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Correlation between the Degree of Hydrolysis and the Peptide Profile of Whey Protein Concentrate Hydrolysates: Effect of the Enzyme Type and Reaction Time

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ABSTRACT

The use of protein hydrolysates for the development of new food products requires their prior characterization including the determination of the degree of hydrolysis (DH) and the distribution of peptides according to the chain length. In this study, the DH of whey protein concentrate hydrolysates was determined by four methods (formol titration, soluble protein content, ortophtalaldehyde and osmometry) and the peptide profile was characterized by size-exclusion high performance liquid chromatography. The correlations among the four methods as well as between the DH and peptide profiles were established. The highest DH values were obtained by the methods of the soluble protein content (44.7%) and the derivatization with ortophtalaldehyde (20.17%) for the proteases from *Bacillus licheniformis* and *Aspergillus sojae*, respectively. The use of the *Bacillus licheniformis* protease was more advantageous because it produced the highest contents of di-and tripeptides (8.79%) and the lowest of large peptides (53.57%). A significant correlation ($p < 0.05$) between the DH and the contents of peptides and free amino acids was observed and the intensity of the correlation varied as a function of the method and the enzyme type analyzed.

Key words: Whey protein concentrate hydrolysates, degree of hydrolysis, peptide profile, protease from *B. licheniformis*, protease from *A. sojae*

INTRODUCTION

Whey is a by product of the dairy industry that has little commercial value and its disposal remains one of the most serious problems in the dairy industry of several countries (Carrasco and Guerra, 2010). Therefore, it is important to create new options for the use of whey which contains several constituents, especially proteins and peptides, that can be of great value for the biotechnology, medical and agroindustrial areas (Smithers, 2008). One method to increase the use of whey is the enzymatic hydrolysis and this process may contribute to the improvement of the nutritional, functional, immunological and biological activity of whey proteins (Pacheco and Sgarbieri, 2005; Cheison *et al*., 2009).

However, the use of protein hydrolysates for the development of new food products or dietary supplements requires the prior characterization of the hydrolysates, including the determination of the degree of hydrolysis (DH) and the distribution of peptides according to the chain length (Silvestre, 1997; Guadix *et al*., 2000; Leonil *et al*., 2000).

According to Cheison *et al.* (2009), the DH is an important parameter to understand and interpret the effects and extent of the hydrolytic process of proteins and is useful to establish the relationships between proteolysis and the improvement of the functional, bioactive and sensory properties of these biomolecules.

Several methods for estimating the degree of protein hydrolysis are available and different approaches are required to determine the DH of different products. These methods are based on four different principles. First of all, the determination of the hydrolytic release of nitrogen which becomes soluble in the presence of a precipitating agent, such as trichloroacetic acid. In the second position comes the determination of free α-amino-groups. The third principle is the titration of protons released after the rupture of peptide bonds. Finally, we can find the measurement of the changes in the freezing point of the protein solution by osmometry (Silvestre, 1997; Nielsen *et al.*, 2001; Spellman *et al.*, 2003).

In the current study, four techniques based on different principles were used for the assessment of DH (1) the formol titration, based on an acid-base titration, (2) the determination of soluble protein content in the presence of a precipitating agent, (3) the reaction of α-amino groups, that were released during hydrolysis, with ortophtalaldehyde and (4) the detection of the freezing point of solutions by osmometry.

The choice of these methods was based on the fact that although there is a broad scientific publication on the determination of DH of whey proteins, few data are found in the literature about the comparison of methods used for assessing this parameter for whey proteins, especially concerning the use of techniques based on different principles. Moreover, in these studies the comparison was carried out only between methods based on the reaction of α-amino groups with either derivatizing agents (ortophtalaldehyde and/or 2,4,6-trinitrobenzene sulfonic acid) (Nielsen *et al.*, 2001) or pH stat method that determines the quantity of alkaline solution (measured by automatic titration) needed to keep the pH constant throughout the reaction (Spellman *et al.*, 2003; Cheison *et al.*, 2009). This fact justifies the performance of the current study which main goal is to establish the DH method that best correlates with the peptide profile. Therefore, this method could be used as a rapid screening of the most appropriate protein hydrolysate to be used in the development of high-nutritional value food products.

Additionally, the current work represents the first time that some methods were used for assessing the degree of hydrolysis of Whey Protein Concentrate (WPC) hydrolysates obtained by the action of *Bacillus licheniformis* and *Aspergillus sojae* proteases. This is the case of the methods of the soluble protein content and osmometry for both enzymes and also the ortophtalaldehyde (OPA) method for the second enzyme.

To our knowledge, the current work represents the first time it was established a correlation among the four methods used in the present work to determine the degree of hydrolysis of WPC hydrolysates obtained by the action of *Bacillus licheniformis* and *Aspergillus sojae* proteases.

Aiming at using a protein hydrolysate for dietetic purpose, a characterization regarding the size distribution of peptides is needed, because the length of the peptide chain influences the rate of absorption. Regarding this subject, several chromatographic techniques have been described in the literature, but they have shown some drawbacks, such as interactions between the solute and the stationary phase and inefficiency in separating small peptides (Silvestre *et al.*, 2011). For these reasons, our group (Silvestre *et al.*, 1994) has developed a method based on the fractionation of peptides by size exclusion high performance liquid chromatography which allowed to separate and quantify peptides with molecular masses smaller than 1,000 Da and this technique was employed in the current work.

