Innovative Food Science and Emerging Technologies 43 (2017) 60–67

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

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

Comparison of the effects of high pressure homogenization and high pressure processing on the enzyme activity and antimicrobial profile of lysozyme

Alline Artigiani Lima Tribst[a,b,*], Luma Rossi Ribeiro[a], 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, S.P., Brazil

[b] Center for Food Studies (NEPA), University of Campinas (UNICAMP), Albert Einstein, 291, 13083–852 Campinas, S.P., Brazil

ARTICLE INFO

Keywords:
Lysozyme
High pressure processing
High pressure homogenization
Enzyme modification
Antimicrobial activity

ABSTRACT

The effects of high pressure homogenization (HPH, < 190 MPa) and high pressure processing (HPP, < 600 MPa) on hen egg white lysozyme muramidase and antimicrobial activities were assessed. The results showed enzyme activation under mild process conditions (< 120 MPa for HPH and < 400 MPa for HPP, both at 20 °C) and mostly for activity measured at non-optimum pH and temperature. When processes were carried out at 50 °C, lower activation were observed (< 18% for HPH and < 13% for HPP), possibly indicating that processes at 50 °C delivered enough energy to promote undesirable unfolding on lysozyme. HPH induced a greater increase in muramidase activity (29%) than HPP (17%), but this not reflected the antimicrobial performance of the processed lysozyme, since only HPP reduced the minimum inhibitory concentration of the lysozyme against *Bacillus cereus* (50%) and *Geobacillus stearothermophilus* (66%). The results highlighted that each process changed differently the lysozyme muramidase and antimicrobial activity.

Industrial relevance: HPP and HPH are generally described as technologies able to increase the activity of several enzymes and are suggested as tools to improve the performance of commercial enzymes. The results showed that although HPP and HPH were able to increase the muramidase activity of lysozyme, improvement of the antibacterial performance was only observed for samples processed by HPP. Therefore HPP was highlighted as the better pressure process to physically modify lysozyme.

1. Introduction

High pressure processing (HPP) and high pressure homogenization (HPH) are non-thermal processing methods aimed the microbial stabilization of foods with minimal nutritional and sensory changes (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015; Huang, Wu, Lu, Shyu, & Wang, 2017; Patrignani & Lanciotti, 2016; Pinela & Ferreira, 2017). Studies concerning these technologies have spread to the field of physicochemical changes in foods, especially in those containing proteins and polysaccharides (Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Dumay et al., 2013; Eisenmenger & Reyes-De-Corcuera, 2009). Several authors have studied the impact of both processes on enzyme activity, and observed there is promotion of enzyme activation or inactivation, depending on the enzyme and matrix characteristics and the process conditions. Regarding to enzyme, it is important to consider its tridimensional structure and bonds and interactions involved in the conformation of its active site. The matrix characteristics (including pH, salt concentration and presence of substrate) affect the enzyme configuration during the process, directly influencing in the enzyme activity alterations (Ribeiro, Júnior, de C., Tribst, & Cristianini, 2017). The intensity of both processes – determined by pressure, temperature and number of cycles applied (HPP and HPH) and time (HPP) – is also responsible by the effects of enzyme activation or inactivation (Eisenmenger & Reyes-De-Corcuera, 2009; Tribst, Cota, Murakami, & Cristianini, 2014; Tribst, Leite Júnior, de Oliveira, & Cristianini, 2016).

For commercial enzymes, activation is desirable, reducing the amount of enzyme necessary in industrial processes and, therefore, reducing its relative costs and increasing its economic viability (Eisenmenger & Reyes-De-Corcuera, 2009; Leite Júnior, Tribst, & Cristianini, 2017).

