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Research Article

Effect of High Pressure Homogenization on the Activity and Stability of Protease from *Bacillus Licheniformis* LBA 46 in Different pH Values

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Abstract

Alkaline proteases have great importance in the pharmaceutical, textile, food industries and research studies. High pressure is an emerging and relatively new technology that is capable of modifying various molecules. The use of High Pressure Homogenization (HPH) has been widely evaluated in the modulation of enzyme activity, either to inactivate, improve or stabilize its activity. The effect of HPH treatment on *Bacillus licheniformis* LBA 46 semi-purified protease activity was investigated at different pH values and temperatures of activity. The enzyme was treated up to 200 MPa at pH 4, 7 and 9 and the residual activity was evaluated on the day of the treatment and after 24 h of refrigerated storage. The protease activity and stability measured at 40, 60 and 90°C between the control and the treated samples showed similar residual activity values. This suggests that the enzyme was resistant to the HPH treatment (50-200 MPa). Though HPH is a promising method to change enzymes characteristics, it was not able to change the protease from *Bacillus licheniformis* LBA 46.

Keywords

Denaturation; Homogenization; Inactivation; Pressure; Protease

Introduction

Pressure is a thermodynamical parameter that has gained importance in a range of fields related to biochemistry and biology [1]. Various studies have been performed to change macromolecules structures, such as proteins, and have explored the use of high pressure, in different aspects, in the field of enzymatic technology [2-4].

High Pressure Homogenization (HPH) is a promising emergent technology, non-thermal, convenient, versatile and environmentally friendly, that can guarantee food safety and stability with reduced sensory and nutritional changes [5-7]. HPH can lead to alterations in the protein structure, stability, and/or enzymatic activity [4,8].

Some of the effects of HPH are mainly attributed to cavitation; shear pressure; and turbulence forces, but, the modification of protein structures using HPH is also caused by treatment time, impact (change in flow direction), friction, and heat, which occur simultaneously during the application of the treatment. These forces have the ability to alter configuration and function of proteins [9].

Proteases are enzymes that catalyze the hydrolysis of peptide bonds of target protein, resulting in smaller peptides and amino acids. This group of enzymes plays a vital role in various industrial applications and represents one of the most important groups of commercial enzymes [10-13]. They are classified as acid, neutral and alkaline, depending on the pH in which they have maximum activity. Proteases are also the main enzymes produced by microbial sources and occupy 60-65% of the global industrial market [14-16].

Enzyme activity is determined by its appropriate conformations that are defined by conditions such as pH and temperature. Pressure can cause changes in secondary, tertiary and quaternary structures of enzymes promoting an increase in hydrophobic sites, exposing amino acid and sulphydryl groups. Pressure can also induce changes in the protein functionality and dissociate protein aggregates. The degree of denaturation and/or aggregation of proteins depends on the intensity of applied mechanical forces [17-21].

There is already a large array of commercially used proteases and there is an incredible landscape of potential uses for proteases and interest for the enhancement of their production, activity and specificity. Based on the importance of such enzymes, the aim of this study was to evaluate the behavior of a novel alkaline semi-purified protease from *B. licheniformis* under the treatment of HPH at different pH values.

Material and Methods

Protease

The protease utilized in this study was produced by *B. licheniformis* LBA 46, from the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, University of Campinas. The microorganism was fermented in 6 L bench reactor containing culture medium as proposed by Contesini [22], with modifications, composed of 32 g/L of sugar cane molasses (Fio de Ouro®); 6 g/L of corn steep liquor (Corn Products®); 2 g/L of yeast extract (Prodex-Lac SD®); and 20 g/L dried whey (Alibra®), adjusted to pH 7 at 30°C and 300 rpm during 48 h. The crude extract was semi-purified, using 80% ammonium sulfate precipitation, dialyzed, freeze-dried, and used for these experiments.

High Pressure Homogenization process

The equipment utilized was a Panda Plus High Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) with a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa at a 9 L/h flow rate.

The protease solution (0.1% w/v) was prepared in 0.05 M acetate buffer (pH 4), in 0.05 M sodium phosphate buffer (pH 7), and in 0.05 M boric acid-borax buffer (pH 9), each mixture was processed under the pressures of 0, 50, 100, 150 and 200 MPa. The enzymatic solutions were collected and immediately cooled in an ice bath. The enzymatic solution without treatment was used as the control. The activities of protease samples, treated at pH 4, 7, and 9, were measured respectively at pH 4, 7, and 9 at different temperatures (40, 60, and 90°C). The inlet and outlet temperature of each mixture were also measured using a digital thermometer.