It is worth stating that as far as we know the correlation between the DH and peptide profile of WPC hydrolysates has never been established before.

This study was conducted to examine (1) the preparation of WPC enzymatic hydrolysates using the proteases from *B. licheniformis* and *A. sojae*, (b) the characterization of these hydrolysates in relation to the DH and peptide profile, (c) the evaluation of the effects of enzyme type and the reaction time on the DH, (d) the comparison of four methods for quantifying the DH and (e) the study of the correlation between the DH and the peptide and free amino acid content.

MATERIAL AND METHODS

Materials: WPC (Kerrylac 750) in powdered form was kindly furnished by Kerry of Brazil Ltda. (Três Corações, MG, Brazil). Proteases from *Bacillus licheniformis* (Alcalase®, activity = 6.22 U mL^{-1}) and *Aspergillus sojae* (Corolase LAP®, activity 0.63 U mL^{-1}) were kindly furnished by Novozymes (Araucária, PR, Brazil) and AB Enzymes (Barueri, SP, Brazil), respectively. In this study, the enzyme activity was defined as the amount of enzyme that liberated 1 g of tyrosine in 1 min at 37°C.

The Folin-Ciocalteu reagent (code F9252), bovine serum albumin (code A2153), ortophtalaldehyde (OPA, Code P0657) and 2-mercaptoethanol (code M6250) were purchased from Sigma (São Paulo, SP, Brazil). The formic acid was purchased from Merck (Whitehouse Station, NJ, USA). The polyvinylidene fluoride membranes used for the filtration of the samples (0.22 μm) and solvents (0.45 μm) as well as the tangential flow system with a 10 kDa cut-off membrane were purchased from Millipore (São Paulo, SP, Brazil). All other reagents used in this study were of analytical grade.

The HPLC system used for fractionating protein hydrolysates consisted of one isocratic pump and a UV-Vis detector (1200 Series, Agilent, Santa Clara, CA, USA) coupled to a computer with ChemStation software for LC Systems (Agilent, Santa Clara, CA, USA). A poly (2-hydroxyethylaspartamide)-silica (PHEA) column (250×9.4 mm, 5 μm and 200 Å pore size) was used for HPLC. The water for HPLC was purified by passage through a MilliQ water purification system (Millipore, Billerica, MA, USA).

Preparation of hydrolysates from whey protein concentrates: Ten hydrolysates from WPC were prepared by varying the enzyme type and the reaction time. The conditions of hydrolysis are listed in Table 1. The pH and temperature values corresponded to the optimal conditions for the enzymes and were provided by the manufacturer.

Table 1: Hydrolytic conditions used in preparing WPC hydrolysates

Hydrolysates	Enzyme type	Reaction time (h)	pH	Temperature (°C)
H1	Protease from *Bacillus licheniformis*	1	8	60
H2		2		
H3		3		
H4		4		
H5		5		
H6	Protease from *Aspergillus sojae*	1	7	50
H7		2		
H8		3		
H9		4		
H10		5		

The 10% (w/v) WPC solutions which corresponded to 3.42% protein (w/v), were prepared in distilled water and the pH was adjusted to 8.0 with a 3 mol L^{-1} NaOH solution. Subsequently, the WPC solutions were heated in an oil bath with continuous stirring (stirrer 752A model from Fisatom, São Paulo, SP, Brazil) and the enzymes were added in an appropriate quantity to attain an enzyme: substrate ratio of 8.0:100. The reaction time varied from 1-5 h and the hydrolytic reaction was stopped by heating the samples at 75°C for 15 sec. The hydrolysates were immediately used for the determination of DH and then freeze-dried (Freeze Dry System/FreeZone 4.5, model 77500, Labconco, Kansas City, MO, USA) for the characterization of peptide profile. The hydrolysates were stored in the freezer (-20°C) until analysis.

Determination of the degree of hydrolysis: In this study, the degree of hydrolysis was evaluated using four different methods. Two methods were based on the determination of α-amino nitrogen (formol titration and derivatization with OPA) and the other methods relied on the depression of the freezing point (osmometry) and on the quantification of the soluble protein content after precipitation with trichloroacetic acid. For all methods, a blank using unhydrolyzed WPC was submitted to the same procedure of the hydrolyzed samples.

