Effects of heat pretreatment on the yield and bioactivity of powdered egg whites hydrolysates by three different proteases.

Efeito do pré-tratamento térmico sobre o rendimento e a bioatividade de hidrolisados de clara de ovo em pó por três diferentes proteases.

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ABSTRACT
Egg white proteins have already shown great potential to generate bioactive peptides after enzymatic hydrolysis. The present study aimed to verify the influence of heat treatment on the efficiency of the hydrolysis of powdered egg white proteins by different proteases. The results were evaluated based on the hydrolysis yield, the antioxidant capacity against ABTS radical and the SDS-PAGE behavior of the hydrolysates. Heat treatment was indispensable for Alcalase to hydrolyse egg white proteins; increased the yield by pepsin hydrolysis and promoted the formation of peptides of lower molecular mass when papain was applied. Regarding the bioactivities tested, heat treatment was favorable for the hydrolysis by Alcalase, indifferent for the hydrolysis by papain and unfavorable for the hydrolysis by pepsin. The highest hydrolysis yield was obtained with pepsin (85.3%); the highest antioxidant capacity was obtained by Alcalase (5,254 μM Trolox /g sample).

Key words: antioxidant; SDS-PAGE; papain; pepsin; alcalase

PRACTICAL APPLICATION
Egg white has a variety of anti-nutritional factors that tend to hinder or prevent the hydrolysis of its proteins by different proteases. However, the permanence of these inhibitors in spray-dried egg whites and their residual influence on the activity of different enzymes had yet to be investigated. In addition, although several alternative treatments have been proposed to eliminate protease inhibitors in egg whites, the heat treatments are still the most efficient and most easily applicable by the egg processing industry, taking advantage of the equipment already installed. Moreover, the great difference found between the heat treatment effects among the tested enzymes activities proved the importance of verifying the need and the effects of the pre-treatments before the use of high cost processing aids as hydrolytic enzymes.

RESUMO
As proteínas da clara do ovo já demonstraram grande potencial para gerar peptídeos bioativos após a hidrólise enzimática. O presente estudo teve como objetivo verificar a influência do tratamento térmico na eficiência da hidrólise de proteínas da clara de ovo em pó por diferentes proteases. Os resultados foram avaliados com base no rendimento da hidrólise, na capacidade antioxidante contra o radical ABTS e no comportamento de SDS-PAGE dos hidrolisados. O tratamento térmico foi indispensável para a Alcalase hidrolisar as proteínas da clara do ovo; aumentou o rendimento pela hidrólise da pepsina e promoveu a formação de peptídeos de menor massa molecular quando a papaina foi aplicada. Quanto às bioatividades testadas, o tratamento térmico foi favorável à hidrólise por Alcalase, indiferente à hidrólise por papaina e desfavorável à hidrólise por pepsina. O maior rendimento de hidrólise foi obtido com pepsina (85,3%); a maior capacidade antioxidante foi obtida por Alcalase (5.254 μM de Trolox / g de amostra).

Palavras chave: antioxidante; SDS-PAGE; papaina; pepsina; alcalase

APLICAÇÃO PRÁTICA
A clara de ovo tem uma variedade de fatores antinutricionais que tendem a dificultar ou impedir a hidrólise de suas proteínas por diferentes proteases. No entanto, a permanência desses inibidores em claras de ovos secas por pulverização e sua influência residual na atividade de diferentes enzimas ainda não haviam sido investigadas. Além disso, embora vários tratamentos alternativos tenham sido propostos para eliminar inibidores de protease em claras de ovos, os
tratamentos térmicos ainda são os mais eficientes e mais facilmente aplicáveis pela indústria de processamento de ovos, aproveitando o equipamento já instalado. Além disso, a grande diferença encontrada entre os efeitos do tratamento térmico entre as atividades enzimáticas testadas comprovou a importância de verificar a necessidade e os efeitos dos pré-tratamentos antes do uso de auxiliares de processamento de alto custo como enzimas hidrolíticas.

