# Degree of hydrolysis and peptide profile of whey proteins using pancreatin

## Grau de hidrólise e perfil peptídico das proteínas do soro de leite utilizando a pancreatina

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## ABSTRACT

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*In the present study, we used pancreatin for hydrolyzing whey proteins and evaluated the degree of hydrolysis (DH) and peptide size distribution. The following methods were used for DH determination: formol titration, soluble protein content, ortho-phthalaldehyde (OPA), and freezing point. Peptide size distribution was conducted by size-exclusion high-performance liquid chromatography (HPLC). The DH varied from 3.26% to 36.41%, and the highest yield was obtained by the soluble protein content method (mean of 35.85%). Formol titration was considered the most suitable method for assessing the DH, because it showed a marked increase with reaction time (from 1h-17.20% to 2h-24.86%). Significant positive correlation of strong intensity was observed between the following methods: formol titration and OPA (r=0.9616; p=0.0090), formol titration and freezing point (r=0.8784; p=0.0493), and OPA and freezing point (r=0.9515; p=0.0127). The highest contents of di- and tripeptides and free amino acids were 9.07% and 8.22%, respectively. A significant positive correlation of strong intensity was also observed between the degree of hydrolysis and the fraction of medium-sized peptides by the formol titration (r =−0.9274; p=0.0232) and OPA (r=−0.8977; p=0.0386) methods.*

Keywords: Whey proteins. Peptides. Pancreatin.

## RESUMEN

*En este trabajo, se utilizó una pancreatina para hidrolizar las proteínas del suero de leche, evaluándose el grado de hidrólisis (GH) y la distribución de tamaño de los péptidos. Los métodos de valoración con formol, determinación de proteínas solubles, ortoftalaldehído (OPA) y disminución del punto de congelación se emplearon para evaluar el GH. La distribución del tamaño de los péptidos se realizó por cromatografía líquida de exclusión molecular. El GH varió de 3,26% a 36,41% y los mejores resultados se obtuvieron con el método de determinación de proteínas solubles (media de 35,85%). La valoración con formol se consideró el método más adecuado para evaluar el GH, en función de los significativos incrementos con el tiempo de reacción (de 17,20% a 24,86%). Se observó una correlación positiva y de fuerte intensidad entre los siguientes métodos: valoración con formol y OPA (r=0,9616, p=0,0090); valoración con formol y disminución del punto de congelación (r=0,8784, p=0,0493); OPA con la disminución del punto de congelación (r=0,9515, p=0,0127). Los contenidos más altos de di-tripéptidos y de aminoácidos libres fueron de 9,07% y 8,22%, respectivamente. Una correlación positiva y de fuerte intensidad se verificó entre la fracción de péptidos medios y el GH evaluado con los métodos de la valoración con formol (r=–0,9274, p=0,0232) y OPA (r=–0,8977, p=0,0386).*

**Palabras clave: Proteínas del suero de leche. Péptidos. Pancreatina.**

## RESUMO

*Neste trabalho, uma pancreatina foi utilizada para hidrolisar as proteínas do soro de leite, avaliando-se o grau de hidrólise (GH) e a distribuição de tamanho dos peptídeos. Os métodos de titulação com formol, determinação de proteínas solúveis, ortoftalaldeído (OPA) e depressão do ponto de congelamento foram empregados para avaliar o GH. A distribuição do tamanho de peptídeos foi realizada por cromatografia líquida de exclusão molecular. O GH variou de 3,26% a 36,41% e os melhores resultados foram obtidos com o método de determinação de proteínas solúveis (média de 35,85%). A titulação com formol foi considerada como o método mais adequado para avaliar o GH, em função dos significativos aumentos com o tempo de reação (de 17,20% a 24,86%). Observou-se uma correlação positiva e de forte intensidade entre os métodos de titulação com formol e OPA (r=0,9616, p=0,0090); titulação com formol e depressão do ponto de congelamento (r=0,8784, p=0,0493); OPA e depressão do ponto de congelamento (r=0,9515, p=0,0127). Os maiores teores de di-tripeptídeos e de aminoácidos livres foram de 9,07% e 8,22%, respectivamente. Correlação positiva e de forte intensidade foi verificada entre a fração de peptídeos médios e o GH avaliado pelos métodos da titulação com formol (r=–0,9274, p=0,0232) e OPA (r=–0,8977, p=0,0386).*