Formol titration: The DH was calculated using the relationship between α-amino nitrogen (AN) and Total Nitrogen (TN) according to Eq. 1:

$$DH\ (\%) = \frac{\text{α-amino nitrogen (AN)}}{\text{Total nitrogen (TN)}} \times 100 \quad (1)$$

The Sorensen method (AOAC, 1995) based on titration with formaldehyde was used for quantifying the AN. The formaldehyde l reagent was prepared by diluting 25 mL of the commercial solution with 50% ethanol to a final volume of 250 mL. The pH was adjusted to 7.0 with 0.2 mol L^{-1} NaOH solution just before use. The pH of the hydrolysate solution was also adjusted to 7.0 with 0.2 mol L^{-1} NaOH solution. Three milliliters of the formaldehyde reagent were added to 3 mL of the hydrolysate solution and the mixture was stirred and then titrated with 0.2 mol L^{-1} NaOH solution with phenolphthalein as an indicator to its final end point. An excess of 0.2 mol L^{-1} NaOH solution was added and the solution was then back-titrated with 0.2 mol L^{-1} HCl solution until colourless. The volumes of NaOH and HCl solutions required were recorded. The TN was determined by the Kjeldahl method (AOAC, 1995).

Derivatization with ortophtalaldehyde: For this analysis, the OPA reagent was prepared according to Church *et al.* (1983). A volume of 25 mL of sodium tetraborate solution (100 mmol L^{-1}) was mixed with 2.5 mL of 20% (w/v) sodium dodecyl sulphate solution, 40 mg of OPA (dissolved in 1 ml of methanol) and 100 μL of 2-mercaptoethanol. The volume was adjusted to 50 mL using type I water. The reagent was always prepared immediately before use and placed in a vial covered with aluminium foil to protect it from light.

The method described by Spellman *et al.* (2003) was used with some modifications for the derivatization. A volume of 10 μL of the sample was mixed with 3.4 mL of the OPA reagent and this mixture was allowed to stand at 25°C for 2 min. Subsequently, the absorbance was read at 340 nm. The degree of hydrolysis was calculated according to Eq. 2:

$$DH\ (\%) = \frac{ABS \times 1{,}934 \times d}{c} \quad (2)$$

where, ABS is the absorbance of the samples, d is the dilution factor and c the protein concentration of the sample (g L^{-1}).

Osmometry: For this analysis, a sample volume of 50 μL was immediately removed after the reaction to determine the freezing point which was measured using a micro-osmometer (Precision System 5004, Spectrun, São Paulo, Brazil). The depression of freezing point was used to determine the degree of hydrolysis, according to Eq. 3, proposed by Adler-Nissen (1986):

$$GH\ (\%) = \left(\frac{\Delta C}{P\% \times f_{osm}}\right) \times \left(\frac{1}{\omega}\right) \times \left(\frac{1}{h_{tot}}\right) \times 100 \quad (3)$$

where, °C is the osmolarity (mOsmol L^{-1}), P% is the protein concentration (g of protein/kg of water), 1/ω is the calibration factor of the osmometer which represents the reciprocal of the osmotic coefficient of peptides that is supposed to be a constant value of 1.04 and h_{tot} is the total number of peptide bonds in the protein which is 8.8 mEq g^{-1} of protein for whey.

Soluble protein content in trichloroacetic acid: The degree of hydrolysis of WPC was also determined by the percentage of soluble protein in 10 g% (w/v) trichloroacetic acid (TCA) in relation to the total protein content of the sample according to Hoyle and Merrit (1994), with modifications. Hydrolysate aliquots of 500 μL were mixed with 500 μL of 20 g% of TCA solution to obtain the soluble and insoluble fractions in 10 g% TCA. After 30 min of rest, the mixture was centrifuged at 3000xg and the soluble protein content of the supernatant was determined by the method of Lowry *et al.* (1951), modified by Hartree (1972) and the result was expressed as mg of protein. Bovine serum albumin was used as the standard. The degree of hydrolysis was calculated according to Eq. 4:

$$DH\ (\%) = \frac{\text{Soluble protein content in 10 g\% TCA (mg)}}{\text{Total protein content (mg)}} \times 100 \quad (4)$$

Characterization of peptide profiles from WPC hydrolysates: The characterization of peptide profiles was performed in two stages which included the fractionation of peptides by size and their subsequent quantification. The fractionation of WPC hydrolysates was performed by Size Exclusion (SE) HPLC on a PHEA column, according to the method previously developed by our group (Silvestre *et al.*, 1994), using 0.05 mol L^{-1} formic acid (pH 2.5) as the mobile phase and isocratic conditions at a flow rate of 0.5 mL min^{-1} for 35 min. The samples were dissolved in the mobile phase (0.1% w/v) and filtered through a membrane of 0.22 μm and 50 μL of these solutions were injected onto the column. The mobile phase was filtered through a membrane of 0.45 μm and degassed by sonication in an ultrasound bath (USC1400 model, Unique, Santo Amaro, SP, Brazil) for 30 min before use.

The rapid method of Correct Fraction Area (CFA) that was previously developed by our group (Silvestre *et al.*, 1994) was used to quantify the peptides and free amino acids in the SE-HPLC

fractions of the WPC hydrolysates. Briefly, five whey standard hydrolysates (two using trypsin and three using pancreatin) were prepared and then fractionated into four fractions by SE-HPLC, as described above. The four fractions were collected (Fraction Collector, CF-1 model, Spectrum Chrom, Houston, TX, USA) and submitted to amino acid analysis following solvent removal (Centrivap, 78100-00D model, Labconco, Kansas City, MO, USA). The calculation of CFA was performed using specific formulas after detection at three wavelengths (230, 280 and 300 nm) to remove the contribution of aromatic amino acids. A standard curve was drawn to correlate CFA with the amino acid contents of the fractions.

