Innovative Food Science and Emerging Technologies 18 (2013) 83–88

Contents lists available at SciVerse ScienceDirect

Innovative Food Science and Emerging Technologies

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

Multi-pass high pressure homogenization of commercial enzymes: Effect on the activities of glucose oxidase, neutral protease and amyloglucosidase at different temperatures

Alline A.L. Tribst [a,*], Pedro E.D. Augusto [b], 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] Technical School of Campinas (COTUCA), University of Campinas (UNICAMP), Campinas, Culto a Ciência, 177, 13020-060, Campinas, S.P., Brazil

ARTICLE INFO

Article history:
Received 10 October 2012
Accepted 13 January 2013

Editor Proof Receive Date 13 February 2013

Keywords:
Ultra high pressure homogenization
Enzyme activity
Non-thermal technology
Multiple homogenization
Homogenization in consecutive cycles
Dynamic high pressure

ABSTRACT

This study evaluated the residual activities of amyloglucosidase (AMG) at 65 and 80 °C, glucose oxidase (GO) at 50 and 75 °C and neutral protease at 20 and 55 °C after 3 passes of high pressure homogenization (HPH) at 150 MPa and 200 MPa (neutral protease and AMG) and at 100 and 150 MPa (GO). The results for AMG and neutral protease showed that the improvement in maximum enzyme activity was reached after one pass at 200 MPa, with an increment in the AMG residual activity measured at 80 °C (activity increased from 13% to 21%) and in the neutral protease residual activity at 20 °C (activity increased from 50% to 64%). However, the multiple passes caused no improvement in the activities of the enzymes. To the contrary, the results obtained for GO showed that HPH at 150 MPa continuously improved the activity at 75 °C up to three passes, reaching an activity three times higher than the native sample. Additionally, it was observed that two passes of GO at 100 MPa resulted in the same level of GO activation reached after a single pass at 150 MPa. These results suggest that multiple HPH effects differ for each enzyme evaluated and can be applied to improve GO activity.

Industrial relevance: Enzymes can be used in several areas of food industry. However, the price of enzymes and their low stability limits the enzymes' application in food industry. The HPH is suggested to process the enzyme prior to application, to increase enzyme activity mainly at non-ideal temperature. Therefore, the application of high pressure homogenization to improve enzymes activity can expand the range of enzyme uses.

1. Introduction

High pressure homogenization (HPH) is an emerging technology developed for food preservation with minimum sensory and nutritional damage (Franchi, Tribst, & Cristianini, 2011; Tribst, Franchi, de Massaguer, & Cristianini, 2011). Recently, HPH was also proposed as a physical method to change the structure of macromolecules, being able to improve (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009; Liu et al., 2010; Tribst, Augusto, & Cristianini, 2012a; Tribst & Cristianini, 2012b,c), reduce (Lacroix, Fliss, & Makhlouf, 2005; Tribst, Augusto, & Cristianini, 2012b; Velázquez-Estrada, Hernández-Herrero, Guamis-López, & Roig-Sagués, 2012; Welti-Chanes, Ochoa-Velasco, & Guerrero-Béltran, 2009) or not alter (Tribst & Cristianini, 2012a) the activity and stability of enzymes. The effects of HPH were dependent on the level of pressure homogenization applied, the temperature of the enzyme during the process, the nature of enzyme studied, pH of homogenization and the presence/absence of substrate during homogenization (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009; Tribst & Cristianini, 2012a,b,c; Tribst et al., 2012a,b).

In addition, multi-pass homogenization was able to improve the activity of polyphenol oxidase from mushrooms and pears after three HPH passes (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009), reaching the same level of activity after 2 cycles at 120 MPa as after one cycle at 140 MPa (Liu, Liu, Xie, et al., 2009). It is important to observe that the lower the homogenization pressure, the smaller the processing costs (equipment and operation). Therefore, the use of multi-passes could be of interest, aiming to optimize processing by HPH (maximizing its effect with lower costs).

