Food Research International 97 (2017) 116–122

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Biophysical evaluation of milk-clotting enzymes processed by high pressure

Bruno Ricardo de Castro Leite Júnior [a], Alline Artigiani Lima Tribst [b], Nicholas J. Grant [c], Rickey Y. Yada [c], Marcelo Cristianini [a,*]

[a] Department of Food Technology (DTA), School of Food Engineering (FEA), University of Campinas (UNICAMP), Monteiro Lobato, 80. PO Box 6121, 13083–862 Campinas, SP, Brazil
[b] Center of Studies and Researches in Food (NEPA), University of Campinas (UNICAMP), Albert Einstein, 291, 13083-852 Campinas, SP, Brazil
[c] Faculty of Land and Food Systems, The University of British Columbia (UBC), MacMillan Building 248, 2357 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada

ARTICLE INFO

Article history:
Received 26 January 2017
Received in revised form 23 March 2017
Accepted 23 March 2017
Available online 27 March 2017

Keywords:
High pressure processing
Milk-clotting enzymes
Intrinsic fluorescence
Surface hydrophobicity
ATR-FTIR spectroscopy

ABSTRACT

High pressure processing (HPP) is able to promote changes in enzymes structure. This study evaluated the effect of HP on the structural changes in milk-clotting enzymes processed under activation conditions for recombinant camel chymosin (212 MPa/5 min/10 °C), calf rennet (280 MPa/20 min/25 °C), bovine rennet (222 MPa/5 min/23 °C), and porcine pepsin (50 MPa/5 min/20 °C) and under inactivation conditions for all enzymes (600 MPa/10-min/25 °C) including the protease from *Rhizomucor miehei*. In general, it was found that the HPP at activation conditions was able to increase the intrinsic fluorescence of samples with high pepsin concentration (porcine pepsin and bovine rennet), increase significantly the surface hydrophobicity and induce changes in secondary structure of all enzymes. Under inactivation conditions, increases in surface hydrophobicity and a reduction of intrinsic fluorescence were observed, suggesting a higher exposure of hydrophobic sites followed by water quenching of Trp residues. Moreover, changes in secondary structure were observed (with minor changes seen in *Rhizomucor miehei* protease). In conclusion, HPP was able to unfold milk-clotting enzymes even under activation conditions, and the porcine pepsin and bovine rennet were more sensitive to HPP.

1. Introduction

Milk-clotting enzymes play an important role in cheese production. These enzymes act during the enzymatic phase of milk coagulation affecting syneresis, yield, flavor and texture of the cheeses (Chitpinityol & Crabbe, 1998; Fox, Guinee, Cogan, & Mc Sweeney, 2000). Traditionally calf rennet and bovine rennet are composed of mixtures of chymosin and pepsin (Fox et al., 2000), with a higher proportion of calf rennet being preferred due to its higher concentration of bovine chymosin. Chymosin is characterized by having a higher milk-clotting activity and lower non-specific proteolytic activity. Other enzymes have also been studied for use in the cheese manufacturing process including porcine pepsin (Nielsen & Foltmann, 1995) and *Rhizomucor miehei* protease (Kumar, Grover, Sharma, & Batish, 2010). With the development of genetic engineering, recombinant chymosin has become prominent with recombinant camel chymosin being considered the best substitute for calf rennet due its high specific activity on the hydrolysis of k-casein and its low general proteolytic activity (Andrén, 2011).

High pressure processing (HPP), also known as high isostatic pressure (HIP) or high hydrostatic pressure (HHP), is an emerging technology known to promote changes in molecular structures of proteins and enzymes (He et al., 2014; Liu, Powers, Swanson, Hill, & Clark, 2005). The effect of the HPP on the molecular structure is a function of the conditions applied. High pressures promote changes to enzyme structure and these are generally intensified with increasing processing time and temperature (Eisenmenger & Reyes-de-Corcuera, 2009). The impact of these changes on enzyme activity cannot be easily established, since it is highly dependent on the enzyme and on the processing conditions and activity measurement (Chakraborty, Kaushik, Rao, & Mishra, 2014; Eisenmenger & Reyes-de-Corcuera, 2009; Tribst, Leite Júnior, Oliveira, & Cristianini, 2016).