Statistical analysis: All determinations were performed in three replicates, each measured by triplicate analysis. To verify significant hydrolysis effects among the different treatments, a completely randomized design was adopted in analysis scheme split-splot (4×6 methods hydrolysis times) and the results were analyzed with software SISVAR version 5.3 (Ferreira, 2011). An experimental design was adopted of completely randomization for factorial analysis (2×6 enzyme hydrolysis times) of the peptide profiles and the results were analyzed with Statistica software (Stat Soft, 2000). The ANOVA and Duncan test at 5% probability were used to determine differences between the means of the degree of hydrolysis as well as the contents of peptides and free amino acids in the WPC hydrolysates. Significant differences ($p<0.05$) between the means were evaluated by Duncan's test (Pimentel-Gomes, 2000). The correlations between the degree of hydrolysis obtained by different methods with the contents of the peptides and free amino acids were obtained by Pearson's correlation coefficient (r) which measures the degree of association between two variables and p was calculated with t-tests, using the software BioStat (Ayres *et al.*, 2007) for data analysis.

RESULTS AND DISCUSSION

In general, the DH evaluation methods that gave the highest results were the soluble protein content and OPA for the hydrolysates prepared with proteases from *B. licheniformis* and *A. sojae*, respectively. A positive and significant correlation of strong intensity was observed in three cases for the *B. licheniformis* protease and in only one case for the *A. sojae* protease. Regarding the peptide profile, three hydrolysates obtained by the action of *B. licheniformis* protease were considered the best from a nutritional standpoint. The correlation between DH and the chromatographic fractions of the WPC hydrolysates ranged with either the method of determination or the enzyme. The results will be discussed in more detail bellow.

Influence of the methods on the degree of hydrolysis: Figure 1 demonstrates the wide range of DH values obtained by the action of the *B. licheniformis* protease using different methods. The results of the DH values of the samples were deducted from the DH values of the blank (26.6% for OPA, 8.4% for soluble protein content, 6.7% for formol titration and 7.4% for osmometry). The highest value (44.7%) was obtained for the hydrolysate H5 using the soluble protein content method. In fact, for all hydrolysates (H1-H5), the highest values were obtained by this method and were much higher than those obtained by the other three methods (OPA, osmometry and formol titration).

In the soluble protein content method, the addition of trichloroacetic acid results in partial or total precipitation of non-hydrolyzed proteins and high molecular mass peptides (Carreira *et al.*, 2003), producing a higher concentration of soluble free amino acids and small peptides that are

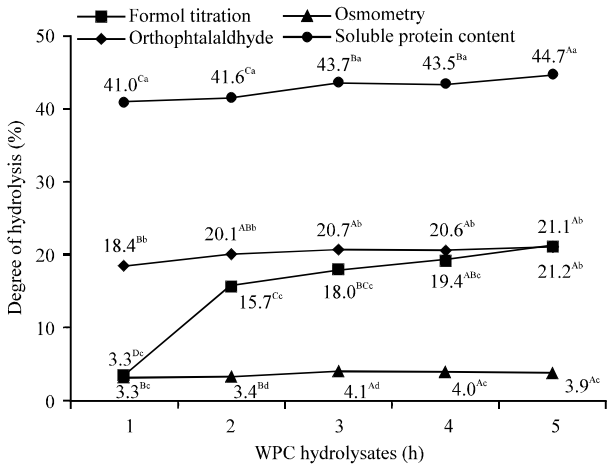

Fig. 1: Degree of hydrolysis of WPC hydrolysates obtained by the action of *Bacillus licheniformis* protease. Different capital letters represent significantly different ($p<0.05$) values for a same method and different hydrolysates. Small letters represent significantly different ($p<0.05$) values for a same hydrolysate and different methods

found in the supernatant. Considering that the protease from *B. licheniformis* is an endopeptidase of broad specificity (Doucet *et al.*, 2003) and also shows high enzymatic activity (6.22 U mL^{-1}), its action may have led to more extensive WPC hydrolysis, thereby resulting in high levels of small peptides. These peptides may have remained soluble after the addition of trichloroacetic acid, contributing to the higher degree of hydrolysis obtained with this method.

After the soluble protein content method, the method that produced the highest values of the DH with the *B. licheniformis* protease was the OPA method, as indicated in Fig. 1. Only for the hydrolysate H5 no significant difference was observed between the results obtained by the OPA and formaldehyde methods. It is worth stating when using the OPA method that the whey proteins have a high percentage of lysine which ε-amino group can also react with the derivatizing agent leading to an overestimation of the DH (Church *et al.*, 1983). This could explain, at least in part, the second position occupied by the OPA method in terms of higher values of DH. However, according to Church *et al.* (1983), this reaction with the ϵ-amino groups would not interfere with the detection of α-amino groups which are exposed during the hydrolysis of WPC proteins.

