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Use of Subtilisin and Pancretin for Hydrolyzing Whey Protein Concentrate

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ABSTRACT

The objective this study was to evaluate the preparation of protein hydrolysates with an appropriate peptide profile as well as the reduction of costs for scaling-up the process, the actions of subtilisin and pancreatin on Whey Protein Concentrate (WPC) were tested. The effect of Enzyme: Substrate ratio (E:S) and Substrate Concentration (SC) was also verified. The hydrolysates were fractionated by size-exclusion-HPLC and the rapid correct fraction area method was used for quantifying peptides and free amino acids. The best peptide profiles were very similar giving rise to 13.17% of di-tripeptides, 46.41% of free amino acids and 12.31% of large peptides on an average. The advantage of using a higher SC was observed only in one case and for the pancretin action. The use of a smaller E:S ratio for subtilisin action was advantageous only when comparing 2:100 with 4:100 while for pancretin this beneficial result was also achieved when comparing either 1:100 with 2:100 or 2:100 with 4:100. It can be inferred that the use of these two enzymes was able to produce some WPC hydrolysates with appropriate peptide profiles for dietary purposes.

Key words: Whey protein concentrate, protein hydrolysates, peptide profile, enzymes, reduction of costs

INTRODUCTION

Whey is the major by-product in the manufacture of cheese or casein from milk, representing 80 and 90% of the volume of transformed milk (Moreno-Indias *et al.*, 2009). Traditionally, it was regarded by cheese producers as waste with little or no commercial value (Walzem *et al.*, 2002), used for animal feed or dumped in sewage without any treatment (Voorbergen and Zwanenberg, 2002).

The use of whey *in natura* is limited although it is considered an important nutritional source of proteins, due to its characteristics of perishable material, the high dilution of its components and the mineral content (Silva, 2010). Despite all the possible applications in food and pharmaceutical industries, its high Biochemical Oxygen Demand (BOD), mainly associated with the presence of lactose and protein, makes the whey a strong agent of environmental pollution

(Moreno-Indias *et al.*, 2009). 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).

Also, it is of great interest to create options to use whey or its isolated proteins and this study represents a contribution in this direction. Among the possibilities that promotes an exponential aggregated value to whey is the use of enzymatic hydrolysis of proteins, since this treatment promotes the breakdown of the protein molecule into smaller units and has been outstanding in improving the nutritional, sensorial and functional properties of proteins (Schmidt and Salas-Mellado, 2009).

In this study, the hydrolytic enzymes of choice were subtilisin and pancreatin. The former is originated from various species of the genus *Bacillus* sp. and its action occur mainly in the peptide bonds involving the amino and carboxyl groups of aromatic or hydrophobic residues, such as tyrosine, phenylalanine and leucine, especially on the C-terminal side (Gupta *et al.*, 2002; Page and Di Cera, 2008). The second is an enzymatic complex consisting of enzymes secreted by the pancreas which have proteolytic, amylolytic and lipolytic activities. These proteases are divided into endopeptidases (trypsin, chymotrypsin and elastase) and exopeptidases (carboxypeptidases A and B) (Park, 2001).

Protein hydrolysates originated by enzymatic treatment have been used for therapeutic purposes for maintaining the nutritional status of individuals with nutritional or physiological needs not covered by conventional foods (Clemente, 2000). Moreover, in the hydrolysed form, proteins may show improved functional properties, depending on the reaction conditions employed (Silva and Silvestre, 2003; Bizzotto *et al.*, 2005; Capobiango *et al.*, 2006; Biasutti *et al.*, 2007; Chicon *et al.*, 2009).

One of the main criteria for characterizing protein hydrolysates for dietary purpose is to establish the distribution of their peptides according to the size. The hydrolysates 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).

Some chromatographic techniques have been described in the literature for fractionating protein hydrolysates. This techniques 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, group has developed an efficient method for fractionating and quantifying peptides from protein hydrolysates, using 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 (Silvestre *et al.*, 1994a). This method was used in this study after having been shown by the group to be an efficient tool in the characterization of hydrolysates from different protein sources (Silvestre *et al.*, 1994b; Morato *et al.*, 2000; Carreira *et al.*, 2004; Lopes *et al.*, 2005; Morais *et al.*, 2005; Lopes *et al.*, 2008; De Souza *et al.*, 2008; Silva *et al.*, 2009).

Thus, the goal of the present study was to optimize WPC hydrolysis, using a subtilisin and a pancreatin, for obtaining an appropriate peptide profile from the nutritional point of view.

MATERIALS AND METHODS

This study was conducted in April 2009 until February, 2011 in Company of Technological Development (EDETEC) and University of Minas Gerais.

Material: Whey Protein concentrate-WPC-(Kerrylac 750), in a powder form, was kindly furnished by Kerry of Brazil Ltda. (Três corações, Minas Gerais, Brazil). A subtilisin (Protemax N200, from *B. subtillis,* activity = 12.4 U mL^{-1}, where one unit of activity is defined as the amount of enzyme that liberates 1 μg of tyrosine in one min at 37°C) was kindly furnished by Prozyn (São Paulo, SP, Brazil). A pancreatin (Corolase PP, from porcine pancreas, activity = 18.9 U mL^{-1}, where one unit of activity is defined as the amount of enzyme that liberates 1 μg of tyrosine in one min at 37°C) was kindly furnished by AB Enzymes® (Barueri, SP, Brazil). The HPLC system consisted of one pump (HP 1100 Series), 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. Also, water for HPLC was purified by passage through a water purification system (Aries-Vaponics, Rockland, EUA). 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).