Protease activity and stability for different pH and temperature values

The protease activity was determined according to Charney and Tomarelli's method [23], modified by Castro and Sato [24] using azocasein as the substrate. The protease activity was measured in different values of pH: 4 (value below the protease stability), 7 (optimum value for the protease activity), and 9 (optimum value for the protease stability). The reaction mixture contained 0.5 mL of 0.5% azocasein (in the adequate pH) and 0.5 mL of the enzyme solution which were incubated for 40 min at the temperature chosen for measuring the enzymatic activity: 40°C (value below the optimum for the protease activity), 60°C (optimum value for the protease activity), and 90°C (temperature of inactivation). The reaction was stopped by adding 0.5 mL of 10% Trichloroacetic Acid (TCA). The reaction mixture was centrifuged at 17,000 x g for 15 min at 15°C. An aliquot of 1 mL of the obtained supernatant was neutralized with 1 mL of 5 M KOH. One unit of protease activity (U) was defined as the amount of enzyme which causes an increase of 0.01 in absorbance at 428 nm.

The analyzes were carried out immediately after processing (time 0 h) and after 24 h of storage under refrigerated conditions (5°C). A non-processed sample was used for a comparative evaluation. The optimum activity condition (pH 7 and temperature of 60°C) represented 100% of the enzymatic activity.

UV- absorption spectra analysis of native and HPH processed protease

The UV-absorption spectra analysis was measured for the unprocessed and processed enzymatic solutions in different pH values (described in item 2.2) just after the HPH treatment. The spectrum was scanned from 200 to 400 nm using a UV-VIS spectrophotometer DU-800 (Beckman Coulter®, Brea, CA) to determine the absorption peak value and its wavelength [3].

Statistical analysis

The analysis, ANOVA and Tukey's test, were carried out in

Minitab 16.1.1 (Minitab Inc., USA) at 95% significance level. The processes and the measurements of the activity were all performed in triplicates.

Results and Discussion

Protease characterization

The enzyme characterization was performed in order to evaluate the protease activity at different pH values and temperatures (Figure 1). The semi-purified protease from *B. licheniformis* LBA 46 presented 100 and 87% activity at 60°C at pH 7 and 9 respectively. After incubation at refrigeration temperature (5°C) for 24 h, the activity decreased to 89 and 86.8%, respectively. The protease presented very low activity in pH 4 at 40, 60 and 90°C (~1.5% of residual activity) and showed low activity at 40°C (15-17%) and at 90°C (6-12%) in pH 7 and 9 just after preparation of the enzyme solution and also after 24 h of refrigeration at 5°C.

solution at pH 9 at 18°C using HPH at 200 MPa increased the temperature to 36.7°C. This protease is stable at 40°C for 1h at pH 7 indicating that the modifications observed in its activity were caused only by HPH. Table 1 presents the inlet and outlet temperatures of homogenized samples after the homogenization process for each pH value studied.

Pressure (MPa)	pH value					
	4	7	9	4	7	9
	Outlet temperature (°C)			Temperature increment (°C)		
0	23.0	24.3	26.7	5.0	6.3	8.7
50	23.3	27.3	28.7	5.3	9.3	10.7
100	24.6	32.0	32.7	6.6	6.0	14.7
150	29.0	33.7	35.7	11.0	15.7	17.7
200	29.0	36.0	36.7	11.0	18.0	18.7

Table 1: Temperature increase during HPH at pH 4, 7 and 9 (inlet temperature ~18°C).

