



Whey protein isolate hydrolysates obtained with free and immobilized Alcalase: Characterization and detection of residual allergens



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ABSTRACT

Protein antigenicity can be reduced by enzymatic hydrolysis, which can be performed either by free or immobilized enzyme. The immobilized enzyme is removed from the reaction medium and reused, while the free enzyme must be inactivated to stop the reaction, generally by heating. Here we have shown that hydrolysates produced with free or immobilized Alcalase on glyoxyl-agarose bead presented different physicochemical properties (hydrophilicity profile, molecular mass distribution, surface hydrophobicity) and different levels of residual milk allergens (α -lactalbumin and β -lactoglobulin). Although, under the studied conditions, the hydrolysis with immobilized enzyme did not reduce the residual allergen levels as efficiently as the free enzyme, the results suggest potential applications of immobilized Alcalase for production of hypoallergenic hydrolysates.

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1. Introduction

Cow's milk allergy (CMA) is an immunologically mediated adverse reaction to one or more milk proteins and is the most common type of food allergy in early childhood (Monaci, Treggoat, Hengel, & Anklam, 2006). The major milk proteins – caseins, β -lactoglobulin (β -Lg) and α -lactalbumin (α -La) – are the main allergens (Wal, 2004). The CMA affects approximately 1.0–3.0% children and less than 0.5% adults (Monaci et al., 2006; Villas-Boas, Vieira, Trevizan, de Lima Zollner, & Netto, 2010). Although this disease is often overcome in the first year, about 15% children remain allergic (Monaci et al., 2006). So far, the treatment of food allergy has been the food elimination diet (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014). However, cow's milk is an important source of nutrients when breastfeeding is not possible (Meulenbroek et al., 2014). Thus, the use of partially or extensively hydrolyzed milk formulas is an alternative for infant with CMA or at risk of developing the disease (Meulenbroek et al., 2014).

Enzymatic hydrolysis is one of the most used methods to reduce the allergenicity of proteins by the cleavage of antigenic regions (epitopes). However, in some cases, only the proteolysis does not fully destroy the epitopes, resulting in the residual presence of allergens in hypoallergenic formulas (El Mecherfi et al., 2011; van Esch et al., 2011). Considering

that minimal amount of allergen can induce severe and even fatal reactions in allergic individuals (Cucu, Jacksens, & De Meulenaer, 2013), detection of allergens in protein hydrolysates is extremely important to ensure the safety of food offered to allergic consumers (Cucu, Platteau, et al., 2013; Pelaez-Lorenzo, Diez-Masa, Vasallo, & de Frutos, 2010).

In a previous work, we found that among the β -Lg hydrolysates produced using Alcalase, bromelain or Neutrase, those obtained with Alcalase exhibited the lowest antigenic response, possibly because of its broad specificity (Sabadin, Villas-Boas, de Lima Zollner, & Netto, 2012). Until now, studies to obtain hypoallergenic hydrolysates have mainly involved the use of soluble enzymes. However, from the industrial point of view, despite the expensiveness, the use of immobilized enzymes is economically viable since they can be recycled and reused (Brena & Batista-Vieira, 2006; Yust, Pedroche, Millán-Linares, Alcaide-Hidalgo, & Millán, 2010). The immobilization of enzymes on solid supports such as glyoxyl agarose has been used to increase the stability and allow reuse of the enzyme (Tavano, 2013; Yust et al., 2010). Different enzymes including Alcalase have been successfully immobilized in this support for various applications (Cabrera-Padilla, Pinto, Giordano, & Giordano, 2009; Coríci et al., 2011; Yust et al., 2010).

In the production of protein hydrolysates, the enzyme in free form must be inactivated, usually by heating, to stop the reaction. Heating may promote the formation of aggregates which in turn may alter the antigenicity of the hydrolysates by exposure or masking epitopes that were not destroyed by hydrolysis (Monaci et al., 2006; Wal, 2004). On the other hand, immobilized enzyme can be easily removed from the

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reaction medium, eliminating the requirement of post-hydrolysis treatment for enzymatic inactivation and the potential change caused by these processes, and allows obtaining enzyme-free hydrolysates. Despite these advantages, we did not find studies on the production of hypoallergenic hydrolysates with immobilized enzymes.

In this paper, we have studied the effect of immobilized Alcalase on the physicochemical characteristics of hydrolysates and the possible benefit of Alcalase immobilization for production of hypoallergenic hydrolysates. For that, hydrolysates were obtained under different pH and temperature conditions, and the effects of Alcalase immobilization on both the physicochemical characteristics and detection of residual allergens (α -La and β -Lg) were investigated.

2. Material and methods

2.1. Material

Whey protein isolate was purchased from PROVON® (Glanbia Nutritionals, Kilkenny, Ireland). Alcalase was purchased from Sigma-Aldrich® (St. Louis, MO, USA). Agarose 4BCL beads were purchased from GE Healthcare (Little Chalfont, UK). Bovine α -lactalbumin ELISA quantitation kit and bovine β -lactoglobulin ELISA quantitation kit were purchased from Bethyl Laboratories, Inc. (Montgomery, USA). All other chemicals were of analytical and chromatographic grade.

2.2. Alcalase immobilization

Glyoxyl-agarose 4BCL beads were activated as described by Guisán (Guisán, 1988). Alcalase and 10 mM sodium bicarbonate buffer pH 10.2 (0.5 mL/105 mL) were mixed, a 5 mL aliquot was removed for control, and 10 g glyoxyl-agarose (10 mL enzyme solution/g support) were added to the solution. The suspension was kept at 4 °C under gently stirring. Aliquots of the supernatant were withdrawn for determination of protein content according to Bradford (1976). After 24 h reaction between the enzyme and the support, derivatives were reduced with sodium borohydride (1 mg/mL suspension), as described by Blanco and Guisán (1989). The gel was washed under vacuum with abundant milli Q water and 0.1 mol/L sodium phosphate buffer, pH 8.0. The immobilized enzyme was stored under refrigeration in 0.1 mol/L sodium phosphate buffer pH 8.0 containing 0.02% (w/v) sodium azide.

2.3. Enzymatic activity

Enzymatic activity was determined in the free (FA) and immobilized Alcalase (ImA) preparations using 1.2% (w/v) WPI solution in 0.1 mol/L sodium phosphate buffer pH 8.0. The reaction was carried out at 60 °C for 10 min and stopped by addition of trichloroacetic acid (TCA, 16%), resulting in a final concentration of 8% (w/v). After centrifugation (31,129 x g) at 25 °C for 10 min, the supernatant was removed and subjected to absorbance readings at 280 nm, using an automated spectrophotometer (Agilent Technologies, Waldbronn, Germany). One unit (U) of enzyme activity was defined as the amount of enzyme that produces 8% TCA soluble peptides equivalent to 1 μ g of tyrosine in 1 min (Emi, Myers, & Iacobucci, 1976).

2.4. Thermal stability and reuse

To evaluate thermal stability, free and immobilized Alcalase were incubated at 65 °C and pH 8.0 (0.1 mol/L sodium phosphate buffer). Aliquots were withdrawn from the reaction medium at different times (0, 15, 30, 60, and 90 min), and the enzyme activities were measured as described above. Residual activity at each time was expressed as percentage of initial enzymatic activity.