**Palavras-chave: Proteínas do soro de leite. Peptídeos. Pancreatina.**

## INTRODUCTION

Enzymatic hydrolysis of proteins releases small peptides and free amino acids, and therefore, may contribute to increase the nutritional value of food proteins. In fact, during metabolism, proteins are first broken into these smaller molecules before being absorbed and used for several functions in the body (HINSBERGER; SANDHU, 2004; GILBERT; WONG; WEBB JUNIOR, 2008).

Several proteases may be used for this purpose, such as pancreatin, which was used in the current work. Pancreatin is an enzymatic complex consisting of enzymes secreted by the pancreas. It has proteolytic, amylolytic and lipolytic activities. These proteases are divided into endopeptidases (trypsin, chymotrypsin and elastase) and exopeptidases (carboxypeptidases A and B) (ANDRIAMIHAJA et al., 2013).

Evaluation of the extent of protein hydrolysis may be conducted by measuring the degree of hydrolysis (DH). In the present study, four techniques based on different principles were used for this purpose: formol titration, determination of soluble protein content, reaction with ortho-phthalaldehyde, and freezing point. These methods were chosen because, to our knowledge, this is the first study where a comparison among them was carried out. In other works, comparisons were carried out between the ortho-phthalaldehyde and 2,4,6-trinitrobenzene sulfonic acid methods (NIELSEN; PETERSEN; DAMBMANN, 2001) or between the ortho-phthalaldehyde and pH stat methods (CHEISON et al., 2009).

The current work is the first to assess the degree of hydrolysis of hydrolyzed whey proteins obtained by the action of pancreatin. Furthermore, this was the first study to use the soluble protein content and freezing point methods for pancreatin. Additionally, to the best of our knowledge, this is also the first report of a correlation among the four methods used in the present work to determine the degree of hydrolysis of hydrolyzed whey proteins obtained by the action of pancreatin.

The peptide composition of hydrolyzed proteins may be evaluated by determining the peptide distribution according to the chain length. The length of peptide chain influences the rate of absorption, and therefore, the nutritional value of food proteins. Our group has developed a method for fractionating peptides with molecular masses smaller than 1,000 Da, and this technique was employed in the current study (SILVESTRE; HAMON; YVON, 1994a, b). Considering that, to our knowledge, the correlation between the degree of hydrolysis and the size distribution of peptides has not been previously established, this investigation was carried out in the present work.

The current study sought to use pancreatin to obtain hydrolyzed whey proteins with proper DH and peptide size distribution.

## MATERIAL AND METHODS

### Materials

Pancreatin (Corolase PP, activity of 5.97U mL$^{-1}$) was kindly provided by AB Enzymes (Barueri, SP, Brazil). In this study, enzyme activity was defined as the amount of enzyme required to release 1g of tyrosine in 1min at 37°C. WPC (Kerrylac 750) in powdered form was kindly supplied by Kerry

of Brazil Ltda. (Três Corações, MG, Brazil). Formic acid was purchased from Merck (Whitehouse Station, NJ, USA). Ortho-phthalaldehyde (OPA, Code P0657), 2-mercaptoethanol (code M6250) and Folin-Ciocalteu reagent (code F9252) were purchased from Sigma (São Paulo, SP, Brazil). The tangential flow system with a 10 kDa cut-off membrane and the polyvinylidene fluoride membranes used for the filtration of samples (0.22μm) and solvents (0.45μm) were purchased from Millipore (São Paulo, SP, Brazil). All other reagents used in this study were of analytical grade. The high-performance liquid chromatography (HPLC) system used for fractionating hydrolyzed proteins 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 (250×9.4mm, 5μm 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).

