more advantageous (smaller final Phe content) than papain when AC was used as the immobilization support and the inverse was observed when using AL. The E:S affected the Phe removal only when the support was AC and the desirable effect associating the decrease of Phe content with E:S reduction was just observed for papain when passing from 2-1%. Also, the use of these two enzymes in an immobilized form was able to produce high oligopeptide (40%) and low aminoacid (2%) contents which is advantageous form the nutritional point of view.

Key words: Whey, enzymatic immobilization, activated carbon, alumina, enzymatic hydrolyze, phenylalanine removal

INTRODUCTION

The necessity of removing phenylalanine (Phe) from protein hydrolysates is associated to fact that these preparations can be used as protein sources for obtaining dietetic supplements for phenylketonuria (PKU). In fact, 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 (Lopez-Bajonero *et al*., 1991; Acosta and Yannicelli, 1997; Shimamura *et al*., 1999).

In fact, free amino acid mixtures (Lajolo and Tirapegui, 1998; Trahms, 1998) or Phe-poor protein hydrolysates (Tesmer *et al*., 1998) may be used in the nutritional therapy of phenylketonurics. However, protein hydrolysates, especially those rich in oligopeptides, present the advantages of being more rapidly adsorbed by the organism, having lower osmolarity and tasting better, besides being more economical than a mixture of free amino acids (Mira and Marquez, 2000).

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Among several protein sources that may be used for preparing dietary supplements for phenylketonurics, isolated casein, the main milk protein, is the choice in most cases (Lopez-Bajonero *et al*., 1991; Outinen *et al*., 1996; Shimamura *et al*., 1999). However, in underdeveloped countries, this protein needs to be imported which represents a considerable increase in production costs. Thus, the use of less expensive alternative sources must be investigated, such as whey, since its proteins are ready assimilated by the organism and shows a high protein efficiency ratio (Nicolau *et al*., 2005). Moreover, the use of whey, a waste of milk industries in some contries, may contribute to reduce the environment pollution.

Considering that in our country the products normally used as dietetic supplements must be imported and, consequently, have high price, our interest turned to the preparation of these preparations, containing protein hydrolysates as the main source of amino acids in a high available form, that is, in oligopeptide form, especially di- and tripeptides. This is the reason we have been preparing several protein hydrolysates and testing different hydrolytic conditions for obtaining peptide profiles appropriate for nutritional purposes (Silvestre *et al*., 1994a,b; Morato *et al*., 2000; Carreira *et al*., 2004, 2004; Morais *et al*., 2005; Lopes *et al*., 2005; Soares *et al*., 2007)

In the present study, we used for the first time immobilized enzymes for preparing protein hydrolysates for phenylketonurics. This is associated to the several advantages of immobilized enzymes over enzymes in bulk solution, especially the reusability of the enzymes which contributes to reduce process costs (Rani *et al*., 2000; Afaq and Iqbal, 2001; Arica and Bayramoglu, 2006; Li *et al*., 2007, Pedroche *et al*., 2007). In fact, we have previously studied the immobilization of pancreatin and papain on activated carbon and alumina and showed that the enzyme activities remained unchanged after 5-15 cycles of use (De Marco *et al*., 2004; Silva *et al*., 2005).

Most of the methods used for Phe removal from protein hydrolysates are based on the principle that a sufficient amount of Phe is liberated by enzymatic hydrolysis and the free Phe is, then, removed by gel filtration, adsorption by activated carbon or resins (Lopez-Bajonero *et al*., 1991; Outinen *et al*., 1996). We have been successfully using activated carbon for removing Phe from different protein hydrolysates since some time ago (Lopes *et al*., 2005; Soares *et al*., 2006; Delvivo *et al*., 2006).

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. Several techniques to quantify Phe among other amino acids are available in the literature, including the ion-exchange chromatography using the amino acid analyzer (Lepage *et al*., 1997), reverse high performance liquid chromatography (Vendrell and Avéles, 1986; Alaiz, 1992; Bank *et al*., 1996), hydrophylic interaction chromatography (Alpert, 1990; Carreira *et al*., 2002) and spectrophotometric methods of second order (SDS) (Silvestre *et al*., 1993; Mach and Middaugh, 1994; 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 (Morais *et al*., 2004), as well as for estimating the Phe removal of different protein hydrolysates (Lopes *et al*., 2005; Soares *et al*., 2006; Delvivo *et al*., 2006).

Normally, tyrosine and tryptophan are also removed by the adsorption media used for removing phenylalanine (Kitagawa *et al*., 1987; Moszczynski and Idziak, 1993; Outinen *et al*., 1996). Thus, it is important to evaluate the losses of these amino acids. Moreover, according to some authors, Tyr is an essential amino acid for phenylketonurics (Trahms, 1998; Surtees and Blau, 2000; Kalsner *et al*., 2001; Van Sprosen *et al*, 2001).