The reuse of immobilized Alcalase was analyzed by measuring the residual activity after six operational cycles. To estimate the residual activity in the other cycles, the initial activity was considered 100%.

2.5. Hydrolysis of WPI

To evaluate the impact of pH and temperature on degree of hydrolysis (DH) using free or immobilized Alcalase, a central composite rotatable design (CCRD) with 2 factors was carried out. The experimental design consisted of eleven experiments per enzyme (Table 1), including 3 central points (level 0), 4 axial points (levels +1.41 and -1.41) and 4 factorial points (levels +1 and -1) (Rodrigues & Iemma, 2014). For all tests, the substrate concentration was set at 3% (w/v) and the enzyme:substrate ratio (E:S) was fixed at 120 U of enzyme/g substrate. To define the amount of enzyme needed for the assays, the activity of free and immobilized enzyme was determined immediately before each trial, as described above. For both forms of enzyme, the hydrolysis reaction was monitored by the pH-stat method using a Mettler DL-21 automated titrator (Schwerzenbach, Switzerland) under agitation. After 180 min, the reaction was stopped by heating at 90 °C for 10 min or by removing the enzyme from reaction medium (by vacuum filtration) for free or immobilized enzyme, respectively. The DH was calculated according to the equation 1 (Adler-Nissen, 1986):

$$DH (\%) = B \times Nb \times \left(\frac{1}{\alpha}\right) \times \left(\frac{1}{MP}\right) \times \left(\frac{1}{htot}\right) \times 100 \quad (1)$$

where B is base volume (mL); Nb is base normality, α is the average degree of dissociation of the α -NH₂ group; MP mass of protein (g); and h_{tot} total number of peptide bonds in the protein substrate (8.8 mmol g⁻¹ for WPI) (Stănciuc, Van Plancken, Rotaru, & Hendrickx, 2008). After stopping the reaction, the pH of hydrolysates was adjusted to 6.5 using 1 mol/L HCl, and the hydrolysates were freeze-dried and stored at -20 °C.

The model proposed for the response variable (Y = DH) as function of independent variables (pH and T) was:

$$Y = \varphi(pH, T) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_1 x_{12} + \beta_2 x_{22} + \beta_{1,2} x_1 x_2 \quad (2)$$

where β_0 , β_1 , β_2 and $\beta_{1,2}$ are the constant, linear, quadratic and interaction coefficients, respectively, and x_1 and x_2 represent the independent variables coded for the model.

2.6. Protein determination

The total nitrogen content of WPI and hydrolysates was determined by micro Kjeldahl procedure, and the protein content was calculated

Table 1
Hydrolysis conditions employed to study the effect of pH and temperature (°C) on the degree of hydrolysis (DH) of WPI using free and immobilized Alcalase.

Trial	Independent variables		Response – DH (%)	
	pH	T (°C)	Free Alcalase	Immobilized Alcalase
1	7.3	48	18.1	14.3
2	8.7	48	18.1	11.4
3	7.3	62	21.0	18.3
4	8.7	62	23.9	17.7
5	7.0	55	22.4	17.4
6	9.0	55	20.1	17.3
7	8.0	45	22.2	9.5
8	8.0	65	18.8	22.2
9	8.0	55	21.5	19.4
10	8.0	55	20.3	17.1
11	8.0	55	22.4	20.0

using 6.38 as conversion factor (AOAC, 2006). The protein content of Alcalase was determined according to Bradford (1976).

2.7. Chromatography

The chromatographic analyses were carried out in Agilent high performance liquid chromatography system consisting of a quaternary and semi preparative pump, and diode array detector (DAD) (Waldbronn, Germany).

The reversed-phase high performance liquid chromatography (RP-HPLC) analyses were performed using a C18 Luna column (250 mm × 4.6 mm DI, 5 µm – Phenomenex – Woburn, MA, USA). The operational conditions were: column at room temperature; flow rate, 1 mL/min; injection volume, 25 µL; detector set at 214 nm; solvent A: 0.04% trifluoroacetic acid (TFA) (Merck, Hohenbrunn, Germany) in Milli-Q water and solvent B: 0.03% TFA in acetonitrile (Merck, Hohenbrunn, Germany) filtered through a membrane 0.45 µm (Schleicher & Schüll, Dassel, Germany). The column was equilibrated with 100% of solvent B. A linear gradient of solvent B in A, from 0% to 70% in 40 min was performed, followed by 5 min at isocratic form and rebalanced to initial conditions of the column for 15 min. α-La and β-Lg were used as standard. Samples were solubilized in 0.03% TFA (3 mg of protein/mL solution) and filtered through 22 µm cellulose acetate membranes (Schleicher & Schüll, Dassel, Germany).

Size exclusion chromatography (SEC-HPLC) was performed using a Protein-Pak™ 60 Å column (7.8 mm × 300 mm–Waters Corporation, Milford, MA, USA) under isocratic conditions. Samples were dissolved in 50 mmol/L sodium phosphate buffer pH 6.8 (3 mg/mL) and filtered as previously described. The operational conditions were: column at room temperature; flow rate, 0.5 mL/min; injection volume, 20 µL; detector set at 214 nm; elution time, 40 min. Mioglobulin (16.95 kDa), α-La (14.437 kDa), aprotinin (6.512 kDa) and bacitracin (1.423 kDa) (Bio-Rad, Hercules, CA, USA) were used as standards. The percentage relative area (corresponding to each peak) was analyzed using software Star Chromatography Workstation (Agilent 1200 series, USA). All chromatographic analyses were performed in duplicate.

2.8. Electrophoresis

The electrophoretic profile was determined using Mini Protein II system (BioRad, Hercules, CA, USA). The SDS-PAGE was performed according to Laemmli (Laemmli, 1970), with separation gel and stacking gel containing 12% and 4% acrylamide, respectively. WPI and hydrolysates were diluted (2% protein) in reducing buffer (0.0625 mol/L Tris–HCl, 2.0% SDS, 20% glycerol, 5.0% β-mercaptoethanol and bromophenol blue, pH 6.8) and heated at 95 °C/4 min. Then, 10 µL aliquots of each sample were applied. The gels were stained with 0.1% Coomassie Blue R-250 (Sigma-Aldrich) in a methanol/acetic acid/water solution (4:1:5) and destained using several changes in methanol/acetic acid/water solution (4:1:5). A 14.4–97.0 kDa MM marker kit (Bio-Rad, Hercules, CA, USA) was used as standard.

For the SDS-PAGE-Tricine, separation gel, spacer gel, and stacking gel with 16.5%, 10%, and 4% acrylamide, respectively, were used. WPI and hydrolysates were diluted (2% protein) in reducing buffer (0.0625 mol/L Tris–HCl, 2.0% SDS, 20% glycerol, 5.0% β-mercaptoethanol and Coomassie Blue G250, pH 6.8) and heated at 95 °C/4 min. Then, 10 µL aliquots of each sample were applied. The gels were fixed with methanol/acetic acid/water solution (5:1:4) for 24 h, stained with 0.025% Coomassie Blue G-250 (Sigma-Aldrich) in 10% acetic acid for 48 h, and destained using 10% acetic acid. The peptides MM was estimated using a 3.5–26 kDa marker kit (Bio-Rad, Hercules, CA, USA) as standard. All electrophoretic analyses were carried out in duplicate.

