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A comparison of dual-functional whey hydrolysates by the use of commercial proteases

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Abstract

Whey is well-known for the functional and bioactive properties of its proteins and peptides, which are of great interest to food and nutraceutical industries. The aim of this study was to investigate the effect of four different commercial proteases (Novo ProD® (NPD), Alcalase® (ALC), Pancreas Trypsin® (TRY), and Flavourzyme® (FLA)) in the generation of hydrolysates with emulsifying and antioxidant activities. Hydrolysis processes were carried out for 5 h, reaching maximum degrees of 18.5, 15.5, 9.2, and 8.7% for NPD, ALC, TRY, and FLA, respectively. All tested enzymes generated very diverse, but conservative peptide profiles when comparing the treatments along the time, with the main enzymatic actions up to 120 min-reaction. An increase in the *in vitro* antioxidant activity was found for all treatments, achieving 46%, 40%, 40% and 22% for ALC, TRY, NDP and FLA, respectively. TRY hydrolysate maintained or slightly increased its emulsifying capacity along the time, however, a decrease in emulsifying capacity was found for ALC and NPD hydrolysates when compared to the non-hydrolyzed whey protein concentrate (WPC). All tested enzymes generated hydrolysates with enhanced antioxidant and/or emulsifying activities, which may be used as food ingredients and the choice of the enzyme will depend on the need.

Keywords: whey protein; hydrolysis; functionality; emulsification; antioxidant activity.

Practical Application: Four proteases were evaluated for the generation of dual functional whey hydrolysates.

1 Introduction

Whey proteins are well known and widely consumed for being a high quality protein source especially incorporated into sports and nutrition products (West et al., 2017). Different commercial ingredients and products are available in the market, including whey powders (WP), whey protein concentrates (WPC) and whey protein isolates (WPI) which are classified according to the protein content, varying from less than 30% for WP to 30-90% for WPC and above 90% for WPI (Whipple & Eckhardt, 2016). In general, higher protein contents will result into better-valued products, mainly to attend nobler uses, as in the market of infant formulas and nutraceutical products.

Annual whey generation is estimated in 240 million metric tons (Mordor Intelligence, 2017). Besides its nutritional and technological properties, whey presents elevated organic matter content, generating high biological and chemical oxygen demands which cause environmental concerns (Sultana et al., 2016). In this sense, industrial interest in the use of whey has been growing, aiming to concentrate and dry whey proteins into different ingredients mainly to attend food industries.

Whey proteins are also known for the bioactivity of its peptides, which can be liberated through hydrolysis processes catalyzed by chemical or enzymatic agents. The use of enzymes has gained popularity since their specificity for some active-sites, results into ingredients and products with enhanced specialized properties, particularly in terms of biological functionalities (Le Maux et al., 2016; Ojha et al., 2016).

Among the main investigated biological properties from whey peptides, which comprise beneficial results for the cardiovascular, nervous, gastrointestinal, and immune systems, antioxidant activity is of relevance (Cánovas et al., 2017). The ability of peptides to interact with radical species or inhibit oxidative reactions is important for the body to prevent a number of age-specific diseases and neurodegenerative disorders caused by the oxidative stress. In food, oxidative reactions may shorten shelf life through deterioration of food quality worsening sensorial and nutritional aspects (Dryáková et al., 2010; Power et al., 2013). Thus, the possibility of using a natural antioxidant from a dietary source can be interesting and may even replace noxious, synthetic compounds.

In addition to the bio-functionalities, whey peptides may also enhance technological properties of food formulations, such as water binding, solubility, gelation, and emulsification (Madadlou & Abbaspourrad, 2017), and the possibility of joining both bio and technological functionalities is also of interest for the food industry sector. In this way, the objective of this study was to compare WPC hydrolyses of four commercial proteases, considering both the antioxidant activity and emulsifying capacity of the hydrolysates.

2 Materials and methods

2.1 Materials

A spray-dried whey protein concentrate (WPC 35) from bovine milk, of 34% (w/w) protein, was gently donated by Alibra Ingredientes Ltda (Paraná, Brazil), and was used as substrate. Enzymes were gently donated from Novozymes Latin America Ltda (Paraná, Brazil) and were used as recommended by the manufacturer in terms of enzyme concentration, optimal temperature, pH and the conditions for enzyme inactivation. Reagents were of high purity and purchased from Sigma-Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany).

