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Determination of the influence of high pressure processing on calf rennet using response surface methodology: Effects on milk coagulation

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ABSTRACT

This study investigated the influence of high pressure processing (HPP) on the proteolytic and milk-clotting activities of calf rennet and milk coagulation using processed enzyme by rheological assay and confocal microscopy. The process was carried out at 25 °C, using pressure range from 50 to 300 MPa and time between 5 and 30 min. It was found that HPP (175–285 MPa for 14–23 min) increased the enzyme proteolytic activity by up to 23% and the milk-clotting activity by up to 17%. Furthermore, the G' values obtained during milk coagulation were higher for calf rennet processed at 280 MPa for 20 min than for the non-processed enzyme, forming more consistent gels (25.8% higher G′ value after 90 min). The evaluation of the milk coagulation by confocal microscopy confirms the results obtained on the rheology. Pretreatment of calf rennet using HPP accelerates the coagulation of milk and produces firmer and more consistent gels.

1. Introduction

Cheese production has been growing about 4% per year (Euromonitor International, 2012). The coagulation step can be carried out using rennet extracted from the calf abomasum (Fox, Mc Sweeney, Cogan, & Guinee, 2004). However, the reduction in slaughtering of young steers due to their low performance in terms of meat production (Food Agriculture Organization [FAO], 2010) is reducing the availability of calf rennet year by year. Currently, only 20–30% of the cheese produced worldwide uses this kind of rennet (Jacob, Jaros, & Rohm, 2011).

Thus, the search for a substitute for rennet enzymes is growing (Walstra, Wouters, & Geurts, 2006; Møller, Rattray, Sørensen, & Ardo, 2012). These substitutes must mimic the calf rennet characteristics, such as high specificity and good proteolytic activity at the usual pH and temperature of cheese manufacture (Dalgleish, 1992; Fox & Kelly, 2004; Bansal et al., 2009). In the last two decades, several animal, microbial (produced by fermentation without using recombinant techniques, e.g. protease from *Rhizomucor miehei*) and plant coagulants have been considered as potential rennet substitutes (Dalgleish, 1992; Kumar, Grover, Sharma, & Batish, 2010; Rolet-Répécaud et al., 2013). However, the application of these enzymes for milk clotting may have some disadvantages, such as a reduction in manufacturing yield and the production of undesirable flavors (Fox et al., 2004; Walstra et al., 2006). Currently the use of genetic engineering to obtain a recombinant enzyme equal to chymosin has appeared as a good option to replace calf rennet in cheese production (Johnson & Lucey, 2006), reaching 70–80% of the current market (Jacob et al., 2011).

Although there is a relatively high availability of recombinant enzyme, calf rennet has kept its importance for traditional consumers that reject products obtained from genetically modified organisms. The alteration of calf rennet by physical or chemical changes, aiming to increase the enzyme activity or stability, represents an interesting process to make it possible to use lower enzyme concentrations during milk clotting for cheese production, reducing the cost of the enzyme for cheesemakers.

High pressure processing (HPP), also known as high hydrostatic

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pressure (HHP) or high isostatic pressure (HIP), is an emerging technology developed for microbial inactivation as an alternative to traditional thermal food processing. However recently, some studies have been dedicated to evaluating the effect of HPP on enzymes. The results indicated that the process is capable of promoting activation (Mozhaev, Lange, Kudryashova, & Balny, 1996; Sila et al., 2007; Eisenmenger & Reyes-De-Corcuera, 2009a,b) and stabilization of enzymes (Mozhaev et al., 1996; Eisenmenger & Reyes-De-Corcuera, 2009a,b), by applying low pressures (of up to 400 MPa) and moderate temperatures (Knorr, 1999) in presence (reaction under pressure) (Mozhaev et al., 1996; Kudryashova, Mozhaev, & Balny, 1998; Eisenmenger & Reyes-De-Corcuera, 2009a,b) or absence of substrate (Sila et al., 2007; Eisenmenger & Reyes-De-Corcuera, 2009a).

The pressurization process follows the "Le Chatelier" principle, inducing a reduction in molecular volume (Knorr, 1999). For several enzymes, this reduction of molecular volume can results in permanent change of the tridimensional structure, which sometimes imparts in increase of enzyme activity (Eisenmenger & Reyes-De-Corcuera, 2009a). The main change in enzyme structure is an increase in enzyme conformational flexibility caused by the hydration of charged groups (Eisenmenger & Reyes-De-Corcuera, 2009a,b). Thus, HPP could be an interesting physical method to increase the enzyme activity and specificity (Eisenmenger & Reyes-De-Corcuera, 2009a,b). It can be an interesting technology to be applied as a to promote increased activity and enzyme stability.