1 INTRODUCTION

The egg is a unicellular body that comprises essential nutrients for the development of the species and consists of approximately 76% water, 13% protein, 10% lipid, 1% salts and small amounts of carbohydrates (Mine, 2008). Egg proteins have important nutritional relevance, which can be attributed to their high biological value, and are predominantly located in the egg white. The most abundant of these proteins are ovoalbumin (54%), ovotransferrin (12%), ovomucoid (11%), lysozyme (3.5%) and ovomucine (2.5%) (Chambers et al, 2017). Several studies report, however, that egg proteins play an important role that extends beyond their nutritional value: they generate bioactive peptides through the hydrolysis of these proteins (Sarmadi, 2010).

Bioactive peptides are small chains of amino acids that may result from digesting or processing proteins in foods (De Mejia and Ben, 2006); these peptides may exhibit antihypertensive, antioxidant, anti-inflammatory, antimicrobial or immunomodulatory capacity, among other biologically relevant activities, with potential benefits to the health of consumers (Abeyrathne, 2018). Antioxidant capacity is of interest not only as nutraceutical but also as a food preservative because of its ability to prevent or delay lipid oxidative reactions (Nimalaratne, 2015). The usual route to obtain antioxidant peptides from proteins is through hydrolysis, which consists of breaking down peptide bonds and is caused by prolonged heating in an acidic environment or is catalysed by proteolitic enzymes. Enzymatic proteolysis generally leads to better results by producing more homogeneous hydrolysates, generating more palatable products and causing less change in the nutritional value of the final product (Udenigwe and Aluko, 2012; Jegannathan, 2013). According to Korhonem (2009), there are three main ways of producing peptides by hydrolysing food proteins: (i) by applying digestive enzymes, (ii) through fermenting (microbial enzymes) or (iii) by applying isolated (commercial) enzymes. The major drawbacks of the use of enzymes are the low yield and low antioxidant capacity of the hydrolysates (Marciniak et al., 2018).
When determining how to improve the yield and performance of the hydrolysates, it is of great importance to consider which enzyme is applied since the specificity of the enzyme will determine the hydrolysis sites and, therefore, the aminoacid sequence of the peptides formed, directly affecting both yield and activity. In addition, previous treatments of the raw material were designed to increase yield and activity of the hydrolysates by eliminating protease inhibitors, promoting the solubilisation and denaturation of the proteins and exposing bonds that are more susceptible to enzymatic action (Liu, 2017; Jovanović et al., 2016). Therefore, the present study aimed to verify the influence of heat treatment on the efficiency of the hydrolysis of dried egg white proteins by three proteases (aspartic protease, cysteine protease and serine protease) from different sources (animal, plant and bacteria) by evaluating the hydrolysis yield and the antioxidant capacity of the generated hydrolysates.

2 MATERIALS AND METHODS

This study was divided into two steps. The first step investigated the effects of heat treatment on the hydrolysis efficiency, and the second evaluated the hydrolysis yield after heat treatment and followed the process over time.

2.1 SAMPLE PREPARATION

Samples consisted of 2.5 g of commercial egg white powder (pasteurised and spray dried) dissolved in 500 mL of ultrapure water and homogenised by magnetic stirring.

2.2 HEAT TREATMENT

The samples were heated in a magnetic stirrer water bath at 90 °C for 10 min. Once treated, the samples were cooled to the reaction temperature in an ice bath and then transferred to a jacketed reactor, which was connected to a thermostatic bath to control the reaction temperature. For each heat-treated (HT) sample, a similar non-treated (NT) sample was prepared and used as control.

2.3 HYDROLYSIS

The hydrolysis reactions were performed using magnetic stirring. The tested enzymes, which were purchased from Merck, were papain (EC 3.4.22.2 – cysteine-protease), porcine pepsin (EC 3.4.23.1 – aspartic protease) and Alcalase® (EC 3.4.21.14 – serine-protease from Bacillus licheniformis) and were applied in the ratio of 1:80 (enzyme: protein). The hydrolysis
conditions were as follows: papain, 60 °C and pH = 7.0; pepsin, 37 °C and pH = 2.0; and Alcalase, 60 °C and pH = 8.0. The pH of the reaction medium was kept constant by the addition of HCl or NaOH throughout the hydrolysis process. The reactions were paralysed by the addition of NaOH (papain and pepsin) or HCl (Alcalase) until the inactivation pH of the enzyme was reached.