Several works have been performed aiming the preparation of protein hydrolysates for phenylketonurics (Lopez-Bajonero *et al*., 1991; Moszczynski and Idziak, 1993; Kasai *et al*., 1994; Outinen *et al*., 1996; Shimamura *et al*., 1999; De Holanda e Vasconcellos *et al*., 2003; Pedroche *et al*., 2004). However, none of them evaluated the peptide profile of these preparations, which is important to have an idea of the nutritional quality of the hydrolysates.

The aim of the present study was to obtain whey hydrolysates with low phenylalaine content using activate carbon as adsorbent support. Immobilized papain and pancreatin, on activated carbon and alumina, were employed for preparing the hydrolysates, whose peptide profiles were investigated. The losses of tyrosine and tryptophan were also evaluated.

MATERIALS AND METHODS

Materials

L-phenylalanine, L-tyrosine, L-tryptophan, pancreatin (P-1500, from porcine pancreas, activity at least equivalent to USP specifications), papain (P-3375, from *Carica papaya*, 1.5 - 3.5 units mg^{-1} solid), alumina (A-5 type) and activated carbon (20-60 mesh) were purchased from Sigma (St. Louis, MO, USA). Whey (powder) was kindly furnished by a food producer (Minas Gerais, Brazil). The stirrer was from Fisatom (São Paulo, Brazil) and the spectrophotometer was CECIL (CE2041, Buck Scientific, England). The HPLC system consisted of one pump (HP 1100 Series) and an UV-VIS detector, coupled to a computer (Hpchemstation HP1100, Germany). A poly (2-hydroxyethylaspartamide)-silica (PHEA) column, 250×9.4 mm, 5 μm, 200 Å pore size (PolylC, Columbia, MD), was used for HPLC. The freeze-dryer was from Labconco (7750 model, Kansas City, MI, EUA). 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. This study was accomplished at the Laboratory of Bromatology R&D of Federal University of Minas Gerais (UFMG), in 2005.

Methods

Determination of the Chemical Composition of Whey

The contents of moisture, protein, lipid, minerals, calcium and lactose of whey were determined according to the Association of Official Agricultural Chemists methods (AOAC, 1995).

Immobilization of the Enzymes

Papain and pancreatin were immobilized by adsorption on two supports: activated carbon (AC) and alumina (AL). Varied volumes of the 0.1% (w v^{-1}) enzymatic solutions in phosphate buffer 0.1 mol L^{-1}, pH 7.5 and quantities of the support were mixed in a beaker, depending on the E:S desired for preparing the protein hydrolysates. Thus, for E:S of 1 and 2%, 5.0 mL of the enzymatic solutions and 1.0 g of the support were used; for E:S of 0.01%, 50 μL of the enzymatic solutions and 10 mg of the support were used. After 30 min of contact at 25°C, the mixtures were centrifuged at 11.000xg for 20 min, at 25°C (Centrifuge Jouan, BR4i model, France) and filtered under vacuum through filter paper. Finally, the enzyme:support complexes hold on the filter were let to dry at room temperature. Blanks were also prepared using only the phosphate buffer 0.1 mol L^{-1}, pH 7.5.

Preparation of Whey Hydrolysates

Initially, a 10% (w v^{-1}) whey solution in 0.01 mol L^{-1} phosphate buffer, pH 7.5, was prepared. The enzyme:support complexes were added to varied volumes of this solution to reach the E:S ratios of 0.01% (42 mL), 1% (42 mL) and 2% (21 mL). These mixtures were stirred for one hour on a magnetic stirrer at 25°C, in such a velocity to keep the complex in suspension. Then, they were centrifuged at 11,000 x g for 20 min, at 25°C, filtered under vacuum through qualitative paper and the filtrates were freeze-dried. The other parameters of hydrolysis are shown in Table 1.

Removal of Phenylalanine from Whey 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

Table 1: Parameters employed for preparing whey hydrolysates using immobilized papain and

		Enzymes/Hydrolysates	
Immobilization supports	E:S (%)	Papain	Pancreatin
Activated carbon	0,01	H1	H7
Activated carbon	1	H2	H8
Activated carbon	2	H3	H9
Alumina	0,01	H4	H10
Alumina	1	H5	H11
Alumina	2	H6	H12

ES = Enzyme Substrate ratio

placed inside a disposable syringe of 20 mL containing a filter of nylon and wool glass, manufactured in our laboratory. Then, a hydrolysate solution (80 mg 100 mL^{-1}) was added to the column and the eluate was collected and filtered through qualitative paper.

