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Phenylalanine Removal from Whey Hydrolysates

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Abstract: The use of activated carbon and a resin was studied as adsorptive media for removing phenylalanine from whey hydrolysates prepared using papain, three enzyme:substrate ratios and two temperatures. Activated carbon led to the lowest final Phe contents. The effect of hydrolytic parameters and ultrafiltration was evaluated. The desirable results associating the least final amount of Phe with the decrease of enzyme:substrate ratios and of the temperature as well as the absence of ultrafiltration was observed in some conditions tested. For all samples, the final Phe contents were inferior to the maximum value established by the Brazilian Legislation for phenylketonurics's products.

Key words: Whey, hydroylysis, phenylalanine removal, activated carbon, amberlite XAD-4

INTRODUCTION

The interest in protein hydrolysates has increased during the last few decades due to the fact that enzymatic hydrolysis shave been shown to be capable of improving the functional properties of proteins (Cândido, 1998; Léonil *et al*., 2000). Similarly, protein hydrolysates have been employed in special foods for premature babies and children with diarrhoea, mal-absorption or phenylketonuria (Smithers and Bradford, 1991; Clemente, 2000) as well as in diets for old people and athletes (Frikjaer, 1994).

The necessity of removing Phenylalanine (Phe) from protein hydrolysates is associated to their use in the preparation of 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). Free amino acid mixtures (Trahms, 1994; Lajolo and Tirapegui, 1998) or Phe-poor protein hydrolysates (Tesmer *et al*., 1998) may be used in the nutritional therapy of phenylketonurics. The 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 amino acids (Mira and Marquez, 2000; Cogan *et al*., 1981).

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; Shimamura *et al*., 1999; Outinen *et al*., 1996). However, in underdeveloped countries, this protein needs to be imported which represents an important increase in production costs. Thus, the use of less expensive alternative sources must be investigated. Among them, the whey may be tested since its proteins are ready assimilated by the organism, showing a high protein efficiency ratio (Nicolau *et al*., 1979). Moreover, the use of whey may contribute to reduce the environment pollution.

Considering that in our country the formulations normally used as dietetic supplements must be imported and consequently, are high-price products, our interest turned to the preparation of these formulations, having 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; Barbosa *et al*., 2004; Carreira *et al*., 2004; Morais *et al*., 2005; Lopes *et al*., 2005a).

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

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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).

In order to evaluate the efficiency of Phe removal, its amount must be determined either in the protein source or in their hydrolysates, after having used an appropriate adsorption method. Several techniques to quantify Phe among other amino acids are available in the literature, including the ion-exchange chromatography using the amino acid analyzer (Kan and Shipe, 1981) reverse high performance liquid chromatography (Badoud and Pratz, 1984; Bidlingmeyer *et al*., 1984; Carisano, 1985; Vendrell and Avéles, 1986; Alaiz *et al*., 1992) hydrophylic interaction chromatography (Alpert, 1990) and spectrophotometric methods of 2nd order (SDS) (Brandts and Kaplan, 1973; Matsushima *et al*., 1975; Silvestre *et al*., 1993; Rojas *et al*., 1998). Some authors reported the great reliability of using SDS, between 250 and 270 nm, for quantifying Phe in proteins, since parameters such as pH and the addition of other elements are controlled (Brandts and Kaplan, 1973; Rojas *et al*., 1998; Ichikawa and Terada, 1979; Cahill and Padera, 1980; Grant and Bhattacharya, 1985). 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*., 2005) as well as for estimating the Phe removal of skim milk hydrolysates (Lopes *et al*., 2005b; Soares *et al*., 2004).

The present research was developed to study the effect of hydrolytic parameters and ultrafiltration on phenylalanine removal from whey hydrolysates, using activated carbon and a resin as adsorption supports.