Methods

Determination of the chemical composition of whey protein concentrate: The contents of moisture, protein, lipid, total ash and lactose were determined according to the Association of Official Agricultural Chemists Methods (AOAC, 1995). The conversion factor of nitrogen to protein was 6.38 (Nielsen, 1998).

Preparation of hydrolysates from whey protein concentrate: Twelve hydrolysates from Whey Protein Concentrate (WPC) were prepared, 06 with subtilisin and 06 with pancreatin. The pH of whey solutions (10 and 15%, w/v) was adjusted to 7.0 with a 3 mol L^{-1} Na_2CO_3 solution. The solutions were heated in an oil-bath at 55°C, under continuous stirring and the enzymes were added in such a concentration to attain the desired enzyme:substrate ratios. The total reaction time was 5 h for all samples and the hydrolytic reactions were stopped by heating at 85°C for 20 min. The hydrolysates were freeze-dried and indentified by different names (Table 1).

Characterization of peptide profiles of the hydrolysates: This characterization was performed in two stages: fractionation of peptides, according to their size, followed by their

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

Hydrolysates	Substrate concentration (w/v)	E:S
S1	10	1:100
S2	10	2:100
S3	10	4:100
S4	15	1:100
S5	15	2:100
S6	15	4:100
P1	10	1:100
P2	10	2:100
P3	10	4:100
P4	15	1:100
P5	15	2:100
P6	15	4:100

E:S = Enzyme:Substrate ratio, S = Hydrolysates prepared with subtilisin, P = Hydrolysates prepared with pancreatin

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 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 to 18.2 min (large peptides with more than 7 amino acid residues); F2, from 18.2 to 21.7 min (medium peptides, with 4 to 7 amino acid residues); F3, from 21.7 to 22.7 min (di-and tripeptides) and F4, from 22.7 to 32 min (free amino acids).

The rapid method of Correct Fraction Area (CFA) developed by 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 *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.

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 (one-way 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 protein concentrate: The results of the analysis of some components of Whey Protein Concentrate (WPC) are shown in Table 2. The values found here for proteins and lactose are close while that one for total ash is smaller in comparison to those of other authors. Although the moisture content agrees with that of the WPC furnisher (> 5%), it is much higher than the values of other authors. Concerning the amount of lipids found here, it is much

Table 2: Chemical composition of whey protein concentrate

Nutrients	Values found[a]	WPC 1	WPC 2	WPC 3
Protein	35.80	---	34.5	38.6
Moisture	8.38	>5	3.5	2.4
Lipids	0.05	---	3.5	2.8
Total ash	5.30	---	6.4	6.5
Lactose	50.18	---	52.1	49.8

All values are in g/100 g. [a]Values found in the present study. WPC 1: Values furnished by the manufacturer of KERRYLAC 750 (Kerry do Brasil Ltda, Três Corações, MG, Brazil). WPC 2: Values found by Sammel and Claus (2003) for WPC Foremost 365 (Foremost Farms, Baraboo, WI, USA). WPC 3: Values found by Mortenson *et al.* (2008)

smaller than that reported in the literature. These differences among the data may be associated to some factors such as variation of raw matter composition as well as the industrial processes used for obtaining WPC.

Chromatographic patterns of protein hydrolysates: 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 1,000 Da. This was previously reported using different protein sources such as casein (Morato *et al*., 2000; Carreira *et al*., 2004; Morais *et al*., 2005), milk (Lopes *et al*., 2005), rice (Lopes *et al*., 2008), whey in a powder form (Silva *et al*., 2007, 2009; De Souza *et al*., 2008) and wheat flour (De Araujo, 2009).

The fractionation of protein hydrolysates based on peptide chain length has been reported by several authors since many years ago (Amiot and Brisson, 1980; Vallejo-Cordoba *et al*., 1986; Deeslie and Cheryan, 1988; Lemieux and Amiot, 1989; Adachi *et al*., 1991; Armstead and Ling, 1991; Lemieux *et al*., 1991; Aubry *et al*., 1992; Visser *et al*., 1992; Parrado *et al*., 1993; Perea *et al*., 1993), however, contrarily to the method used in the present study, most of the described techniques concerns the separation of peptides of high molecular mass higher than 1,000 Da and showed some inconvenients like molecular weight overlaps, inefficiency for separating small peptides as well as hydrophobic and electrostatic interactions between solute molecules and the matrix (Kopaciewicz and Regnier, 1982; Lemieux *et al*., 1991; Golovchenko *et al*., 1992).