HPH involves considerable mechanical forces (Keerati-U-Rai & Corredig, 2009), which results in an intense and abrupt energy input into the homogenized samples with consequent molecular changes. The changes in enzyme activity/stability were linked with conformational alterations caused to the enzymes by the HPH process, which is able to modify the quaternary, tertiary and secondary structures (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009; Liu et al., 2010). The main structural effects described are: (i) increase in the hydrophobic sites on the enzyme and exposure of amino acids (Liu, Liu, Liu, et al., 2009; Liu et al., 2010; Tribst et al., 2012a), (ii) increase in exposure of SH groups due to unfolding of the protein and a reduction

* Corresponding author at: Monteiro Lobato, 80, PO Box 6121, 13083–862, Campinas, S.P., Brazil. Tel.: +55 193521 4021; fax: +55 19 3289 3617.
E-mail address: alline.lima.tribst@gmail.com (A.A.L. Tribst).

1466-8564/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ifset.2013.01.002

in the total SH content due to new disulphide bonds formation (Liu, Liu, Liu, et al., 2009; Liu et al., 2010) and (iii) changes in the α-helix and β-sheet ratio composition due to alterations of the secondary structure (Liu, Liu, Liu, et al., 2009). Despite all these changes, the process is apparently unable to alter the enzyme molecular weight (Liu et al., 2010).

In addition to the enzymes, the effects of HPH were also measured on the proteins in general. The results obtained indicated that the process provided enough energy to disrupt the tertiary and quaternary structures of most of the globular proteins (Subirade, Loupil, Allain, & Paquin, 1998), induce protein rearrangement and aggregation (Gracia-Julia et al., 2008; Keerati-U-Rai & Corredig, 2009; Luo et al., 2010; Yuan, Ren, Zhao, Luo, & Gu, 2012), and increase the protein exposure area (Dong et al., 2011), hydrophobic interactions (Gracia-Julia et al., 2008; Luo et al., 2010; Yuan et al., 2012), reducing power of the hydroxyl radical scavenging (Dong et al., 2011) and even cause protein broken (Luo et al., 2010). On the contrary, other authors did not observe changes in the protein conformation (Bouaouina, Desrumaux, Loisel, & Legrand, 2006).

Although some studies evaluated the effect of multi-passes of HPH on enzymes activity, no previous investigations have explained the effect of multiple HPH passes on the structure of enzymes or proteins. For polysaccharides, multiple HPH passes induced continuous depolymerization, broken chains and a reduction in molecular size. However, the main effects occurred in the first steps (Kivelä, Pitkänen, Laine, Aseyev, & Sontag-Strohm, 2010; Lagoueyte & Paquin, 1998; Villay et al., 2012) and additional passes had less and less impact on the average molecular weight (Lagoueyte & Paquin, 1998) and viscosity (Villay et al., 2012).

Considering previous results, this research studied how multi-pass through the homogenizer changed the activity of three commercial enzymes. These enzymes had previously been reported as activated by a single pass through the HPH equipment (Tribst et al., 2012a; Tribst & Cristianini, 2012b,c) and are very important for food industry. The neutral protease is applied for milk protein modification, nitrogen control, mash extraction and chill-haze removal in brewing, soy modification for the use as flavors and in animal feeds (Tribst et al., 2012a). The amyloglucosidase is applied for starch saccarification and reduction of juice consistency (Tribst & Cristianini, 2012b) and the glucose oxidase is used to reduce glucose (liquid egg) or oxygen content (juices, yogurt, probiotic food) in food (Tribst & Cristianini, 2012c).

2. Materials and methods

2.1. Amyloglucosidase

The amyloglucosidase (AMG) used in these experiments was a commercial enzyme from Prozyn Biosolutions® (São Paulo, Brazil). The enzyme is presented as a yellow powder obtained as a fermentation product from *Aspergillus niger*. It has an expected molecular weight of 70–90 kDa, and optimum activity in the pH range from 4.4 to 6.0 and temperature range from 40 to 65 °C.

The enzymatic activity was determined following the method previously described (Rami, Das, & Satyanarayana, 2000) with a few modifications: 500 μL of enzyme solution (0.1 grams of dried enzyme diluted in one liter of 0.05 M acetate buffer at pH 4.3) was added to 4 mL of a 0.5% (w/v) soluble starch (for analysis degree with purity of 99.6%, (Synth, Brazil)) solution. The reaction was carried out at 65 and 80 °C for 10 min and stopped by the addition of 3 mL of 1 M Tris–HCl buffer at pH 7.5. Starch hydrolysis was determined from the release of glucose, measured using a glucose oxidase enzyme kit (Laborlab, Guarulhos, SP, Brazil) by way of a colorimetric reaction (Fleming & Pegler, 1963). Sample absorbance was measured at 510 nm using a DU 800 UV–VIS spectrophotometer (Beckman Coulter®, Brea, CA, USA). One unit of enzyme (U) was defined as the amount of enzyme able to produce 1 μmol of glucose during the reaction time. Tubes containing only starch and only enzyme were used as the controls.