MATERIALS AND METHODS

L-pheylalanine, L-tyrosine, L-tryptophan, adsorption resin XAD-4, pancreatin (P-1500) and activated carbon (20-60 mesh) were purchased from Sigma (St. Louis, MO, USA). The whey (powder) was kindly furnished by a food industry (Minas Gerais, Brazil). 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, 5μm, 200 Åpore 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.

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

Preparation of whey hdrolysates: Twelve hydrolysates were prepared from solutions of whey (1.06 g 100 mL^{-1}) in 0.01 mol L^{-1} phosphate buffer (pH 7.5). Initially, they were pre-heated in a water-bath, at 80°C for 10 min. Then, the temperature was adjusted to 25 or 50°C and the enzyme was added in such a concentration to attain the desired enzime:substrate ratios. The total reaction time was 5h for all samples. The hydrolytic reactions were stopped by the reduction of pH to 3.0 by adding formic acid and the samples were freeze-dried. Six hydrolysates were submitted to ultrafiltration (Amicon 8400, Millipore Corporation, MA, USA), using 10,000 Da cut-off membranes. The other parameters of hydrolysis are listed in Table 1.

Removal of phenylalanine from whey hydrolysates

Use of activated carbon: The activated carbon, previously hydrated for 10 min, was 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 (Whatman, number 1, Maidstone, England).

Use of adsorption resin: An amount of 5.0 g of XAD-4 was added to the hydrolysate solutions (80 mg 100 mL^{-1}). After stirring in a beaker for 10 min at 30°C, the mixture was filtered through qualitative paper (Whatman, number 1, Maidstone, England).

Evaluating the efficiency of phe removal: For evaluating the efficiency of Phe removal, its content in whey and in

Table 1: Hydrolytic conditions employed for preparing whey hydrolysates

Hydrolysate	E:S (%)	Temperature (°C)	Use of Ultrafiltration
H1	0.01	25	No
H2	0.01	25	Yes
H3	0.01	50	No
H4	0.01	50	Yes
H5	0.1	25	No
H6	0.1	25	Yes
H7	0.1	50	No
H8	0.1	50	Yes
H9	1	25	No
H10	1	25	Yes
H11	1	50	No
H12	1	50	Yes

E:S = Enzyme:Substrate ratio

its hydrolysates was estimated by second derivative spectrophotometry, as described before by our group (Lopes *et al*., 2005b). 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 to 280 nm. Second derivative spectra were drawn (CECIL spectrophotometer, CE2041 model, Buck Scientific, England) and the areas of negative peaks were used to calculate the amount of Phe in the samples, employing a standard curve. In case of protein hydrolysates, this same procedure was employed after the treatment with activated carbon and the 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:

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

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

Statistical analysis: All experiments were carried out in triplicate. Differences between means of areas of negative peaks 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.

RESULTS AND DISCUSSION

Chemical composition of whey: As shown in Table 2, the amount of the components evaluated in this research are close to the values of the United States Department of Agriculture (USDA, 2001) as well as to those cited by other authors (Berlot *et al*., 1996) although several factors may influence the composition of whey, such as original milk, the type of cheese and the fabrication process.

Removal of phenylalanine: The data in Table 3 show that the activated carbon was efficient to remove Phe from whey hydrolysates.

The reduction changed from 64 to 98% and the final content of Phe from 4.2 (H2) to 49.1 mg 100 g^{-1} (H4) of hydrolysate. The amount of Phe in the whey was 456 mg 100 g^{-1}. Similar results were found among five hydrolysates (H5, H7, H8, H10 and H11), for which a significant difference was observed only between H5 and H7. Also, no significant difference was shown between the results of hydrolysates H6 and H12 as well as of H1 and H3.

