Influence of high pressure homogenization on commercial protease from *Rhizomucor miehei*: Effects on proteolytic and milk-clotting activities

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**Abstract**

This work studied the influence of high pressure homogenization (HPH) on a commercial fungal protease. The enzyme solutions (2 and 20 g/100 mL) were processed up to 190 MPa and the proteolytic activity (PA), milk-clotting activity (MCA) and the rheological behavior of the milk coagulation phase were evaluated. The effects of multi-pass (three cycles) HPH at 25 and 190 MPa was evaluated for enzyme processed at concentration of 2 g/100 mL. No differences in PA and MCA were observed for the samples of 2 g/100 mL of enzyme concentration processed by HPH. On the other hand, increase in PA (~3%) and MCA (~10%) were observed for the enzymes processed at 190 MPa at high concentration, which consequent faster clotting and higher consistency of the milk gel. The multi-pass increased PA (~20%) but did not alter MCA nor improved the milk coagulation phase. The results highlight that the energy supplied from HPH to enzyme at low concentration is not enough to promote positive changes in the enzyme coagulant profile; however, the HPH of solution with high enzyme concentration showed a positive effect, indicating that the collisions between enzymes during the process was important to reach the observed changes.

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1. Introduction

High pressure homogenization (HPH) has been proposed as a physical process capable of modifying the structure of enzymes, with consequent improvement of their activity, specificity and stability (Leite Júnior, Tribst, & Cristianini, 2014; Tribst, Augusto, & Cristianini, 2013). For other enzymes, however, just inactivation is observed. The effect of HPH on milk coagulant were only evaluated for calf rennet and the results showed a significant reduction of unspecific proteolytic activity associated with an increment on milk-clotting rate, resulting in a more consistent curd (Leite Júnior et al., 2014). These results highlight HPH as a promising process to improve the performance of milk coagulants; however, it is not possible to generalize the results since it depends on the pressure applied and type of enzyme.

Based on the lack of information about the effect of HPH on milk coagulant and the potential effect of this technology for improving the enzyme performance, the present study evaluated the influence of HPH on a commercial *Rhizomucor miehei* protease, which is the coagulant that dominates the market of non-genetically modified microbial coagulant (35% of the coagulants used in France, according to Andrén (2011)).

2. Material and methods

2.1. *Rhizomucor miehei* protease and high pressure homogenization processing

A commercial preparation of *Rhizomucor miehei* protease was used in the experiments (commercial name: Marzyme 150 MG Powder Microbial Rennet, Danisco, Vinay, France). This preparation contains ≥28,000 mg of protease from *R. miehei* per kg of product with activity of, at least, 2200 IMCU/g. The commercial product package has 500 g.

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the trials. 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 a 2.0 g/100 mL of commercial *Rhizomucor miehei* protease solution prepared in 0.2 mol/L sodium acetate buffer (pH 5.6), was processed at pressures of 0, 50, 100, 150 and 190 MPa, using an inlet temperature of 23 °C. Samples (200 mL) were collected and cooled to 23 °C. The temperatures were measured before and immediately after HPH using a digital T type thermocouple (Multithermometer®, Porto Alegre, Brazil). A non-processed sample of *R. miehei* protease was evaluated as the control sample.

The relative proteolytic activity (RPA, Section 2.4) and relative milk-clotting activity (RMCA, Section 2.5) were carried out immediately after the end of the process (time 0 h) and after 1, 2, 3, 4, 5 and 30 days. The rheological assays were performed at time 0 h. The samples were stored under refrigeration (4 °C) throughout the period.

### 2.2. High pressure homogenization processing with multi-passes

A sample was prepared as described in item 2.1 and high pressure homogenized at 25 and 190 MPa for 3 consecutive passes.

After each pass, the samples were immediately cooled to 23 °C using a shell and tube heat exchanger, aiming to guarantee that the final temperature after each homogenization step was the same. The residence time at the temperature reached after the HPH valve (<10 s) was estimated considering the equipment flow and the distance between the homogenization valve and the heat exchange inlet. The time between consecutive processes was less than 5 min and the final process time was around 15 min. The temperatures were measured using a digital T type thermocouple (Multithermometer®, Porto Alegre, Brazil).

After each pass (one, two and three), a total of 100 mL sample was collected and the non-processed enzyme was evaluated as the control. The RPA, RMCA and rheological assays were carried out immediately after the end of the process.

### 2.3. Effect of HPH processing when process was carried out in high enzyme concentration

A volume of 1 L enzyme solution at a concentration of 20 g/100 mL (tenfold than the concentration used in the other assays) was prepared as described in item 2.1 and high pressure homogenized at 25 and 190 MPa using an inlet temperature of 23 °C. Samples (200 mL) were collected and cooled to 23 °C.

A non-processed enzyme was evaluated as the control. The RPA, RMCA and rheological assays were carried out immediately after the end of the process.

