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WPC Hydrolysates Obtained by the Action of a Pancreatin: Preparation, Analysis and Phenylalanine Removal

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ABSTRACT

The aim of this work was to use a pancreatin to obtain Whey Protein Concentrate (WPC) hydrolysates with high degree of hydrolysis, appropriate peptide profiles from the nutritional point of view as well as with reduced Phenylalanine (Phe) content. Six hydrolysates were prepared by varying the enzyme: Substrate ratio and the substrate concentration. The degree of hydrolysis was calculated by the ratio between α-amino and total nitrogen. The analysis of peptide profile involved the fractionation of hydrolysates by high performance size-exclusion liquid chromatography and the rapid correct fraction area method was used to quantify the chromatographic fraction components. The activated carbon was used to remove Phe and the efficiency of this procedure was evaluated by measuring the amount of this amino acid by second derivative spectrophotometry. The results showed that the degree of hydrolysis changed from 15 to 30%. An appropriate peptide profile was obtained with high free amino acid (55.43%) and low large peptide (15.75%) contents as well as an amount of di and tripeptides greater than 6%. Also, Phe removal changed from 59.1 to 81.3%. The economical advantage of using the smallest enzyme: Substrate ratio (1:100) was associated to the achievement of the best peptide profile. Thus, the use of a pancreatin in the hydrolytic conditions tested in the current study produced WPC hydrolysates with high degree of hydrolysis, suitable peptide profile and reduced Phe content.

Key words: WPC, enzyme, hydrolysis, peptide profile, phenylalanine

INTRODUCTION

Several protein sources may be used for preparing protein hydrolysates. Among them, 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 emergent 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 readily assimilated by the organism and shows a high protein efficiency ratio (Nicolau *et al*., 1997). Moreover, the use of whey, a waste of milk industries in some contries, may contribute to reduce the environment pollution (Smithers, 2008; Barbosa *et al*., 2010).

The use of whey *in natura* is limited although it is considered as an important nutritional source of proteins, due to its characteristics of perishable material, the high dilution of its

components and the mineral content. Thus, different technologies have been developed in order to add value to this raw material notably those involving the separation of proteins by membranes giving rise to Whey Protein Concentrate (WPC) and whey protein isolate (Brans *et al*., 2004).

Among the possibilities that promotes an exponential aggregated value to WPC is the enzymatic hydrolysis of its proteins. This treatment promotes the breakdown of the protein molecule into smaller units and has been outstanding in improving its nutritional, sensorial and functional properties (Boza *et al*., 2000; Gad and Sayed, 2009). It allows a wide application of WPC in food industries either in the development of dietetic preparations or as ingredient in a variety of products (Brans *et al*., 2004; Onwulata and Tomasula, 2006; Mongo, 2006; Asghar *et al*., 2009).

Several proteolytic enzymes have been used for preparing protein hydrolysates with dietary applications (Boza *et al*., 2000; Clemente, 2000; Morato *et al*., 2000; Carreira *et al*., 2004; Lopes *et al*., 2005a; Soares *et al*., 2006). After having tested some enzymes for this purpose, in this work was decided to use a pancreatin which is an enzymatic complex consisting of enzymes secreted by the pancreas having proteolytic, amylolytic and lipolytic activities. These proteases are divided into endopeptidases (trypsin, chymotrypsin and elastase) and exopeptidases (carboxypeptidases A and B) (Park, 2001).

The interest in protein hydrolysates is associated with the preparation of dietetic supplements for several purposes. In this way, different protein sources, enzymes and hydrolytic conditions have been testing in order to have an appropriate peptide profile from the nutritional point of view, i.e., a high oligopeptide content. Also, since long time ago some studies have been examining several analytical methods of protein hydrolysates (Silvestre *et al*., 1993a, b; Silvestre *et al*., 1994a, b; Silvestre, 1997).

One of the main criteria for characterizing protein hydrolysates for dietary purpose is related to the evaluation of the degree of hydrolysis. Another important one is related to the establishment of the distribution of their peptides according to the size, since those containing high content of oligopeptides, especially di and tripeptides are used more effectively (Keohane *et al*., 1985; Grimble *et al*., 1986; Rerat, 1993; Boza *et al*., 2000).

The Degree of Hydrolysis (DH) or the extent of proteolysis may be assessed by the calculation of the percentage of peptide bond cleavage in protein molecules. This evaluation depends on three basic principles: The quantification of the nitrogen released by protein hydrolysis, the determination of free amino groups and the titration of protons released (Wang and Wang, 2001).