2.9. Zeta potential

The isoelectric point (pI) of hydrolysates was determined using Zetasizer equipment (Malvern, Worcestershire, UK). The hydrolysates were resuspended in deionized water (0.1% w/v) and pH was adjusted to 3.0–7.0 using 0.2 mol/L HCl. The analyses were carried out in triplicate.

2.10. Surface hydrophobicity

The surface hydrophobicity (S_0) was determined using the anionic fluorescence probe 1-anilino-naphthalene-8-sulfonate (ANS) as reported by Alizadeh-Pasdar & Li-Chan (2000), with slight modifications. Hydrolysates were diluted in 0.01 mol/L sodium phosphate buffer (pH 7.4) at concentrations ranged from 0.005 to 0.025 g/mL, while the ANS concentration was maintained at 8 mmol/L in 0.1 mol/L sodium phosphate buffer (pH 7.4). The excitation wavelength was 390 nm and the emission wavelength was 470 nm. A 10 µL aliquot of ANS solution was added to 2 mL hydrolysate solutions and mixed. Then, 200 µL aliquots were transferred to microplates (96 wells). After 15 min in dark environment, the relative fluorescence intensity (RFI) of each solution was measure against the blank (buffer with ANS) using a Synergy™ HT Multi-Mode Microplate Reader (BioTek®, Vermont, USA). The S_0 was determined from the initial slope of linear regression analysis of the plot of RFI against protein concentration (%). Each sample was analyzed in triplicate.

2.11. Turbidity

Turbidity of hydrolysates solutions was analyzed as described by Groleau, Morin, Gauthier, et al. (2003). The hydrolysates were rehydrated (1% protein w/v) in citrate–phosphate buffer pH 4.0 (0.1 mol/L citric acid and 0.2 mol/L sodium phosphate), or pH 7.0 (0.2 mol/L sodium phosphate). For turbidity measurements, the buffered hydrolysates solutions were analyzed in the presence of NaCl (0.5 and 1 mol/L), urea (6 mol/L), SDS (5%) or a mixture of SDS (5%)/β-mercaptoethanol (5%). All solutions were maintained for 90 min at room temperature, except solutions with SDS and β-mercaptoethanol (β-ME), which were boiled for 2 min. Protein aggregation was estimated by optical density measurements at 500 nm (A_{500}) using a Agilent 8453 spectrophotometer (Agilent Technologies, Waldbronn, Germany). The absorbance measurements were carried out in triplicate and absorbances of all buffers was measured and consider as nil.

2.12. Residual allergens detection

The determination of residual whey proteins (α-La and β-Lg) in the hydrolysates obtained with FA and ImA was performed using ELISA kits according to the manufacture's protocol (Bethyl Laboratories Inc., Montgomery, USA).

2.13. Statistical analysis

For CCRD, analysis was performed using the Statistica 7.0 (Statsoft, Tulsa, USA) software package. To analyze data obtained from CCRD validation, *t*-test was employed. Turbidity results were analyzed using One-Way ANOVA and Tukey's test. Surface hydrophobicity and ELISA results were analyzed using the non-parametric Mann–Whitney test. For all analyses, *p* values ≤0.05 were considered significant.

3. Results and discussion

3.1. Alcalase immobilization

The Alcalase used in this study is an enzyme complex containing 1.94 ± 0.05 mg protein/mL. The amount of immobilized enzyme was

0.028 mg protein/g of support, determined indirectly by the protein remaining in the supernatant. Despite the low yield immobilization, the ImA showed high activity retention, with approximately 85% of the initial activity of the free enzyme. In the reuse test, the ImA retained activity in 6 operating cycles, with losses of only 15% after the third treatment cycle (see supplementary data, Fig. 1A in the online repository). In the thermal stability test, the ImA retained 15% of its initial activity after 90 min incubation (65 °C, pH 8.0), while the FA completely lost activity in 60 min (see supplementary data, Fig. 1B in the online repository). This result suggests that the Alcalase immobilization onto glyoxyl agarose bead increased enzyme stability at 65 °C. Long immobilization periods (~24 h) allow the enzyme to rearrange so as to bind through more points (multipoint covalent attachment), which can increase its thermal stability due to rigidification of the protein chain (Pedroche et al., 2007).

3.2. Production of the WPI hydrolysates

The hydrolysis conditions and DH of each trial of the CCRD performed for free and immobilized enzyme are shown in Table 1. In the experimental design, all trials were performed with the same E:S ratio (120 U/g protein), which enable comparison between the performance of free and immobilized enzyme.

The regression coefficients for the response DH in the trials with FA and ImA are shown in Table 2. Statistical analysis of the results of the CCRD conducted with the FA showed that none of the evaluated parameters was statistically significant. Thus, it was not possible to establish a model for the response DH as function of the variables pH and temperature. To validate the results obtained by the CCRD with FA, the trial 2 (pH 8.7 and 48 °C) was performed in triplicate, and the results ($\text{DH} = 17.7\% \pm 0.6\%$) were compared to the results at the central point ($\text{DH} = 21.4\% \pm 1.05\%$) using unpaired *t*-test. Once no difference ($p > 0.05$) was observed between them, the results of the experimental design were confirmed.

The CCRD with ImA resulted in the reparametrization of the model represented by Equation 3.

$$Y = \varphi(\text{pH}, T) = 17.83 + 3.53 T - 1.46 T^2 \quad (3)$$

Despite the R^2 value was relatively low, F_{cal} was significant, thus it can be stated that the model fits the experimental data quite well. The response surface for ImA (Fig. 1) showed that the pH did not affect the DH for the conditions studied. However, the DH increased with the increase in temperature, indicating that the ImA activity has increased at temperatures above 60 °C.

The model generated for the results with the ImA was validated by the hydrolysis assay carried out in triplicate at 60 °C and pH 8.5. The

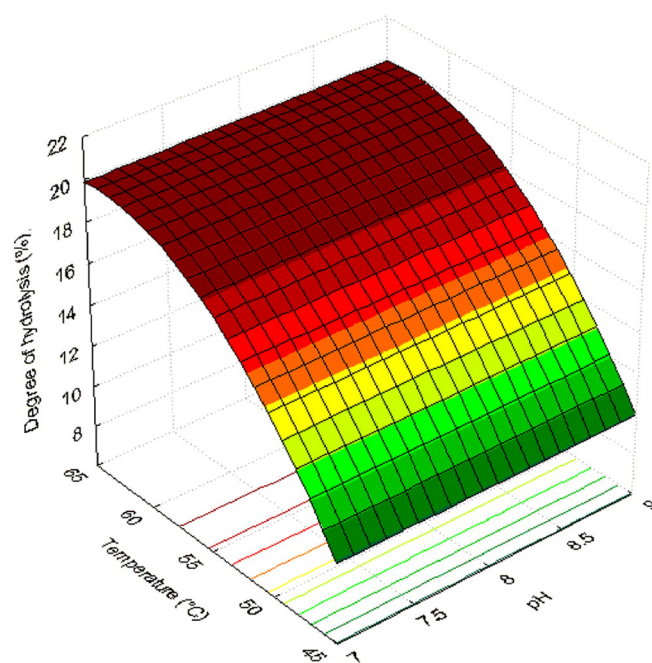


Fig. 1. Response surface for the effect of pH and temperature on degree of hydrolysis (%) of WPI hydrolysates obtained with immobilized Alcalase.

temperature was selected according to the region with the highest DH (Fig. 1), while pH was chosen according to the optimal Alcalase activity (pH 6.5 to 8.5), taking into account the upper limit, since the immobilization increases enzyme stability to pH. The experimental DH was $18.4 \pm 0.4\%$ while the value predicted by the reparametrization of the model was 19.6%. The prediction error was small ($\pm 0.84\%$), indicating that the model is effective (Rodrigues & lemma, 2014).