To determine the effects of heat treatment on hydrolysis, 5 mL aliquots were taken at 0, 1, 30, 60, 120, 180 and 240 min after enzyme addition, and then the decrease in protein concentration was evaluated.

To examine the yield and antioxidant capacity over time, 5 mL aliquots were taken at 30, 60, 120, 180, 240, 300 and 360 min after enzyme addition.

All tests were performed in triplicates.

2.4 PROTEIN CONCENTRATION

Using the method developed by Bradford (1976), colorimetric assay for total proteins was performed with a standard curve using bovine serum albumin (BSA) in a UV-Vis spectrophotometer (Shimadzu UV-2700). For the analyses, the aliquotted samples were centrifuged at 14,000 x g for 10 min at 5 ºC (Thermo Fischer Scientific, USA) and the supernatant was collected. The samples were evaluated in triplicates.

2.5 PEPTIDE CONCENTRATION

Aliquots consisting of 2.0 mL of hydrolysate were added to an equal volume of trichloroacetic acid solution (TCA at 0.44 mol.L⁻¹) to evaluate the TCA soluble protein, according to Pericin et al. (2009). This mixture was incubated overnight under refrigeration (8 ºC) and then centrifuged at 14,000 x g for 10 min at 5 ºC. The supernatant was measured using the method devised by Lowry (1951), and the result was quantified based on a standard curve using BSA. All samples were evaluated in triplicates.

2.6 YIELD

The yield in peptides of the enzymatic hydrolysis was calculated based on the total protein and TCA soluble protein, according to Equation 1:

\[
yield\% = \frac{[TCA \text{ soluble protein}]}{[total \text{ protein}]} \times 100 \quad [\text{Equation 1}]
\]
2.7 SDS-PAGE

Based on the method described by Laemmli (1970), the egg white powder and the HT and NT samples were analysed using SDS-PAGE both before and after the hydrolysis.

A 12% resolution gel and a 5% packing gel were applied. The samples were mixed with a sample buffer (8% SDS, 40% glycerol, 0.2% bromophenol blue and 20% β-mercaptoethanol in 250 mM TRIS-HCl buffer pH 6.8) in a ratio of 1: 4 (buffer: sample) and then heated at 100 °C for 5 min before gel application. The gels were stained with Coomassie Brilliant Blue G-250. A molecular mass standard (BioRad) ranging from 6.1 kDa to 210 kDa was also applied to the mixture.

Three different reaction times were analysed: 0 min, 60 min and the time of best yield for each tested enzyme.

2.8 ANTIOXIDANT CAPACITY

The test was performed according to the works of Rufino et al. (2007) and Re et al. (1999), with modifications. A cationic solution of ABTS (2.2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) was prepared by mixing 2.5 mL of 7.0 mM ABTS solution and 44 μL of 140 mM potassium persulfate solution. This mixture reacted for 16 hours at room temperature and in the absence of light. After ABTS** radical was formed, ethanol was added to the solution until an absorbance value of 0.700 (± 0.05) was obtained at 734 nm. In a test tube, 2.5 mL of ABTS** solution was added, followed by the sample in the following volumes: 5, 10 and 15 μL; the final volume was filled up to 3 mL with ethanol. After 6 min of reaction, the determination of the absorbance of the samples was performed at room temperature, 23 °C (±1), in a spectrophotometer (SHIMADZU UV-200, Japan). Trolox was used as the reference antioxidant. The results were expressed in TEAC (Trolox Equivalent Antioxidant Activity).

2.9 STATISTICS

The results were expressed as mean ± standard deviation of, at least, triplicates. An analysis of variance was performed, followed by the Tukey’s test, and results with a p-value < 0.05 were considered significant.
3. RESULTS

3.1 EFFECTS OF HEAT TREATMENT ON THE ACTIVITY OF PROTEOLYTIC ENZYMES

Figure 1 shows the decrease in protein concentration during the hydrolysis process, comparing HT and NT samples.