### 2.4. Relatives proteolytic (RPA) and milk-clotting activities (RMCA) of the protease

The proteolytic activity and milk-clotting activity of the *Rhizomucor miehei* protease was based on Arima, Yu, and Iwasaki (1970), and followed the methods described by Leite Júnior et al. (2014).

The relative proteolytic activity (RPA) was calculated considering the activity of the HPH and non-processed samples according to Equation (1):

\[
RPA = \left( \frac{\text{enzyme activity}_{\text{after HPH and/or storage}}}{\text{enzyme activity}_{\text{non-processed sample at 0 h}}} \right) \times 100
\]  

The relative MCA (RMCA) was calculated considering the MCA of the HPH processed and non-processed samples, according to Equation (2):

\[
\text{RMCA} = \left( \frac{\text{MCA}_{\text{after HPH and/or storage}}}{\text{MCA}_{\text{non-processed sample at 0 h}}} \right) \times 100
\]

The RMCA and RPA of samples after storage were also calculated using the stored non-processed sample as the reference, aiming to differentiate the effect of HPH and natural activity loss over the time. The ratio of MCA/PA was calculated for all samples.

### 2.5. Rheological assays

The milk coagulation was 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 *Rhizomucor miehei* protease samples, following the method described by Leite Júnior et al. (2014).

### 2.6. Statistical analysis

The processes were carried out with three repetitions at different days and analyses (proteolytic activity; milk-clotting activity; rheological assays) were carried out with three repetitions and each experimental unit was carried out in triplicate. The analysis of variance (ANOVA) was used to compare the effects of the different treatments and its effects on enzyme RPA and RMCA at different times. The Tukey test was performed to determine the differences between the samples at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 7.0 software—(StatSoft, 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 relative proteolytic activity and relative milk-clotting activity

The fast decompression during homogenization promotes intense shear and friction with consequent heating of the homogenized fluid. Considering that enzymes can be affected by heating, the sample temperature reached at each pressure was measured and the results showed that temperature increases linearly with pressure. For samples processed at low (2 g/100 mL) and high concentration (20 g/100 mL), the temperature after HPH was described by the Equations (3) and (4), respectively.

\[
\text{Temperature (°C)} = 0.169 + 22.931 \times \left( \frac{\text{Pressure (MPa)}}{R^2 = 0.999} \right)
\]  

\[
\text{Temperature (°C)} = 0.177 + 23.497 \times \left( \frac{\text{Pressure (MPa)}}{R^2 = 0.999} \right)
\]

Therefore, the maximum temperatures reached after process at 190 MPa were 55.2 and 57.1 °C for samples processed at low and high concentration, respectively. The residence time at those temperatures was <10 s. Considering that D-value (decimal reduction time) of this enzyme is 30 min at 72 °C in pH 5.5 (Hyslop, Swanson, & Lund, 1979), it was concluded that no thermal effect impacted in the responses obtained after HPH process.

The PA and MCA of enzymes processed at 2 g/100 mL of concentration showed no differences (p < 0.05) with the non-processed sample immediately after process and for 30 days of...
refrigerated storage. During the storage, the non-processed enzyme decreased around 5% of its activity, showing that this fungal protease is a high stable enzyme. Similarly, the ratio of MCA/PA showed no differences between native and HPH samples immediately after HPH but, after 30 days of storage, a ratio 10% higher was observed for samples processed at 100 and 150 MPa. This indicates that, for the enzyme processed at these pressures, the reduction of PA was higher than the MCA after storage.

Fig. 1A and B shows the results obtained for RPA and RMCA for the enzyme processed with up to three passes at 25 MPa and 190 MPa. A significant increment in RPA (106.0%) was only observed after 3 passes at 190 MPa (p < 0.05). For RMCA, only the results for the sample processed at 25 MPa showed a significant change; with a sequential reduction after 2 and 3 passes, reaching a minimum of 90.7% (p < 0.05). Thus, the results corroborate that this enzyme is highly stable to the HPH process, requiring 3 consecutive passes to obtain minimal changes in the RPA and RMCA values.

To the contrary, for the assay that HPH was performed for enzyme solution at high concentration (20 g/100 mL), a small but significant increase in proteolytic activity (102.7%) as well as the milk clotting activity (110.0%) was observed after process at 190 MPa (Fig. 1C and D). Tribst et al. (2013) found that the processes at 150 and 200 MPa with up to three passes reduced in 60% the activity of a neutral protease at 60 °C. On the other hand, a high increase in the protease activity was observed at 20 °C after two passes at 200 MPa. Other studies demonstrated that the activity of pectin methyltransferase was not affected by five HPH passes at 100 MPa (Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009) or at 170 MPa (Laboriau, Fliss, & Makhlouf, 2005), while the polyphenol oxidases from mushrooms and from pears showed a significant improvement in activity after three HPH passes at 150 and 160 MPa, respectively (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009). Therefore, it is not possible to establish a standard behavior for enzymes processed with multi-passes by HPH.

