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The effect of high pressure processing on recombinant chymosin, bovine rennet and porcine pepsin: Influence on the proteolytic and milk-clotting activities and on milk-clotting characteristics

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ABSTRACT

This study evaluated the effect of high pressure processing (HPP) on the proteolytic and milk-clotting activities of recombinant chymosin, bovine rennet and porcine pepsin. The optimum process conditions were established considering the maximum increase on milk-clotting activity, which was 10% for recombinant chymosin (212MPa/5min/10 °C) and the bovine rennet (222 MPa/5min/23 °C) and 6% for the porcine pepsin (50MPa/5min/20 °C). The high pressure (HP) processed enzymes promoted faster milk-clotting and the gels produced were more consistent (values of G' >6% and k' >5%). Processed enzymes produced gels with higher Δ backscattering measured using near-infrared (NIR) light (at least 6%), indicating higher degree of protein aggregation. Images obtained by confocal microscopy showed a faster reduction in the total number of pores, with an increased average pore area for gels obtained with the HP processed enzymes. Furthermore, the wet yields of the gels obtained from HP processed enzymes were up to 4.3% greater than gels obtained with non-processed enzymes. Thus, HPP emerges as an interesting alternative to improve the performance of milk-clotting enzymes, accelerating milk-clotting and allowing for an increase in cheese yield.

1. Introduction

The milk coagulation is promoted by clotting enzymes and this coagulation process can be divided into two phases: specific enzymatic hydrolysis of the κ-casein (cleavage preferably at the Phe_{105}-Met_{106} site) and aggregation of the para-κ-casein (formed from the cleaved casein micelles) in the presence of calcium ions (Fox, Mc Sweeney, Cogan, & Guinee, 2004). Characteristics such as the water holding capacity, strength and porosity are important in the cheese making process, once they affect parameters such as the yield, moisture content and texture of the product (Pandey, Ramaswamy, & St-Gelais, 2000).

Several enzymes have the ability to promote milk-clotting, however different enzymes have different abilities to cleave the κ-casein specifically at the Phe_{105}-Met_{106} site, and also to cleave the casein in other unspecific fractions. Chymosin (obtained from the abomasum of ruminants or by fermentation using recombinant microorganisms) is the milk-clotting enzyme with the greatest specificity, which is widely in cheese production. Bovine rennet (~80% of pepsin and 20% of chymosin) can be used as an optional milk-clotting enzyme, especially for the production of cheese destined for markets that reject the use of genetically modified microorganisms and to produce cheeses with a desirable degree of proteolysis during maturation. Pepsin is not commonly used in cheese production, due to its low specific activity, resulting in cheeses with low yield and undesirable sensory attributes.

High pressure processing (HPP), also known as high isostatic pressure (HIP) or high hydrostatic pressure (HHP), is an emerging technology able to promote changes in enzyme activity. The activation or inactivation effect is dependent on the conditions applied such as pressure, time, temperature, pH of the solution and food matrix (Eisenmenger & Reyes-De-Corcuera, 2009; Knorr, 1999). In molecular terms, the pressurization process promotes an increase in the conformational flexibility of the enzyme (Eisenmenger & Reyes-De-Corcuera, 2009) which may increase the exposure of active sites with subsequent activation. Thus, HPP could be an

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interesting tool to improve the hydrolytic characteristics of enzymes. Therefore, this study evaluated the effect of HPP on the proteolytic and milk-clotting activities of recombinant chymosin, bovine rennet and porcine pepsin and effect on milk-clotting process and the milk gel characteristics.

2. Materials and methods

2.1. Enzymes

The enzymes used in the assays were: recombinant chymosin with activity of 2500 IMCU g^{-1} (CHY-MAX® M 2500 Power NB, Chr Hansen, Hoersholm, Denmark); adult bovine rennet with activity of 200 IMCU mL^{-1} (Coalho Líquido BV®, Bela Vista Ltda, Santa Catarina, Brazil, containing 80–90% of bovine pepsin and 10–20% of bovine chymosin) and porcine pepsin protease with activity of 3000 IMCU g^{-1} (freeze dried powdered Porcine Pepsin, PEPSINA SUINA TS®, Bela Vista Ltda, Santa Catarina, Brazil).

2.2. Sample preparation

Aliquots of 100 mL of the enzyme solutions at a concentration of 1.5 IMCU mL^{-1} for recombinant chymosin, adult bovine rennet and porcine pepsin were prepared in sodium acetate buffer (0.2 M, pH 5.6) – conditions preliminarily stablished as optimum for enzyme activity (data non showed) – and vacuum-packed in plastic bags (LDPE-Nylon-LDPE).