Other authors have also used the OPA method for evaluating the DH of WPC proteins. Spellman *et al.* (2003) studied the action of a *B. licheniformis* protease in the hydrolysis of WPC 75 using reaction conditions (pH 7, 50°C, 6 h) differing from the current work and reported a maximum DH of 13.3%. This is lower than the values obtained in this study which ranged from 18.4-21.1%, although the same method was used to determine the DH. According to Spellman *et al.* (2003), the low DH values found in their work may be explained by the fact that whey proteins are rich in cysteine which reacts weakly and unstably with OPA. Alternatively, the higher results obtained here could be explained by the addition of 2-mercaptoethanol and the preparation of the hydrolysates at pH 8.0. In fact, according to Roth (1971), the reaction of cysteine with OPA can be optimized in the presence of a strong reducing agent and an alkaline medium contributes to increase of the stability of the compound formed.

The formaldehyde method produced the third highest DH values, as demonstrated in Fig. 1 for the *B. licheniformis* protease. Significant differences were observed between the results obtained

by the formaldehyde method and by osmometry, with the exception of the H1 hydrolysate. The ranking of the formaldehyde method among all of the methods used could be explained, at least in part, by the fact that the action of *B. licheniformis* protease increased the release of hydrophobic or aromatic residues (Gupta *et al.*, 2002; Rawlings *et al.*, 2010) which may have interfered with the titration of protons released by the addition of formaldehyde (Levy, 1935) and resulted in an underestimation of DH values. Although this interference is inconvenient, several authors claim that the technique of formaldehyde, if properly standardized, represents an efficient, fast and cost-effective method for monitoring the hydrolytic process of proteins and has already been used in different protein sources (Denis *et al.*, 2008; Cao *et al.*, 2009; Zheng *et al.*, 2009; Zhao and Hou, 2009).

To the authors' knowledge, with respect to the DH of WPC, titration with formaldehyde was only used in another work by our group. Silva *et al.* (2009), using the proteases from *B. subtilis* and *B. amyloquefaciens* to hydrolyse WPC under different conditions than those used here (pH 9, 55°C and pH 7, 55°C), obtained DH values of 20% and 14%, respectively which are lower than the maximum value obtained in the current study (21.2%) with the formaldehyde method. These differences in DH values obtained by the same research group can be explained by the variety of parameters used in the preparation of the hydrolysates (pH, temperature and enzyme: substrate ratio), as well as the use of different lots of WPC and enzyme.

Among all of the methods used in the current work, osmometry exhibited the lowest DH results (from 3.3-4.1%) when using the *B. licheniformis* protease (Fig. 1). A probable explanation for these low values could be related to the fact that the *B. licheniformis* protease is an endopeptidase (Gupta *et al.*, 2002; Rawlings *et al.*, 2010) and therefore releases more peptides than free amino acids, thus reducing the DH determined by the osmometry. It is well known that osmometry produces higher values when the number of soluble molecules in the sample is greater which decreases the freezing point of the solution (Wong and Boyce, 1988). Also, according to some authors (Ju *et al.*, 1995; Otte *et al.*, 1996; Doucet *et al.*, 2001), the increase in the number of peptides in a solution can produce aggregation between the peptides which would result in a gradual increase in the viscosity and turbidity of the samples, interfering with the determination of DH by osmometry.

No report was found in the literature on the use of the methods of the soluble protein content and osmometry for assessing the degree of hydrolysis of WPC by the action of a *B. licheniformis* protease.

Figure 2 demonstrates the wide range of DH values observed for the action of *A. sojae* protease, as shown for the *B. licheniformis* protease which varied by the method used. Also, the results of the DH values of the samples were deducted from the DH values of the blank (25.5% for OPA, 8.6% for soluble protein content, 4.6% for formol titration and 7.9% for osmometry). The highest value (20.2%) was obtained for the hydrolysate H10 using the OPA method.

For all hydrolysates (H6-H10), a significant variation was observed in the DH values obtained by the OPA method and these values were much greater than those obtained by the other three methods. Considering that the *A. sojae* protease is an exopeptidase capable of releasing N-terminal amino acids (NC-IUBMB, 2010), the results obtained by the OPA method were expected; the greater the release of free amino acids, the higher is the number of free α-amino groups available to react with OPA and therefore the greater the DH obtained with this method.

After the OPA method, the method that produced the higher values of DH with the *A. sojae* protease was the formaldehyde method, as shown in Fig. 2. The results obtained by the

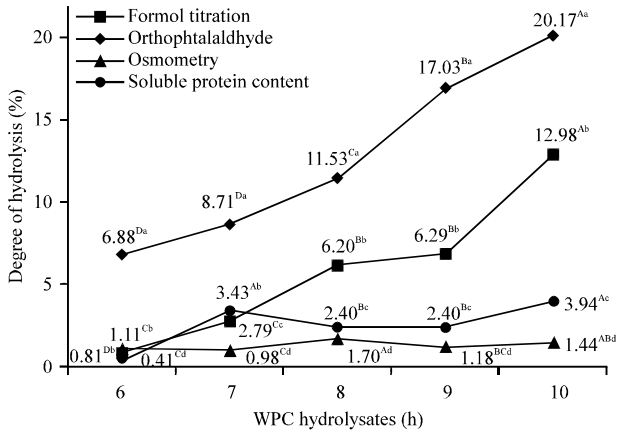

Fig. 2: Degree of hydrolysis of WPC hydrolysates obtained by the action of *Aspergillus sojae* protease. The results represent the means of triplicate experiments. Different capital letters represent significantly different ($p<0.05$) values for the same method and different hydrolysates. Small letters represent significantly different ($p<0.05$) values for the same hydrolysate and different methods