More recently, some authors have also reported the use of size-exclusion chromatography to characterize the peptide profile of protein hydrolysates, according to the size of peptides. However, contrarily to the method used in the present study, in none of these works it was possible to separate the di-and tripeptides from larger ones. Thus, Kammoun *et al*. (2003) used the Shodex KW 802.5 column to characterize the peptide profile of protein hydrolysates from wheat yielding four fractions: Fraction 1 (0-1,000 Da), Fraction 2 (1-2,000 Da), Fraction 3 (2-3,000 Da). Also working with wheat flour, Akiyama *et al*. (2006) used the Superdex™ Peptide HR column for fractionating protein hydrolysates and were able to separate peptides with molecular mass over 1,000 Da. Kong *et al*. (2007) evaluated the distribution of molecular mass of peptides obtained from hydrolysis of wheat gluten, using the Sephadex G -15 column and were able to notice only that the peptides showed molecular mass below 1,355 Da. The BioSepSec-4000 column used by Wang *et al*. (2007) to fractionate protein hydrolysates from wheat gluten led to the separation of peptides in 3 fractions: higher than 1,500 Da, between 15 and 10,00 Da and lower than 5,00 Da.

In another study (Peng *et al*., 2009), the molecular mass distribution of whey protein isolate hydrolysates was evaluated by Sephadex G-10 gel filtration chromatography yielded four fractions (I, II, III and IV), corresponding to molecular weigths of >40, 2.8-40, 0.1-2.8 and <0.1 kDa. However, the fraction III was comprised of two peaks that were not well resolved. The weight distribution of whey protein hydrolysates using a Sephadex G25 column (Li-Jun *et al*., 2008) showed the presence of short peptides which masses ranged from 600 to 1400 Da (Su *et al*., 2008) examined the molecular weight distribution of bovine casein pancreatic hydrolysates by high-performance size exclusion chromatography, coupled with Multiangle Laser Light Scattering (MALLS) and dynamic light scattering and obtained 6 peaks: 20.0-7.0, 7.0-3.0, 3.0-2.0; 2.0-0.8,

0.8-0.4 and <0.4 kDa. However, these authors affirmed that the absorption at 214 nm, employed in this work, was mainly caused by peptide bonds and therefore the concentrations of small peptides could be underestimated. Besides, it was difficult to use a correction factor, since the exact composition of each molecular weight fraction was not known. Therefore, the peak area of UV chromatograms at 214 nm was applied to examine the changes in the protein/peptide concentration in spite of its imperfection.

Other techniques were tested by some authors aiming the characterization of the peptide profile of protein hydrolysates, according to the size of peptides. Thus, Li-Jun *et al.* (2008) used the HPLC with electro spray coupled with mass spectrophotometer to evaluate the molecular distribution of peptides obtained from whey. However, according to them, this method was able to separate peptides with molecular mass from 300 to 1,300 Da from the largest ones. Saint-Sauveur *et al.* (2008) used the isoelectric focus in a size-exclusion mode for characterizing the peptide profile of WPI hydrolysates and reported that this method was not able to separate di- and tripeptides, giving rise to the following fractions of peptides: larger that 10 kDa, between 5 and 10 kDa, between 2 and 5 kDa and smaller than 2 kDa.

In the study of Contreras *et al.* (2010) the permeate from whey protein concentrate hydrolyzed with thermolysin was subjected to RP-HPLC coupled on line to a mass spectrometer. Nineteen small peptides containing between 3 and 10 amino acid residues were identified. Pouliot *et al.* (2009) characterized by LC-MS a total of 24 peptide sequences from a tryptic hydrolysate of whey proteins with molecular mass ranging from 208.2 to 2928.4 Da.

Peptide and amino acid contents of WPC hydrolysates: The amount of peptides and free amino acids of the hydrolysates is shown in Table 3. In order to choose the hydrolysate which showed the best peptide profile from the nutritional point of view, the statements of some authors must be considered. 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.

Thus, it can be inferred that among the hydrolysates prepared with subtilisin and pancreatin, S3 (substrate concentration = 10%; E:S = 4:100) and P6 (substrate concentration = 15%; E:S = 4:100) showed the best peptide profiles which were very similar, because they showed the smallest large peptide content (12.28 and 12.34%, respectively) and one of the highest amount of di- and tripeptides (13.34 and 13.00%, respectively) as well as free amino acids (45.56 and 47.26%, respectively). Moreover, P2 showed a peptide profile very close to these two ones, losing only with respect to its little higher amount of large peptides (14.55%). Other two hydrolysates (P1 and P5), also obtained by the action of pancreatin, showed good peptide profiles and the only disadvantages compared to S3 and P6, were their higher content of large peptides and lower of di- and tripeptides (Table 3).

Relating to the former enzyme, Morato *et al.* (2000) evaluated the effect of a subtilisin (Carlsberg, Sigma, from *B. Licheniformis*) different from that used here for preparing casein hydrolysates. These authors obtained a better peptide profile concerning their much higher di- and tripeptides contents (36%) and much lower amount of large peptides (14%). However, their free amino acid content was strongly low (4%). Also, it is worth stating that the substrate concentration