## METHODS

### *Hydrolysis of whey protein concentrate (WPC)*

WPC solutions (10%, w/v) were prepared. After adjustment of pH to 7.0 with a 3mol L$^{-1}$ NaOH solution, the WPC solutions were heated in oil bath at 50°C with continuous stirring (stirrer 752A model from Fisatom, São Paulo, SP, Brazil), and the pancreatin was added in appropriate quantity to attain an enzyme:substrate ratio of 8.0:100 (w/w). The reaction times were 1h (P1), 2h (P2), 3h (P3), 4h (P4) and 5h (P5) and the hydrolytic reaction was stopped by heating the samples at 75°C for 15 sec. The hydrolyzed proteins were immediately used for the determination of the degree of hydrolysis and then freeze-dried (Freeze Dry System/FreeZone 4.5, model 77500, Labconco, Kansas City, MO, USA) and stored in a freezer (–20°C) until size distribution evaluation. The pH and temperature values corresponded to the optimal conditions for the enzyme and were provided by the manufacturer.

### *Evaluation of the degree of hydrolysis*

#### Formol titration

The α-amino nitrogen (AN) was titrated by formaldehyde as described in the Sorensen method (ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, 1995). Total nitrogen (TN) was determined by the Kjeldahl method (ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, 1995). Then, the degree of hydrolysis (DH) was calculated using the ratio between AN and TN according to Equation 1:

$$\% \, DH = \frac{\alpha\text{-amino nitrogen (AN)}}{\text{total nitrogen (TN)}} \times 100 \tag{1}$$

#### Reaction with ortho-phthalaldehyde

The method described by Spellman et al. (2003) was used, with some modifications, for the reaction of amino nitrogen with ortho-phthalaldehyde (OPA). A volume of 10μL of the sample was mixed with 3.4mL of the OPA reagent, and this mixture was allowed to stand at 25°C for

2min. Subsequently, the absorbance was read at 340nm and the DH was calculated according to Equation 2:

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

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

## Freezing point measurement

The measurement of the freezing point depression of the solution, called Osmometry, was conducted by using a micro-osmometer (Precision System 5004, Spectrun, São Paulo, Brazil). For this analysis, a sample volume of 50μL was immediately removed after the hydrolytic reaction and the DH was calculated according to Equation 3, proposed by Adler-Nissen (1986):

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

Where $\Delta C$ is the osmolarity (mosmol L$^{-1}$); P% is the protein concentration (g of protein/kg of water); $1/\omega$ 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 8.8mEq g$^{-1}$ of protein for whey.

## Quantification of the soluble protein

The percentage of solubilized protein in 10g% trichloroacetic acid (TCA), in relation to the total protein content of the sample, was measured by the method of Hoyle and Merritt (1994), with modifications. Aliquots of 500μL of the hydrolyzed protein were mixed with 500μL of 20g% of TCA solution to obtain the soluble and insoluble fractions in 10 g% TCA. After 30 minutes of rest, the mixture was centrifuged at 3000 x *g*, and the soluble protein content of the supernatant was determined by the method of Lowry et al. (1951), modified by Hartree (1972) - the result was expressed as mg of protein. Bovine serum albumin was used as the standard. The DH was calculated according to Equation 4:

$$DH\,(\%) = \frac{\text{soluble protein content in 10 g\% TCA (mg)} \times 100}{\text{total protein content (mg)}} \tag{4}$$

## Distribution of peptide size

Initially, the peptides were fractionated according to chain length by size exclusion (SE) HPLC on a PHEA column, using the method previously developed by our team (SILVESTRE; HAMON; YVON, 1994a). A 0.05mol L$^{-1}$ formic acid solution (pH 2.5) was the mobile phase and isocratic conditions at a flow rate of 0.5mL min$^{-1}$ for 35min were used. The samples were dissolved in the mobile phase (0.1% w/v) and filtered through a membrane of 0.22μm, and 50μL of these solutions were injected onto the column. The mobile phase was filtered through a membrane of 0.45μm and degassed by sonication in ultrasound bath (USC1400 model, Unique, Santo Amaro, SP, Brazil) for 30min before use.