formaldehyde and osmometry methods differed significantly, with the exception of the H6 hydrolysate. The release of α-amino groups by the action the *A. sojae* protease could explain the lower DH values obtained by the formaldehyde method when compared to the OPA method; according to Spellman *et al.* (2003), these α-amino groups have pK values considerably higher as free amino acids than within polypeptides. Additionally, as described above for the *B. licheniformis* protease, some interference may have occurred with the titration of the protons released by the addition of formaldehyde, resulting in an underestimation of the DH values obtained with the formaldehyde method.

As far as the authors are aware with respect to the DH of WPC, formol titration was only used in another work by our group. Silva *et al.* (2009) used an *A. sojae* protease for the hydrolysis of WPC, similar to the current study, under different hydrolytic conditions than those used here (pH 9, 55°C) and obtained a DH of 2.5% which is lower than almost all of the values obtained using the same method in this study, i.e., from 2.79-12.98% for hydrolysates H7-H10, respectively. As cited above, these differences in the values of DH obtained by the same research group can be explained by the variety of parameters used in the preparation of the hydrolysates (pH, temperature and enzyme: substrate ratio) as well as the use of different lots of WPC and enzyme.

The soluble protein content method ranked just after formaldehyde in terms of DH values for the *A. sojae* protease, as demonstrated in Fig. 2. Considering that the use of trichloroacetic acid causes the precipitation of the intact protein and high molecular weight peptides (Carreira *et al.*, 2003), the values obtained by the soluble protein method could be explained, at least in part, by the specificity of the *A. sojae* protease, an exopeptidase only capable of releasing N-terminal amino acids. Further, the low activity (0.63 U mL^{-1}) of this enzyme could have contributed to a smaller rupture of the protein molecules, leading to a decreased amino acid content in the supernatant and therefore a smaller DH than the *B. licheniformis* protease.

As shown for the *B. licheniformis* protease, the lowest DH values were observed when the osmometry was used to evaluate the DH by the *A. sojae* protease (Fig. 2). According to

Dzwolak and Ziajka (1999), the osmotic coefficient (ω) of a sample varies with the concentration and type of solute and is higher for amino acids than peptides and intact proteins. Because *A. sojae* protease is an exopeptidase, the release of amino acids by the action of this enzyme can increase the osmotic coefficient of the sample, changing the viscosity and consequently reducing the DH measured by osmometry.

No report was found in the literature on the use of the methods of OPA, soluble protein content and osmometry for assessing the degree of hydrolysis of WPC by the action of an *A. sojae* protease.

Influence of the reaction time on the degree of hydrolysis: Figure 1 demonstrates that the reaction time with the *B. licheniformis* protease influenced the DH obtained by the soluble protein content method, especially when it increased from 2-3 h and from 4-5 h, where there was a concomitant increase from 41.6-43.7% and from 43.5-44.7%, respectively. When using the OPA method, an increase in the DH was observed when increasing from 1-3 h (from 18.4-20.7%, respectively) and subsequently remained unchanged after this time. For the formaldehyde method, there was also a marked increase in the DH (from 3.4-15.7%) when the reaction time increased from 1-2 h, with a slight further increase observed until the maximum value of 21.2 % was obtained at 5 h. For the osmometry method, only a slight increase in the DH was detected when the reaction time changed from 2 h (3.4%) to 3 h (4.1%).

These results indicate that the formaldehyde method is the most suitable among those that have been tested here for evaluating the DH of WPC hydrolysates obtained by the action of the *B. licheniformis* protease because this method exhibited more pronounced differences in the DH values with the reaction time.

No literature report was found in evaluating the hydrolysis WPC by the *B. licheniformis* protease under the same hydrolytic conditions as the current study. However, in contrast to the result found in the current study, Spellman *et al.* (2003), using a *B. licheniformis* protease in the hydrolysis of a sample of WPC 75 (73.96% protein) under different hydrolytic conditions than those used here (E:S 0.25:100; pH 7.0; 50°C; 6 h), reported a change in the DH during the first hour of reaction when using the OPA method. These differences between these two studies could be explained by the use of different WPC and hydrolytic conditions.

In the case of the *A. sojae* protease, the effect of reaction time on the degree of WPC hydrolysis can be observed in Figure 2. Initially, one can observe that the reaction time influenced the DH for all methods. When using the OPA method, there was an increase in the DH from 2 h (11.53%) to the maximum at 5 h (20.17%). With the formaldehyde method, the DH increased from 1 h (1.11%) to 3 h (6.20%), remained constant between 3 h and 4 h (6.92%) and increased sharply at 5 h (12.98%). For the soluble protein content method, there was an increase in the DH between 1 h (0.41%) and 2 h (3.43%) followed by a decrease between 2 h and 3 h (2.40%). No change in the DH occurred between 3 h (2.40%) and 4 h (2.40%) and the DH increased again to reach 3.94% at 5 h, a value similar to that obtained at 2 h. For the osmometry method, the DH varied over the course of the hydrolytic reaction between 2 h (0.98%) and 3 h (1.70%) and between 3 h and 4 h (1.18%).