Under the conditions of the CCRD, the hydrolysis carried out with ImA resulted in lower DH values than those obtained with the FA, with the exception of trial 8, in which the DH reached with ImA was higher (Table 1). Since, under the conditions of the experiment, the FA proteolytic activity was not affected either by pH or temperature and ImA was affected only by temperature (Table 2), the increased performance of ImA at 65 °C is related to its maximum proteolytic activity, which may be up to 10 °C higher than the free enzyme (Tardioli, Pedroche, Giordano, Fernández-Lafuente, & Guisán, 2003). At 65 °C ImA is quite stable while FA loses activity (see supplementary data, Fig. 1B in the online repository), therefore FA may have lost activity along the hydrolysis reaction. Moreover, higher temperature may increase the energy of the system, thus facilitating the contact between enzyme and substrate.

For the further steps of this study, hydrolysates obtained under hydrolysis conditions that led to different DH values were selected. These conditions were of trials 2 (pH 8.7; 48 °C), 5 (pH 7.0; 55 °C), and 8 (pH 8.0; 65 °C). For each hydrolysis conditions, the experiments were carried out with free and immobilized Alcalase, using the same E:S ratio. The hydrolysates obtained with the FA were named as FAH, and those produced with the ImA were named as ImAH, identified by the hydrolysis assay number in the CCRD. Thus, the hydrolysates FAH2, FAH5, FAH8, ImAH2, ImAH5, and ImAH8 were selected for further analysis.

3.3. Characterization of hydrolysates

3.3.1. Electrophoretic and chromatographic characterization

The chromatographic profiles (RP-HPLC) of the hydrolysates are shown in Fig. 2. The α -La and β -Lg peaks can be observed in the WPI chromatogram, with retention times (RT) of 27.2 and 28.0 min, respectively (Fig. 2A). The profiles of FAH obtained under various hydrolysis

Table 2
Regression coefficients and statistical analysis for the response DH (%) of WPI obtained with free and immobilized Alcalase.

Factors	Regression coefficient for DH (%)	
	Free Alcalase	Immobilized Alcalase
Constant	21.333*	18.836*
Linear		
pH	−0.061	−0.454
T	0.486	3.534*
Quadratic		
pH ²	−0.172	−1.063
T ²	−0.622	1.778**
Interaction		
pH × T	0.675	0.570
F _{cal}	0.19	16.08
R ²	16.08	81.49*

R² = % variation explained by model;

** Significant at $p \leq 0.01$;

* Significant at $p \leq 0.05$.

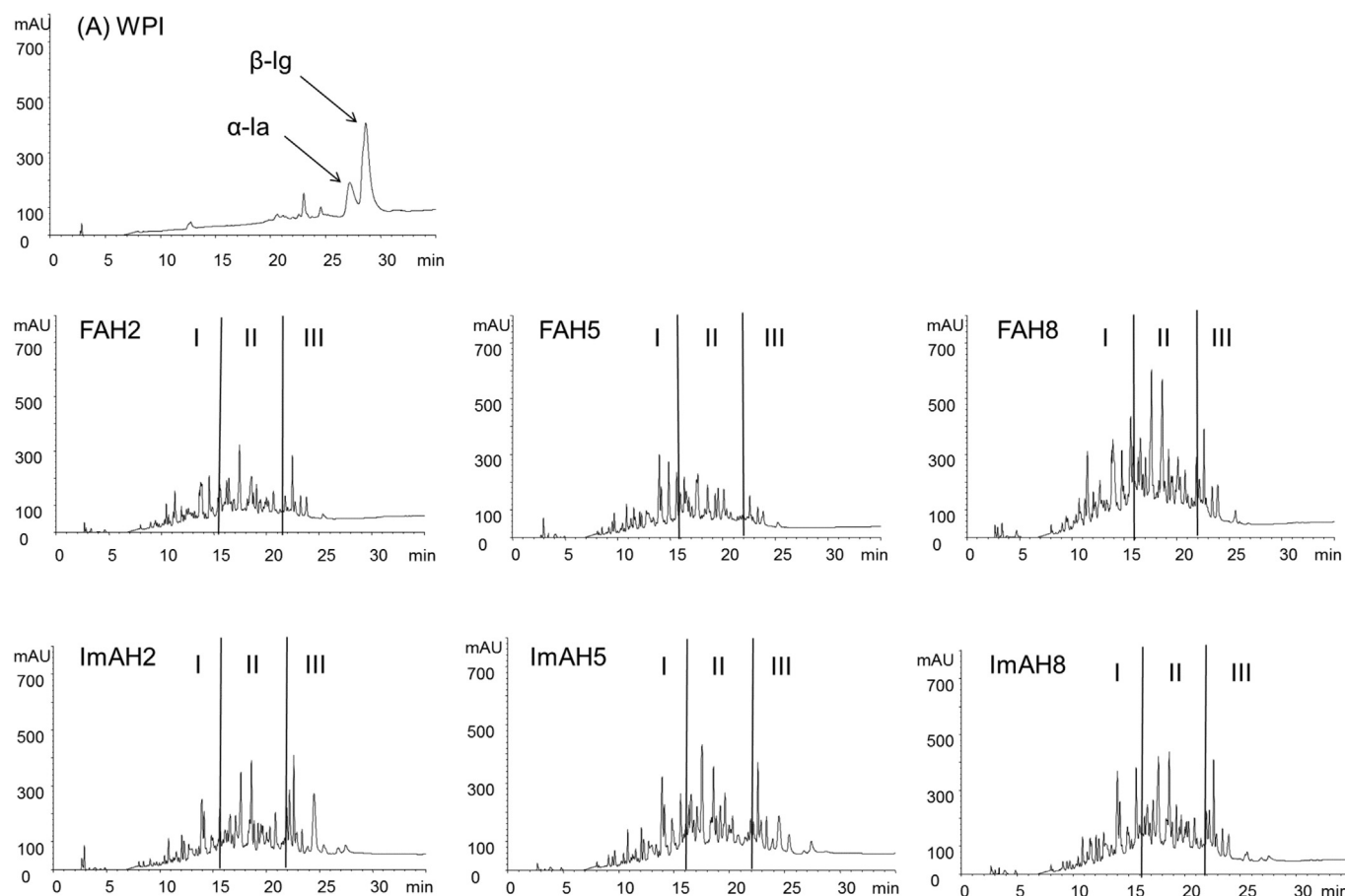


Fig. 2. RP-HPLC profile of WPI hydrolysates with free (FAH) and immobilized Alcalase (ImAH) obtained at conditions of pH and temperature of CCRD: (2) pH 8.7/ 48 °C; (5) pH 7.0/ 55 °C; and (8) pH 8.0/ 65 °C. (A) Untreated WPI. Region I: high hydrophilicity; region II: medium hydrophilicity; region III: low hydrophilicity.