Although HPP and HPH are both processes involving the application of pressure to a product, the physical effects on processed fluids are different (Balasubramaniam et al., 2015). For HPP, it is the

* Corresponding author at: Albert Einstein, 291, Campinas 13083-852, S.P., Brazil.
E-mail address: tribst@unicamp.br (A.A.L. Tribst).

http://dx.doi.org/10.1016/j.ifset.2017.07.026
Received 7 March 2017; Received in revised form 14 July 2017; Accepted 20 July 2017
Available online 24 July 2017
1466-8564/

maintenance of the products at high pressure that alters the enzyme configuration, due to the reduction in molecular volume, following the principle of *Le Chatelier* (Bermúdez-Aguirre & Barbosa-Cánovas, 2011). At lower pressures, this alteration results in an increase in conformational flexibility due to hydration of the charged groups (Eisenmenger & Reyes-De-Corcuera, 2009), increasing the enzyme activity. Additionally, HPP can results in exposure of hydrophobic protein core, with consequent increase of molecular surface hydrophobicity (Leite Júnior, Tribst, Grant, Yada & Cristianini, 2017) leading to its denaturation. Moreover, thermal enzyme stability is favored by stabilization of the hydrogen bonds and the increase in charged groups that occurs after HPP processing (Eisenmenger & Reyes-De-Corcuera, 2009).

To the contrary, in the homogenization process, it is the fast reduction in pressure that induces unfolding of the enzymes. Several authors have described the HPH process as being able to (i) increase the exposure of sulfhydryl groups at the molecular surface and reduce the available SH- groups, resulting in molecular unfolding and the formation of new disulfide bonds (Liu et al., 2009; Liu et al., 2010); (ii) increase the exposure of tyrosine and tryptophan residues, with the consequent exposure of hydrophobic groups (Liu et al., 2009; Liu et al., 2010; Tribst et al., 2014); (iii) reduce the number of inter- and intramolecular hydrogen bonds (Liu et al., 2009; Liu et al., 2010); and (IV) change the proportions of the secondary structures (α-helix and β-sheet). (Liu et al., 2009; Liu et al., 2010; Tribst et al., 2014).

Lysozyme is an *N*-acetylmuramide glycanhydrolase (E.C. 3.2.1.17) that hydrolyses the 1,4-beta-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in a peptidoglycan, resulting in microbial wall lysis and consequent cell death (Masschalck & Michiels, 2003; Nakimbugwe, Masschalck, Anim, & Michiels, 2006; Iucci, Patrignani, Vallicelli, Guerzoni, & Lanciotti, 2007; Tribst, Franchi, & Cristianini, 2008). The enzyme is a low-cost antimicrobial agent (Chung & Hancock, 2000) generally recognized as safe (GRAS) by the FDA, and is primarily extracted from hen egg white (Masschalck & Michiels, 2003). The main use of lysozyme is to prevent late blowing in cheese due to the growth of *Clostridium tyrobutyricum* (Masschalck & Michiels, 2003).

Although several authors have studied the impact of HPH and HPP on lysozyme (Tribst et al., 2008; Nakimbugwe et al., 2006; Diels, Taeye, & Michiels, 2005; Iucci et al., 2007; Masschalck, Houdt, Haver, & Michiels, 2001), the trials were normally only carried out under optimum conditions for enzyme activity, and using different process conditions, making it difficult to compare the two technologies. Therefore considering the importance of lysozyme as an antimicrobial agent and as a model enzyme, the aim of this work was to compare the impact of HPH and HPP on the muramidase and antimicrobial activities of lysozyme, allowing for a final comparison between these two non-thermal technologies and the establishment of process conditions that could improve the performance of the lysozyme depending on the expected activity conditions.