The standard curve was obtained using 0.125, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 mmol of glucose solution. The glucose reacted with the glucose oxidase enzyme kit and sample absorbance was measured at 510 nm in triplicate.

2.2. Glucose oxidase

The glucose oxidase (GO) evaluated in these experiments was a commercial enzyme from Prozyn Biosolutions® (São Paulo, Brazil). It is a yellow powder obtained as a fermentation product from *Aspergillus niger*. It has an expected molecular weight of 150 kDa, contains 16% of carbohydrate and is active in the pH range from 3.5 to 7.0 and at temperatures up to 60 °C (lowest temperature with activity is 15 °C), with optimum activity at 50 °C.

GO activity was determined using the method previously described (Kona, Quereshi, & Pai, 2001) with a few modifications: 400 μL of enzyme solution (0.3 g of dried enzyme per liter of 0.1 M acetate buffer, pH 5.0 containing 0.02 g L^{-1} of sodium nitrate) was added in a tube containing 400 μL of a 4 g L^{-1} glucose (for analysis degree with purity of 99.8%) solution (Synth, Brazil) and 1.2 mL of 0.1 M acetate buffer pH 5.0. The reaction was carried out at 50 °C and 75 °C for 30 min. 1.5 mL of DNS (dinitrosalicylic acid) solution was then added followed by heating at 100 °C for 5 min to stop the reaction. A control sample was obtained using a similar procedure, but without the addition of the GO solution. After heating, the samples were cooled using an ice bath and 6.5 mL of 0.1 M acetate buffer (pH 5.0) added. The absorbance was measured at 547 nm in a DU 800 spectrophotometer (Beckman Coulter®, Brea, CA, USA).

The standard curve was obtained using glucose solutions at concentrations of 0.5, 1, 2, 3, 4 and 6 g L^{-1} prepared in 0.1 M acetate buffer pH 5.0. The glucose was reacted with the DNS following the procedure described above, and absorbance measured at 547 nm in triplicate. The absorbance of the samples was converted to glucose concentration using the standard curve, and the GO activity calculated from the difference in glucose concentration between the control and the GO samples. One enzyme unit was defined as the amount of enzyme which converted 1 μg of glucose per minute. The final GO activity was calculated per gram of commercial dried enzyme.

2.3. Neutral protease

The neutral protease used in these experiments was a commercial metalloprotease enzyme from Prozyn Biosolutions® (São Paulo, Brazil). The enzyme is presented as a yellow powder obtained as a fermentation product from *Bacillus subtillis*. The enzyme has an expected molecular weight of 19–37 kDa, an optimum pH at 7.5 and optimum temperature at 55 °C.

The protease activity was determined using the method previously described (Merheb, Cabral, Gomes, & Da-Silva, 2007) with a few modifications: 200 μL of enzyme solution (0.1 g of dried enzyme per liter of 0.1 M phosphate buffer pH 7.5) was added to 400 μL of casein solution at 0.5% (w/v) (97.5% of purity, Synth, Brazil) and to 400 μL of the same buffer. The reaction was carried out at 20 °C and 55 °C for 30 min and then 1 mL of trichloroacetic acid (TCA) 10% (w/v) was added to stop the reaction. The samples were centrifuged at 9500 g/5 min/10 °C and the absorbance measured at 275 nm in a DU 800 UV–VIS spectrophotometer (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme was defined as the amount of enzyme required to increase the absorbance at 275 nm by 0.1 unit under the assay conditions. The control samples were prepared by adding the TCA to the tubes before adding the enzyme solution, and the ΔAbs_{275nm} was determined from the difference in absorbance between the sample and the control. The enzyme activity was calculated according to Eq. (1).

$$U/g = \Delta Abs_{275nm} \cdot 10 \cdot \text{dilution factor}/(0.2) \quad (1)$$