Afterwards, the peptides and free amino acids were quantified using the rapid method of Correct Fraction Area (CFA), which had been previously developed by our team (SILVESTRE; HAMON; YVON, 1994b). Briefly, five whey standard hydrolysates (two using trypsin and three using pancreatin) were prepared and then fractionated into four fractions by SE-HPLC, as previously described. 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 300nm) 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 for analysis in split-plot (4×6, methods - hydrolysis times) scheme, and the results were analyzed using SISVAR version 5.3 software (FERREIRA, 2010). A completely randomized experimental design was adopted for factorial analysis (2×6, enzymes - hydrolysis times) of the peptide profiles, and the results were analyzed using Statistica software (STATSOFT, 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 and the contents of peptides and free amino acids were calculated by Pearson's correlation coefficient (r), which measures the degree of association between two variables, and $p$ was calculated with t-tests using BioStat software (AYRES et al., 2007) for data analysis.

## RESULTS

### DEGREE OF HYDROLYSIS OF WHEY PROTEINS

The DH yields obtained by the four methods are shown in Figure 1. They varied from 3.26% to 36.41%. The results of the DH of the samples were deducted by 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 soluble protein content method gave rise to a much higher yield compared with the other methods for all samples, and no significant difference was observed among P1, P2, P3 and P4 samples (mean of 35.85%). Only P5 showed a smaller yield (34.28%).

The formol titration method produced the second highest values of the DH, ranging from 17.20% to 30.52%. The third highest DH values were reached by the OPA method, producing similar values for all samples (mean of 21.99%). The lowest DH values were found by the freezing point method, and no significant difference was observed among the samples (mean of 3.60%).

No report has been found in the literature describing the use of soluble protein content, osmometry or formol titration for evaluating the DH of WPC proteins by the action of pancreatin.
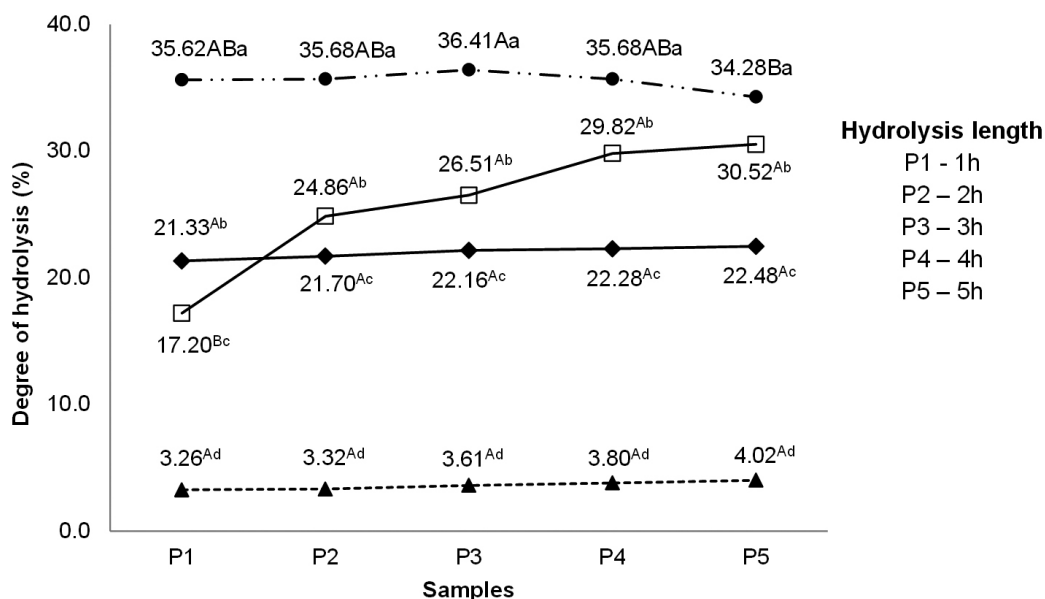
**Figure 1 - Degree of hydrolysis of WPC hydrolysates obtained by the action of pancreatin. Methods of formol titration (□), reaction with ortho-phthalaldehyde (♦), freezing point (○), and soluble protein content (●). The results represent the means of triplicate experiments. Different capital letters 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.**

## EFFECT OF HYDROLYSIS LENGTH ON THE DEGREE OF HYDROLYSIS

In general, the DH of WPC proteins is weakly affected by hydrolysis length (Figure 1). Only one important modification was observed for the formol titration method, where the DH increased sharply as the reaction time increased from 1h (17.20%) to 2h (24.86%). For the soluble protein content method, a slight decrease of the DH was observed when the reaction time increased from 4h (35.68%) to 5h (34.28%).