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

Hydrolysates	F1 (> 7 AA residues)	F2 (4-7 AA residues)	F3 (2-3 AA residues)	F4 (Free AA)
S1	51.91 b/1	19.49 d,e,f/3	6.51 d/4	22.08 e,f/2
S2	49.29 b,c /1	21.18 d/3	6.18 d/4	23.34 e/2
S3	12.28 g/3	28.80 a,b/2	13.34 a/3	45.56 a,b,c/1
S4	50.13 b,c/1	21.99 d/2	7.89 c/4	19.97 f/3
S5	48.28 c/1	22.65 c,d/2	7.80 c/3	21.25 f/2
S6	43.36 d/1	23.92 c/2	6.43 d/3	26.27 d/2
P1	18.02 e/3	29.06 a/2	8.16 c/4	44.75 a,b,c/1
P2	14.55 f/3	29.38 a/2	12.13 a,b/4	43.92 b,c/1
P3	45.37 d/1	21.79 d/3	6.34 d/4	26.48 d/2
P4	68.67 a/1	17.13 f/2	3.88 e/4	10.30 g/3
P5	16.34 e/3	27.96 b/2	11.61 b/4	44.07 b,c/1
P6	12.34 g/3	27.37 b/2	13.00 a/3	47.26 a/1

Values are in % of nmoLs of the four fractions and represent the means of triple repetition. 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. AA = Amino acid. S = Hydrolysates prepared with subtilisin. P = Hydrolysates prepared with pancreatin

used in the study with casein was 80-120 times lower (0.125% of casein) than that one used here (10 and 15% of whey) which would increase the costs associated to the drying stage, a serious inconvenient for scaling-up the process.

In another study, Biasutti *et al.* (2008) using the same subtilisin of the present work to hydrolyze whey, giving rise to a poorer peptide profile than S3, mainly in relation to the free amino acid (35.29%) and large peptide (22.91%) contents.

Concerning the pancreatin action, three works were carried out. 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 P6. However, this procedure gave rise to an amount of free amino acids around the half (23%) of P6. Another inconvenient of this study refers to fact that the whey concentration used for preparing the hydrolysates (1.06%) is almost ten times smaller than the one used here, 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%) and lower free amino acid (2%) contents than P6 (Silva *et al.*, 2007). The small superiority of this peptide profile compared to P6 refers to its amount of di-tripeptides, which was a little higher (15%) than P6. Finally, Biasutti *et al.* (2008) used the same enzyme of the present work for hydrolyzing proteins of whey and, although a similar di- and tripeptides content (12.42%) to the one obtained here for P6 was found, the amount of free amino acids (22.02%) was the half and that of large peptides (28.93%) was more than the double of P6.

Comparison between different enzymatic treatments: The results of this study were compared only with those obtained in the same laboratory, since no report coming from other authors involving the action of subtilisin or pancreatin on peptide profiles of whey or WPC hydrolysates was found in the literature.

For the analysis of the effect of some parameters on peptide profile, the reduction of costs for scaling-up the process was also considered. In this way, the use of high initial substrate

concentration is associated with the reduction of final volume and therefore with the time and the investment for the drying stage. Also, a small Enzyme:Substrate (E:S) ratio is associated to the reduction of the enzyme quantity.

Effect of enzyme type: Although the comparison between the best results obtained by the use of the two enzymes (S3 with P6) showed no effect of the type of enzyme on peptide profiles of WPC hydrolysates, it can be inferred by the data in Table 3 that, in terms of number of samples that presented an appropriate peptide profile, the action of pancreatin was more advantageous than subtilisin. Among the six samples prepared, four showed a good peptide profile, from the nutritional point of view (P1, P2, P5 and P6), while the same happened only with one hydrolysate prepared by using subtilisin (S3).

In a previous study of Biasutti *et al.* (2008), these same enzymes were used for hydrolyzing whey and all the six hydrolysates prepared with pancreatin showed good peptide profiles, close to those of S3 and of pancreatic hydrolysates of the present work, while none of the ones using subtilisin could be considered satisfactory, from the nutritional point of view.

Effect of substrate concentration: Concerning the action of subtilisin, the following samples must be compared in order to analyze the effect of Substrate Concentration (SC) on peptide profile: S1 with S4 (E:S = 1:100), S2 with S5 (E:S = 2:100) and S3 with S6 (E:S = 4:100). As shown in Fig. 1a, no advantage of using higher substrate concentration was observed, since the use of a value of 15% produced similar (S1 with S4 and S2 with S5) or poorer (S3 with S6) peptide profiles compared to 10% (Fig. 1a). However, in a previous study of Biasutti *et al.* (2008), this same enzyme was used for hydrolyzing whey and the advantage of using 15% of raw matter compared to 10% occurred in one case (E:S = 1:100), related to its lower large peptide and higher di-tripeptides contents.

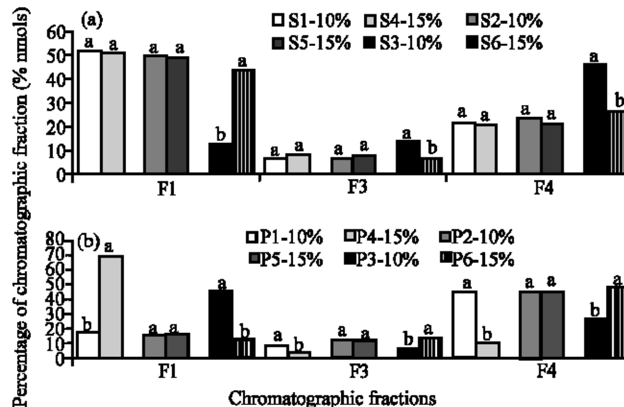

Fig. 1: Effect of substrate concentration on peptide profile of hydrolysates from whey protein concentrate. (a): hydrolysates prepared with subtilisin, using the concentrations of 10% (S1, S2 and S3) and 15% (S4, S5 and S6). (b): hydrolysates prepared with pancreatin, using the concentrations of 10% (P1, P2 and P3) and 15% (P4, P5 and P6). F1: large peptides (> 7 amino acid residues); F3: di- and tripeptides; F4: free amino acids. Different letters are significantly different ($p<0.05$) for the same fraction of different hydrolysates

