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Proteolytic and milk-clotting activities of calf rennet processed by high pressure homogenization and the influence on the rheological behavior of the milk coagulation process

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ABSTRACT

This work studied the effects of high pressure homogenization (HPH) on the performance of a commercial calf rennet. The enzyme solution was processed at 50, 100, 150 and 190 MPa and then stored for up to five days at 4 °C. The effects were evaluated on the proteolytic and milk-clotting activities of the enzyme and on the rheological behavior of the milk gels obtained using the processed enzyme. HPH caused a reduction in proteolytic activity with increase in pressure (up to 52% loss of activity at 190 MPa). At the first 48 h of storage, the processed enzyme maintained its activity while the non-processed sample showed a continuous reduction in activity. The results showed that HPH processing did not alter the milk-clotting activity and the rheological tests showed high G' values for the milk coagulated with the enzyme processed at 190 MPa (7% higher after 84 min of coagulation). Thus, HPH improves the milk coagulation by calf rennet and reduces the proteolytic activity of enzyme, which may improve the quality of fresh cheeses, especially after their storage.

Industrial relevance: The changes caused by HPH in the calf rennet improved the cheese manufacturing process and the quality of the product (higher consistency of the milk gel and lower proteolysis during storage). Therefore, the HPH processing of calf rennet can improve its application in the dairy industry to obtain high quality fresh cheese and an extended shelf-life.

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1. Introduction

Cheese production is growing annually at a rate of 4% throughout the world (Euromonitor International — Related Analysis, 2012). However, the supply of calf rennet is decreasing year by year due to the tendency to reduce the early slaughter of steers because of low achievement in terms of meat production (Food Agriculture Organization [FAO], 2010). Currently, only 20–30% of the cheeses produced worldwide use calf rennet (Jacob, Jaros, & Rohm, 2011).

Potential substitutes should emulate their specific properties, with high specificity and good proteolytic activity at the pH and temperature of cheese manufacture (Fox & Kelly, 2004; Kumar, Grover, Sharma, & Batish, 2010). Several coagulants from animals, microorganisms and plants have been considered as potential substitutes for rennet (Rolet-Répécaud et al., 2013; Walstra, Wouters, & Geurts, 2006). However, the application of these enzymes in the coagulation of milk could result in problems such as a reduction in manufacturing yield (due to the intense activity of these proteolytic enzymes) and the appearance of defects in flavor and texture (especially a bitter taste and crumbly texture) (Møller et al., 2012). Thus, it can be seen that the coagulation step is critical for cheese production and that there is a real demand for new coagulants with better activity and stability.

High pressure homogenization (HPH) or dynamic high pressure (DHP) is a non-thermal process primarily developed to ensure the microbiological quality of food without affecting its sensory and nutritional attributes (Tribst, Sant'Ana, & Massaguer, 2009). The effect of HPH on enzymes has been studied by some authors (Lacroix, Fliss, & Makhlouf, 2005; Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009; Liu, Zhang, et al., 2010; Liu, Zhong, et al., 2010; Welti-Chanes, Ochoa-Velasco, & Guerrero-Béltran, 2009), who found that the process was capable of promoting the activation and stabilization (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009; Liu, Zhang, et al., 2010; Tribst, Augusto, & Cristianini, 2012; Tribst & Cristianini, 2012b) or inactivation (Lacroix et al., 2005; Welti-Chanes et al., 2009) of enzymes, the effect being associated with the type of enzyme and the pressure applied (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009). These changes in enzyme performance are normally linked to changes in the tertiary and quaternary structures of the enzymes, with the exposure of hydrophobic amino acids to a less hydrophobic environment (Liu, Liu, Liu, et al., 2009).

Thus HPH may be an interesting tool for calf rennet modification, which can increase its milk-clotting activity and stability, and

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consequently reduce the amount of enzyme required for cheese production, affecting directly the enzyme relative costs. Therefore, this study aimed to evaluate the influence of high pressure homogenization on the proteolytic activity, milk-clotting activity and stability of the commercial calf rennet used in cheese manufacture.

2. Material and methods

2.1. Calf rennet and high pressure homogenization processing

A commercial calf rennet was used in the experiments (freeze dried powder Carlina™ Animal Rennet 1650 — Danisco, Vinay, France). This enzyme is composed of 94% chymosin and 6% pepsin.

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the assays. This equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa and operates at a flow rate of 9 $L.h^{-1}$.

A volume of 2 L of the calf rennet solution was prepared at 1.5% (w/v) in 0.2 M sodium acetate buffer (pH 5.1) and homogenized under pressures of 50, 100, 150 and 190 MPa, using an inlet temperature of 23 °C. Samples (200 mL) were collected and cooled immediately in an ice bath after exiting the homogenizing valve. A non-processed sample of calf rennet was evaluated as the control sample.