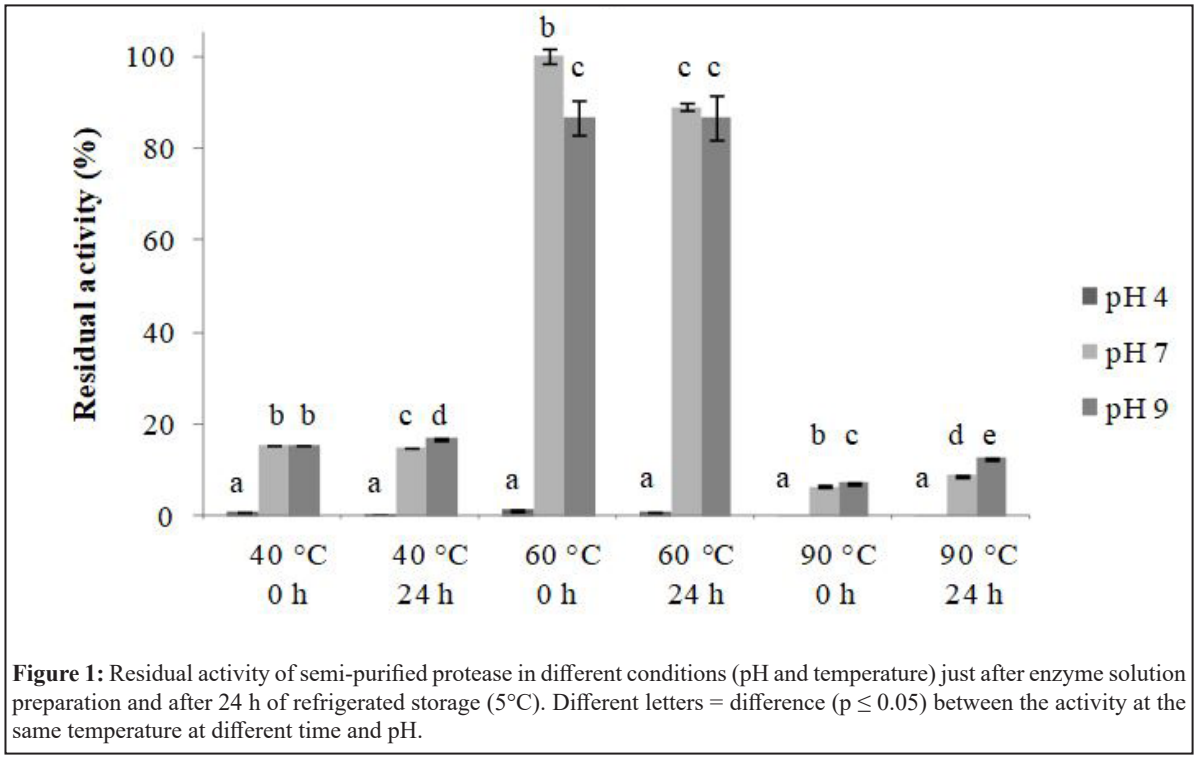

Figure 1: Residual activity of semi-purified protease in different conditions (pH and temperature) just after enzyme solution preparation and after 24 h of refrigerated storage (5°C). Different letters = difference ($p \leq 0.05$) between the activity at the same temperature at different time and pH.

Effect of HPH process on semi-purified protease from *B. licheniformis* LBA 46 activity and stability

Treatment of samples using HPH results in a heating effect because the rapid decompression promotes intense shear and friction forces [25]. The samples were cooled before being placed in the homogenizer device to minimize any damage caused by heating during the process. Treatment of the protease

No significant differences ($p \leq 0.05$) were observed between the triplicates of processed samples, indicating good repeatability of the process. The temperature increase could be related to differences in the residence time of the different protease solutions within the homogenization valve during the process.

Figure 2A-C illustrated, respectively, the protease residual activity determined at 40, 60 and 90°C, after HPH treatment

(0-200 MPa) in different pH values (4, 7 and 9). The enzyme residual activity was determined at the same pH used in the high pressure treatment, as described in item 2.3. At pH 4, the residual activity was approximately 1%, determined at 40, 60, and 90°C. Figure 2A showed that protease treated at pH 7 and 9 had no activity increase at 40°C, obtaining residual activity of approximately 15-17%.

at pH 7 and 9 respectively at 60°C. After HPH treatment, the protease showed low residual activity of approximately 5-7% at pH 7 and 9 at 90°C (Figure 2C).

The treatment at 0 MPa, when the solution passes through the valve into the equipment without pressure application, can promote small changes in enzyme configuration due to shear, friction and others stress caused by the process. According