According to the results in Figure 1, it is clear that heat treatment was indispensable for the activity of Alcalase; heat treatment was not significant for the activity of papain, and, while it was beneficial for the activity of pepsin, it was not indispensable for the occurrence of the hydrolysis.

Figure 1. Decrease in protein concentration over time. A. Alcalase; B. Papain; C. Pepsin. NT = non-treated; HT = heat treated.
3.2 HYDROLYSIS YIELD AND ANTIOXIDANT CAPACITY

The results in Figure 1 also suggested that the yield using papain, regardless of the application of heat treatment, was lower than when the other enzymes were applied, as the consumption of the initial protein was clearly lower.

Figures 2, 3 and 4 show the SDS-PAGE and antioxidant capacities of the HT and NT samples and controls, evaluated by their ability to scavenge ABTS** radicals and expressed as Trolox equivalents.

It is possible to observe that the treatment at 90 °C for 10 min did not induce visible changes in the initial protein profile of the egg whites, with the exception of ovotransferrin. The corresponding band showed a partial reduction after the treatment (Lanes 6 and 8), which was possibly related to the high thermosensitivity of this protein as it suffers denaturation in temperatures above 60 °C, whereas ovalbumin supports temperatures higher than 80 °C (Powrie and Nakai, 1985). It is also visible that, although the egg white samples showed some antioxidant capacity, this feature was not significantly affected by heat treatment or by the hydrolysis conditions in the absence of proteases (Lanes 5 to 8).

**Alcalase.** Lanes 1 and 3 in Figure 2A show that, even after 60 and 240 min of hydrolysis, Alcalase was not able to significantly hydrolyse the ovalbumin present in the reaction medium. Other important proteins, such as ovotransferrin, were effectively hydrolysed without showing any detectable impact on total protein decrease (Figure 1), peptide yield or antioxidant capacity (Figure 2C and 2B, respectively). Lanes 2 and 4 (Figure 2A) show that, after heat treatment, the hydrolysis of most egg white proteins by Alcalase was successfully achieved. However, Lane 4 illustrates that, after a shorter period of hydrolysis (60 min), the HT sample presented some visible low molecular mass fractions that disappeared after 240 min (Lane 2). This result indicated that these small protein fragments were further hydrolysed to lower mass peptides, which may have been responsible for the higher antioxidant capacity and yield of the HT240 hydrolysate (Figures 2B and 2C).
Figure 2. Hydrolysis of egg white powder by Alcalase. A: SDS-PAGE. Lane 1: NT sample after 240 min hydrolysis; Lane 2: HT sample after 240 min hydrolysis; Lane 3: NT sample after 60 min hydrolysis; Lane 4: HT sample after 60 min hydrolysis; Lane 5: NT sample, no hydrolysis; Lane 6: HT sample, no hydrolysis; Lane 7: NT egg white (EW) powder; Lane 8: HT egg white (EW) powder; Lane 9: Molecular mass marker. B: Antioxidant capacity. Different letters are significantly different (p < 0.05). C: Hydrolysis yield. Different letters are significantly different (p < 0.05).

**Papain.** Although heat treatment had no influence on the ability of papain to reduce the initial concentration of proteins in the reaction medium (Figure 1), the efficiency of the hydrolysis was greatly affected, as can be observed in Lanes 1 to 4 of Figure 3A. The hydrolysis occurred independently of the treatment, but the profile of the products formed was noticeably different: after heat treatment, the hydrolysis generated products of lower molecular mass. This result, however, did not seem to influence the antioxidant capacity of the hydrolysates; both HT and NT samples showed similar scavenging activity after 120 min of hydrolysis.
Figure 3. Hydrolysis of egg white powder by Papain. A: SDS-PAGE. Lane 1: NT sample after 120 min hydrolysis; Lane 2: HT sample after 120 min hydrolysis; Lane 3: NT after 60 min hydrolysis; Lane 4: HT sample after 60 min hydrolysis; Lane 5: NT sample, no hydrolysis; Lane 6: HT sample, no hydrolysis; Lane 7: NT egg white (EW) powder; Lane 8: HT egg white (EW) powder; Lane 9: Molecular mass marker. B: Antioxidant capacity. (*) samples are significantly different (p < 0.05). C: Hydrolysis yield. Different letters are significantly different (p < 0.05).