2.3. High pressure processing

The experiments were carried out in a high pressure equipment (QFP 2L-700 Avure Technologies, OH, USA). This equipment works at pressures of up to 690 MPa and temperatures up to 90 °C. The temperature of the equipment chamber block was set for different process conditions. The initial temperature of the water in the chamber was set considering the rate of temperature increase expected to occur under adiabatic conditions in this equipment (3 °C/100 MPa). The control (non-processed) was not subjected to pressure. Each sample was processed in triplicate under each process condition.

2.3.1. Experimental design

The samples were subjected to the HP process to evaluate the simultaneous effect of three independent variables: isostatic pressure (X1) ranging from 50 to 600 MPa, process time (X2) ranging from 5 to 30min and temperature (X3) ranging from 15 to 55 °C on the proteolytic profile of the enzyme. The variables were studied using a central composite rotational design (CCRD) with eight linear levels +1 and −1, six axial points (α ±1.682) and three assays at the central point, totalizing 17 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)).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y was the predicted response, β_0 the constant (intercept), β_i the linear coefficient, β_{ii} the quadratic coefficient and β_{ij} the cross product coefficient. X_i and X_j were levels of the coded independent variables. The dependent variables were RPA and RMCA and these variables were analyzed according to 2.4.

The results were analyzed using the software Statistica® 7.0 and the responses were: significance of the effects and analysis of variance (ANOVA). In the ANOVA it was used 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 mathematical model (Myers & Montgomery, 2002). Validation of the mathematical models obtained was carried out in sextuplicate under the optimal process conditions (the point of maximum milk-clotting activity) for each enzyme, by evaluating the proteolytic activity and milk-clotting activity immediately after the process (time 0 h) and after 7, 14, 21, 28 and 35 days of storage at 4 °C. Furthermore, non-processed samples were also prepared on day 0 and assessed over time for a comparative evaluation.

2.4. Determination of the proteolytic and milk-clotting activities

The proteolytic (PA) and milk-clotting activities (MCA) of the samples were assessed in sextuplicate immediately after the HP process (time 0 h). Both activities were measured using the methods described by Leite Júnior, Tribst, and Cristianini (2014). The relative proteolytic activity (RPA) and relative milk-clotting activities (RMCA) were calculated by using the following equations:

$$RPA = \left(\frac{\text{PA after } {}_{\text{HPP}_{\text{and/or}_{\text{storage}}}}}{\text{PA non} - \text{processed}_{\text{sample}_{\text{at}_{0h}}}} \right) \cdot 100 \quad (2)$$

$$RMCA = \left(\frac{\text{MCA after } {}_{\text{HPP}_{\text{and/or}_{\text{storage}}}}}{\text{MCA non} - \text{processed}_{\text{sample}_{\text{at}_{0h}}}} \right) \cdot 100 \quad (3)$$

2.5. Evaluation of milk-clotting process and milk gels formed using HP processed enzymes under the optimal conditions

Aliquots of 100 mL of enzyme solutions were prepared according to section 2.2. The HP processes were carried out under the optimal conditions for each enzyme, which were established as 212 MPa/5 min/10 °C for recombinant chymosin; 222 MPa/5 min/23 °C for bovine rennet and 50 MPa/5 min/20 °C for porcine pepsin. A control for each enzyme sample (non-processed enzyme) was also prepared for a comparative evaluation.

2.5.1. Rheological profile of milk-clotting induced by the addition of non-processed and HP processed clotting enzymes

The milk-clotting process was monitored by way of a time sweep using a low deformation oscillatory test in a rheometer with controlled stress (AR2000ex, TA Instruments, USA) using the procedure described by Leite Júnior, Tribst et al. (2014) for 60 min of clotting at 35 °C. After, the formed gel was evaluated using frequency sweep assays according to the procedures described by Oliveira, Augusto, Cruz, and Cristianini (2014). For the evaluation of the experimental results, values obtained before breaking of the gel were used according to the power law model (Eq. (4)).

$$G' = k' . \omega^{n'} \quad (4)$$

where G' is the storage modulus, which represents the elastic solid behavior (Pa), k' is the consistency index ($Pa \cdot s^{n'}$), ω is the oscillation frequency (Hz) and n' is the behavior index. The parameters of the model were obtained by linear regression using the software Microsoft Excel 2007, with a significant probability level of 95%.