conditions exhibited, in general, few peaks in the region of low hydrophilicity (III), with the majority of the peptides eluted in the regions of high (I) and medium (II) hydrophilicity. This suggests preferential formation of small hydrophilic peptide resulting from the broad specificity of Alcalase. Although the FAH showed similar DH, the reaction conditions led to different hydrolysis patterns, resulting in differences in the hydrophilicity profile, in particular in the region III. The hydrolysate FAH8, for example, presented a higher number of peaks in this region than the hydrolysates FAH2 and FAH5.

In general, the chromatograms of ImAH presented more peaks in the region of low hydrophilicity (III) when compared to the FAH, suggesting that these hydrolysates comprise larger and more hydrophobic peptides. Similarly to that observed in the hydrolysis with FA, the reaction conditions also influenced the pattern of peptides released by ImA. The hydrolysate ImAH8 exhibited fewer peaks in the region of low hydrophilicity when compared to the hydrolysates ImAH2 and ImAH5, which may indicate the presence of smaller and more hydrophilic peptides due to the highest DH obtained in this hydrolysis condition. Moreover, high temperatures (65 °C) may have promoted conformational changes that facilitated substrate translocation, resulting in an increase in the enzyme-substrate interaction (Benjakul & Morrissey, 1997).

The steric hindrance caused by immobilization may affect enzyme performance (Brena & Batista-Vieira, 2006). It is possible that the accessibility of the enzyme to the substrate or even the affinity of the enzyme towards a specific substrate changes due to immobilization (Rocha, Gonçalves & Teixeira, 2011). Furthermore, it worth highlighting that depending on initial performance of the enzyme (taking into account the way that enzyme is used – free or immobilized) and the diverse reaction conditions, different products might be released. And those products, in turn, can modify the kinetic behavior of the hydrolysis.

Hydrolysis and MM distribution profile of the hydrolysates were evaluated by two electrophoresis techniques, SDS-PAGE and SDS-PAGE/Tricine (Fig. 3A and B). The electrophoretic profiles of the hydrolysates obtained with FA showed almost total disappearance of β -Lg and α -La whereas the hydrolysates ImAH2 and ImAH5 presented more intense bands corresponding to β -Lg and α -La, and peptides with greater MM. The hydrolysate ImAH8 was the exception, showing profiles similar to the hydrolysates obtained with FA. No band was observed in the FAH8 profile in both electrophoretic systems, which suggests the presence of peptides smaller than 3 kDa that may not have been stained (Claeys, De Smet, Balcaen, Raes, & Demeyer, 2004). A band corresponding to BSA was observed in all profiles, except in the sample FAH8, and was more intense in the hydrolysates produced with ImA (Fig. 3A). Although some hydrolysates show similar DH values, they exhibited different MM profiles, which indicate that cleavage pattern was dependent on the hydrolysis conditions. Whereas the hydrolysate FAH2 (DH 18.1%) showed a band corresponding to the residual BSA, the hydrolysate FAH8 (DH 18.8%) showed absence of BSA (Fig. 3). Possibly, the temperature to produce the hydrolysates FAH8 and ImAH8 (65 °C) increased the flexibility of whey proteins, resulting in a conformation more suitable for Alcalase cleaving the hydrolytic sites previously hidden (O'Loughlin, Murray, Kelly, FitzGerald, & Brodtkorb, 2012).

According to apparent MM distribution obtained by HPLC-SEC (Table 3), none of the hydrolysates showed fractions lower than 7 kDa. Hydrolysis with FA and ImA produced hydrolysates mainly formed by fractions of apparent MM between 12 and 14 kDa (Table 3), however ImA produced higher amounts of fractions of MM larger than 14 kDa than FA. The exception was ImAH8, which showed distribution more similar to those observed for hydrolysates obtained with FA. Since peptides of lower MM were observed in

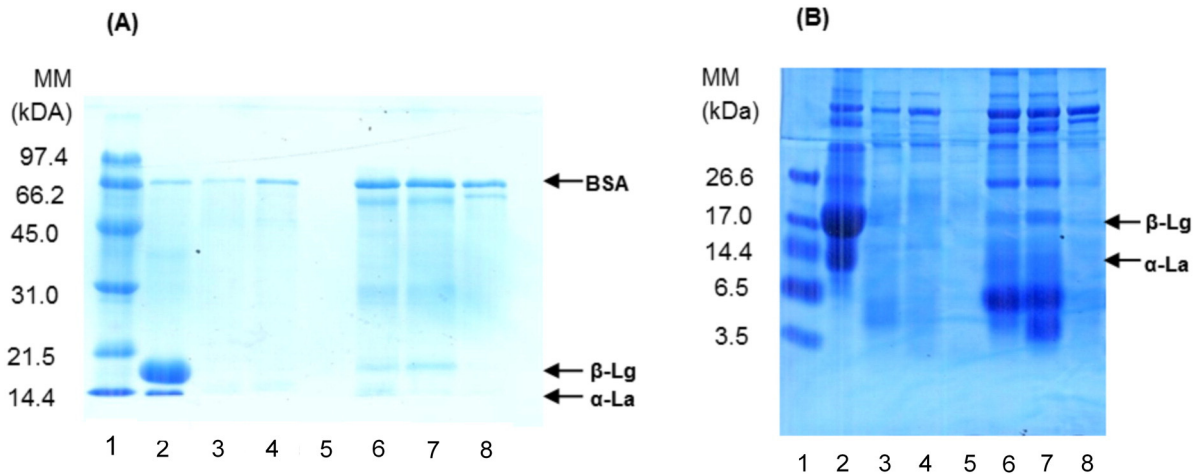


Fig. 3. Electrophoresis patterns of untreated WPI and hydrolysates with free and immobilized Alcalase obtained under the conditions of pH and temperature of CCRD: (2) pH 8.7/ 48 °C; (5) pH 7.0/ 55 °C; and (8) pH 8.0/ 65 °C. (A) SDS-PAGE (polyacrylamide gel – 12%): (1) Molecular mass (MM) standard (14.4–97.4 kDa); (2) untreated WPI; (3) FAH2; (4) FAH5; (5) FAH8; (6) ImAH2; (7) ImAH5; (8) ImAH8. (B) SDS-PAGE/Tricine under reducing conditions: (1) MM standard (3.5–26 kDa); (2) untreated WPI; (3) FAH2; (4) FAH5; (5) FAH8; (6) ImAH2; (7) ImAH5; (8) ImAH8.

electrophoretic profiles using reducing and denaturing conditions (Fig. 3), these results indicate formation of different types of aggregates under the chromatographic conditions (pH 6.8) (O'Loughlin et al., 2012).