2. Material and methods

2.1. Lysozyme and enzyme activity

Lysozyme was obtained from the hen egg white commercial preparation Lysozyme G (Germinal – Ashland – lot 119491/1-1). The lysozyme activity was measured from the lysis of the *Micrococcus lysodeikticus* cell wall, using the method described by Tribst et al. (2008) with several modifications: A 150 $mg{\cdot}L^{-1}$ suspension of *M. lysodeikticus* ATCC 4698 (M3770, Sigma-Aldrich) was prepared by diluting the microbial cells in 0.05 M citrate buffer adjusted to different pH values (3.0–6.5). Two milliliters of the suspension were incubated at the temperature of the activity measurement (10–70 °C) for 5 min, and then 0.1 mL of a 100 $mg{\cdot}L^{-1}$ lysozyme solution diluted in the same buffer was added. Immediately after addition of the lysozyme, the decrease in absorbance at 450 nm was measured for 2 min at 15 s intervals. Samples only containing the *M. lysodeikticus* suspension were used as the control for the decrease in absorbance. One unit of activity (UA) was defined as the amount of enzyme which reduced 0.001 units of absorbance of the suspension per minute. The optimum pH and temperature were the conditions under which the enzyme showed the maximum activity.

2.2. Microorganisms

Escherichia coli ATCC 11229, *Enterococcus faecalis* ATCC 29212 and *Geobacillus stearothermophilus* ATCC 10149 – obtained from the Tropical Culture Collection (Campinas, Brazil) - and *Bacillus cereus*, isolated from a Brazilian dairy industry (IFRP0815 - culture collection of the Federal Institute of Rio Pomba, MG - Brazil), were used as the targets for the determination of the minimum inhibitory concentration of the lysozyme and for growth curve assays at sub-inhibitory concentrations. For the growth curve evaluations, the microorganisms were grown in TSB broth for 18 h at 30 (*B. cereus*), 37 (*E. coli* and *E. faecalis*) and 55 °C (*G. stearothermophilus*).

2.3. High pressure processing

The high pressure processing was carried out using an Avure (QFP 2 L-700) system (Avure Technologies, OH, USA). The compression come-up time as a maximum of 122.7 s and the decompression phase was up to 2.2 s. Water was used as the compression-transmitting medium. The temperature of the equipment chamber block was set at 25 °C or 50 °C. The initial temperatures of the samples and of the water in the chamber were set considering (i) the normal adiabatic heating of the equipment (3 °C/100 MPa) that occurs due to compression and (ii) the desirable process temperature (25 °C or 50 °C). For the HPP process, a 0.04% (*w/v*) lysozyme solution was prepared using 0.05 M citrate buffer pH 5.5 and packaged in flexible bags (LDPE-Nylon-LDPE, 16-μm thickness - TecMaq, Brazil) under vacuum. The processes were carried out in triplicate at 100, 200, 300, 400, 500 and 600 MPa for 10 min. The control (unprocessed) sample was not subjected to pressure. Each process was carried out in triplicate.

After processing, the enzyme activity of each sample was measured in quadruplicate at pH 4.5, 5.5 and 6.5 and temperatures of 25 and 50 °C, following the methodology described in Section 2.1. For each condition, the residual activity was calculated as described in Eq. (1).

$$\text{Residual activity (\%)} = (\text{activity}_{\text{sample,after process}}/\text{activity}_{\text{control,sample}})^{*}100 \quad (1)$$

2.4. High pressure homogenization

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi) was used for these 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 $L{\cdot}h^{-1}$.

A volume of 500 mL of lysozyme solution (400 $mg{\cdot}L^{-1}$), prepared using 0.05 M citrate buffer pH 5.5, was homogenized under pressures of 40, 80, 120, 160 and 190 MPa using inlet temperatures of 25 °C and 50 °C. After homogenization, samples (50 mL) were collected and cooled in an ice bath. These processes were carried out in triplicate. An unprocessed lysozyme sample (native) was evaluated as the control. After processing, the enzyme activity of each sample was measured in quadruplicate at pH 4.5, 5.5 and 6.5 and temperatures of 25 and 50 °C, following the methodology described in item 2.1. The residual activity was calculated according to Eq. (2).

2.5. Minimum inhibitory activity (MIC)

A lysozyme solution with a concentration of 5000 $mg{\cdot}L^{-1}$ was