Enzymatic hydrolysis

WPC hydrolysates were prepared by suspending the powder in ultrapure water at a concentration of 24% (w/v), corresponding to 8.2 g of protein in 100 mL of suspended whey. The suspension was then continually stirred (350 rpm) and the hydrolysis conditions were kept as indicated in Table 1. Prior the reaction, the systems were allowed to rehydrate and solubilize for 30 min. The suspension was then adjusted to the adequate pH using aq. NaOH (1.0 M). At this point, an aliquot (control – C) was collected. The enzymes were then added to each system as recommended by the manufacturer. Values of pH and temperature were monitored along the reaction. Aliquots were collected after 1 min (T0), 60 min (T1), 120 min (T2), 180 min (T3), 240 min (T4), and 300 min (T5) of hydrolysis. All hydrolyzed samples were heated at 85 °C for 15 min to inactivate the enzymes, and then cooled down under running tap water (18 °C). Samples were freeze-dried (Liotop, São Paulo, Brazil) then kept at -20 °C for further analyses. Degree of hydrolysis (DH %) was determined by the volume of base consumed, according to the pH-stat method (Adler-Nissen, 1986).

Chromatographic analysis of peptides and proteins

RP-HPLC was used to analyze the peptide profiles generated from the hydrolysis processes. An analytical HPLC unit (Waters – Alliance, separation module 2695, São Paulo, Brazil) with a C18 column, held at 30 °C, was used. Gradient elution was carried out by the use of two solvents. Solvent A: 0.1% trifluoroacetic acid (TFA) in ultrapure water (v/v), and Solvent B: 0.1% TFA in acetonitrile (ACN) (v/v). Hydrolysates were eluted as follows: 0 - 2 min, 95% A; 2 - 15 min, 95 - 80% A; 15 - 20 min, 80 - 70% A; 20 - 25 min, 70 - 60% A; 25 - 28 min, 60 - 50% A; 28 - 32 min,

50 - 40% A; 32 - 34 min, 40 - 30% A; 34 - 36 min, 30 - 20% A; 36 - 38 min, 20 - 95% A; 38 - 40 min, 95%A. The flow rate was 1.0 mL min-1, and the detection was at 216 nm (Waters PDA Detector, 2996). Total running time was 40 min. For the chromatographic analysis, freeze-dried samples were suspended into ultrapure water (2 mg mL^{-1}) and 20 µL was injected. Bovine α-lactoalbumin (α-la) and β-lactoglobulin (β–lg) were used as standards, and the retention times were 28.20 and 31.85 min, respectively.

In vitro Antioxidant Activity

The in vitro antioxidant activity of hydrolysates were measure by scavenging of the ABTS radical (ABTS•+) (2,2'-azinobis (3-ethylbenzothiazoline-6-solfonic acid)) using a decolorization assay (Re et al., 1999). 0.1M aq. phosphate-buffered saline (PBS) was used in pH 7.4. Spectrophotometric (Micronal, São Paulo, Brazil) reading was done 6 min after the addition of ABTS•+, in triplicate (Dryáková et al., 2010). The antioxidant activity was calculated as antioxidant activity % (AA %), following the equation 1:

$$AA \% = \left[\left(Abs\ blank - Abs\ sample \right) / Abs\ blank \right] * 100 \qquad (1)$$

Emulsifying Capacity (EC)

Hydrolysates EC were evaluated by the emulsifying index of the sample in hexadecane according to Mellinger-Silva et al. (2015), right after hydrolyses processes, as soon as the hydrolysates cooled down from the enzyme inactivation. 1 mL of each hydrolysate was added of 1 mL of hexadecane in a tube test, and the tube was submitted to vigorous and non-interrupted stirring for 3 min. Then, tubes reposed for 24h. The emulsifying capacity (EC%) was calculated by using digital pachymetric measures (200 mm, IP67, PD202, Vonder, PR, Brazil) of the total solution height (Ht) and the emulsified phase height (He), as shown by the equation 2:

$$EC\ (\%) = \left(He / Ht \right) * 100 \qquad (2)$$

3 Results and discussion

3.1 Hydrolysis of WPC and peptide profile of the hydrolysates

The degree of hydrolysis (DH) of WPC differed significantly when comparing the results among the tested enzymes (Figure 1). NPD and ALC presented higher DH%: 18.5 and 15.5, respectively. In both curves it was possible to observe a rapid and intense enzymatic activity until 90 min-reaction, heading to a plateau after 120 min reaction. NPD and ALC were both obtained from Bacillus licheniformis and present endopeptidase activity, with a broad possibility of cleavage. The peptide profiles of NPD and ALC (Figure 2) were similar, with intense signals from 4 to 22 min of the chromatograms. HPLC analyses corroborated with the data from DH, showing that the main peaks were generated from 1 to 120 min and maintained up to 300 min-reaction. Both enzymes rapidly degraded α-la, while β-lg was just partially hydrolyzed along the reaction. β-lg is a globular protein which exists as a dimer at physiological pH and temperature, and two

Table 1. Hydrolysis conditions of WPC 35 by the use of different enzymes.