The effect of this parameter, in case of pancreatin action, may be analyzed by comparing P1 with P4 (E:S = 1:100), P2 with P5 (E:S = 2:100) and P3 with P6 (E:S = 4:100), in Fig. 1b. It can be observed that, contrarily to subtilisin, the advantage of using higher substrate concentration occurred, at least, in one case (P3 with P6), since the use of a concentration of 15%, compared to 10%, produced smaller amount of large peptides, as well as larger di- and tripeptide and free amino acid contents. In the other two cases, the results obtained with 15% were either similar (P2 with P5) or inferior (P1 with P4) to those with 10% (Fig. 1b). The use of this same pancreatin for hydrolyzing whey was tested by Biasutti *et al.* (2008) and contrarily to the result of the present work, the peptide profiles of all hydrolysates prepared with 10 or 15% of whey were similar.

Effect of E:S ratio: Aiming the evaluation of the effect of E:S ratio of subtilisin action on peptide profiles of WPC hydrolysates, two groups containing three samples each must be compared, in Fig. 2a: S1 with S2 and S3 (SC = 10%) as well as S4 with S5 and S6 (SC = 15%). The advantage of using a smaller E:S ratio was observed in some cases and for both groups this occurred only when comparing 2:100 with 4:100. Thus, for the first group (SC = 10%) the use of this lower value was more advantageous since it gave rise to a smaller amount of large peptides as well as higher di- and tripeptides and free amino acid contents. In case of the second group (SC = 15%), the same was observed in relation to the amount of large peptides and free amino acids, but no significant difference was found for di- and tripeptides contents (Fig. 2).

Using this same enzyme for hydrolyzing whey, Biasutti *et al.* (2008) showed that, contrarily to this present work, the use of an E:S of 2:100 for a SC of 10% was a more disadvantageous condition than 4:100, since it gave rise to a poorer peptide profile. However, similar result of this work was observed for a SC of 15%.

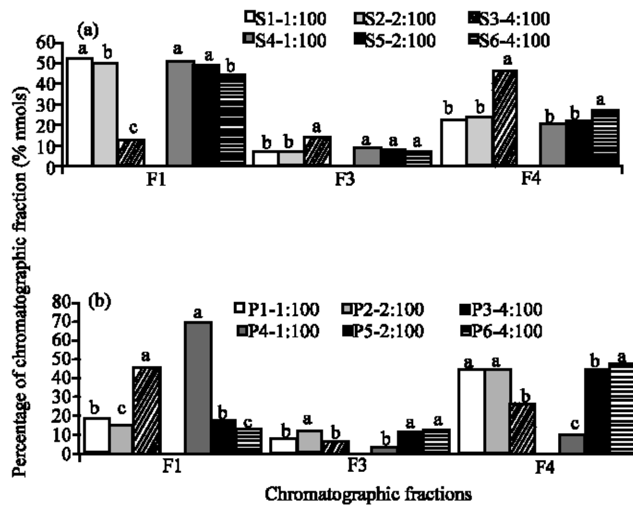

Fig. 2: Effect of Enzyme:Substrate (E:S) ratio on peptide on peptide profile of hydrolysates from whey protein concentrate. (a): hydrolysates prepared with subtilisin, using the E:S ratios of 1:100 (S1 and S4), 2:100 (S2 and S5) and 4:100 (S3 and S6) and (b) hydrolysates prepared with pancreatin, using the E:S ratios of 1:100 (P1 and P4), 2:100 (P2 and P5) and 4:100 (P3 and P6). F1: large peptides (> 7 amino acid residues); F3: di- and tripeptides; F4: free amino acids. Different letters are significantly different ($p<0.05$) for the same fraction of different hydrolysates

In study of Morato *et al.* (2000) the action of another subtilisin (Carlsberg, from Sigma Co.) for preparing casein hydrolysates was evaluated and it was reported that the use of an E:S of 2:100 produced a better peptide profile than 4:100 only in terms of its higher amount of free amino acids, since its large peptide content was higher and no difference in the di- and tripeptides contents was observed.

The influence of E:S ratio in the hydrolytic process of WPC proteins by pancreatin may be evaluated in Fig. 2b, where two groups containing three samples each must be compared, in Fig. 2a: P1 with P2 and P3 (SC = 10%) as well as P4 with P5 and P6 (SC = 15%). For the first group, the advantage of using a smaller E:S ratio was observed only when comparing 1:100 with 2:100, since the use of the lower value gave rise to a smaller amount of large peptides and higher of free amino acids. In case of the second group, this advantage was achieved comparing either 1:100 with 2:100 or 2:100 with 4:100, giving rise to a smaller amount of large peptides and higher of free amino acids, in both cases and also to a higher di- and tripeptides content in the first case (Fig. 2).

The use of this same pancreatin for hydrolyzing whey showed no advantage when comparing E:S of 2:100 with 4:100, since the peptide profiles of hydrolysates were similar (Biasutti *et al.*, 2008).