Table 2: Chemical composition of whey

Nutrients	Values found*	USDA [42]**	Berlot *et al*., (1996)
Moisture (g%)	3.51±0.07	3.51	-
Protein (g%)	11.82±0.38	11.73	11.41-15.71
Lipids (g%)	0.85±0.03	0.54	0.4-1.0
Total ash (g%)	8.72±0.02	10.11	8.30-11.33
Total sugars (g%)	67.47±0.64	73.45	63.20-72.12
Calcium (mg%)	981.5±0.06	2.054	790-2,400

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

Other authors also used activated carbon to remove Phe from protein hydrolysates and reported results near to those of the present research and to the other research of our group. Thus, after hydrolysing whey proteins with actinase, in pH 6.5 at 37°C, these preparations were treated with activated carbon and 97% of Phe were removed (Kitagawa *et al*., 1987). However, the conditions for the treatment with activated carbon were not mentioned. Around 92% of Phe were reduced from hydrolysates of skim milk or sodium caseinate obtained by the action of papain and a protease from *Aspergillus orysae* (Lopez *et al*., 1991). The use of a mixture of three enzymes (chymotrypsin, carboxypeptidase A and leucine aminopeptidase), led to 95% of Phe removal from casein hydrolysates (Moszczynski and Idziac, 1993). However, in this research more severe conditions than those used here were employed, i.e., a very long time for hydrolysis (72 h) and for the treatment with activated carbon (5.5 h).

The hydrolysates containing the lowest Phe amount (H1, H2, H3, H6, H9 e H12), after the treatment with activated carbon, were submitted to Phe removal process using XAD-4 resin. The results in Table 3 show that this resin was efficient having removed from 80 to 95% of Phe from the hydrolysates, corresponding to final Phe contents of 18.1 (H3) to 41.6 mg 100 g^{-1} (H6), respectively. No significant difference was observed between hydrolysates H1 and H12 as well as H2 and H9. Similar results for casein hydrolysates obtained by the action of pancreatin (E:S = 3%, 6 h, pH 7,0) were reported before (Outinen *et al*., 1996). Thus, the treatment with XAD-4 removed 92 to 100% of Phe from the hydrolysates. However, in this study the amount of enzyme used was at least 3 times higher than those of the present work, which increases the costs of the process. Other authors described Phe removal lower than those found here, using pepsin (De Holanda *et al*., 2005) and pronase E (De Holanda, 1989) for hydrolysing casein. However, in both cases, only around 62% of Phe were removed after using XAD-4 resin.

Finally, the data in Table 3 show that the activated carbon was much more efficient than the resin for

Table 3: Efficiency of Phe removal from whey hydrolysates by activated carbon and adsorbent resin

	Removal percentage		Final Phe content* (mg Phe/100g of hydrolysate)	
Hydrolysates	AC	XAD-4®	AC	XAD-4®
H1	98 $^{a/1}$	92 $^{b/2}$	9.2$^{d/1}$	30.1$^{b/2}$
H2	97 $^{a/1}$	80 $^{d/2}$	4.2$^{e/1}$	26.6$^{c/2}$
H3	97 $^{a/1}$	95 $^{a/2}$	10.1$^{d/1}$	18.1$^{d/2}$
H4	64 $^{d/1}$	-	49.1^{a}	-
H5	93 $^{b/1}$	-	19.4^{b}	-
H6	88 $^{c/1}$	-	14.4^{c}	41.6$^{a/2}$
H7	93 $^{b/1}$	-	22.5^{b}	-
H8	86 $^{c/1}$	-	21.4^{b}	-
H9	97 $^{a/1}$	92 $^{b/2}$	7.5^{f}	23.4$^{c/2}$
H10	86 $^{c/1}$	-	21.7^{b}	-
H11	94 $^{a,b/1}$	88 $^{c/2}$	20.7^{b}	-
H12	95 $^{a,b/1}$	89 $^{c/2}$	13.7^{c}	29.9$^{b/2}$

AC = Activated Carbon; XAD-4 = Adsorption resin. *Final Phe content = Phe content after treatment with activated carbon or XAD- resin. Different letters are significantly different ($p<0.05$) for different hydrolysates and the same removal medium. Different numbers are significantly different ($p<0.05$) for different removal media and the same hydrolysate

removing Phe from the six hydrolysates submitted to both treatments. Thus, the final content of Phe in the hydrolysates using activated carbon was much lower than using the resin: two times for H3 and H12; three times for H1, H6 and H9 and six times for H2.