	Hydrolysis Conditions[a]			
Enzymes[b]	[E/S][c]	pH	T (°C)	EIC[d]
ALC	2% (w/v)	8.0	65.0	85 °C/15'
NPD	7% (w/v)	9.0	50.0	80 °C/15'
TRY	1% (w/v)	8.5	47.0	80 °C/15'
FLA	3% (w/v)	7.0	50.0	80 °C/10'

(a) hydrolysis conditions were set according to the manufacturer recommendation; (b) ALC = Alcalase 2.4L FG®; NPD = Novo-Pro D®; TRY = Pancreas Trypsin 6.0S®; FLA = Flavourzyme1.000L®. (c) E/S = enzyme: substrate concentration; (d) EIC = Enzyme Inactivation Condition; T (°C) = temperature in Celsius degrees.
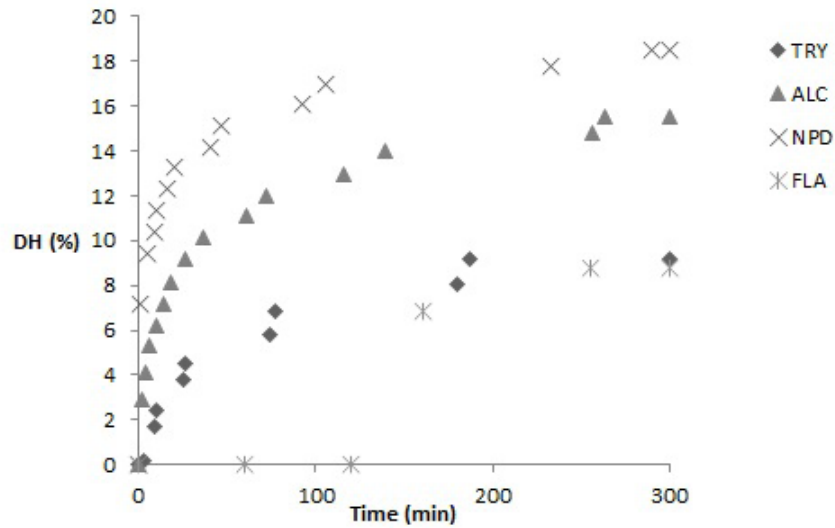
Figure 1. Degree of hydrolysis of whey protein concentrate (WPC) by the use of commercial proteases. DH% was determined by the volume of base consumed, according to the pH-stat method. (ALC) alcalase, (NPD) novo pro D, (TRY) pancreas trypsin, and (FLA) flavourzyme.
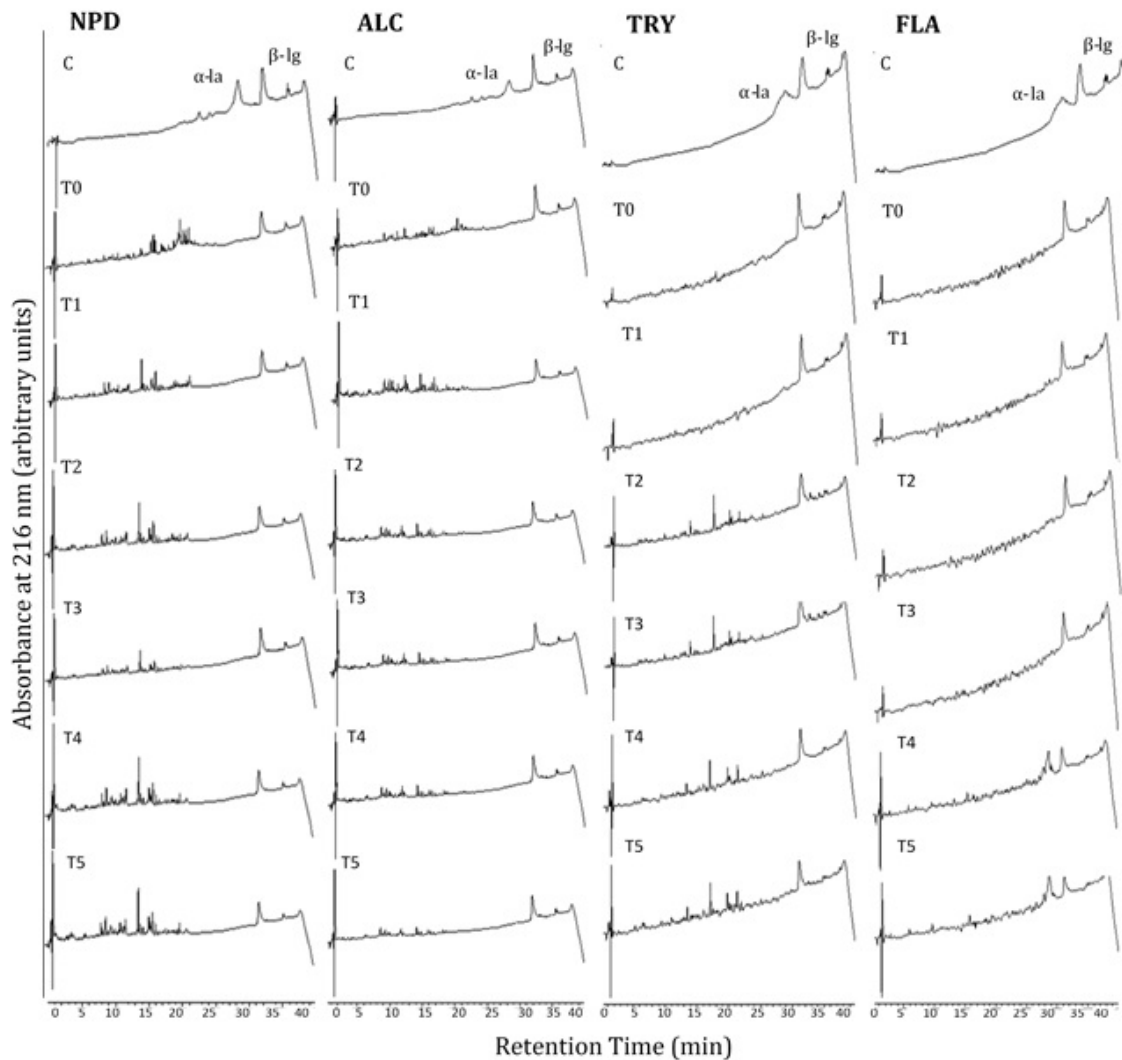