In two other studies, a different pancreatin (Carlsberg, from Sigma Co.) which was previously immobilized on activated carbon, was used for hydrolyzing whey. In these works, the peptide profiles were analyzed after the treatment of whey hydrolysates by Activated Carbon (AC) for removing phenylalanine. Thus, using a SC of 10%, it was shown by Silva *et al.* (2007) that the use of a smaller E:S of 1:10,000 produced a better peptide profile than 1:100, notably concerning the higher di- and tripeptides content. In the second study, using a much more diluted substrate solution (SC of 1%), Delvivo *et al.* (2006) reported that E:S (1:10,000 and 1:100) showed no effect on the peptide profiles of whey hydrolysates.

CONCLUSION

The action of pancreatin and subtilisin in the hydrolysis of WPC produced good and similar peptide profiles, from the nutritional point of view. However, an advantage of the former enzyme over the second one was observed in terms of number of samples that showed this result (6 against 1). The economical benefit of using a higher substrate concentration and a smaller E:S ratio was observed in few cases for both enzymes. It can be inferred that some of the conditions tested here for hydrolysing WPC showed to be an alternative process for the utilization of this derivative of an industrial by-product (whey).

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REFERENCES

AOAC, 1995. Official Methods of Analysis of AOAC international. 16th Edn., AOAC International, Arlington pp: 1298.

Adachi, S., Y. Kimura, K. Murakami, R. Matsuno and H. Yokogoshi, 1991. Separation of peptide groups with definite characteristics from enzymatic protein hydrolysate. Agric. Biol. Chem., 55: 925-932.

Akiyama, H., K. Sakata, Y. Yoshika, Y. Murata and Y. Ishihara *et al*., 2006. Profile analysis and immunoglobulin E reactivity of wheat protein hydrolysates. Int. Arch. Allergy Immunol., 140: 36-42.

Amiot, J. and G.J. Brisson, 1980. Continuous automatic nitrogen determination for gel chromatography of protein enzymatic hydrolysates. J. Chromatogr., 193: 496-499.

Armstead, I.P. and J.R. Ling, 1991. Chromatographic separation of mixed peptides from amino acids in biological digests with volatile buffers. J. Chromatogr., 586: 259-263.

Aubry, A.F., M. Caude and R. Rosset, 1992. Separation and identification of dipeptides in an hydrolyzed brain extract. Chromatography, 33: 533-538.

Biasutti, E.A.R., C.R. Vieira, M. Capobiango, V.D.M. Silva and M.P.C. Silvestre, 2007. Study of some functional properties of casein: Effect of pH and tryptic hydrolysis. Int. J. Food Prop., 10: 173-183.

Biasutti, E.A., W.O. Afonso, C.O. Lopes Jr., J.V. Coelho, V.D.M. Silva and M.P.C. Silvestre, 2008. Action of pancreatin in obtaining protein hydrolysates with high whey oligopeptide. Braz. J. Pharm. Sci., 44: 51-60.

Bizzotto, C.S., M. Capobiango and M.P.C. Silvestre, 2005. Evaluation of functional properties of a blood protein. Pak. J. Nutr., 4: 11-16.

Boza, J.J., D. Moennoz, J. Vuichoud, A.R. Jarret, D. Gaudard-de-Weck and O. Ballevre, 2000. Protein hydrolysate vs. free amino acid-based diets on the nutritional recovery of the starved rat. Eur. J. Nutr., 39: 237-243.

Brans, G., C.G.P.H. Schroen, R.G.M. Van der Sman and R.M. Boom, 2004. Membrane fractionation of milk: State of the art and challenges. J. Membr. Sci., 243: 263-272.

Capobiango, M., C.S. Bizzotto, E.A.R. Biasutti and M.P.C. Silvestre, 2006. Action of pepsin on emulsifying properties of globin. Int. J. Food Prop., 9: 357-364.

Carreira, R.L., L.M. De Marco, D.R. Dias, H.A. Morais and M.P.C. Silvestre, 2004. Analysis of peptide profiles of casein hydrolysates prepared with pepsin, trypsin and subtilisin. Acta Farm. Bonaerense, 23: 17-26.

Chicon, R., J. Belloque, E. Alonso and R. Lopez-Fandino, 2009. Antibody binding and functional properties of whey protein hydrolysates obtained under high pressure. Food Hydrocolloids, 23: 593-599.

Clemente, A., 2000. Enzymatic protein hydrolysates in human nutrition. Trends Food Sci. Technol., 11: 254-262.

Contreras, M.D.M., B. Hernandez-Ledesma, L. Amigo, P.J. Martin-Alvarez and I. Recio, 2010. Production of antioxidant hydrolysates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. LWT-Food Sci. Technol., 44: 9-15.

Davis, M.T. and T.D. Lee, 1992. Analysis of peptide mixtures by capillary high performance liquid chromatography: A practical guide to small-scale separations. Protein Sci., 1: 935-944.

De Araujo, R.L.B., 2009. Obtaining enzyme extract from pineapple peel and its use in the preparation of protein hydrolysates of wheat flour with reduced phenylalanine content. Ph.D. Thesis, Universidade Federal de Minas Gerais.