Evaluating the Efficiency of Phe Removal

The efficiency of Phe removal was evaluated by measuring the free Phe content in whey and in hydrolysates (after carbon treatment) using the second derivative spectrophotometry, as described before by our group (Lopes *et al*., 2005). The free Phe content corresponds to the Phe that was released by acid hydrolysis.

Briefly, the samples were hydrolysed (5.7 mol L^{-1} HCl, 110°C, 24 h), their pH was adjusted to 6.0 and their absorbance measured from 250-280 nm. Second derivative spectra were drawn and the area of one of the negative peaks was 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 and resin. 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. 1

$$\text{Phe Removal (\%)} = \frac{\text{Initial amount of Phe - final amount of Phe}}{\text{Initial amount of Phe}} \times 100 \qquad (1)$$

Where,
Initial amount of Phe = amount of Phe in whey.
Final amount of Phe = amount of Phe in hydrolysates treated by activated carbon.

Evaluating the Losses of Tryptophan and Tyrosine

The losses of Trp and Tyr due to their adsorption on activated carbon were evaluated by SDS. In case o Trp, the loss was estimated in terms of reduction of exposure rate (ER), calculated as 100 % ER, while for Tyr, in addition to this parameter, its loss was also evaluated by the determination of the free Tyr content, i.e., the amount of Tyr that was released by the acid hydrolysis. The ER corresponds to the amount of the amino acids that was exposed by the enzymatic hydrolysis and was calculated according to the Eq. 2.

$$\text{ER (\%)} = (B/A)\times 100 \qquad (2)$$

Using the Second Derivative Spectrophotometry (SDS)

In the present study, the SDS was used for quantifying free amino acids, Phe, Tyr and Trp as well as for evaluating the ER of Tyr and Trp.

Quantification of Free Phenylalanine

For quantifying free Phe, initially a standard curve was prepared, in the presence of Tyr and Trp. 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 0.01 mol L^{-1} phosphate buffer, pH 6.0. Then, 10 mL of each solution were mixed and successive dilutions of this mixture were made to have Phe concentrations in a range from $0.13\text{-}1.01\times10^{-4}$ mol L^{-1}.

The absorbance of these solutions were measured from 250-280 nm. Second derivative spectra were drawn and the area of the third negative peak or peak c (Fig. 1) was used to calculate the amount of Phe in the samples, employing a standard curve. A software GRAMS-UV was used to draw the second derivative spectra.

For quantifying free Phe in the samples, the spectra of whey and its hydrolysates after the treatment with AC were drawn in the same wavelength cited above. Then, the area of the third negative peak was taken to the standard curve in order to find the Phe content.

Quantification and Measurement of the Exposure Rate of Free Tyrosine and Tryptophan

First, a standard curve of Tyr and Trp was prepared, in the presence of Phe. Stock solutions of Tyr (7.0×10^{-5} mol L^{-1}), Trp (1.3×10^{-5} mol L^{-1}) and Phe (9.6×10^{-5} mol L^{-1}), were prepared in 0.01 mol L^{-1} phosphate buffer, pH 13.0. Then, 10 mL of each solution were mixed and successive dilutions of this mixture were made to have Tyr concentrations in a range from $4.2\text{-}21.2\times10^{-6}$ mol L^{-1} and Trp varying from $7.4\text{-}36.9\times10^{-7}$ mol L^{-1}. Then, the absorbance of these solutions were measured from 250-280 nm. Second derivative spectra were drawn and the area of the peak a and of the broad band b, corresponding to Trp and Tyr (Fig. 2), respectively, were used to draw the standard curves of these amino acids in function of their concentrations.

For quantifying the free Tyr content in the whey and its hydrolysates, after the treatment with AC, the samples were first submitted to the acid hydrolysis (5.7 mol L^{-1} HCl, 110 °C, 24 h) and the pH was adjusted to 13.0. The spectra of the solutions were drawn in the same wavelength cited above and the area of the broad band b was taken to the standard curve in order to find the Tyr content.