Some chromatographic techniques have been described in the literature for fractionating protein hydrolysates according to their peptide size. However, they have shown several drawbacks, such as interactions between the solute and the stationary phase and the inefficiency for separating the small peptides (Lemieux *et al*., 1991; Schmidt and Poll, 1991; Davis and Lee, 1992; Golovchenko *et al*., 1992; Visser *et al*., 1992).

Aiming to reduce this problem (Silvestre *et al*., 1994a) developed an efficient method for fractionating and quantifying peptides from protein hydrolysates. They used a molecular exclusion chromatography column containing the complex poly (2-hydroxyethyl-aspartamide)-silica (PHEA column) which allowed the separation of peptides with molecular mass smaller than 1.000 Da. This method was used in this study after having been shown by some works to be an efficient tool in the characterization of hydrolysates from different protein sources such as whey (Biasutti *et al*., 2007; Lopes *et al*., 2007; Silva *et al*., 2007; Afonso *et al*., 2008; Souza *et al*., 2008), casein (Morato *et al*., 2000; Morais *et al*., 2002; Barbosa *et al*., 2004; Carreira *et al*., 2004), skim milk (Lopes *et al*., 2005a; Soares *et al*., 2006; Soares *et al*., 2007), rice (Lopes *et al*., 2008) and wheat flour (De Araujo, 2009).

Phenylketonuria 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). Therefore, the development of WPC with low Phe content is of great interest.

The method for removing Phe from proteins involves initially an enzyme assisted process and some studies employed different proteases and reaction conditions for hydrolyzing proteins from varied sources (Morato *et al*., 2000; Carreira *et al*., 2004; Lopes *et al*., 2005a; Morais *et al*., 2005; Silva *et al*., 2007; Soares *et al*., 2007). Then, Phe removal is achieved using varied adsorbent supports. Among them, different studies have already tested the activated carbon (Lopes *et al*., 2005b; Soares *et al*., 2006; Capobiango *et al*., 2007; Silva *et al*., 2007; Lopes *et al*., 2008) and a resin (Delvivo *et al*., 2006).

The evaluation of the efficiency of Phe removal is achieved by quantifying this amino acid in the protein source and in its hydrolysates after activated carbon treatment. The second derivative spectrophotometry has been successfully used for this purpose in different foods (Delvivo *et al*., 2006; Soares *et al*., 2006; Lopes *et al*., 2008; Carreira *et al*., 2009).

This work has represented an important step for obtaining low-Phe WPC, since the hydrolysis process of its proteins using a pancreatin was studied in terms of degree of hydrolysis, peptide profile and Phe removal where the activated carbon was used as the adsorbent support. Also, the effect of some parameters, such as enzyme: Substrate ratio (E:S) and substrate concentration was evaluated.

MATERIALS AND METHODS

Materials: The WPC in powder form (Kerrylac 750) was kindly furnished by Kerry do Brazil Ltda (Tres Coraçoes, MG, Brazil). This product contains 32.6% of proteins, 5.1% of moisture, 0.2% of lipids, 7.4% of ash and 54.8% of lactose. Pancreatin (Corolase® PP) was kindly furnished AB Enzymes (Darmstadt, Germany). The HPLC system consisted of one pump (HP 1100 Series), an UV-VIS detector, coupled to a computer (Hp-chemstation HP1100, Germany). A poly (2-hydroxyethylaspartamide)-silica (PHEA) column, 250×9.4 mm, 5 μm, 200 Å pore size (PolyIC, Columbia, MD), was used for HPLC. Also, water for HPLC was purified by passage through a water purification system (Aries-Vaponics, Rockland, USA). All solvents used for the HPLC were carefully degassed by sonication for 10 min before use. The freeze dryer was from Labconco (77500 model, Kansas City, MI, USA) and the stirrer from Fisatom (São Paulo, SP, Brazil). The amino acids L-phenylalanine, L-tyrosine and L-tryptophan were purchased from Sigma-Aldrich (St. Louis, MO, USA). The activated carbon (granular N° 119.20×50, 12×25, 6×12 mesh Tyler Series) was purchased from Carbomafra SA (Curitiba, PR, Brazil). All reagents used were of analytical grade.