Effect of different treatments on the phenylalanine removal: This study was performed with all whey hydrolysates submitted to the treatment with activated carbon.

Effect of E:S ratio: For evaluating the effect of E:S ratio, the hydrolysates were divided in four groups in order to keep constant the other parameters (temperature and treatment by ultrafiltration-UF): group 1 = 25°C and no UF; group 2 = 25°C and UF; group 3 = 50°C and no UF and group 4 = 50°C and UF.

The data in Fig. 1 show that the E:S ratio affected the Phe removal of whey hydrolysates. The desirable effect associating the decrease of E:S with the least final amount of Phe (in mg 100 mg^{-1} of hydrolysate), related to the reduction of production costs of dietary supplements for phenyketonurics, occurred in three groups (1, 2 and 3). For groups 1 and 3, this benefit happened when passing from 0.1 to 0.01%, while for group 2 when passing from 1 to 0.1% as well as from 0.1 to 0.01%. On the other side, the reduction of E:S ratio increased the Phe content in group 1 when passing from 1 to 0.1% and in group 4 when passing from 1 to 0.1% as well as from 0.1 to 0.01%.

In a previous study of our group with skim milk, the 10-fold decrease of E:S ratio from 10 to 1% and from 20 to 2% for a protease from *Aspergillus oryzae* and for papain, respectively, used in association, was advantageous leading to a decrease in the final content of Phe from 0.82 to 0.21 mg 100 g^{-1} of hydrolysate (Lopes *et al*., 2005b). No report concerning the effect of E:S ratio on the Phe removal was found in the literature.

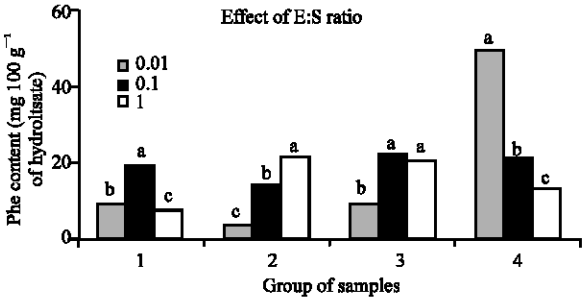

Fig. 1: Effect of E:S ratio over Phe removal. Groups of samples, according to the conditions employed for preparing the hydrolysates: group 1 = 25°C and no UF; group 2 = 25°C and UF; group 3 = 50°C and no UF and group 4 = 50°C and UF. UF = ultrafiltration. Different letters are significantly different ($p<0.05$) within each group

Effect of the temperature: In this case, the hydrolysates were divided in 6 groups in order to keep constant the other parameters (E:S ratio and treatment by ultrafiltration- UF): group 1 = 0.01% and no UF; group 2 = 0.01% and UF; group 3 = 0.1% and no UF; group 4 = 0.1% and UF; group 5 = 1.0% and no UF; group 6 = 1.0% and UF. As shown in Fig. 2, the Phe removal was also influenced by the temperature. The desirable effect associating the decrease of the temperature with the least final amount of Phe (in mg 100 mg^{-1} of hydrolysate), related to the reduction of production costs of dietary supplements for phenyketonurics, occurred in groups 2, 4 and 5. For groups 1 and 3 this procedure had no effect on Phe removal and the decrease of the temperature was disadvantageous for group 6.