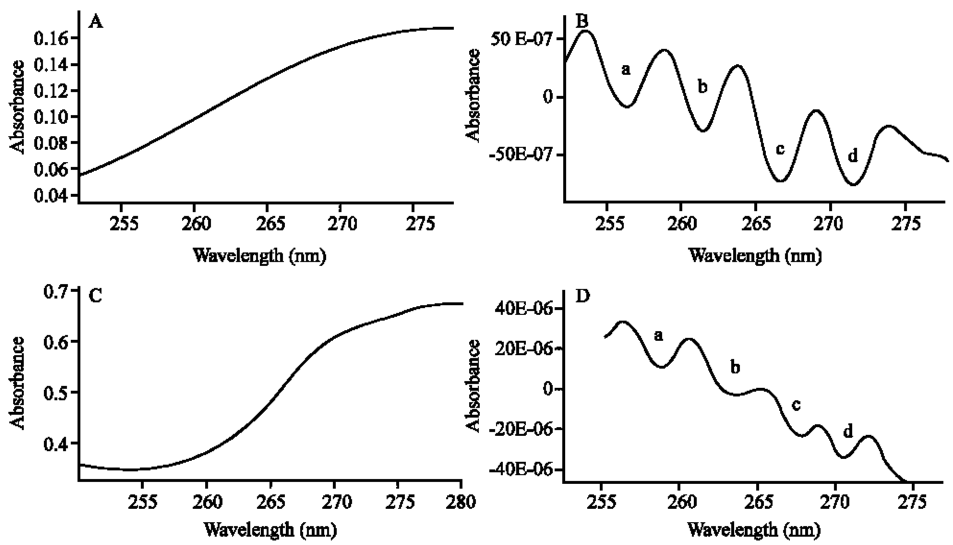

Fig. 1: Absorbance and second derivative spectra of Phe in a solution containing Tyr and Trp (A and B, respectively) and of a hydrolysate obtained after the second hydrolysis (C and D, respectively), in pH 6.0

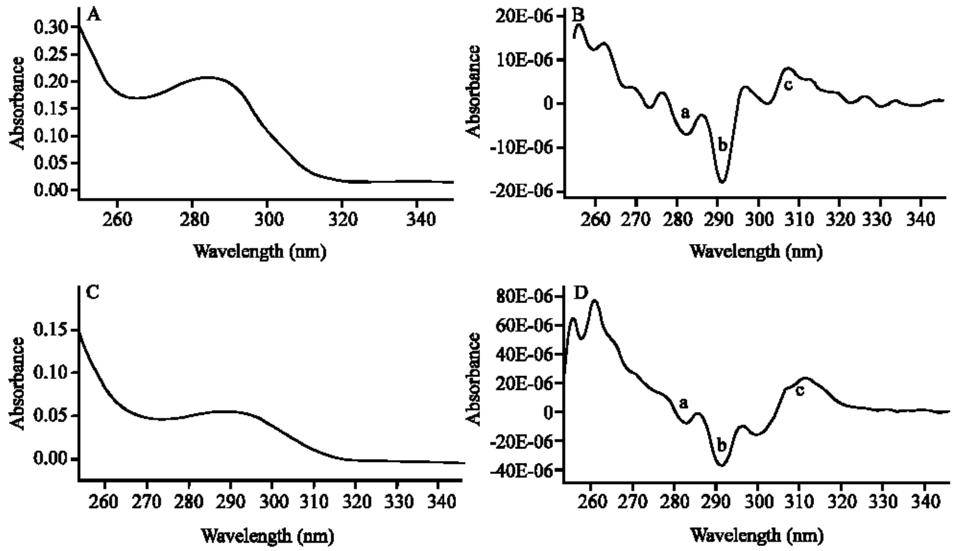

Fig. 2: Absorbance spectra, at pH 13.0: (A) Tyr and Trp in a solution containing also Phe; (C) hydrolysate H1. Second derivative spectra, at pH 13.0: (B) Tyr and Trp in a solution containing also Phe; (D) hydrolysate H1

The exposure rates of Tyr and Trp were measured in the hydrolysate solutions (0.8% w v^{-1}, in water), pH 13.0, before and after the treatment with AC. The spectra of the solutions were drawn in the same wavelength cited above and the areas of the peak a and of the broad band b were taken to the standard curve in order to find the amount of Trp and Tyr that were exposed by the enzymatic hydrolysis.

Characterization of Peptide Profiles of Whey Hydrolysates

This characterization was performed in two stages: fractionation of the peptides, according to their size and their quantification.

The fractionation of whey 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 three 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 to 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 whey as the substrate. Briefly, five whey standard hydrolysates (two using trypsin and three using pancreatin) were prepared and then fractionated in four fractions by SE-HPLC, as described above. The four fractions were collected and submitted to an amino acid analysis. The calculation of CFA was performed using the formulas described by Silvestre (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 whey hydrolysates prepared by using papain or pancreatin, their CFA were taken to this curve.

Statistical Analysis

All experiments were replicated three 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 Whey

As shown in Table 2, the amount of the nutrients evaluated in this work are close to the values of the United States Department of Agriculture (USDA, 2003), although several factors may influence the composition of whey, such as the original milk, the type of cheese and the fabrication process.