Methods

Preparation of protein hydrolysates: Six WPC hydrolysates were prepared varying the following parameters: E:S and concentration of WPC (Table 1). A volume of 100 mL of a 10% (w/v) WPC solution was initially placed in a flask and the pH and temperature were adjusted to the optima values of pancreatin given by its supplier. Then, the enzyme was added in an appropriate quantity to obtain the E:S ratio desired. After 5 h, the reaction was stopped by heating the flask in a water bath at 75°C for 15 sec, in order to inactivate the enzyme which was confirmed by measuring the enzyme activity before and after the enzymatic treatment using the method described by Dias *et al*. (2008) without addition of casein.

Table 1: Hydrolytic conditions employed for preparing hydrolysates from whey protein concentrate using a pancreatin

Hydrolysates	pH	Temperature (°C)	E:S	WPC concentration (w/v)
H1	7	50	1:100	10%
H2	7	50	2:100	10%
H3	7	50	4:100	10%
H4	7	50	1:100	7%
H5	7	50	1:100	8%
H6	7	50	1:100	9%

E:S: Enzyme:substrate ratio, WPC: Whey protein concentrate

Determination of the degree of hydrolysis: The DH was calculated using the relationship between α-Amino Nitrogen (AN) and Total Nitrogen (TN) according to Eq.1:

$$\%\,DH = \frac{\alpha\text{-Amino Nitrogen (AN)}}{\text{Total Nitrogen (TN)}} \times 100 \quad (1)$$

The Sorensen method (AOAC, 1995) based on titration with formaldehyde was used for quantifying the AN. The formaldehyde reagent was prepared by diluting 25 mL of the commercial solution with 50% ethanol up to 250 mL final volume. The pH was adjusted to 7.0 with 0.2 mol L^{-1} NaOH just before use. As the pH of a sample of hydrolysed solution was also adjusted to 7.0 with 0.2 mol L^{-1} NaOH. Ten millilhter of the formaldehyde reagent was added to 10 mL of the hydrolysed solution, the mixture was stirred and then titrated with 0.2 mol L^{-1} NaOH, with phenolphthalein as indicator, to its final end-point. An excess of 0.2 mol L^{-1} NaOH was added and then back-titrating with 0.2 mol L^{-1} HCl until colourless. The volumes of NaOH and HCl required were recorded. The TN was determined by the Kjeldahl method (AOAC, 1995).

Characterization of peptide profiles of the hydrolysates: This characterization was performed in two stages: Fractionation of peptides, according to their size, followed by their quantification. The fractionation of WPC 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 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 WPC hydrolysates. The samples were fractionated and the CFA values calculated with aid of a standard curve, prepared by using WPC as the substrate. Briefly, five WPC 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 our group (Silvestre *et al.*, 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 WPC hydrolysates prepared by using the subtilisin and the pancreatin described below, their CFA were taken to this curve.

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 WPC and in its hydrolysates after AC treatment, using the Sec 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. 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 as well as to verify the effect of some parameters over DH, peptide profiles and Phe removal. 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). The software used to analyse the data was Microsoft Office Excel 2003.

RESULTS AND DISCUSSION

Degree of hydrolysis: The results of the DH of WPC hydrolysates are shown in Table 2. It can be observed that the values showed a wide range of values ranging from 15 to 30%. As can be seen on Table 1, the reaction condition used for preparing H3 produced the highest DH and corresponds with an E:S ratio of 4:100 and a concentration of WPC of 10% (w/v). No report concerning the DH of WPC hydrolysates prepared by the action of a pancreatin was found in the literature.

Peptide profiles of protein hydrolysates: As illustration, the chromatographic pattern of hydrolysate H1, at 230 nm, is shown in Fig. 1. The hydrolysates were resolved in four fractions: Fraction 1 corresponding to peptides containing more than 7 amino acid residues, fraction 2 to those containing from 4 to 7 residues, fraction 3 to di and tripeptides and fraction 4 to free amino acids. The last two peaks in fraction 4 correspond to free tyrosine (peak Y) and tryptophan (peak W). The SE-HPLC technique used here showed to be efficient in fractionating protein hydrolysates, especially peptides of molecular mass lower than 1.000Da, as reported successfully by some studies

Table 2: Degree of hydrolysis of WPC hydrolysates

Hydrolysates	DH (%)
H1	19^{c}
H2	23^{b}
H3	30^{a}
H4	15^{d}
H5	$17^{c,d}$
H6	$17^{c,d}$