## CORRELATION AMONG THE DEGREE OF HYDROLYSIS METHODS

Given that the values found for some correlations were above 0.7 with $p<0.05$ (SAMPAIO, 2002), significant positive correlation of strong intensity was found among the following methods: formol titration with OPA (r=0.9616; p=0.0090), formol titration with freezing point (r=0.8784; p=0.0493), and OPA with freezing point (r=0.9515; p=0.0127) (Table 1).

To our knowledge, this was the first time correlation among the degree of hydrolysis methods of whey proteins was established.

## PEPTIDE SIZE OF HYDROLYZED PROTEINS

The hydrolyzed proteins were fractionated in four fractions: large peptides (with more than 7 amino acid residues); medium peptides (with 4 to 7 amino acid residues); di- and tripeptides and free amino acids (Table 2).

**Table 1 - Correlation among methods for the determination of the degree of hydrolysis**

| Comparisons | Pancreatin | |
|---|---|---|
| | **r**[a] | **p**[b] |
| Formol titration with OPA[c] | 0.9616 | 0.0090 |
| Formol titration with Freezing point | 0.8784 | 0.0493 |
| Formol titration with Soluble Protein | –0.3337 | 0.5831 |
| OPA[c] with Freezing point | 0.9515 | 0.0127 |
| OPA[c] with Soluble Protein | –0.3552 | 0.5575 |
| Freezing point with Soluble Protein | –0.5659 | 0.3199 |

[a]r: correlation; [b]p: determined by t-*Student* test with significant differences for $p<0.05$; [c]OPA: ortho-phthalaldehyde.

**Table 2 - Peptide and free amino acid contents of hydrolyzed whey proteins**

| Samples | Reaction time (h) | Large peptides[a] | Medium-sized peptides | Di- and tri- peptides | Free amino acids |
|---|---|---|---|---|---|
| P1 | 1 | 56.69±1.94[C] | 35.63±2.03[B] | 4.22±0.23[D] | 3.46±0.34[D] |
| P2 | 2 | 45.54±0.58[D] | 42.02±0.63[A] | 6.43±0.70[B] | 6.01±0.84[B] |
| P3 | 3 | 42.43 2.82[DE] | 44.65±2.00[A] | 9.07±0.70[A] | 3.85±0.15[CD] |
| P4 | 4 | 41.33±4.83[E] | 43.57±4.79[A] | 7.12±0.73[B] | 7.98±0.63[A] |
| P5 | 5 | 41.13±2.56[E] | 44.18±3.41[A] | 6.46±0.15[B] | 8.22±1.08[A] |

Large peptides: with more than 7 amino acid residues; medium-sized peptides: with 4 to 7 amino acid residues.

P3 sample showed the highest di- and tri- peptide content (9.07%), but one of the lowest free amino acid contents (3.85%). P4 showed the second di- and tri- peptide content (7.12%) and one of the highest free amino acid contents (7.98%). P2 and P5 occupied the third position in terms of di- and tri- peptide contents with no significant difference between them (6.43% and 6.46%, respectively). Regarding the free amino acid contents, P5 showed higher result (8.22%, similar to P4) than P2 (6.01%, the second position). No significant difference was observed among the large peptide contents of P2, P3, P4 and P5 samples (mean of 42.63%). P1 ranked in the last position with the lowest di- and tri- peptide content (4.22%), one of the lowest free amino acid contents (3.46%, similar to P3), and the highest amount of large peptides (56.69%).