Spectra and Standard Curves of Phenylalanine, Tyrosine and Tryptophan

The absorbance and SDS spectra of Phe, in a mixture of aromatic amino acids and of hydrolysate H2, in pH 6.0, are shown in Fig. 1. In case of Phe (Fig. 1b), we can see four 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 H2 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 (Morais *et al*., 2004) and skim milk (Lopes *et al*., 2005; Soares *et al*., 2006).

Concerning the standard curve of Phe, its linear regression 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 (Morais *et al*., 2004; Lopes *et al*., 2005, Soares *et al*., 2006), since in all these works a linearity for the standard curve of Phe, in presence of Tyr and Trp in several concentrations, was shown.

The absorbance spectra, at pH 13.0, of Tyr and Trp are shown in Fig. 2A and that of hydrolysate H1 in Fig. 2C. The maxima values for Trp were observed at 278, 287 and 297 nm while the minima were at 283 and 292 nm. The second derivative spectra, at pH 13.0, of Tyr and Trp are shown in Fig. 2B and that of hydrolysate H1 in Fig. 2D. The negative peaks are indicated by the letters a and b. Tyr is presented with a broad band (c) between 305 and 319 nm with a maximum near 31 nm, which corresponds to the dissociation of phenolic OH group that takes place at pH 13.0 (Ichikawa and Terada, 1977). Also, the similarity between the SDS spectrum of a casein hydrolysate and those of standard amino acids can be shown.

Table 2: Chemical composition of whey

Nutrients	Values found*	USDA (2003)**
Moisture (g %)	3.51	3.19
Protein (g %)	11.82	12.93
Lipids (g %)	0.85	1.07
Total ash (g %)	8.72	8.35
Total sugars (g %)	67.47	74.46

*Values found in the present work, **USDA Nutrient database for standard reference

According to Ichikawa and Terada (1977), at pH 13.0 Phe has no optical effect and Tyr has a very little influence on the Trp spectrum in the range of 286-300 nm. This allows Trp to be determined in a mixture of aromatic amino acids. For quantifying Tyr, the band at 310 nm may be used, since the absorption of Trp is very low and Phe has no influence.

The standard curves of Tyr and Trp showed the following equations and correlation coefficients, respectively: $y = 2.288x$ and $R^2 = 0.993$; $y = 0.5563x+0.2699$ and $R^2 = 0.9869$. Also, their linear regressions were highly significant ($p<0.01$).

Efficiency of Phenylalanine Removal

The data in Table 3 show that activated carbon was efficient to remove Phe from whey hydrolysates prepared with papain and pancreatin immobilized on activated carbon and on alumina. Also, no great differences were observed among the results showed for both enzymes. Thus, for papain, Phe removal changed from 84-95% on AC and was of 97% on AL. For pancreatin, Phe removal changed from 84-95% on AC and was of 93% on AL.

It is worth stating that the highest final Phe content found (55 mg 100 g^{-1}) was around half of the maximum value established by the Brazilian Legislation for products intended for phenylketonurics, that is 0.1 g 100 g^{-1} (Anonymous, 2001).

In a previous study in our laboratory, using the same pancreatin in solution, the removal of Phe from whey hydrolysates by the activated carbon varied in the same range (from 75-99%) as that obtained in the present work (Delvivo *et al*., 2006).

Other authors also used activated carbon to remove Phe from protein hydrolysates and reported results near to those of the present work and to the other works of our group. Thus, Kitagawa *et al*. (1987), after hydrolysing whey proteins with actinase, at pH 6.5 and 37 °C, treated these preparations with activated carbon and removed 97% of Phe. However, the conditions for the treatment with activated carbon 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 three 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, i.e., a very long time for hydrolysis (72 h) and for the treatment with activated carbon (5.5 h).

Comparing to the previous study, the great advantage of the present one is related to the process optimization from the technological and economical points of view. Thus, favorable results of Phe removal were obtained using only one enzyme in an immobilized form and smaller hydrolysis time as well as amount of activated carbon and E:S ratio.

Moreover, as shown before by our group, the immobilization of papain and pancreatin on AC showed a reusability of 5 times, while on AL it was of 15 and 2 times, respectively (De Marco *et al*., 2004; Silva *et al*., 2005).