As described before for E:S ratio and temperature, the UF also influenced the Phe removal (Fig. 3). The desirable effect associating the absence of UF with the

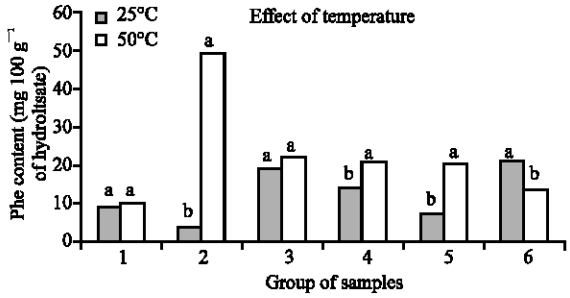

Fig. 2: Effect of the temperature over Phe removal. Groups of samples, according to the conditions employed for preparing the hydrolysates: group 1 = 0.01% and no UF; group 2 = 0.01% and UF; group 3 = 0.1% and no UF; group 4 = 0.1% and UF; group 5 = 1.0% and no UF; group 6 = 1.0% and UF. UF = ultrafiltration. Different letters are significantly different ($p<0.05$) within each group

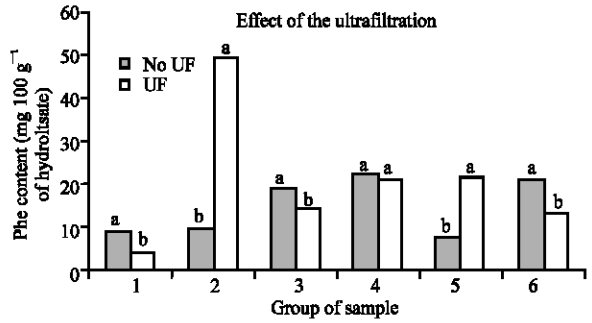

Fig. 3: Effect of ultrafiltration over Phe removal. Groups of samples, according to the conditions employed for preparing the hydrolysates: group 1 = 0.01% and 25°C; group 2 = 0.01% and 50°C; group 3 = 0.1% and 25°C; group 4 = 0.1% and 50°C; group 5 = 1.0% and 25°C; group 6 = 1.0% and 50°C. UF = ultrafiltration. Different letters are significantly different ($p<0.05$) within each group

least final amount of Phe (in mg 100 mg^{-1} of hydrolysate), related to the reduction of production costs of dietary supplements for phenyketonurics, occurred in groups 2 and 5. No report concerning the effect of the temperature on the Phe removal was found in the literature.

Effect of the ultrafiltration: The estimation of this treatment required that the hydrolysates were divided in 6 groups in order to keep constant the other parameters (E:S ratio and the temperature): group 1 = 0.01% and 25°C; group 2 = 0.01% and 50°C; group 3 = 0.1% and 25°C; group 4 = 0.1% and 50°C; group 5 = 1.0% and 25°C; group 6 = 1.0% and 50°C. As described b efore for E:S ratio and temperature, the UF also influenced the Phe removal (Fig. 3). The desirable effect associating the absence of UF with the least final amount of Phe (in mg 100 mg^{-1} of hydrolysate), related to the reduction of production costs of dietary supplements for phenyketonurics, occurred in groups 2 and 5. No effect of the UF was observed in group 4 and the absence of UF was disadvantegous for groups 1, 3 and 6.

On the other hand, not only the economical aspect of UF but also other approaches may be taken into consideration. Although the UF is an expensive process, it shows some immunological and nutritional advantages such as reduction of the allergenicity of proteins and their enzymatic hydrolysates as well as the improvement of dietary formulas by increasing their oligopeptide content (Van Beresteijn *et al*., 1994; Exl, 2001; Chandra, 2002). Thus, a precise evaluation of the costs and benefits must be undertaken before choosing the best procedure to follow. No report concerning the effect of ultrafiltration on the Phe removal was found in the literature.

CONCLUSION

It was possible to remove up to 98 or 95% of phenylalanine from whey hydrolysates using activated carbon and the adsorption resin XAD-4, respectively. The E:S ratio, the temperature of hydrolysis and the ultrafiltration affected diversely the Phe removal, depending on the conditions employed for preparing the hydrolysates, but for all samples the use of either AC or resin led to final Phe contents inferior to the maximum value established by the Brazilian Legislation for products intended for phenylketonurics.

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