Figure 2. Chromatographic peptide profiles of whey protein concentrate (WPC) hydrolysates. (ALC) alcalase, (NPD) novo pro D, (TRY) pancreas trypsin, and (FLA) flavourzyme. (a) α-lactoalbumin; (b) β-lactoglobulin, C = control sample; T0, T1, T2, T3, T4, T5 = aliquots collected in 1, 60, 120, 180, 240 and 300 min-reaction.

disulfide bonds are responsible for its globular stable structure. However, the exposure of β-lg to high temperatures or basic pH (pH>8) generate certain instability to protein's structure, causing dimer's dissociation, which is probably related to the greatest degrees found for the WPC hydrolyses with NPD (pH 9.0) and ALC (pH 8.0) (Cheison et al., 2010; Hernández-Ledesma et al., 2008).

TRY and FLA showed lower DH% of 9.2 and 8.7%, respectively. Hydrolytic curves (Figure 1) were similar among themselves, but different from the previous ones. Intense hydrolyses were observed up to 180 min reactions for the treatments with both enzymes. TRY is an endoprotease from animal origin that hydrolyses the carboxyl-side of lysine and arginine, and such amino acid specificity may explain a lower DH. Complementing this result, the peptide profile of TRY (Figure 2) was not so varied, but showed an intense peak at 17.17 min that was maintained from the first hour of hydrolysis on.

On the other hand, FLA, a crude fungal enzyme formula from *Aspergillus oryzae*, declared by the manufacturer to present both endo- and exo-peptidase activities, showed the lowest DH and the poorest peptide profile, with only partial cleavage of α-la and no action on the β-lg structure. The stability of the β-lg structure varies according the pH, while in solutions with pH>8 it shows certain instability, in pH<8 it tends to form molecular aggregates, preventing enzyme's access to the amino acid residues that has affinity, which may explain the lowest degree of hydrolysis presented by FLA. (Cheison et al., 2010).