Table 3: Efficiency of Phe removal from whey hydrolysates obtained by action of immobilized papain and pancreatin, after activated carbon treatment

	Immobilization of papain						Immobilization of pancreatin					
	Activated carbon			Alumina			Activated carbon			Alumina		
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Phe removal (%)	$84^{b,2,x}$	$95^{a,1,x}$	$89^{b,2,y}$	$97^{a/1}$	$96^{a/1}$	$97^{a/1}$	$84^{f,2,x}$	$93^{e,1,x}$	$95^{e,1,x}$	$92^{e/1}$	93^{fe1}	$95^{e/1}$
Final Phe content, (mg 100 g^{-1})	$36^{a,1,y}$	$23^{b,1,x}$	$33^{a,1,x}$	$12^{a,2,y}$	$14^{a,2,y}$	$10^{a,2,y}$	$55^{e,1,x}$	$15^{f,1,y}$	$13^{f,1,y}$	$15^{e,2,x}$	$17^{e,1,x}$	$13^{e,1,x}$

Final Phe content = Phe content after treatment with activated carbon. For the same enzyme, different letters (a,b) are significantly different ($p<0.05$) for the same support and hydrolysate and different E:S ratio and different numbers are significantly different ($p<0.05$) for the same E:S ratio and different support and hydrolysate. For different enzymes, different letters (x,y) are significantly different ($p<0.05$) for the same support and E:S ratio

Effect of Different Parameters on the Phenylalanine Removal

Effect of the Enzyme Type

The effect of the enzyme type can be evaluated by comparing the results of H1 with H7, H2 with H8, H3 with H9, H4 with H10, H5 with H11 and H6 with H12 (Table 3). It can be inferred that, when the immobilization support used was AC, pancreatin was more advantageous (smaller final Phe content) than papain in two of the three cases, where E:S was of 1 and 2%. Contrarily, using AL as support, papain yielded better results (smaller final Phe content) than pancreatin in all the three cases studied (E:S of 0.01, 1 and 2%).

Effect of E:S Ratio

For evaluatin the effect of E:S ratio, the following groups must be compared : H1, H2 and H3; H4, H5 and H6; H7, H8 and H9; H10, H11 and H12. The data in Table 3 show that, for papain, the E:S ratio affected the Phe removal of whey hydrolysates, when the immobilization support was AC. The desirable effect associating the decrease of E:S with the smallest final amount of Phe, related to the reduction of production costs of dietary supplements for phenyketonurics, occurred when passing from 2 to 1%. This effect was not observed for pancreatin, since passing from 2-1% no significant difference was shown among the results, while passing from 1% to 0.01% led to an increase in the final Phe content. When the immobilization support was AL, the E:S ratio had no effect on the Phe removal, for both enzymes. In this case, the best condition would be that using E:S ratio of 0.01%, since it is the most economical one. On the other hand, no effect of E:S ratio was observed on Phe removal when using AL as immobilization support.

We have been evaluating the effect of E:S ratio on Phe removal, using these enzymes in solution and AC as Phe adsorbent. Thus, using papain associated with a protease from *Aspergillus oryzae* for hydrolysing skim milk, we s howed that the 10-fold decrease of E:S ratio from 20-2% and from 10-1%, respectively, was advantageous leading to a decrease in the final content of Phe from 0.82-0.21 mg 100 g^{-1} of hydrolysate (Lopes *et al*., 2005). In another study of our group, the effect of E:S on the Phe content of whey hydrolysates using pancreatin was evaluated (Delvivo *et al*., 2006). Several hydrolysis conditions were tested in this study and the desirable effect associating the decrease of E:S with the least final amount of Phe was observed when passing from 1-0.1% where the Phe content passed from 37.2-8.6 mg 100 g^{-1} of hydrolysate (temperature = 25°C and using ultrafiltration) and from 37.8-4.3 mg 100 g^{-1} of hydrolysate (temperature = 25°C and not using ultrafiltration). The further reduction of E:S ratio from 0.1-0.01% was beneficial for the samples using temperature of 50°C and ultrafiltration, where the Phe content passed from 24.9-18.7 mg, 100 mg^{-1} of hydrolysate.

No other report was found in the literature concerning the effect of the E:S ratio on the Phe removal, using enzymes either immobilized or in solution.

Effect of the Immobilization Support

The effect of the immobilization support can be evaluated by comparing the results obtained by the hydrolysates H1 with H4, H2 with H5, H3 with H6, H7 with H10, H8 with H11 and H9 with H12. The data in Table 3 show that the immobilization support affected the Phe removal. Thus, for papain, AL was more advantageous than AC for all three cases, i.e., when using E:S of 0.01, 1 and 2%, leading to smaller Phe contents. In case of pancreatin, the only difference observed between the supports was for E:S of 0.01%, where the use o AL yielded much smaller Phe content.

A probable explanation for these results would be associated to the fact that, depending on the concentration used, these enzymes would have a more favorable conformation after immobilization on the support which favorized the protein hydrolysis and, therefore, Phe removal.