WPC: Whey protein concentrate, DH(%): Degree of hydrolysis in percentage of cleavage of peptide bonds. Different letters are significantly different ($p<0.05$)

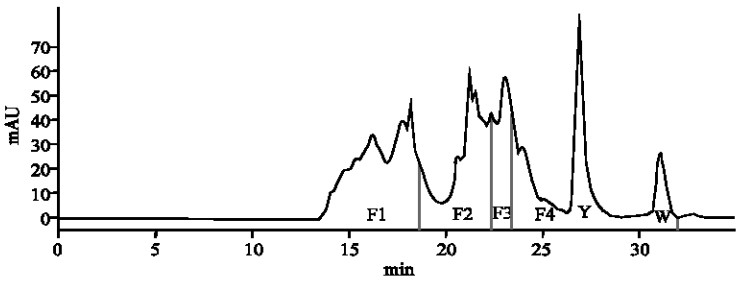

Fig. 1: Chromatographic profile of hydrolysate H1 at 230 nm. PHEA column. Mobile Phase: 0.05 mol L^{-1} formic acid. F1: large peptides (> 7 amino acid residues), F2: medium peptides (4 to 7 amino acid residues), F3: di and tripeptides, F4: free amino acids, Y: tyrosine peak, W: tryptophan peak. Hydrolysate H1: substrate concentration = 10% (w/v), E:S ratio = 1:100

using different protein sources such as casein (Morato *et al.*, 2000; Carreira *et al.*, 2004; Morais *et al.*, 2005), milk (Lopes *et al.*, 2005a), rice (Lopes *et al.*, 2008), whey (Silva *et al.*, 2007; Souza *et al.*, 2008; Silva *et al.*, 2009) and wheat flour (Carreira *et al.*, 2010).

Peptide and amino acid contents of WPC hydrolysates: The amount of peptides and free amino acids of the hydrolysates is shown in Table 3. The best peptide profile, from the nutritional point of view, was achieved for the hydrolysate H1, since it showed the highest amount of free amino acid (55.43%), one of the highest di and tripeptide contents (6.51%) as well as the lowest large peptide (15.75%) content. In this case, an E:S ratio of 1:100 and a concentration of WPC of 10% were used. The peptide profiles of hydrolysates H4 and H5 come just after H1 and were very close, with the only advantage of H4 over H5 concerning its higher di and tripeptide content.

According to Frenhani and Burini (1999), during the metabolism of proteins, the first stage of their hydrolysis leads to the formation of oligopeptides containing 2 to 6 amino acid residues and free aminoacids. Then, these peptides are broken to di and tripeptides. Finally, the proteins are absorbed in the form of di and tripeptides as well as of free amino acids where the absorption of the formers is quicker. Gonzalez-Tello *et al.* (1994) also reported the advantage of the di and tripeptides over the free amino acids, concerning the velocity of absorption.

No report of other authors concerning the action of a pancreatin on the peptide profile of WPC hydrolysates was found in the literature. Thus, the results of the present work were compared with those obtained in three works previously carried out for whey hydrolysates, having been found several results.

In the first one, a different pancreatin (Sigma, P-1500) was used and the whey hydrolysates were treated by activated carbon and ultrafiltrated, before the characterization of peptide profiles (Delvivo *et al.*, 2006). As expected, ultrafiltration enriched the hydrolysates in di-and tripeptides whose content was much higher (21%) than H1. However, this procedure gave rise to an amount of free amino acids around the half (23%) of H1. Another inconvenient of this study refers to the fact that the whey concentration used for preparing the hydrolysates (1.06%) is almost ten times smaller than the one used here for WPC which would largely increase the cost for a scaling-up process.

The use of this same pancreatin immobilized on activated carbon was tested in the second work for hydrolysing whey and produced a peptide profile containing much higher large peptide (58%)

Table 3: Peptide and free amino acid contents of chromatographic fractions of WPC hydrolysates