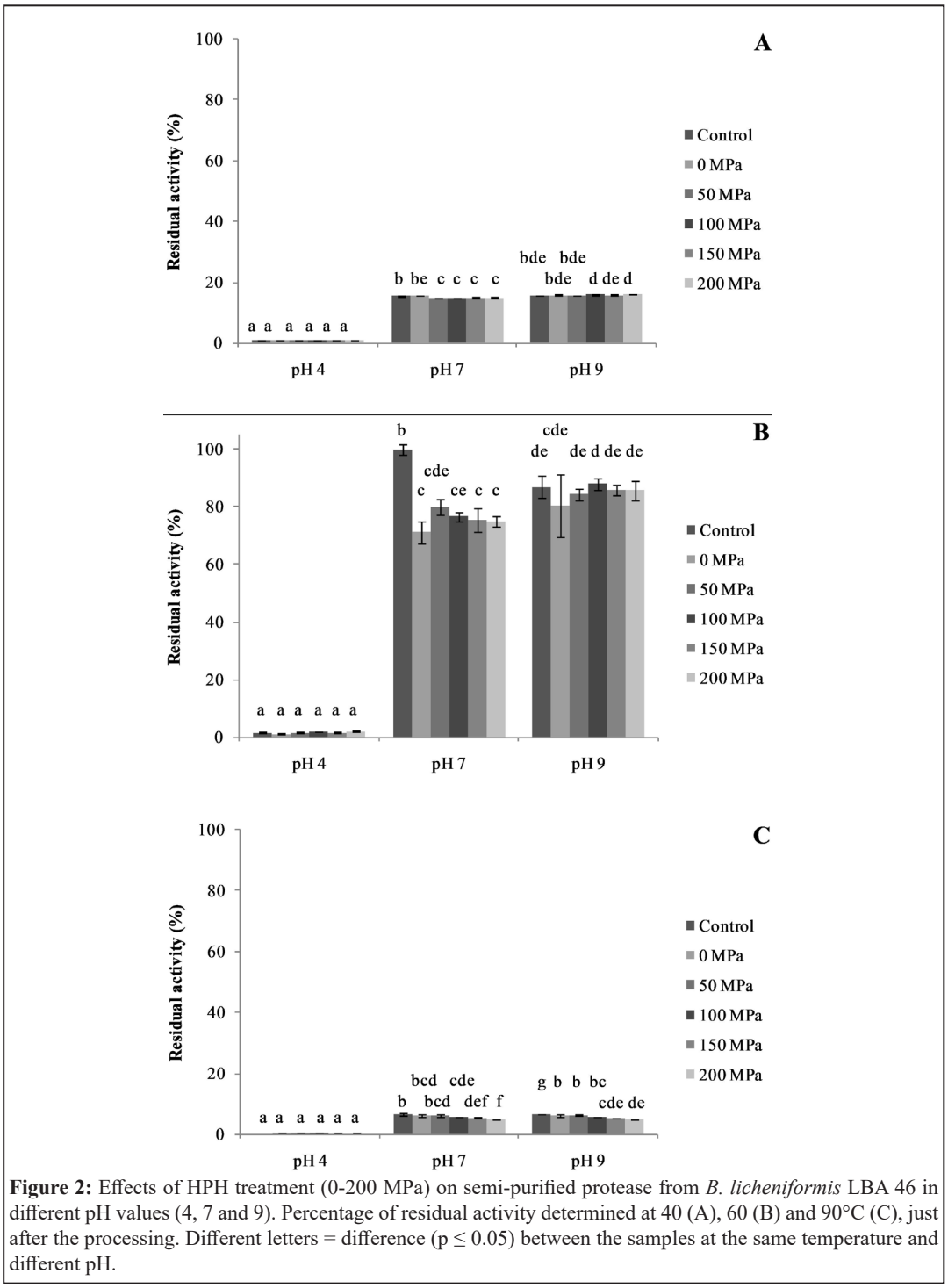

Figure 2: Effects of HPH treatment (0-200 MPa) on semi-purified protease from *B. licheniformis* LBA 46 in different pH values (4, 7 and 9). Percentage of residual activity determined at 40 (A), 60 (B) and 90°C (C), just after the processing. Different letters = difference ($p \leq 0.05$) between the samples at the same temperature and different pH.

Figure 2B showed that protease at pH 7 and 9, just after HPH treatment, retained 70-80% and 80-88% of residual activity

to Benjakul and Morrissey, the conformation and charge distributions are altered under acidic conditions making the

enzyme unable to bind to the substrate properly [26]. Klomklao et al., related that in a very acidic or alkaline solution, most enzymes experience an irreversible denaturation which causes loss of stability [27]. This indicated that the pH could have changed the enzyme native configuration and, consequently, the enzyme susceptibility to HPH treatment.

Figure 3A-C shows that after 24 h of incubation at 5°C, the HPH treated protease samples at pH 4 did not result in increased activity compared to the control. The protease was unstable at pH 4 obtaining approximately 1% of the initial activity under the tested conditions.