Pepsin. Figure 4A illustrates that pepsin was able to hydrolyse all egg white proteins in the absence of heat treatment except for ovalbumin and lизozime (Lanes 1 and 3) and that heat treatment allowed for the complete hydrolysis of the ovalbumin present after the first 60 min of reaction (Lane 4). This did not seem to interfere with the hydrolysis yield, which appeared to be time-dependent and was only slightly, not significantly, greater after heat treatment. The effect of heat treatment on the antioxidant capacity was not consistent: After 60 min of hydrolysis, heat treatment seemed to impair the development of this ability in the hydrolysate, but after 180 min, the treatment seemed beneficial.
4 DISCUSSION

When hydrolysing egg whites, heat treatment prior to enzymatic hydrolysis may have two distinct purposes: to promote the thermal destruction of protease inhibitors and to foster the denaturation of globular proteins, which enable the enzymes to access formerly protected peptide bonds.

Chicken eggs exhibit a wide range of protease inhibitors; of which, the main ones are ovomucoid, ovo inhibitor and ovostatin, all of them specific inhibitors to serine-proteases such as trypsin, and subtilisins, such as Alcalase. Other types of inhibitors, such as cystatin, which is specific to cysteine-proteases, such as papain, are also found in egg whites. Ovomucoid accounts for about 10% of all egg white proteins, whereas cystatin is found in concentrations as low as 80 μg / mL. And, while ovomucoid can be inactivated by treatments with temperatures around 80 °C, cystatin depends on long treatment times at 100 °C for complete inactivation (Saxena & Tayyabb, 1997; Van der Plancken et al., 2003).

4.1 ALCALASE

The positive and indispensable effects of heat treatment on Alcalase activity can be explained by the possible presence of serine-protease inhibitor activity in the commercial, pasteurised and spray-dried egg white product used as the raw material. According to Geveke...
and Torres (2013), pasteurization of egg whites is recommended under the following conditions: 55.6 ºC / 6.2 min or 56.7 ºC / 5.3 min. This treatment, although enough to ensure microbial safety, is not enough to promote the thermal inactivation of the major egg white protease inhibitors. The spray-drying process is believed to cause low thermal damage to products being dried. While the drying air can reach 190 ºC, there is very little contact time with the product, and the latent heat, which is necessary for the evaporation of the water, so the solid particles cool off (Ma et al., 2013). Therefore, this process is not able to promote the inactivation of the protease inhibitors present in the egg white. Thus, it can be inferred that heat treatment applied at 90 ºC for 10 min was efficient for reducing the activity of serine-protease inhibitors present in the raw material, significantly favouring Alcalase activity. In a study about the heat-induced changes in the susceptibility of egg white proteins to trypsin hydrolysis, Van der Plancken et al. (2003) pointed out that not only was the heat inactivation of ovomucoid and ovoinhibitor expected to enable the trypsinization of egg albumen, but also the heat denaturation of the proteins occurring during treatments above 75 ºC. According to these authors, the higher the temperature applied, the higher the degree of hydrolysis, up to 92 ºC (the highest temperature tested). However, Castro and Sato (2015) did not report any previous treatment for the hydrolysis of fresh egg whites by three microbial proteases, including Alcalase. In this work, although neither the degree of hydrolysis nor the peptide yield were evaluated at this stage, hydrolysis by Alcalase resulted in the least antioxidant hydrolysate, possibly because of its low activity.

Similar to the present study, in Abeyrathne et al. (2016), Alcalase hydrolysed isolated ovomucine without previous heat treatment and, in an earlier study conducted by Abeyrathne et al. (2014), the same enzyme was not able to significantly hydrolyse purified, untreated albumin. Ours and others’ findings corroborate the conclusions of Van der Plancken et al. (2003) in that heat treatment up to 90 ºC was distinctly beneficial for serine-proteases activity by destroying serine-protease inhibitors and by denaturing the ovalbumin structure, thereby increasing protease penetration.