Hydrolysates	F_1 >7 AA	F_2 4-6 AA	F_3 2-3 AA	F_4 free AA
H1	$15.75^{d/3}$	$22.29^{c/2}$	$6.51^{a,b/4}$	$55.43^{a/1}$
H2	$53.99^{b/1}$	$19.39^{c/3}$	$2.76^{c/4}$	$23.86^{c/2}$
H3	$80.64^{a/1}$	$9.81^{d/2}$	$5.75^{b/3}$	$3.80^{e/4}$
H4	$21.35^{c/3}$	$31.49^{a/2}$	$9.12^{a/4}$	$38.04^{b/1}$
H5	24.20^{c2}	$33.23^{a/1}$	$5.37^{b/3}$	$37.17^{b/1}$
H6	$52.27^{b/1}$	$27.10^{b/2}$	$5.14^{b/4}$	$15.47^{d/3}$

Values are in (%) of nnmols of the four fractions and represent the means of triple repetition. AA: Amino acids residues. Different numbers are significantly different ($p<0.05$) for different fractions of the same hydrolysate. Different letters are significantly different ($p<0.05$) for the same fraction of different hydrolysates

and much lower free amino acid (2%) contents than H1 (Silva *et al.*, 2007). The only advantage of the peptide profile obtained in this study compared to H1 refers to its amount of di-tripeptides which was more than the double (15%) of H1.

Finally, Biasutti *et al.* (2008) used the same enzyme of the current work for hydrolyzing proteins of whey. The only advantage of the peptide profile obtained in this study was related to the di and tripeptides content (12.42%) which was the double of H1. However, the amount of large peptides was much higher (28.93%) and that one of free amino acids the half (22.02%) of H1.

Efficiency of phenylalanine removal: The results obtained for Phe removal from WPC hydrolysates are shown in Table 4. The Phe content of WPC was 2106.7 mg Phe 100 g^{-1} product. It can be noted that Phe removal changed from 59.1 to 81.3% and the final Phe content from 394.1 to 861.8 mg 100 g^{-1} of product. The condition that produced the best result was the one using an E:S ratio of 4:100 and a WPC concentration of 10% (w/v).

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

No work was found in the literature concerning Phe removal from WPC hydrolysates. Instead, other milk proteins or milk products were reported, using activated carbon as adsorbent. Thus, Kitagawa *et al.* (1987), after hydrolysing whey proteins with actinase, at pH 6.5 at 37°C, treated these preparations with activated carbon and removed 97% of Phe. However, the conditions for the treatment with this adsorbent were not mentioned. 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 Idziac (1993) removed 95% of Phe from casein hydrolysates. However, these authors employed more severe conditions than those used by our group, that is, a very long time for hydrolysis (72 h). The differences observed among all these results and those obtained here, may also be associated to other factors such as type of proteolytic enzymes, protein: AC ratios and protein sources.

Also, the activated carbon has been previously used by our group for removing Phe from hydrolysates prepared by using different protein sources, such as milk (93.6 to 99%) (Soares *et al.*, 2006; Lopes *et al.*, 2006), whey (75 to 99%) (Silva *et al.*, 2007; Delvivo *et al.*, 2006), rice grains (85 to 100%) (Lopes *et al.*, 2008), rice flour (25.7 to 94.1%) (Silvestre *et al.*, 2009a,b), corn flour (68.63 to 97.55%) (Capobiango *et al.*, 2007) and beans (25.4 to 81.5%) (Lopes *et al.*, 2009).

Table 4: Phe removal and Phe contents of samples

Samples	Phe removal (%)	Final Phe content (mg Phe 100 g^{-1} of sample)
WPC	-	2106.7
H1	63.2[b]	775.3
H2	63.0[b]	778.6
H3	81.3[a]	394.1
H4	62.7[b]	785.5
H5	62.3[b]	794.8
H6	59.1[b]	861.8

WPC: Whey protein concentrate, Phe: Phenylalanine. Different letters are significantly different ($p<0.05$) for different hydrolysates

Effect of some parameters on degree of hydrolysis, peptide profile and phenylalanine removal: Two of the parameters which may influence these three characteristics of WPC hydrolysates 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 desired since it is associated with the use of a smaller amount of enzyme needed to hydrolyze proteins. Also, a higher Substrate (WPC) Concentration (SC) for preparing the hydrolysates is advantageous because it is associated with the reduction of final volume and therefore with the time and the investment for the drying stage.

Effect of E:S ratio: The effect of E:S ratio on these three characteristics of WPC hydrolysates may be evaluated by comparing the results obtained for hydrolysates H1 (1:100), H2 (2:100) and H3 (4:100). First of all, one can observe in Table 2 that the higher the E:S ratio, the larger was the DH of the hydrolysates which indicates that the advantage of using a lower E:S ratio was not observed. No report concerning the effect of E:S ratio on DH of protein hydrolysates was found in the literature.

