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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.

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, Tregoat, 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, Jacxsens, & 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; Corîci 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 purchase 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 μg 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:

$$Y = \varphi(\mathrm{pH}, \mathrm{T}) = \beta o + \beta 1\ x1 + \beta 2\ x2 + \beta 1\ x12 + \beta 2\ x22 + \beta 1,2\ x1x2 \quad (2)$$

where βo, β1, β2 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