Although the process has already been identified as capable of promoting an increase or decrease in the enzymatic activity, no work had studied the effect of HPP in milk-clotting enzymes, which is a gap in the knowledge about the effect of this technology. Additionally, the majority of results were obtained by processing enzyme and substrate together, which may have a different effect to applying HPP on the enzyme alone. Therefore, this study aimed to evaluate the influence of high pressure processing on the proteolytic and milk-clotting activities of a commercial calf rennet and the milk clotting profile by using processed enzyme measured by rheological assay and confocal microscopy.

2. Materials and methods

2.1. Enzyme

A commercial powder calf rennet containing 94 g/100 g of chymosin and 6 g/100 g of pepsin (freeze-dried Carlina™ Animal Rennet 1650, Danisco, Vinay, France) was used in the assays. The activity of the enzyme was 1700 IMCU per gram of powder.

2.2. High pressure processing

The experiments were carried out in a pilot scale high pressure equipment (High Pressure Equipment Company, Model 37-5-75-60, Erie, Pennsylvania, USA). This equipment has a cylindrical chamber of 15 mm in diameter and 35 mm in height, with 10 mL of capacity. The equipment works at pressures of up to 350 MPa and ethanol was used as the pressurizing fluid. The come up time to reach 350 MPa was 3 min and the decompression process took 1 min.

2.3. Experimental design

Aliquots of 2 mL of enzyme solution (3 g/100 mL) prepared in sodium acetate buffer (0.2 mol/L, pH 5.5) were vacuum-packed in sterile plastic polyolefin bags (BagLight® Polysilk® 132025, Interscience, Saint-Nom-la-Bretèche, France) at 4 °C. Subsequently, the samples were subjected to the HPP at room temperature (25 °C) to evaluate the simultaneous effect of two independent variables: isostatic pressure (X1) and process time (X2) on the enzyme. The variable were studied using a central composite rotational design (CCRD) with four linear levels +1 and −1, four axial points (α ±1.414) and three assays at the central point, totalizing 11 experiments. Furthermore, a control enzyme sample (non processed enzyme) was also prepared for comparative evaluation. The assays were performed in random order, and the data fitted to a second order polynomial model (Equation (1)).

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i} \sum_{j=i+1} \beta_{ij} X_i X_j \quad (1)$$

where Y was the predicted response, β_0 was the constant (intercept), β_i the linear coefficients, β_{ii} the quadratic coefficients and β_{ij} the cross product coefficient. X_i and X_j were the independent variables.

Table 1 shows the CCRD with the coded and real values of the independent variables and their levels.

The proteolytic and milk-clotting activities of the samples were assessed in quadruplicate immediately after the HPP (time 0 h) and after 24 h of storage at 4 °C. The results were analyzed using the software Statistica® 7.0 and the responses were: significance of the effects ($p \leq 0.10$), analysis of variance (ANOVA) using the F test to assess the significance of the regression and the lack of fit (pure error), calculated using a 95% confidence level ($p \leq 0.05$). The R^2 values were also calculated. The regressions approved in the F test (for regression and lack of fit) were used to generate a response surface (Myers & Montgomery, 2002). The validation of the mathematical models obtained was carried out in quadruplicate at a pressure of 280 MPa and time of 20 min (the point of maximum activity based on the optimization), by evaluating the proteolytic activity and milk-clotting activity. Additionally, the performance of the HPP treated enzyme in forming milk gels was evaluated by rheological and confocal microscopy assays.

2.4. Proteolytic activity determination

The proteolytic activity was based on Arima, Yu, and Iwasaki (1970), and followed the methods described by Leite Júnior, Tribst, and Cristianini (2014), using sodium caseinate as substrate. The activity was determined spectrophotometrically by measurement of the release of peptides. The proteolytic activity was calculated according to Equation (2).

Table 1
Variables and levels used in CCRD for the independent variables.

Standard order	Run order	Coded variables		Real values	
		X1	X2	X1	X2
1	8	−1	−1	86	8.60
2	10	1	−1	264	8.60
3	7	−1	1	86	26.40
4	1	1	1	264	26.40
5	3	−1.414	0	50	17.50
6	4	1.414	0	300	17.50
7	11	0	−1.414	175	5.00
8	5	0	1.414	175	30.00
9	9	0	0	175	17.50
10	2	0	0	175	17.50
11	6	0	0	175	17.50
Control (non- processed)		–	–	0	0.00

X1: Pressure (MPa); X2: Time (min).