3.2 In vitro antioxidant activity of whey hydrolysates

All hydrolysates showed an increase in the *in vitro* antioxidant activity (AA) when measured by the ABTS assay, which is a colorimetric method widely used for food matrices and is applicable to water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts. It is based on the reduction of the radical 2,2´-azinobis (3-etilbenzotiazolin-6-sulfonic acid - ABTS.+),

which can be generated through chemical, electrochemical or enzymatic reaction. The quantity of radical consumed by the antioxidant substance is expressed as TEAC - trolox equivalent antioxidant capacity (Dryáková et al., 2010; Power et al., 2013; Zulueta et al., 2009).

All control samples were submitted to the same conditions of other samples with pH and temperature variations, which may impact the AA, justifying the differences in the non-hydrolyzed samples. ALC presented the highest AA, followed by TRY, NPD and FLA. ALC achieved 46% after 300 min-reaction, while TRY and NPD presented similar profiles, ranging from 25% and 20% to 40% in both, respectively, as shown in Figure 3. FLA showed the lowest AA, with 22% after 300 min of reaction. Corroborating with the degree of hydrolysis and peptide profiles, samples presented a tendency to stabilize after 120 min-reaction.

Comparing hydrolysis processes with different enzymes, Dryáková et al. (2010) also found alcalase hydrolysates as the most effective in scavenging the ABTS+ radical, ranging from 19.8% to 54.2%, for non-hydrolyzed to 180 min hydrolyzed samples, respectively. Although the degree of hydrolysis impacts the antioxidant activity, there is also a structure-activity relationship between both, justifying the lower DH of ALC when compared to NPD, and the greater antioxidant activity of the further. (Dryáková et al., 2010; Madureira et al., 2010; Power et al., 2013). In this manner, according to the results presented, the hydrolysate obtained with alcalase may be considered as the most efficient when the aim is to produce a whey hydrolysate with high antioxidant capacity.

3.3 Emulsifying capacity of whey hydrolysates

The emulsifying capacity of proteins and peptides is related to their capacity to lower interfacial tension between hydrophobic and hydrophilic components into a food system (Bos & van Vliet, 2001; Lam & Nickerson, 2013).
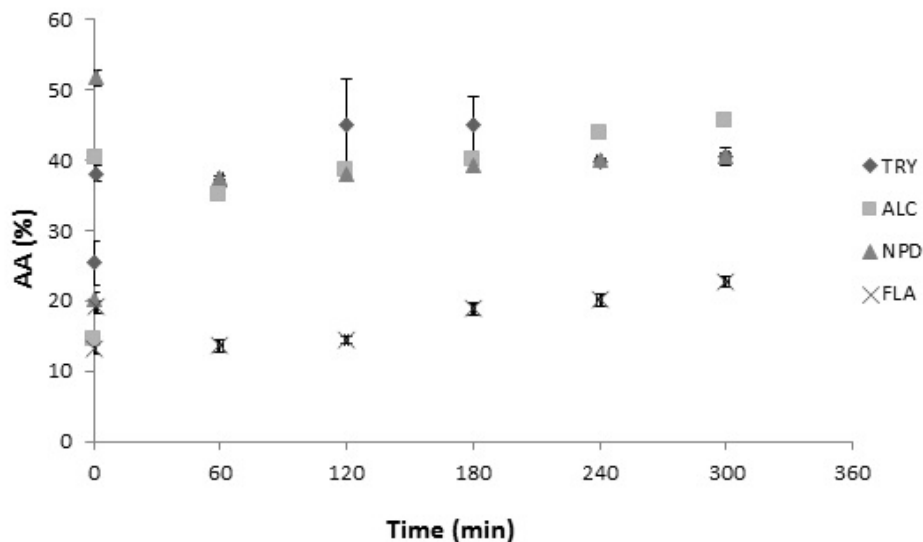