4.2 PAPAIN

Heat treatment was not significant for papain activity; there was no difference between the hydrolysis of the egg white proteins in the HT and NT samples (Figure 1A). Papain, a cysteine-protease, is little or not at all affected by the presence of the trypsin inhibitors found in chicken egg whites. Papain activity was also not significantly affected by the presence of cystatin,
possibly because of the low concentration of this inhibitor in the reaction medium. Chen et al. (2009) found similar results when they hydrolysed duck egg whites without previous heat treatment. Of the various proteases tested, including trypsin, Alcalase and Flavourzyme, only papain was able to perform the hydrolysis.

Despite being a cysteine-protease, papain exhibits trypsin-like specificity, preferring to hydrolyse bonds involving arginine and lysine (Brenda, 2017). Thus, since thermal denaturation of egg white proteins proved to be beneficial for trypsin activity in Van der Plancken et al. (2003), it is possible that this denaturation similarly affected papain activity in the present study, which could explain the difference in the peptide profile displayed by SDS-PAGE.

4.3 PEPSIN.

Pepsin activity was significantly affected by heat treatment. However, unlike when Alcalase was tested, significant hydrolysis of egg white proteins by pepsin occurred even in the NT samples, which suggests the existence of a mechanism of influence different from protease inhibitors. In addition, although there is mention of egg white inhibitors for all classes of proteases in the literature (Saxena & Tayyabb, 1997), no details are reported on specific aspartic-protease inhibitors, such as pepsin. The main protein present in egg white is ovalbumin, which corresponds to more than 50% of these proteins. Ovalbumin is found in three different isoforms, specifically A1, A2 and A3, in the following proportions, respectively: 85:12:3. This protein is characterised by a strongly packed globular structure and contains more than 50% hydrophobic amino acids, which are mostly protected within this globular structure (Zhao et al., 2016; Sheng et al., 2018). Pepsin is an aspartic protease that exhibits low specificity but has a high affinity for hydrophobic amino acids (Brenda, 2017). The thermal denaturation of the proteins leads to the unfolding of their structures and exposes different cleavage sites for the enzymes during hydrolysis (Van der Plancken et al., 2003); thus, it is possible to infer that heat treatment was responsible for the denaturation of the proteins in the egg whites, causing changes in the structure of ovalbumin and facilitating the access of pepsin to the peptide bonds inside the molecules (involving hydrophobic amino acids), which favoured the efficiency of the hydrolysis. Wang et al. (2017), in addition to the effects of dry heating on egg white powder, verified the increase in hydrophobic residues exposure by analysing surface hydrophobicity and highlighted how heating exposed more pepsin activity sites on the surface of the protein. Abeyrathne et al. (2014, 2015, 2016), however, did not find it difficult to hydrolyse the isolated
proteins ovalbumin, ovomucoid and ovomucine, respectively, by pepsin and in the absence of heat treatment.

Comparing the three enzymes tested with respect to yield and hydrolysis time, pepsin achieved the highest yield (85.3%) in the shortest time (180 min); Alcalase produced a lower yield (65.9%) and needed a much longer reaction time (240 min), while the application of papain resulted in the lowest hydrolysis yield (58.3%) in a time-independent manner. In particular, hydrolysis by papain reached its maximum value after 120 min of reaction and did not change, even after double the time. This behaviour was expected, as papain is the most specific of the three enzymes tested, which means that, after hydrolysing the recognisable bonds in the primary structure of a substrate, this enzyme will not be able to hydrolyse it any further. The behaviour of Alcalase was also expected. As an enzyme of low specificity, once freed from specific inhibitors, Alcalase should be able to hydrolyse the several different proteins of the egg white into low mass peptides. The behaviour of pepsin, however, was somewhat unexpected. This enzyme is considered to be of low specificity and tends to be less effective than Alcalase (Shazly et al., 2019). In this case, the higher yield and faster hydrolysis achieved by the use of pepsin may be credited to the predominant presence of peptide bonds susceptible to hydrolysis by this enzyme (involving hydrophobic amino acids) in the most abundant egg white proteins (e.g. albumins) (Brenda, 2017). Other studies have also pointed to the higher efficiency of pepsin in obtaining high yields of hydrolysis of isolated egg proteins when compared to Alcalase (or other subtilisins) and trypsin, which is an enzyme with papain-like specificity (Hiidenhovi et al., 2005; Noh & Suh, 2015, Horimoto & Lim, 2017).