Milk-clotting enzymes (calf rennet, bovine rennet, recombinant camel chymosin, and porcine pepsin), intracellular peptidases, and glycolytic enzymes from *Lactococcus lactis* subsp. *cremoris* have been shown to be activated at pressures <400 MPa (Malone, Wick, Shellhammer, & Courtney, 2003; Eisenmenger & Reyes-de-Corcuera, 2009; Leite Júnior, Tribst, Bonafe and Cristianini, 2016; Leite Júnior, Tribst, & Cristianini, 2017), while other enzymes such as Aminopeptidase C (Malone et al., 2003), polyphenolxidase and peroxidase (Tribst et al., 2016) are activated at higher pressures (600–700 MPa). On the

* Corresponding author at: Monteiro Lobato, 80, School of Food Engineering, University of Campinas, 13083-862 Campinas, SP, Brazil.
E-mail address: olecram@unicamp.br (M. Cristianini).

http://dx.doi.org/10.1016/j.foodres.2017.03.042
0963-9969/

other hand, enzymes such as amylase (Riahi & Ramaswamy, 2004) and m-Calpain, μ-Calpain, and Calpastatin (Homma, Ikeuchi, & Suzuki, 1995) are inactivated even at relative low pressures (300–400 MPa).

Previous studies involving milk coagulants established optimized processes conditions (pressure, time and temperature) in order to increase milk clotting activity for 10% recombinant camel chymosin (212 MPa/5 min/10 °C), 8% calf rennet (280 MPa/20 min/25 °C), 9% bovine rennet (222 MPa/5 min/23 °C), and 5% porcine pepsin (50 MPa/5-min/20 °C) (Leite Júnior et al., 2017; Leite Júnior, Tribst, Bonafe et al., 2016) which resulted in faster milk coagulation, gels with higher consistency and lower syneresis (Leite Júnior, Tribst, Bonafe et al., 2016; Leite Júnior et al., 2017). Conversely, a drastic reduction in MCA was obtained at 600 MPa/10 min/25 °C (Leite Júnior, Tribst & Cristianini, 2016; Leite Júnior et al., 2017).

The effect of HPP results on enzyme activation for monomeric, dimeric and tetrameric enzymes (Eisenmenger & Reyes-De-Corcuera, 2009; Knorr, 1999) indicated that the changes were primarily due to electrostatic and hydrophobic interactions, as well as hydrogen bonding (Chakraborty et al., 2014; Mozhaev, Heremans, Frank, Masson, & Balny, 1994) resulting from greater exposure of aromatic residues to more polar solvents, as well as the formation of intermolecular disulfide bounds due to cysteine exposure followed by oxidation of sulfhydryl groups to disulfide (Chakraborty et al., 2014).

In addition, it was observed that each enzyme had an upper limit of pressure, temperature and process time that would result in an increase in activity. Above this limit, the enzyme would unfold, leading to a denatured structure with a loss of activity (Chakraborty et al., 2014). For enzymes processed in absence of substrate, the activation observed is possibly related to the increase in molecular conformational flexibility after HPP due to hydration of the charged groups, activation of the latent isoenzymes, and/or exposure of active sites (Chakraborty et al., 2014; Eisenmenger & Reyes-de-Corcuera, 2009). Activation may also be due to stabilization of the enzyme caused by an increase of intramolecular interactions, hydration of non-polar and charged groups, as well as the stabilization of hydrogen bridges (Eisenmenger & Reyes-de-Corcuera, 2009; Kudryashova, Mozhaev, & Balny, 1998). On the other hand, enzyme inactivation is linked to the formation and/or disruption of hydrogen bonding, and Van der Waals, hydrophobic, and electrostatic interactions with a change in structure due to folding and/or unfolding without affecting covalent linkages (Chakraborty et al., 2014).

Although previous research has shown that HP processing is able to alter the structure of proteins and enzymes, no detailed work on the molecular structures of milk coagulants has been reported. Therefore, this work was carried out in order to differentiate the structural changes of milk-clotting enzymes after HPP under activation conditions (previously optimized in order to obtain the maximum increase in activity) and as well as inactivation conditions (Leite Júnior, Tribst, Bonafe et al., 2016; Leite Júnior, Tribst, & Cristianini, 2016; Leite Júnior et al., 2017).