These results indicate that the OPA and formaldehyde methods are the most suitable of those tested here for evaluating the DH of WPC hydrolysates obtained by the action of the *A. sojae* protease because they exhibited more pronounced changes in the DH values with reaction time.

No report was found in the literature evaluating the hydrolysis extent of WPC by the *A. sojae* protease under the same hydrolytic conditions as the current study. However, Spellman *et al.* (2003) used an *Aspergillus niger* protease (Debitrase HYW20) which is also an exopeptidase, in the

Table 2: Correlation among the methods for the evaluation of the degree of hydrolysis

	Bacillus licheniformis		*Aspergillus sojae*	
Comparisons	r	p	r	p
Formaldehyde with OPA	0.6492	0.0088	0.9300	<0.0001
Formaldehyde with osmometry	0.6641	0.0069	0.5201	0.0468
Formaldehyde with soluble protein	0.8245	0.0002	0.6868	0.0047
OPA with osmometry	0.7650	0.0009	0.3259	0.2357
OPA with soluble protein	0.5471	0.0347	0.6243	0.0128
Osmometry with soluble protein	0.7305	0.0020	0.1550	0.5812

r: Correlation, p: Determined by t-student test with significant differences for $p<0.05$, OPA: Ortophtalaldehyde

hydrolysis of WPC 75 (73.96% protein) under different hydrolytic conditions than those used here (E:S of 1:100, pH 7.0, 50°C, 1-6 h). These authors reported a variation in the DH, measured by the OPA method, during the first hour of reaction, a result that differs from that observed in the present work. This is likely related to differences in the WPC samples as well as in the proteolytic activities of the enzymes which were not mentioned in the work of Spellman *et al.* (2003).

Correlation among the methods for determining the degree of hydrolysis: The results of this analysis for each of the two proteases are shown in Table 2. A positive and significant correlation of strong intensity was observed in three cases for the *B. licheniformis* protease (formaldehyde with soluble protein content, OPA with osmometry and osmometry with soluble protein content) and in only one case (formaldehyde with OPA) for the *A. sojae* protease because, according to Sampaio's statement (Sampaio, 2002), the values for these correlations are above 0.7, with $p<0.05$.

For the *B. licheniformis* protease, no report was found in the literature on the correlation among the methods used here for evaluating the DH of WPC. However, Spellman *et al.* (2003) observed a correlation between the DH values WPC obtained by the TNBS and pH stat methods using this same enzyme under different hydrolytic conditions. However, no mention was made by these authors about the data for their statistical analysis. For the *A. sojae* protease, no data were found in the literature concerning the correlation among methods for assessing the DH of WPC.

Peptide profile of WPC hydrolysates: The technique used here efficiently fractionated the protein hydrolysates, especially peptides of molecular mass lower than 1,000 Da. The hydrolysates were resolved in four fractions, F1: large peptides, with more than 7 amino acids residues, F2: medium peptides, with 4-7 amino acids residues, F3: di- and tripeptides and F4: Free amino acids. These results are similar to results obtained with different protein sources, such as casein (Morais *et al.*, 2005), milk (Lopes *et al.*, 2005), rice (Lopes *et al.*, 2008), whey (De Souza *et al.*, 2008), wheat flour (Carreira *et al.*, 2011) and WPC (Silvestre *et al.*, 2011).

As shown in Table 3, there was a significant variation in the peptide and free amino acid contents among the different WPC hydrolysates. To choose the most appropriate hydrolysates for the development of nutritional supplements for clinical use, previous studies must be considered. During the metabolism of proteins, the first stage of hydrolysis leads to the formation of free Amino Acids (AA) and small peptides (2-6 AA residues) which can be further hydrolyzed by the brush border peptidases to produce AAs and di- and tripeptides (Hinsberger and Sandhu, 2004). The AA may be absorbed either in free form, by simple or facilitated diffusion (membrane transporters), or

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

Hydrolysates	Reaction time (h)	F1	F2	F3	F4
Bacillus licheniformis					
H1	1	63.08^{C}	36.27^{B}	0.37^{E}	0.27^{E}
H2	2	60.26^{C}	35.24^{B}	3.90^{B}	0.60^{E}
H3	3	50.89^{D}	39.36^{B}	8.47^{A}	1.28^{D}
H4	4	53.57^{D}	36.87^{B}	8.25^{A}	1.30^{D}
H5	5	44.61^{E}	45.40^{A}	8.79^{A}	1.20^{D}
Aspergillus sojae					
H6	1	97.13^{A}	1.23^{C}	1.50^{D}	0.13^{F}
H7	2	95.42^{A}	1.98^{C}	0.41^{E}	2.18^{BC}
H8	3	94.81^{A}	2.25^{C}	0.65^{E}	2.28^{B}
H9	4	92.90^{AB}	2.87^{C}	2.45^{C}	1.77^{C}
H10	5	89.73^{B}	4.63^{C}	0.83^{E}	4.81^{A}

Values are in % of nmol of the four fractions and represent the means of triplicate, F1: Large peptides (>7 amino acid residues), F2: Medinm peptides (4-7 amino acid residues), F3: di- and tripeptides, F4: Free amino acids, Different superscripts represent significantly different ($p<0.05$) values for the same fraction of different hydrolysates

as di- and tripeptides (oligopeptide carriers) (Gilbert *et al*., 2008). Further, according to Frenhani and Burini (1999), the di- and tripeptides are more efficiently absorbed than an equivalent mixture of free amino acids.