2.2. Relative proteolytic activity (RPA) determination

The proteolytic activity of calf rennet was measured according to Merheb-Dini, Gomes, Boscolo, and da Silva (2010): the enzyme solution (1.5% w/v) was prepared in a 0.2 M acetate buffer (pH 5.1). 600 μL of the enzymatic solution was added to 400 μL of a 0.5% (w/v) sodium caseinate solution (Sigma Aldrich®, USA) prepared in the same buffer. The reaction was carried out at 35 °C/40 min in a shaken water bath (62 rpm) and 1 mL of 10% (w/v) trichloroacetic acid (TCA) then added to stop the hydrolysis. The samples were centrifuged at 2300 *g*/5 min/10 °C and the absorbance was measured at 280 nm in a DU 800 UV–VIS spectrophotometer (Beckman Coulter®, Brea, CA, USA). One unit of enzyme was defined as the amount required to increase the absorbance at 280 nm by 0.1 under the assay conditions. The blank samples were prepared by adding the TCA to the tubes before the addition of the enzymatic solution, and the $\Delta Abs_{280\ nm}$ was determined from the difference in absorbance between the sample and the blank. The enzymatic activity was calculated according to Eq. (1).

$$U/mL = (\Delta Abs_{280\,nm} \times 10 \times \text{dilution factor}/0.6 \times 40). \quad (1)$$

RPA assays were carried out immediately after processing (time 0 h) and after 12 h, 24 h, 48 h, 72 h, 96 h and 120 h. The samples were stored under refrigeration (4 °C) throughout this period. A non-processed sample was used for a comparative evaluation. The relative proteolytic activity (RPA) was calculated considering the activity of the HPH processed and non-processed samples, according to Eq. (2):

$$RPA = (\text{enzyme activity}_{\text{after\_HPH\_and/or\_storage}} / \text{enzyme activity}_{\text{non-processed\_sample\_at\_0h}}) * 100. \quad (2)$$

2.3. Relative milk-clotting activity (RMCA) determination

The milk-clotting activity was determined according to Merheb-Dini et al. (2010). A volume of 5 mL of skimmed milk powder, reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids) was added to a 0.01 M $CaCl_2$ solution and pre-incubated at 35 °C/10 min. An aliquot of 0.5 mL of the enzyme solution (0.003%, w/v, prepared in 0.1 M sodium acetate buffer, pH 5.1) was then added and the time count started. Clot formation was determined by manual tube rotation and the time taken for the first particles to form measured. One milk-clotting activity unit (MCA) was defined as the amount of enzyme required to clot 1 mL of substrate in 40 min at 35 °C. The MCA was calculated using Eq. (3):

$$\text{Unit of milk-clotting activity(MCA)} = 2400/t \times S/E \quad (3)$$

where t is the time (seconds) necessary for clot formation, S is the milk volume and E is the enzyme volume.

MCA assays were carried out immediately after processing (time 0 h) and after 24 h, 48 h, 72 h, 96 h and 120 h. The samples were stored under refrigeration (4 °C) throughout this period. A non-processed sample was also used for a comparative evaluation. The relative MCA (RMCA) was calculated considering the MCA of the HPH processed and non-processed samples, according to Eq. (4):

$$RMCA = \left(MCA_{\text{after\_HPH\_and/or\_storage}} / MCA_{\text{non-processed\_sample\_at\_0h}}\right) * 100. \quad (4)$$

2.4. Rheological assays

The milk coagulation kinetics were evaluated by monitoring the milk coagulation process by way of a time sweep using a low deformation oscillatory test in a rheometer with controlled stress (AR2000ex, TA Instruments, USA). These assays were carried out with the processed and non-processed calf rennet.

The experiments were determined according to Lee and Lucey (2003) using 60 mL of skimmed milk powder reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids) with the addition of a 0.01 M $CaCl_2$ solution. This mixture was pre-incubated at 35 °C/10 min, and subsequently 0.8 mL of enzyme solution (0.03%, w/v) prepared in 0.1 M acetate buffer (pH 5.1) was added. The mixture was immediately transferred to the rheometer cup (30 mm diameter and 80 mm height), which had a *vaned quarter* geometry (with 28 mm of diameter and 42 mm of length) and a 4 mm *gap*. The stress was set at 0.1 Pa and frequency at 0.1 Hz. The parameter G' (storage modulus) was measured at 3 min intervals for 84 min of the clotting process at 35 °C. The temperature was controlled by a *Peltier* system. The rheological assays were carried out immediately after processing (time 0 h) and after 120 h. The samples were stored under refrigeration (4 °C) throughout this period.

Furthermore, the rate of milk-clotting was calculated as the variation of G' with variation in time as the log (dG'/dt) at three minute intervals, and expressed in $Pa.min^{-1}$. The storage modulus (G') describes the elastic (solid) behavior of the product, and consequently the energy stored and released in each oscillatory cycle.

2.5. Statistical analysis

The processes and analyses were carried out with three repetitions and each experimental unit was carried out in quadruplicate. The analysis of variance (ANOVA) was used to compare the effects of the different treatments and the Tukey test to determine the differences between them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 7.0 software (StatiSoft, Inc., Tulsa, Okla., U.S.A.) and the results were presented as the mean ± standard deviation.

3. Results and discussion

3.1. Effect of high pressure homogenization on the proteolytic activity

The fast decompression during HPH promotes intense shear and friction, with consequent heating of the product. Since enzymes can be affected by heating, the temperature reached under each set of process conditions was also measured. The residence time at those temperatures was less than 10 s, and Eq. (5) can be used to estimate the temperatures reached after homogenization. The increase in pressure promoted a

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