3.3.2. Physicochemical characterization

In general, the S_0 values of WPI and hydrolysates differed significantly ($p < 0.05$) in the following order: ImAH > WPI > FAH (Fig. 4). The highest S_0 values of ImAH may be due to the exposition of hydrophobic sites, since hydrolysis with ImA released larger and more hydrophobic peptides than with FA (Figs. 2 and 3). The lowest S_0 values of the FAH may suggest the formation of peptide aggregates by hydrophobic interactions, which may be favored by the heat treatment (Damodaran, 2010). Therefore, while the hydrophobic groups of the hydrolysates with free enzyme possibly were hidden within aggregates, the hydrophobic groups of the hydrolysates obtained with ImA remained exposed on surface. Furthermore, the presence of some peptides such as β -Lg AB [f1–45], β -Lg AB [f90–108], and α -La [f50–113] in WPI hydrolysates can increase the tendency to form aggregates (Creusot & Gruppen, 2007).

The turbidity of the hydrolysate solutions was determined to estimate the aggregation and the nature of their interactions. Fig. 5 shows the turbidity of hydrolysate solutions (1% w/v) as a function of pH, NaCl concentration, and presence of denaturing/dissociating agents. At pH 4.0, all hydrolysates presented higher turbidity than at pH 7.0, which can be explained by the pI value of the hydrolysates (from 3.6 to 4.6, Fig. 4). Under this condition the electrostatic repulsion is reduced, favoring approximation of charges and increasing interactions between peptides (Muschiolik, 1997). A slight decrease in turbidity was observed

with addition of 0.5 or 1 M NaCl as compared to the control (buffer), which suggests little contribution of the electrostatic interactions to peptide aggregation at both pH studied (Groleau, Morin, Gauthier, & Pouliot, 2003).

Turbidity decreased significantly ($p \leq 0.05$) in the presence of urea and more expressively SDS and SDS + β -ME, for both pH values (4.0 and 7.0). The significant lower turbidity ($p \leq 0.05$) in the presence of urea for all hydrolysates suggests that hydrogen bonds and hydrophobic interactions are involved in the formation of aggregates (Otte et al., 1997). The SDS, despite significantly decreased turbidity of all hydrolysates solutions, alone was not able to completely solubilize the aggregates, suggesting that other interactions may be involved in the aggregation. At pH 7, the most significant decrease in turbidity was observed in the presence of SDS + β -ME, indicating that the disulfide bonds play an important role in aggregation. However, the FAH2 and FAH5 solutions showed different behaviors, and the decrease in turbidity was greater in the presence of urea or SDS, suggesting that, for these hydrolysates, hydrogen bonds and hydrophobic interactions are the main interactions involved in the formation of aggregates. At pH 7.0, the presence of SDS + β -ME decreased turbidity, but not as evident as observed at pH 4.0. This may be due to the higher reactivity of the free thiol groups of cysteine residues near pH 8.0 which can result in the formation of new disulfide bonds (Andersson, 1970).

The various combinations of pH and hydrolysis temperature, as well as the enzyme form, possibly altered the hydrolysis dynamics and/or protein structure in different ways, resulting in different accessibility of the substrate by the enzyme and, consequently, in different cleavage patterns (O'Loughlin et al., 2012; Rocha, Gonçalves, & Teixeira, 2011; Tardioli, Sousa Jr., Giordano, & Giordano, 2005). For example, changes in pH modify the charge of the proteins (substrate and enzyme), which may affect the enzyme selectivity, defined as the rate that the enzyme breaks each cleavage site within its specificity (Butré, Sforza, Gruppen, & Wierenga, 2014). Therefore the effect of the hydrolysis conditions on the substrate and enzyme selectivity resulted in distinct cleavage patterns, releasing peptides with different physicochemical and aggregation behavior due to their different composition.

3.4. Detection of allergenic proteins

Immunoassays are adequate tools to assess products allergenicity, as they are highly sensitive, and the antibodies recognize specific proteins even in food matrices composed of various ingredients (Monaci, Brohée, Tregot, & van Hengel, 2011). In this sense, the detection of α -La and β -Lg in the hydrolysates was carried out using commercial sandwich

Table 3

Molecular mass (MM) distribution of WPI hydrolysates with free (FAH) and immobilized Alcalase (ImAH) obtained from trials 2, 5 and 8 of CCRD.

MM Distribution (kDa) ^a	Area (%)					
	FAH2	FAH5	FAH8	ImAH2	ImAH5	ImAH8
< 7 kDa	nd	nd	nd	nd	nd	nd
7–10 kDa	3.5	0.7	9.3	8.7	7.7	8.7
10–12 kDa	28.7	31.0	26.6	18.4	20.3	26.3
12–14 kDa	45.7	51.7	47.1	29.1	30.7	37.6
14–16 kDa	22.1	15.8	17.0	29.0	23.8	21.7
> 16 kDa	nd	0.8	nd	14.8	17.6	5.7
Degree of hydrolysis (%)	18.1	22.4	18.8	11.4	17.4	22.2

nd = not detected.

^a Molecular mass (MM) distribution determined by HPSEC.

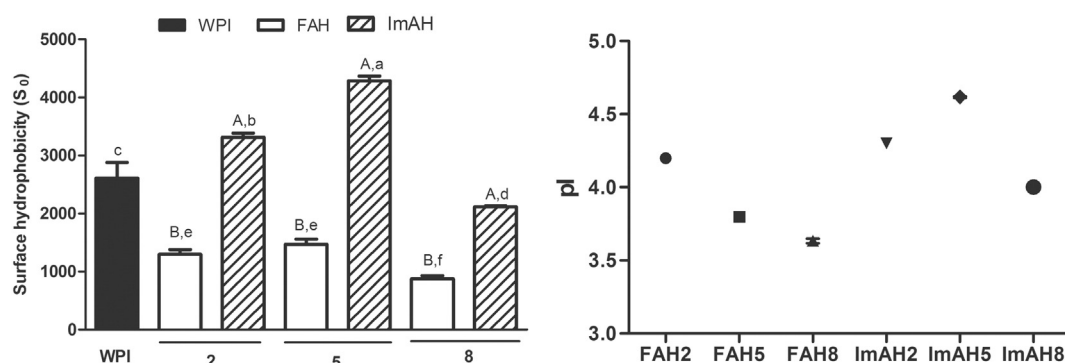


Fig. 4. Surface hydrophobicity (S_0) and isoelectric point (pI) of WPI, free Alcalase hydrolysates (FAH) and immobilized Alcalase hydrolysates (ImAH) obtained under conditions of trial (2) pH 8.7/ 48 °C; (5) pH 7.0/ 55 °C; and (8) pH 8.0/ 65 °C of the CCRD. Results are expressed as mean $\pm$ standard deviation of three determinations. Different letters express significant differences ($p < 0.05$ - Mann-Whitney test). Uppercase letters compares means between FAH and ImAH under the same hydrolysis condition (2, 5 or 8); lowercase letters compares means between WPI, FAH and ImAH.