Regarding the effect of E:S ratio on the peptide profiles, it can be observed in an association between the results of Table 1 and 3 that the use of the lowest E:S ratio (1:100) was beneficial because, compared with 2:100 and 4:100 produced much smaller amount of large peptides and much greater free amino acid content. In respect to the quantity of di and tripeptides, the value of 1:100 gave rise to a similar amount of 4:100 but the double of 2:100.

No report of other authors concerning the effect of E:S ratio on peptide profiles of protein hydrolysates was found in the literature. However, some studies has already evaluated this effect for protein hydrolysates from varied protein sources and hydrolytic conditions. In some of these studies, the use of a lower E:S improved the peptide profile of protein hydrolysates (Barbosa *et al.*, 2004; Silva *et al.*, 2010) while in others this benefit was not observed (Morato *et al.*, 2000).

As shown in Table 1 and 4, the desired effect of using a lower E:S ratio to produce a higher Phe removal was not found, because the value of 4:100 led to the highest percentage. Three studies reported concerning the effect of E:S ratio on Phe removal of protein hydrolysates. In the first one was used the corn flour as raw-matter and in some cases the advantage of using lower E:S ratio was observed, notably when comparing Phe removal between 1:100 and 2:100 which were 86.68% and 79.01%, respectively (Capobiango *et al.*, 2007). For the protein hydrolysates of rice the E:S ratio had no effect on Phe removal (Lopes *et al.*, 2008). In the study where protein hydrolysates from beans were analyzed, the advantage of using a lower E:S ratio was not observed, since the highest Phe removal was achieved with an E:S ratio of 10:100, compared with 4:100, 5:100 and 7:100 (Lopes *et al.*, 2009).

Effect of substrate concentration: The effect of SC on these three characteristics of WPC hydrolysates may be evaluated by comparing the results obtained for hydrolysates H4 (7%), H5 (8%), H6 (9%) and H1 (10%). It can be observed in Table 2 that the highest DH (30%) was obtained when the more elevated WPC concentration (H1-10%) was used and no significant difference was observed among the results found for the other values of concentration (H4-15%, H5-17% and H6-17%) These results show that the advantage of using a lower WPC concentration was not observed. No report concerning the effect of substrate concentration on DH of protein hydrolysates was found in the literature.

The analysis of the effect of SC on peptide profiles of WPC hydrolysates may be done by evaluating the results shown in Table 3 where it can be seen that the best peptide pattern was achieved for the highest SC value (H1-10%), followed by 7% (H4) and 8% (H5) for which the only difference was observed in relation to di and tripeptide contents that was bigger for the first one. Thus, the advantage of using a smaller SC concentration was not observed. No report of other authors concerning the effect of substrate concentration on peptide profiles of protein hydrolysates was found in the literature. Our group carried out one study concerning this subject with whey hydrolysate and contrarily to the current work it was demonstrated that a SC concentration of 10% produced a better peptide profile than 15% (Souza *et al.*, 2008).

The values of Table 4 indicate that the SC had no effect on Phe removal from WPC hydrolysates, because no significant difference was observed among the results obtained for H4 (7%), H5 (8%), H6 (9%) and H1 (10%).

However, in two previous studies, contrarily to the current work, the SC affected Phe removal and in one of them the advantage of using a smaller value was shown because a SC of 1 g 100 mL^{-1} of corn flour led to a Phe removal of 97.3% while only 68.7% of Phe were removed using a SC of 2 g 100 mL^{-1} (Capobiango *et al.*, 2007). Working with rice, the opposite happened and 95% of Phe were removed with a SC of 3 g 100 mL^{-1} and 90% with an SC of 2 g 100 mL^{-1} (Lopes *et al.*, 2008).

CONCLUSION

The parameters tested in the current work in the action of a pancreatin for hydrolysing WPC were able to produce high degree of hydrolysis (up to 30%), high free amino acid (55.43%) and low large peptide (15.75%) contents as well as a fair amount of di and tripeptides (6.51%). Also, it was possible to obtain a level of Phe removal over 80%. The benefit of using the smallest E:S ratio (1:100), it terms of reduction of costs for scaling up the process, was observed in the achievement of the best peptide profile. However, this advantage was not shown in any case for the SC, since the best results for DH, peptide profile and Phe removal were found for the highest SC (10%).

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