2. Materials and methods

2.1. Enzymes

Five commercial enzymes were examined: recombinant camel chymosin with an activity of 2500 IMCU·g^{-1} (CHY-MAX® M 2500 Power NB, Chr Hansen, Hoersholm, Denmark); calf rennet with an activity of 1700 IMCU·g^{-1} (freeze-dried Carlina™ Animal Rennet 1650, Danisco, Vinay, France, containing 94% of chymosin and 6% of pepsin); adult bovine rennet with an 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); porcine pepsin protease with an activity of 3000 IMCU·g^{-1} (freeze dried powdered porcine pepsin, PEPSINA SUINA TS®, Bela Vista Ltda, Santa Catarina, Brazil), and *Rhizomucor miehei* protease with an activity of 2200 IMCU·g^{-1} (Marzyme 150 MG Powder Microbial Rennet, Danisco, Vinay, France, which contains ≥28,000 mg of protease per kg of product). These enzymes were chosen to represent the most commonly used enzyme enzymes used for cheese making including recombinant versions, those from natural sources i.e., non-genetically modified enzymes (calf and bovine rennet and *Rhizomucor miehei* protease) and a general protease (pepsin).

2.2. High pressure processing

The experiments were carried out in a static high pressure equipment (QFP 2L-700 Avure Technologies, OH, USA). This equipment consists of a cylindrical chamber with a 2 L capacity and works at pressures of up to 100,000 psi (690 MPa), using deionized water as the pressurizing fluid. The temperature of the chamber was measured by a type K thermocouple set inside the chamber and the pressure was measured by an internal pressure transducer. The temperature of the equipment chamber block was set for different processing conditions. The initial temperature of the water in the chamber was set according to the rate of temperature increase under adiabatic conditions for this equipment (3 °C/100 MPa). The control (non-processed) was not subjected to pressure processing.

Aliquots of 100 mL of recombinant chymosin (10.0% w/v), calf rennet (10.0% w/v), bovine rennet (10.0% w/v), porcine pepsin (10.0% w/v), and protease from *R. miehei* (10.0% w/v), solutions were prepared in sodium acetate buffer (0.2 M, pH 5.6) and vacuum-packed in plastic bags (LDPE-Nylon-LDPE, 16 μm thickness - TecMaq, São Paulo, Brazil). The HP processes were carried out under conditions determined in previous studies as capable of inducing the maximum increase in milk clotting activity for each enzyme: 212 MPa/5 min/10 °C for recombinant chymosin (Leite Júnior et al., 2017); 280 MPa/20 min/25 °C for calf rennet (Leite Júnior, Tribst, Bonafe et al., 2016); 222 MPa/5 min/23 °C for bovine rennet (Leite Júnior et al., 2017) and 50 MPa/5 min/20 °C for porcine pepsin (Leite Júnior et al., 2017). Additionally, the effects on structures caused by inactivation process for all enzymes (recombinant chymosin, calf rennet, bovine rennet, porcine pepsin and also for protease from *R. miehei*, which has not been activated by the HPP process) were assessed after process at 600 MPa/10 min/25 °C (a condition able to reduce activity to ~0% for all enzymes examined in this study - Leite Júnior, Tribst, & Cristianini, 2016; Leite Júnior et al., 2017). A control for each enzyme sample (non-processed enzyme) was also prepared. After processing, the samples were lyophilized and stored at −80 °C, in order to prevent any further changes prior to structural evaluation.

Prior to analysis, the lyophilized samples were reconstituted at 0.05% (w/v) in sodium acetate buffer (pH 5.6, 0.02 M) and filtered by passing through a 0.22 μm syringe filter (EMD Millipore, Billerica, Massachusetts, USA). Samples were then dialyzed with the same extraction buffer using dialysis-tubing units with a 6–8 kDa molecular cutoff – 5.1 mL/cm (Spectra/Por®, Spectrum Laboratories Inc., USA) in order to remove any low molecular weight compounds that are possibly present in the commercial enzymes preparation which could interfere with the biophysical analysis (e.g., low molecular weight compounds which could absorb either UV and/or infrared light).

2.3. Structural evaluation of purified enzymes processed by high isostatic pressure

2.3.1. Intrinsic fluorescence spectroscopy

Intrinsic emission fluorescence spectra of enzymes samples were carried out according to the procedures described by Dee et al. (2006). For this, the intrinsic fluorescence was measured using a Shimadzu RF-540 spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) with a 1-cm quartz cell at room temperature and the following settings: excitation at 295 nm (the optimal excitation wavelength for measurements of Trp fluorescence), emission scan from 305 to 450 nm, and excitation and emission slit widths of 5 nm.