**DEGREE OF HYDROLYSIS *VERSUS* PEPTIDE SIZE**

According to Sampaio (2002), *r* values greater than 0.7 with $p<0.05$ indicate a strong and significant association between the data. Therefore, it can be observed in Table 3 that some correlations of different types were found between the degree of hydrolysis and peptide size. Thus, a significant positive correlation of strong intensity was observed between the degree of hydrolysis and the fraction of medium-sized peptides for the formol titration (r=–0.9274; p=0.0232) and OPA (r=–0.8977; p=0.0386) methods. A non-significant correlation (r=0.7221; p=0.1683) was

Table 3 - Correlation between the degree of hydrolysis and the peptide size of hydrolyzed whey proteins

| Degree of hydrolysis (method) | Peptide size | Correlation parameters | |
|---|---|---|---|
| | | r | p |
| Formol titration | large peptides | −0.9736 | 0.0051 |
| | Medium-sized peptides | 0.9274 | 0.0232 |
| | Di- and tri- peptides | 0.6428 | 0.2420 |
| | Free amino acids | 0.8145 | 0.0931 |
| OPA[c] | large peptides | −0.9273 | 0.0233 |
| | Medium-sized peptides | 0.8977 | 0.0386 |
| | Di- and tri- peptides | 0.6529 | 0.2274 |
| | Free amino acids | 0.7161 | 0.1736 |
| Freezing point | large peptides | −0.7808 | 0.1190 |
| | Medium-sized peptides | 0.7221 | 0.1683 |
| | Di- and tri- peptides | 0.4129 | 0.4896 |
| | Free amino acids | 0.7686 | 0.1288 |
| Soluble Protein Content | large peptides | 0.1503 | 0.8094 |
| | Medium-sized peptides | −0.0580 | 0.9261 |
| | Di- and tri- peptides | 0.4117 | 0.4910 |
| | Free amino acids | −0.6634 | 0.2222 |

r: correlation; p: determined by t-*Student* test with significant differences for $p<0.05$; OPA: ortho-phthalaldehyde; Large peptides: with more than 7 amino acid residues; medium-sized peptides: with 4 to 7 amino acid residues.

observed between the degree of hydrolysis and the fraction of medium-sized peptides in the case of the freezing point method. Similarly, non-significant correlation was observed between the degree of hydrolysis and the free amino acid fraction for the formol titration (r=0.8145; p=0.0931), OPA (r=0.7161; p=0.1736), and freezing point methods (r=0.7686; p=0.1288). Additionally, a significant negative correlation of strong intensity was found between the fraction of large peptides and the degree of hydrolysis for the formol titration (r=−0.9736; p=0.0051) and OPA (r=−0.9273; r=0.0233) methods. Non-significant correlation (r=−0.7808; p=0.1190) was observed between the degree of hydrolysis and the fraction of large peptides for the freezing point method.

## DISCUSSION

The fact that the soluble protein content method produced the highest hydrolysis yield may be associated with the action of pancreatin. The pancreatin used was an enzymatic preparation containing enzymes with endopeptidase (trypsin and chymotrypsin) and exopeptidase (carboxypeptidases) activities (INTERNATIONAL UNION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, 2010) and may have extensively hydrolyzed the WPC proteins,

producing high content of small peptides that are not precipitated by TCA, and therefore, contributed to increase the DH obtained by this method.

The higher DH values obtained by the formol titration method in relation to OPA for most of the samples could be explained by the action of two enzymes found in the pancreatin preparation. Trypsin, which preferably cleaves peptide bonds containing residues of lysine or arginine, and chymotrypsin, which hydrolyzes peptide bonds with basic or aromatic amino acids (GUPTA; BEG; LORENZ, 2002; HINSBERGER; SANDHU, 2004; PAGE; DI CERA, 2008). The action of these enzymes most likely increased the exposure of basic amino acid residues whose side chains also have protons that can be released by the addition of formaldehyde (LEVY, 1935; TAYLOR, 1957).