In this way, it can be inferred that the best peptide profiles, from the nutritional point of view, were obtained for the hydrolysates H3, H4 and H5, prepared using the *B. licheniformis* protease, because they exhibited the highest content of di-and tripeptides and the smallest of large peptides. Additionally, the results in Table 3 confirm the low DH values obtained by the osmometry method when using the *B. licheniformis* protease.

Correlation between the degree of hydrolysis and the peptide profiles: The results of the correlation (r) between the DH and the chromatographic fractions of the WPC hydrolysates are shown in Table 4. For some hydrolysates, a significant correlation ($p<0.05$) between the DH and the content of peptides and free amino acids can be observed. Additionally, the intensity of the correlation changed with either the method of determination or the enzyme. According to Sampaio (2002), r values above 0.7 with $p<0.05$ indicate a strong association between the two parameters, i.e., the DH and the peptide distribution.

Regarding the *B. licheniformis* protease, a strong positive correlation between the contents of di- and tripeptides (F3) and the DH obtained by all the methods is observed. Additionally, for three of the tested methods (formaldehyde, osmometry and soluble protein content), the same type of correlation occurred between the DH and the free amino acid content (F4) and a negative correlation was observed between the DH and the large peptide content (F1). With respect to the *A. sojae* protease, there was a strong positive correlation between the di- and tripeptide content (F3) and the DH obtained by the three methods (formaldehyde, OPA and soluble protein content). Furthermore, the soluble protein content method was negatively correlated with the large peptide content (F1).

In summary, these results indicate that in most cases, the increase in the hydrolysis time of WPC proteins produced more small peptides and free amino acids and less large peptides.

Considering that among the fractions for which it was detected a strong positive correlation with the DH, the F3 (di- and tripeptides) is the most important to evaluate the nutritional value

Table 4: Correlation between the degree of hydrolysis and the peptide profiles of WPC hydrolysates

		Bacillus licheniformis		*Aspergillus sojae*	
Degree of hydrolysis (method)	Chromatographic fractions	r	p	r	p
Formaldehyde	F1	-0.7726	0.0007	-0.3742	0.1693
	F2	0.4573	0.0865	-0.0504	0.8586
	F3	0.9195	<0.0001	0.9142	<0.0001
	F4	0.8125	0.0002	0.2120	0.4481
OPA	F1	-0.0606	0.0166	-0.3087	0.2629
	F2	0.3648	0.1812	-0.0260	0.9267
	F3	0.7073	0.0032	0.7933	0.0004
	F4	0.6769	0.0056	-0.2615	0.3464
Osmometry	F1	-0.6135	0.0150	-0.1132	0.6879
	F2	0.2368	0.3955	-0.1539	0.5838
	F3	0.8719	<0.0001	0.4553	0.0881
	F4	0.8877	<0.0001	-0.0620	0.8264
Soluble protein content	F1	-0.8683	<0.0001	-0.7498	0.0013
	F2	0.6088	0.0160	0.4673	0.0789
	F3	0.9133	<0.0001	0.8798	<0.0001
	F4	0.8326	0.0001	-0.6755	0.0057

r: Correlation, p: Determined by t-student test with significant differences for $p<0.05$, OPA: Ortophtalaldehyde, F1: Large peptides (>7 amino acid residues), F2: Medium peptides (4-7 amino acid residues), F3: di- and tripeptides, F4: Free amino acids

of protein hydrolysates, it can be concluded that for the *B. licheniformis* protease all tested methods could be used to screen hydrolysates for the preparation of food formulas, especially the formaldehyde method which had the highest r value (0.9195). For *A. sojae* protease, only the osmometry method would not be suitable for this purpose.

No report was found in the literature correlating the DH of WPC hydrolysates and the peptide profile.

CONCLUSION

The degree of hydrolysis of WPC hydrolysates changed with the type of method used to evaluate this parameter and the highest values were obtained with the soluble protein content method for the *B. licheniformis* protease and with the OPA method for the *A. sojae* protease. For both enzymes, the formaldehyde method was considered the most suitable for evaluating the DH of WPC hydrolysates because it showed the most pronounced variation of the results with the reaction time. For the *A. sojae* protease, the same was observed when using the OPA method. With respect to the peptide profile, the action of the *B. licheniformis* protease was more advantageous than *A. sojae* protease because it produced protein hydrolysates with higher content of di- and tripeptides and lower of large peptides.

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