The effects of HPH were dependent on the pressure level of homogenization applied, the temperature of the enzyme during the process, the nature of the enzyme studied, the pH of homogenization and the presence/absence of substrate during homogenization (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie, et al., 2009; Tribst, Augusto, & Cristianini, 2012). The global evaluation of the results obtained for PA and MCA of this fungal protease indicates that this enzyme is more resistant to HPH than other enzymes previously studied like calf rennet (Leite Júnior et al., 2014), trypsin (Liu et al., 2010), or commercial neutral protease from Bacillus subtilis (Tribst et al., 2012) for which were observed higher changes at pressures between 50 and 200 MPa. These results indicate that R. miehei protease requires more energy input to change its performance than the other enzymes, which might be related to the enzyme conformation or to the capability of HPH to cause reversible modification. The high resistance to HPH might be explained due the fact that the R. miehei protease is the most glycosylated enzyme among aspartic proteases. These carbohydrates are flexible and were described as energy reservoir able to stabilize conformation of this protease against thermal inactivation (Yang, Teplyakov, & Quail, 1997). Therefore, it is possible that the high level of R. miehei protease glycosylation also protects this enzyme against the denaturation caused by HPH. In general, enzymes with no quaternary structure and showing good thermal resistance were also resistant to HPH processing (Tribst et al., 2012).

This hypothesis is corroborated by the results of multi-pass, which showed a positive effect just after 3 consecutive processes at 190 MPa. Among the variables evaluated, the most important was the concentration of the enzyme solution subjected to HPH processing. It might be linked to the increase of the molecules
collisions after the homogenization valve, which possibly enhances the impact of HPH process on the molecules (Dumay et al., 2013).

3.2. Rheological evaluation

Rheological parameters of storage modulus ($G'$) — which describes the elastic (solid) behavior of the product and can represent the phenomenon of milk coagulation — and storage modulus derivate ($dG'/dt$) — which represents milk-clotting rate, which has higher speed at the moment that aggregation began — can be used as indirect tools to evaluate the milk coagulation process and compare the results obtained by different enzymes.

Results obtained for milk coagulated with enzyme processed in lower concentration at 190 MPa showed a slight improvement of $G'$ value when compared with the non-processed sample (Fig. 2A), although no differences have been observed in MCA responses. The disparity between rheological and enzyme activity data can be attributed to the sensibility of the methods, since MCA is a visual evaluation while rheological is an instrumental method with high sensibility. By the rheological data, it was observed that 190 MPa positively changed the protease from R. miehei, reducing the time of coagulation and improving the gel consistence. Multi-passes were not able to promote changes in the $G'$ value of the sample processed at 190 MPa neither in the time required to the start coagulation (27 min), indicating that the RPA increase of 6% observed after three passes did not affected the milk gel formation. Additionally, the milk-clotting rate (Fig. 2B) was not affected by the number of passes and the results obtained for samples processed at 190 MPa (0.57 Pa.min$^{-1}$) was equal to the obtained for non-processed sample (0.56 Pa.min$^{-1}$).

On the other hand, samples processed at 25 MPa showed a gradual reduction of $G'$ value after each pass and the time spent to start coagulation was 29–30 min. These results can be directly correlated to the gradual reduction on MCA caused by HPH at 25 MPa. Additionally, multi-passes at 25 MPa negatively impacted the rate of milk-clotting, reducing it from 0.54 to 0.24 Pa.min$^{-1}$ after three consecutive passes.

The results obtained for milk gels prepared with enzyme processed by HPH at high concentration (Fig. 3A and B) showed that the gel formed using the enzyme processed at 190 MPa starts aggregation 3 min before non-processed or processed sample at 25 MPa. After 90 min coagulation, $G'$ value of the gel obtained using enzyme processed at 190 MPa was significantly higher (16.32 Pa) than non-processed (14.69 Pa) and processed sample at 25 MPa (14.54 Pa). Again, the comparison between results obtained for samples processed at low and high concentrations evidenced that HPH is more effective when enzyme is processed at high concentration.

Fig. 2. Effects of the number of passes on (a) the milk coagulation and (b) the milk-clotting rate of Rhizomucor miehei protease at 2 g/100 mL evaluated after processing at 25 and 190 MPa. Samples. (— control); (— 25 MPa-1 pass); (— 25 MPa-2 pass); (— 190 MPa-1 pass); (— 190 MPa-2 pass); (— 190 MPa-3 pass). (n = 9).
The overall evaluation of the results of this study highlights that the better effect of HPH on this milk coagulant occurred for the enzyme at 20 g/100 mL processed at 190 MPa.

4. Conclusions

The fungal protease from *Rhizomucor miehei* is highly resistant to the HPH process, with no changes in the PA and MCA when processed at 2 g/100 mL at pressures of up to 190 MPa. Even multi-passes cause slight changes on the enzyme processed at this concentration. On the other hand, for enzyme processed at high concentration (20 g/100 mL) the process increased MCA, improved the coagulation phase and the quality of the product (faster clotting and higher consistency of the milk gel). Therefore, the HPH can be used as a unitary operation to improve the competitiveness of this fungal protease as milk coagulant.

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