Figure 3. *In vitro* antioxidant activity of whey protein concentrate (WPC) hydrolysates. (ALC) alcalase, (NPD) novo pro D, (TRY) pancreas trypsin, and (FLA) flavourzyme. Measured by scavenge of the ABTS radical (ABTS•+). The antioxidant activity was calculated as AA%.
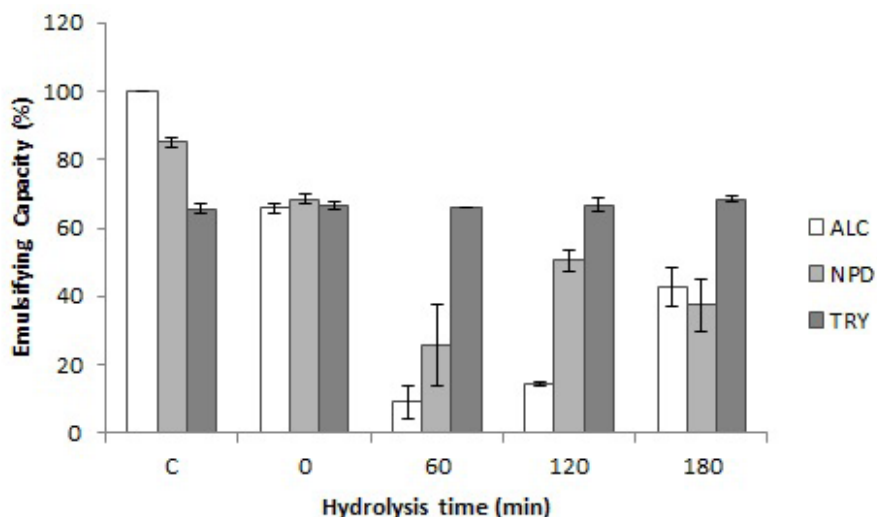
Figure 4. Emulsifying capacity of whey protein concentrate (WPC) hydrolysates. (ALC) alcalase, (NPD) novo pro D, (TRY) pancreas trypsin.

According to the results presented above, as FLA showed the lowest DH and AA, this sample was not included into the EC activity. ALC, NPD and TRY had their EC% assessed until 180 min-reaction (Figure 4), since after this time all the hydrolysates produced presented a tendency to stabilize their DH and AA, as shown in Figures 1 and 3. TRY hydrolysate showed a stable emulsifying capacity, which may be related with the low DH observed that promoted polypeptides generation. Larger size peptides are more likely to have both hydrophobic and hydrophilic residues on the same molecule, in which the hydrophobic residues interact with the oil droplets, while the hydrophilic ones will favor the aqueous phase and stabilize the droplets through steric effects. Adjonu et al. (2014) also related trypsin hydrolysates with high interfacial adsorption, emulsifying capacity and good storage stability. NPD presented an intense decrease on EC after 60 min, going from 85 to 26%, and after 120 - 180 min-hydrolysis the EC increased, and reached 50% within 120 minutes. ALC emulsifying capacity assays also presented certain instability, with a high decrease in 60-min hydrolysis, reaching 9%, followed by a significant increase at 180 min, rising from 9% to 42% EC.

Different protein characteristics may affect its emulsifying activity, including pH, temperature and protein hydrolysis. In general, low DH retain or increases this functionality, whereas intense hydrolysis diminishes it since peptides are too short to act as effective emulsifiers, saturating the continuous phase rather than adhering to the water–oil interface (Lam & Nickerson, 2013; Wani et al., 2015). Dual-functional peptides are of great interest for food industries, since it aggregates both technological and biological functions, increasing the added value of a product. Among the hydrolysates obtained, the fraction hydrolyzed with trypsin presented a stable emulsifying capacity along the experiment and a tendency to stabilize its antioxidant activity after 2h-reaction. In this sense, the 120 min tryptic hydrolysate may be considered the best condition for obtaining peptides with both antioxidant and emulsifying capacities.

4 Conclusion

This study dealt with a comparison of WPC hydrolysates by the use of commercial proteases. Alcalase and Novo pro-D presented the highest hydrolysis degrees, corroborating with their peptide profiles, which showed some similarities. According the RP-HPLC chromatograms, all enzyme treatments presented a tendency to stabilize after 120 min-reaction. The same tendency was observed for the antioxidant activity analyses, which revealed ALC hydrolysate as the most capable of scavenging the radical ABTS+, followed by TRY, NPD and FLA hydrolysates. Pancreas Trypsin showed an average emulsifying activity, while Novo Pro-D and Alcalase showed satisfactory results towards this property. In this sense, among tested enzyme, ALC, TRY and NPD could be used for producing dual-functional hydrolysates, especially the 120 min fraction of TRY hydrolysate, which presented high antioxidant activity and emulsifying capacity.

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