ELISA kits. In these kits, the capture antibodies are generally polyclonal, once they are capable of identifying a number of different epitopes on the target protein (Monaci et al., 2011).

WPI hydrolysis with FA or ImA significantly decreased ($p \leq 0.05$) the detection of α -La and β -Lg when compared to the intact WPI (Fig. 6), suggesting that the epitopes that bind to antibodies in the kits were destroyed by partial hydrolysis (Cucu, Platteau, et al., 2013). The hydrolysates produced with ImA presented higher detection of allergenic proteins as compared to those produced with FA. The higher concentration of α -La and β -Lg detected in ImAH2 and ImAH5 may be related to the presence of unhydrolyzed proteins, as was evidenced by their electrophoretic profiles (Fig. 3). Additionally, the RP-HPLC profiles of the hydrolysates obtained with ImA (Fig. 2) showed more peaks in the low

hydrophilicity region, suggesting that larger peptides were released, which may have retained some specific sequences recognized and detected by the antibodies in the ELISA kits (Diez-Masa, Frutos, & Puerta, 2006).

Between the hydrolysates which present similar DH (ImAH8 - DH 22.2% versus FAH5 - DH 22.4%; FAH2 - DH 18.1% versus FAH8 - DH 18.8%), FAH5 and FAH8, which presented lower S_0 (Fig. 4), also presented lower detection of allergens (Fig. 6). Low S_0 values suggest the formation of aggregates stabilized by hydrophobic interactions, which may hide epitopes recognized by the IgG in the kits. Unfortunately, commercial kits generally do not provide information on the antibodies specificity, which complicates the assessment of given effect in the allergens detection (Cucu, Platteau, et al., 2013). Thereby, the epitopes recognized by the antibodies in the kits may be not the same epitopes

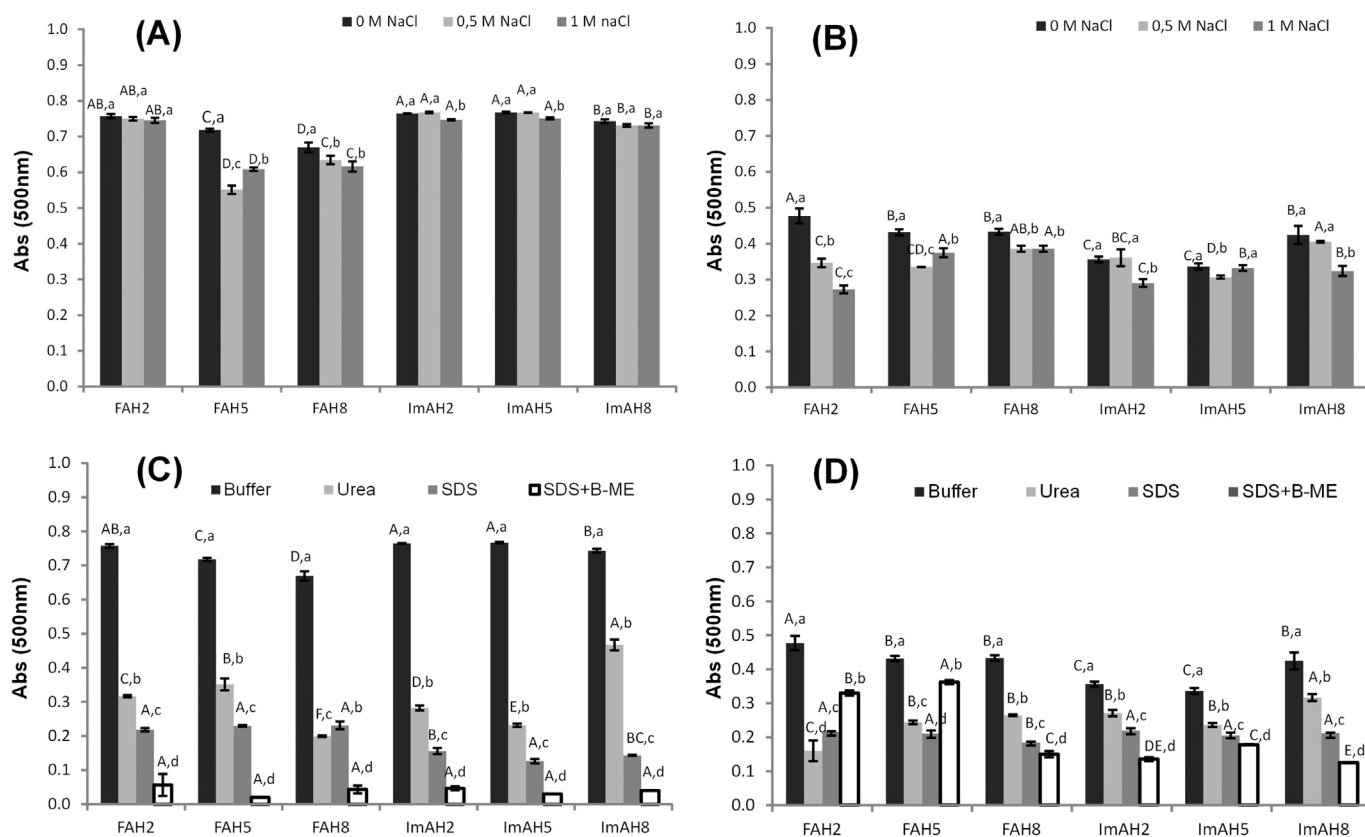


Fig. 5. Turbidity (Abs 500 nm) of solutions (1% w/v) of hydrolysates obtained with free Alcalase (FAH) and immobilized Alcalase (ImAH) solubilized in the presence of NaCl (0.5 and 1 mol/L), urea (6 mol/L), SDS (5%) or a mixture of SDS (5%)/ β -mercaptoethanol (5%). A and C: pH 4.0; B and D: pH 7.0. The hydrolysates were obtained under the conditions of trials (2) pH 8.7/ 48 °C; (5) pH 7.0/ 55 °C; and (8) pH 8.0/ 65 °C of CCRD. Different lowercase letters in columns of the same sample express significant differences ($p < 0.05$). Uppercase letters compares means among hydrolysates under the same solvent condition ($p < 0.05$).