De Souza, M.W.S., E.A.R. Biasutti, R.L. Carreira, W.O. Afonso, V.D.M. Silva and M.P.C. Silvestre, 2008. Obtaining oligopeptides from whey: Use of subtilisin and pancreatin. Am. J. Food Technol., 3: 315-324.

Deeslie, W.D. and M. Cheryan, 1988. Functional properties of soy protein hydrolysates from a continuous ultrafiltration reactor. J. Agric. Food Chem., 36: 26-31.

Delvivo, F.M., C.R. Vieira, E.A.R. Biasutti, M. Capobiango, V.D.M. Silva, W.O. Afonso and M.P.C. Silvestre, 2006. Evaluating the effect of adsorption medium, hydrolytic parameters and ultrafiltration on the phenylalanine removal from pancreatic whey hydrolysates. Am. J. Food Technol., 1: 94-104.

Frenhani, P.B. and R.B. Burini, 1999. Mechanisms of absorption of amino acids and oligpeptideos. Arq. Gastroenterol., 36: 227-237.

Golovchenko, N.P., I.A. Kataeva and V.K. Akimenko, 1992. Analysis of pH-dependent protein interactions with gel filtration medium. J. Chromatogr., 591: 121-128.

Gonzalez-Tello, P., F. Camacho, E. Jurado, M.P. Paez and E.M. Guadix, 1994. Enzymatic hydrolysis of whey proteins. II. Molecular-weight range. Biotechnol. Bioeng., 44: 529-532.

Grimble, G.K., P.P. Keohane, B.E. Higgins, M.V. Kaminski Jr. and D.B. Silk, 1986. Effect of peptide chain length on amino acid and nitrogen absorption from two lactoalbumin hydrolysates in the normal human jejunum. Clin. Sci., 71: 65-69.

Gupta, R., O.K. Beg and P. Lorenz, 2002. Bacterial alkaline proteases: Molecular approaches and industrial applications. Applied Microbiol. Biotechnol., 59: 15-32.

Kammoun, R., S. Bejar and R. Ellouz, 2003. Protein size distribution and inhibitory effect of wheat hydrolysates on Neutrases. Bioresour. Technol., 90: 249-254.

Keohane, P.P., G.K. Grimble, B. Brown, R.C. Spiller and D.B. Silk, 1985. Influence of protein composition and hydrolysis method on intestinal absorption of protein in man. Gut, 26: 907-913.

Kong, X., H. Zhou and H. Qian, 2007. Enzymatic hydrolysis of wheat gluten by proteases and properties of the resulting hydrolysates. Food Chem., 102: 759-763.

Kopaciewicz, W. and F.E. Regnier, 1982. Nonideal size-exclusion chromatography of proteins: Effects of pH at low ionic strength. Anal. Biochem., 126: 8-16.

Lemieux, L. and J. Amiot, 1989. Application of reversed-phase high-performance liquid chromatography to separation of peptides from phosphorylated and dephosphorylated casein hydrolysates. J. Chromatogr. A., 473: 189-206.

Lemieux, L., J.M. Piot, D. Guillochon and J. Amiot, 1991. Study of the efficiency of a mobile phase used in size-exclusion HPLC for the separation of peptides from a casein hydrolysate according to their hydrodynamic volume. Chromatographia, 32: 499-504.

Li-Jun, L., Z. Chuan-He and Z. Zheng, 2008. Analyzing molecular weight distribution on whey protein hydrolysates. Food Bioprod. Process, 86: 1-6.

Lopes, D.C.F., F.M. Delvivo and M.P.C. Silvestre, 2005. Hydrolysates of skim milk powder: Peptide profiles for dietetic purposes. Br. Food J., 107: 42-53.

Lopes, D.C.F., C.S. Bizzotto, R.L. Carreira, W.O. Afonso, C.O. Lopes Jr. and M.P.C. Silvestre, 2008. Removal of phenylalanine from protein hydrolysates prepared with rice. J. Food Technol., 6: 57-65.

Morais, H.A., L.M. De Marco, M.C. Oliveira and M.P.C. Silvestre, 2005. Casein hydrolysates using papain: Peptide profile and encapsulation in liposomes. Acta Alimentaria, 34: 59-69.

Morato, A.F., R.L. Carreira, R.G. Junqueira and M.P.C. Silvestre, 2000. Optimization of casein hydrolysis for obtaining high contents of small peptides: Use of subtilisin and trypsin. J. Food Compos. Anal., 13: 843-857.

Moreno-Indias, I., N. Castro, A. Morales-delaNuez, D. Sanchez-Macias, P. Assuncao, J. Capote and A. Arguello, 2009. Farm and factory production of goat cheese whey results in distinct chemical composition. J. Dairy Sci., 92: 4792-4796.

Mortenson, M.A., Z.M. Vickers and G.A. Reineccius, 2008. Flavor of whey protein concentrates and isolates. Int. Dairy J., 18: 649-657.

Nielsen, S.S., 1998. Food Analysis. 2nd Edn. Aspen Publisher, Gaithersburg, pp: 630.

Page, M.J. and E. Di Cera, 2008. Serine peptidases: Classification, structure and function. Cell. Mol. Life Sci., 65: 1220-1236.