When using the OPA method, it is important to note that although whey proteins have a high percentage of lysine, whose ε-amino group can also react with the derivatizing agent leading to an overestimation of DH (CHURCH et al., 1983), they are also rich in cysteine, which reacts weakly and unstably with OPA, and may reduce the DH of WPC hydrolysates (SPELLMAN et al., 2003). This explains, at least in part, the third position occupied by the OPA method in terms of DH value.

A likely explanation for the low values showed by the freezing point method is related to the ability of pancreatin to increase the exposure of aromatic or basic amino acid residues (GUPTA; BEG; LORENZ, 2002; RAWLINGS; BARRETT; BATEMAN, 2010). This would cause a pH modification in the solution, leading to the aggregation of peptides and, consequently, gradually increasing the viscosity and turbidity of the solution (DOUCET et al., 2003).

To our knowledge, this is the first time the soluble protein content, freezing point, or the formol titration methods were used to assess the degree of hydrolysis of WPC through the action of pancreatin.

Only one work in the literature reports the use of the OPA method. Smyth and FitzGerald (1998) reported a maximum DH of 11.5% when using the OPA method for evaluating the DH of WPC proteins hydrolyzed by pancreatin, which is half of the values obtained in the current study (mean of 21.99%). This difference could be explained either by the hydrolytic conditions used or by the presence of a reducing agent. In fact, contrarily to the present work, these authors carried out the reaction at pH 8, 37°C, E:S ratio of 1:50, for 8:30 h, in absence of 2-mercaptoethanol. According to Roth (1971), the reaction of cysteine with OPA can be optimized when the reagent is mixed with a solution of amino acids in the presence of a strong reducing agent.

Considering that formol titration was the only method that showed a marked increase with reaction time, it could be inferred that it is the most suitable among the methods tested for assessing the DH of hydrolyzed whey proteins obtained by the action of pancreatin, with the caveat that hydrolysis proceeds for a maximum of 2h.

Regarding the correlation among the degree of hydrolysis methods, it is worthy stating that no reports have been found in the literature investigating the correlation among DH methods for WPC hydrolyzed proteins obtained by pancreatin.

Peptide size is associated to the nutritional value of hydrolyzed proteins. In truth, during protein metabolism, the first stage of hydrolysis leads to the formation of free amino acids (AA) and peptides with 2 to 6 AA residues, which can be further hydrolyzed by brush border peptidases to produce AA and di- and tri- peptides (HINSBERGER; SANDHU, 2004). The

amino acids can be absorbed either in the free form, by simple and facilitated diffusion (membrane transporters), or in the di- and tri- peptide form (oligopeptide transporters) (GILBERT; WONG; WEBB JUNIOR, 2008). Furthermore, according to Frenhani and Burini (1999), the di- and tri-peptides are more efficiently absorbed than an equivalent mixture of free amino acids. Therefore, the enzymatic hydrolysis of proteins can improve their nutritional value by reducing the peptide size and releasing small peptides and free amino acids, which can be readily absorbed. In this way, from a nutritional point of view, P2, P3, P4 and P5 samples showed similar peptide profiles, which were superior to P1.

The evidence of the correlations between the degree of hydrolysis and the peptide size indicate that some methods were able to show that, as expected, an increase in the degree of hydrolysis of WPC proteins produced more medium-sized peptides and free amino acids but fewer large peptides. This was especially the case of the formol titration and OPA methods. Consequently, these two methods could be used as a rapid screening for choosing the hydrolyzed proteins with the best nutritional profile or peptide size distribution.

No report has been found in the literature correlating the DH of WPC hydrolysates with peptide size.

## CONCLUSION

The use of pancreatin at an E:S ratio of 8:100 from 1h to 5h was able to produce hydrolyzed whey proteins with proper peptide size distribution, from a nutritional point of view. Formol titration and OPA seemed to be the best methods for the rapid screening of hydrolyzed whey proteins with this type of profile. Formol titration was the most suitable method for assessing the DH of hydrolyzed whey proteins. Correlations found between the DH and the peptide size showed that a DH increase of whey proteins leads to a peptide size distribution of higher nutritional value, with more medium-sized peptides (4 to 7 amino acid residues) and fewer large peptides (>7 amino acid residues).

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