Comparing the three tested enzymes with respect to the antioxidant capacity of the hydrolysate, the best results were obtained with Alcalase (5,254.23 μM Trolox / g sample), followed by the hydrolysates obtained with pepsin (4,029.81 μM Trolox / g sample) and papain (3,876.34 μM Trolox / g sample), although the latter two did not differ from each other (p > 0.05). Ren et al. (2014) and Shazly et al. (2019) have pointed to Alcalase as an excellent enzyme for obtaining peptides with high antioxidant capacity. This characteristic has been attributed to the low specificity of Alcalase, which is capable of producing low molecular mass peptides (Shazly et al., 2019; Liu et al., 2015). This low molecular mass has been considered an important feature in the peptide component of hydrolysates with high antioxidant activity (Guo et al., 2009; Nilmarante et al., 2015). Although this may be considered true for hydrolysates obtained with Alcalase (Figures 2A and 2B), this result did not repeat itself for the hydrolysates obtained with papain or pepsin. In both of these cases, the higher antioxidant capacity seemed to be more
related to the higher yield than to the molecular mass of the peptides in the hydrolysate (Figures 3 and 4). In Graszkiewicz et al. (2010), egg white protein precipitate was hydrolysed with trypsin and chymotrypsin. Although the smallest peptides were obtained with chymotrypsin, the highest antioxidant activity was detected in the hydrolysate produced with trypsin. Like in Sun et al. (2014), the egg white hydrolysate obtained with pepsin was fractionated into four fractions according to the molecular mass of the obtained peptides, and the fraction with the highest antioxidant activity contained the second largest peptides.

According to Jovanovic et al. (2016), the use of heat treatment prior to enzymatic hydrolysis is recommended to increase the susceptibility of egg white proteins to the actions of different proteases. However, the application of heat is often associated with negative effects, such as changes in solubility and color (Stefanović et al., 2014). Consequently, different pretreatment methods have already been tested to replace the use of heat as a method of prehydrolysis treatment of egg white proteins (Lei et al., 2011; Wang et al., 2013). Although several treatments, especially the use of ultrasound (Jovanovic et al., 2016) and pulsed electric fields (Liu et al., 2017), have been efficient in allowing the hydrolysis of the egg white proteins by different proteases, when compared to effective thermal treatments (in temperatures over 80 ºC), the alternative treatments presented, at most, heat-treatment-like efficiency. If the lower cost and ease of application (e.g. using equipment already installed in the industries) are taken into account, heat treatment is still the best option and, therefore, it is important to gain a better understanding of its effects on the hydrolysis of egg whites by different proteases.

5 CONCLUSION

Overall, for the hydrolysis of reconstituted egg white powder in the same manner as for fresh egg whites, heat treatment prior to enzymatic hydrolysis was particularly important for serine-proteases, such as trypsin and subtilisins, because of the need to inactivate specific, highly efficient inhibitors present in the raw material; this requirement was not true for aspartic-proteases, such as pepsin, or for cysteine-proteases, such as papain.

While heat treatment was indispensable for the occurrence of the hydrolysis by Alcalse, it increased the yield of hydrolysis by pepsin and promoted the formation of peptides of lower mass when papain was applied.

Regarding the tested bioactivity (antioxidant capacity), heat treatment benefitted hydrolysis by Alcalase and did not affect hydrolysis by papain. While heat treatment was
harmful for hydrolysis by pepsin after 60 min of reaction, it was beneficial after longer hydrolysis times (180 min).

The best hydrolysis yield was obtained with pepsin (85.3%) after 180 min, generating 4,029.81 μM Trolox / g sample activity. The best activity was obtained with Alcalase (5,254.23 μM Trolox / g sample), yielding 65.9% after 240 min of reaction.

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CONFLICTS OF INTEREST (REQUIRED)

There are conflicts of interest to declare.

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