Table 4: Losses of tyrosine and tryptophan of whey hydrolysates, after activated carbon treatment

Hydrolysates	Tyr removal (%)	Reduction of ER of Tyr (%)	Reduction of ER of Trp (%)
H2 (Pap-AC)	$69^{a/1}$	$67^{a/1/x}$	$59^{d/y}$
H4 (Pap-AL)	$58^{b/1}$	$57^{b/1/y}$	$78^{a/x}$
H8 (Panc-AC)	$45^{c/1}$	$47^{c/y/1}$	$68^{b/x}$
H10 (Panc-AL)	$70^{a/1}$	$70^{a/x/1}$	$63^{c/y}$

Pap-AC: papain immobilized on activated carbon; Pap-AL: papain immobilized on alumina; Panc-AC: pancretin immobilized on activated carbon; Panc-AL: pancreatin immobilized on alumina. ER = Exposure Rate. Reduction of ER = 100-ER (%). Different letters (a, b) are significantly different ($p<0.05$) for the same method and different hydrolysate for evaluating the Tyr and Trp losses. Different letters (x, y) are significantly different ($p<0.05$) for the same hydrolysate using the Reduction of ER Method, for evaluating the Tyr and Trp losses. Different numbers are significantly different ($p<0.05$) for the same hydrolysate and different methods, for evaluating the Tyr loss

Losses of Tyrosine and Tryptophan

The losses of tyrosine and tryptophan were evaluated in the hydrolysates that, using different immobilization supports, showed the highest Phe removal and whose preparation employed the greatest economical condition (the smallest E:S value). Thus, for papain the hydrolysates chosen were H2 and H4 while for pancreatin H8 and H10 were analysed.

According to Table 4, for all analysed hydrolysates, no significant difference was observed between the results obtained by both methods for evaluating the Tyr losses. Thus, the measurement of the ER reduction could be used as a simple, rapid and economical method for evaluating aromatic amino acid losses, avoiding the time-consuming and expensive acid hydrolysis stage.

Also, concerning Tyr losses, it can be observed in Table 4 that it changed from 47-70% and the condition that yielded the smallest loss was that using pancreatin immobilized on AC and the highest loss was obtained by the use of pancreatin on AL and papain on AC. In case of Trp, the losses changed from 63-78% and the condition that yielded the smallest loss was that using papain immobilized on AC, while that using papain on AL provided the most unfavorable result.

The Tyr losses by adsorption on AC were similarly evaluated by other authors interested in removing Phe from protein hydrolysates and some of them reported values higher (91%, Kitagawa *et al*., 1987) and others near (60%, Moszczynski and Idziak, 1993) those obtained here. On the other hand, no work was found in the literature about the Trp losses from protein hydrolysates associated to Phe removal by AC.

Effect of Different Parameters on the Losses of Tyrosine and Tryptophan

Effect of the Enzyme Type

The effect of the enzyme type can be evaluated by comparing the results of H2 with H8 and H4 with H10 (Table 4). Concerning the Tyr losses, pancreatin yeilded better results than papain when the E:S used was of 1%. Contrarily, papain was more advantageous than pancreatin when the E:S used was of 0.01%. For Trp losses, these results were inverted. Thus, the effect of the enzyme on Tryr and Trp losses depends on the E:S ratio used.

Effect of the Immobilization Support

For evaluating the effect of the immobilization support, the following comparaisons must be done: H2 with H4 and H8 with H10. The data in Table 4 show that AL yielded smaller losses of Tyr than AC, when papain was the enzyme used. Contrarily, AC produced better results than AL, when pancreatin was the enzyme used. For Trp losses, these results were inverted, as shown for the enzyme effect above. Thus, the effect of the immobilization support on Tryr and Trp losses depends on the enzyme used.

Peptide Profiles of Whey Hydrolysates

The same hydrolysates chosen for the evaluation of Tyr and Trp losses were analysed for their pepetide profiles. The chromatographic pattern of a whey hydrolysate is shown in Fig. 3.

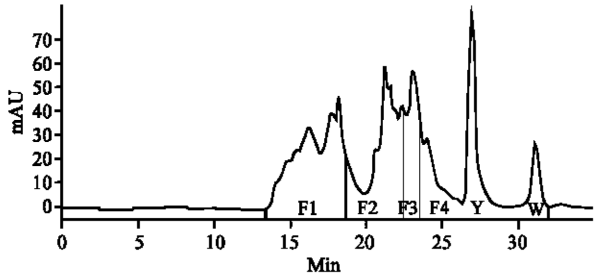

Fig. 3: Chromatographic profile of the whey hydrolysate (H2). Conditions used: PHEA column (250 ×9.4 mm, 5 μm e 200 Å - PolylC); Detection wavelength = 230 nm. Hydrolysate P1: hydrolysis for 6h with pancreatin, in sodium phosphate buffer, pH 7.5; E:S = 4% at 37°C. F1: large peptide (> 7 amino acid residues); F2: medium peptides (4 to 7 amino acid residues); F3: di- and tripeptides; F4: free amino acids. Y = tyrosyl peak, W = tryptophanyl peak