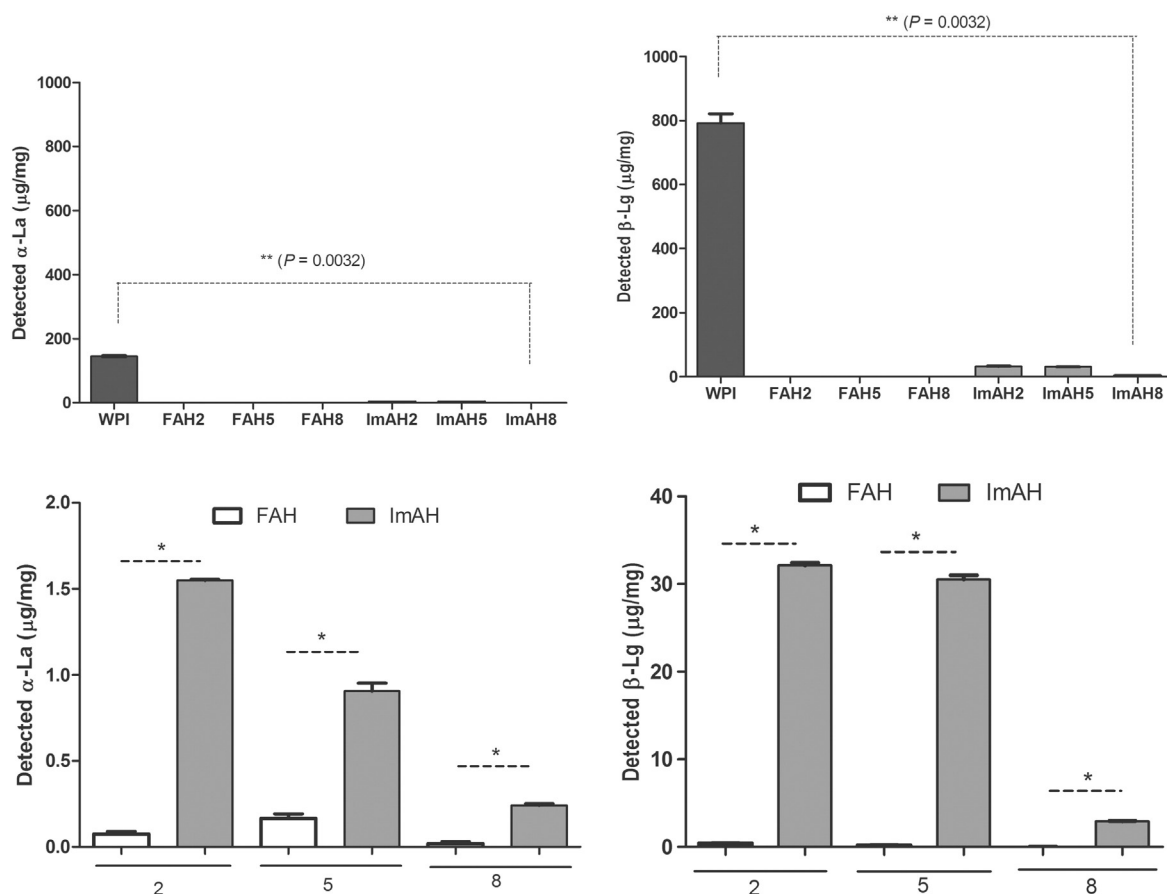


Fig. 6. Detection of α -La and β -Lg in hydrolysates obtained with free (FAH) and immobilized Alcalase (ImAH) under the conditions of trials (2) pH 8.7/48 °C; (5) pH 7.0/55 °C; (8) pH 8.0/65 °C of CCRD. Results are expressed as mean $\pm$ standard deviation of three determinations ($p < 0.05$ - Mann-Whitney test).

involved in IgE binding in allergic reactions (Taylor, Nordlee, Niemann, & Lambrecht, 2009). Although the hydrolysis with FA or ImA decreased the allergens detection, it is not possible to state that allergenicity decreased. To do so, the hydrolysates should be evaluated by IgE binding capacity, because partially hydrolyzed proteins may retain their allergenicity due to the presence of specific IgE-binding epitopes (Taylor et al., 2009).

4. Conclusions

According to our results, under the same hydrolysis conditions, the use of Alcalase in free or immobilized form impacts on hydrolysates characteristics. Differences in enzyme performance resulting from enzyme immobilization and heating to inactivate the free enzyme resulted in different physicochemical characteristics and detection of residual allergen (α -La and β -Lg) of the protein hydrolysates. Although the use of immobilized Alcalase has proven to be a promising strategy for the production of hydrolysates with hypoallergenic purposes, additional studies on enzyme immobilization must be performed to improve the performance of the immobilized enzyme (decreasing steric hindrances and optimum pH and temperature values). In addition, it is worth emphasizing that the hydrolysates produced with the immobilized Alcalase can be interesting for other applications using more hydrophobic peptides.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.foodres.2016.02.015>.

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Supplementary material

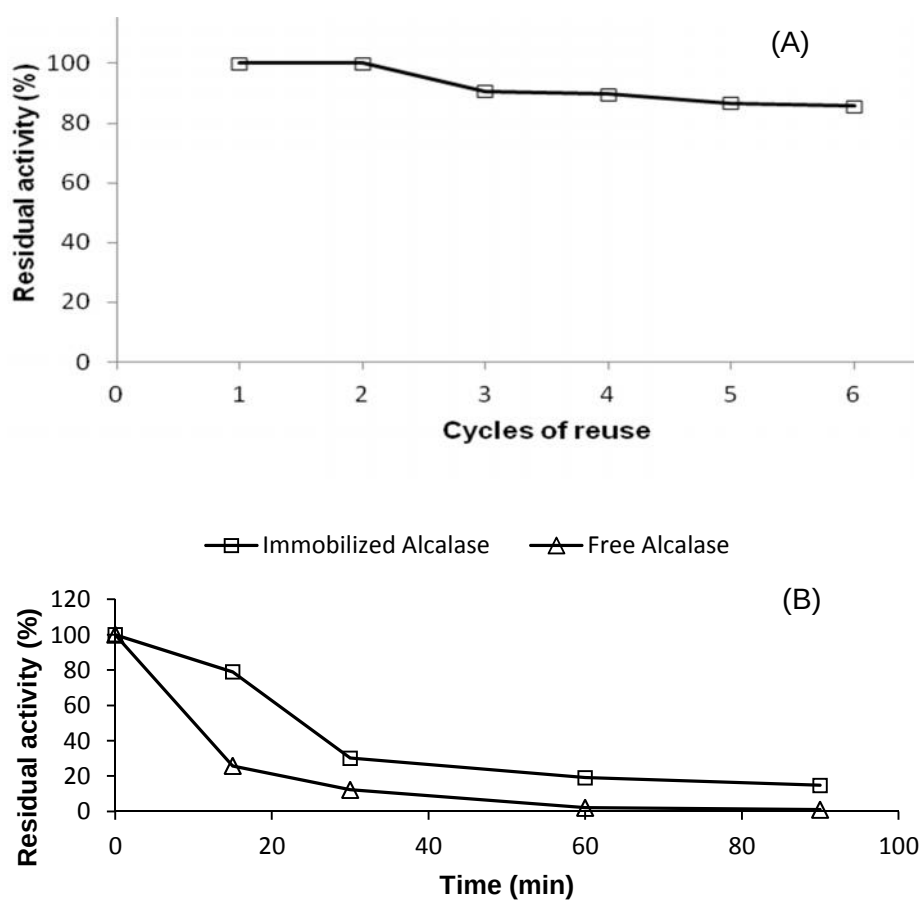


Fig. 1. (A) Reuse of Alcalase immobilized on glyoxyl-agarose bead analyzed in six cycles at 60°C, pH 8. (B) Thermal stability (65 °C and pH 8) of free and immobilized Alcalase onto glyoxyl-agarose bead expressed as residual activity (% of initial activity).