Park, Y.K., 2001. Producao de Enzimas Industriais de Origem Animal. In: Biotecnologia Industrial: Processos Fermentativos e Enzimaticos. Lima, U.A., E. Aquarone, W. Borzani and W. Schmidell (Eds.). Edgard Blugher, Sao Paulo, pp: 363-371.

Parrado, J., F. Millan, I. Hernandez-Pinzan, J. Bautista and A. Machado, 1993. Characterization of enzymatic sunflower protein hydrolysates. J. Agric. Food Chem., 41: 1821-1825.

Peng, X., Y.L. Xiong and B. Kong, 2009. Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. Food Chem., 113: 196-201.

Perea, A., U. Ugalde, I. Rodriguez and J.L. Serra, 1993. Preparation and characterization of whey protein hydrolysates: Applications in industrial whey bioconversion processes. Enzyme Microbial Technol., 15: 418-423.

Pimentel-Gomes, F., 2000. Curso De Estatística Experimental. 14th Edn., World Health Organization, Piracicaba.

Pouliot, Y., M.M. Guy, M. Tremblay, A.C. Gaonach, B.P.C.P. Ting, S.F. Gauthier and N. Voyer, 2009. Isolation and characterization of an aggregating peptide from a tryptic hydrolysate of whey proteins. J. Agric. Food Chem., 57: 3760-3764.

Rerat, A.A., 1993. Nutritional supply of proteins and absorption of their hydrolysis products: Consequences on metabolism. Proc. Nutr. Soc., 52: 335-344.

Saint-Sauveur, D., S.F. Gauthier, Y. Boutin and A. Montoni, 2008. Immunomodulating properties of a whey protein isolate, its enzymatic digest and peptide fractions. Int. Dairy J., 18: 260-270.

Sammel, L.M. and J.R. Claus, 2003. Whey protein concentrates effects on pink color development in a cooked ground Turkey breast model system. Meat Sci., 65: 1293-1299.

Schmidt, D.G. and J.K. Poll, 1991. Enzymatic hydrolysis of whey proteins. Hydrolysis of á-lactoalbumin and beta-lactoglobulin in buffer solutions by proteolytic enzymes. Neth. Milk Dairy J., 45: 225-240.

Schmidt, C.G. and M. Salas-Mellado, 2009. Influence of action of the enzymes Alcalase Flavourzyme and the degree of hydrolysis of proteins of chicken. Quim. Nova, 32: 1144-1150.

Silva, V.D.M. and M.P.C. Silvestre, 2003. Functional properties of bovine blood plasma intended for use as a functional ingredient in human food. Lebensm. Wiss. Technol., 36: 709-718.

Silva, V.D.M., L.M. De Marco, W.O. Afonso, D.C.F. Lopes and J.N. Januario *et al*., 2007. Preparation of low-phenylalanine whey hydrolysates, using papain and pancreatin immobilized on activated carbon and alumina. Am. J. Food Technol., 2: 327-341.

Silva, M.C.D., V.D.M. Silva, A.M.Q. Lana and M.P.C. Silvestre, 2009. Degree of hydrolysis and peptide profile of hydrolysates of whey protein concentrate. Aliment. Nutr., 20: 395-402.

Silva, M.R., 2010. Obtaining hydrolysates of whey protein concentrate from milk with high content of oligopeptides and high inhibitory activity on angiotensin converting enzyme, using pancreatin and papain. Master's Thesis. Federal University of Minas Gerais

Silvestre, M.P.C., M. Hamon and M. Yvon, 1994a. Analyses of protein hydrolysates. 1. Use of poly (2-hydroxyethyl-aspartamide)-silica column in size-exclusion chromatography for the fracionation of casein hydrolysates. J. Agric. Food Chem., 42: 2778-2782.

Silvestre, M.P.C., M. Hamon and M. Yvon, 1994b. Analyses of protein hydrolysates. 2. Characterization of casein hydrolysates by a rapid peptide quantification method. J. Agric. Food Chem., 42: 2783-2789.

Su, R., Z. He and W. Qi, 2008. Pancreatic hydrolysis of bovine casein: Changes in the aggregate size and molecular weight distribution. Food Chem., 107: 151-157.

Vallejo-Cordoba, B., S. Nakai, W.D. Powrie and T. Beveridge, 1986. Protein hydrolysates for reducing water activity in meat products. J. Food Sci., 51: 1156-1161.

Visser, S., C.J. Slagen and A.J.P.M. Robben, 1992. Determination of molecular mass distributions of whey protein hydrolysates by high-performance size-exclusion chromatography. J. Chromatogr., 599: 205-209.

Voorbergen, M. and A. Zwanenberg, 2002. Whey-ing up the future: As world cheese production increases, so does production of whey products. Mark Voorbergen and Adrie Zwanenberg look at the rise in the trade of whey products. (Whey Markets). Dairy Ind. Int., 67: 25-28.

Walzem, R.L., C.J. Dillard and J.B. German, 2002. Whey components: Millennia of evolution create functionalities for mammalian nutrition: What we know and what we may be overlooking. Crit. Rev. Food Sci. Nutr., 42: 353-375.

Wang, J., M. Zhao and Y. Jiang, 2007. Effects of wheat gluten hydrolysates and its ultrafiltration fractions on dough properties and bread quality. Food Technol. Biotechnol., 45: 410-415.