The standard hydrolysates were resolved in four fractions (Silvestre *et al*., 1994b; Morato *et al*., 2000; Carreira *et al*, 2002; Lopes *et al*., 2005; Morais *et al*, 2005; Soares *et al*., 2006). Fraction 1 corresponding to peptides containing more than 7 amino acid residues, fraction 2 to those containing from 4-7 residues, fraction 3-2 and 3 residues and fraction 4 to free amino acids. The last two peaks in fraction 4 correspond to tyrosine (peak Y) and tryptophan (peak W).

The peptide and free amino acid contents found in the chromatographic fractions of whey hydrolysates are shown in Table 5. In agreement with the statement of González-Tello *et al*. (1994) about the characteristics of hydrolysates to be used in special diets, the best results were shown by hydrolysate H8 (pancreatin immobilized on AC), since it presented the highest di- and tripeptide contents (15 %) and of peptides with molecular mass of 500 Da (25%), as well as the lowest level of free amino acids (2%).

No reports concerning the peptide profile of protein hydrolysates from whey were found in the literature.

Effect of Different Parameters on the Peptide Profiles of Whey Hydrolysates

Effect of the Enzyme Type

The effect of the enzyme type can be evaluated by comparing the results of H2 with H8 and H4 with H10. It can be shown in Table 5 that the use of pancreatin was more advantageous than papain in terms of peptide distribution, when using AC or AL as immobilization support. Considering the use of AC, it can be observed that, although the large peptide content of H8 was higher than that of H2, its peptide profile is much better since its di- and tripeptide contents were the triple, its amount of peptides with molecular mass of 500 Da was higher and its level of free amino acids was extremely lower than H2. When the support used was AL, the same kind of results was obtained. Thus, although the large peptide content of H10 was higher than that of H4, its peptide profile is much better since its di- and tripeptide contents were almost six time higher, its amount of peptides with molecular mass of 500 Da was higher and its level of free amino acids was almost six time lower than H4.

Effect of the Immobilization Support

For analysing the effect of the support type on the peptide profile, one has to compare the results obtained for H2 (papain on AC) with H4 (papain on AL) and H8 (pancreatin on AC) with H10 (pancreatin on AL). In both cases, it can be observed that the use of AC was more advantageous than AL. Thus, in the former case, H2 showed the same free amino acid content of H4, but it showed smaller large peptide and higher di- and tripeptide and medium peptide contents. In the second case, H8 showed smaller large peptide and free amino acid contents as well as higher amount of di- and tripeptide and medium peptides than H10.

Table 5: Peptide and free amino acid contents in chromatographic fractions of whey hydrolysates

	Fractions			
Hydrolysates	F1	F2	F3	F4
H2 (Pap-AC)	$45^{c/1}$	$17^{b/3}$	$5{,}0^{b/4}$	$33^{a/2}$
H4 (Pap-AL)	$55^{b/1}$	$8{,}0^{d/3}$	$3{,}0^{c/4}$	$34^{a/2}$
H8 (Panc-AC)	$58^{b/1}$	$25^{a/2}$	$15^{a/3}$	$2^{c/4}$
H10 (Panc-AL)	$64^{a/1}$	$13^{c/3}$	$17^{a/2}$	$6^{b/4}$

The values are in nmols % (porcentage of nmoles of the four fractions). Each value represents the mean of triple determination. Different numbers are significantly different ($p \leq 0.05$) for different fractions of the same hydrolysate. Different letter(s) are significantly different ($p \leq 0.05$) for the same fraction of different hydrolysates. F1: large peptide (>7 amino acid residues); F2: medium peptides (4 to 7 amino acid residues); F3: di- and tripeptides; F4: free amino acids

CONCLUSION

Using immobilized papain and pancreatin on AC and AL for preparing whey hydrolysates and AC as adsorbent support, it was possible to obtain whey hydrolysates with low phenylalanine content. The best results (average of 12 mg of Phe 100 g^{-1} of product) were shown for the hydrolysates prepared with papain immobilized on AL in the three E:S ratios tested (0.01, 1 and 2%). The use of pancreatin and of papain, both immobilized on AC, lead to the smallest losses of Tyr (47%) and Trp (59%). The highest oligopeptide content (40%) was obtained when using pancreatin immobilized on AC.

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