HPH treatment of the protease at pH 7 and 9 with subsequent refrigeration for 24 h did not result in increased activity at any of the analyzed temperatures. The protease retained approximately 15-17% of the initial activity at 40°C (Figure 3A), 80-90% at 60°C (Figure 3B), less than 12% at 90°C (Figure 3C).

Similar protease behavior was observed at 40 and 90°C (Figures 2 and 3) and could be explained because in these temperatures the enzyme is outside its zone of activity, having low activity at 40 and being inactivated at 90°C. Then, the treatment was not able to modify the enzyme temperature of activity, which remained at 60°C.

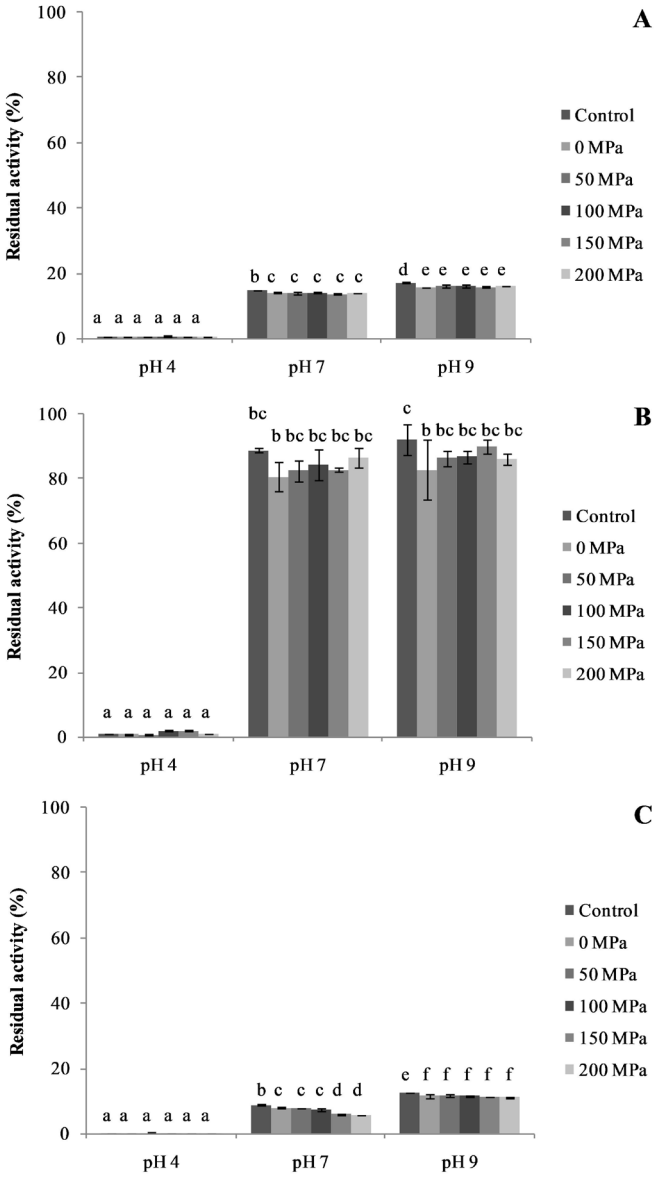

Figure 3: Effects of HPH treatment (0-200 MPa) on semi-purified protease from *B. licheniformis* LBA 46 in different pH values (4, 7 and 9). Percentage of residual activity determined at 40 (A), 60 (B) and 90°C (C), after 24 h of refrigerated storage at 5ºC. Different letters = difference ($p \leq 0.05$) between the samples at the same temperature and different pH.

According to Cheftel, the pressurization can generate reversible or irreversible, partial or complete enzyme inactivation because of the changes generated in the protein structure [28]. High pressures could lead to the inactivation or reduction of enzymatic activity [29]. HPH treatment is a process able to improve [8,30], decrease [31,32], or not change [3] the enzymatic activity.

After the homogenization, the enzymatic activity of the treated samples remained very close to the enzymatic activity of the native enzyme. This could be due to the resistance of the enzymatic structure to modification caused by pressure. Probably the treatment, even at high pressures (up to 200 MPa), was not sufficient to cause the unfolding of the enzyme, alterations in molecular conformation or reduction of the exposed active sites, resulting in no activity alterations just after the application of treatment and after the refrigerated storage. The protease from *B. licheniformis* LBA 46 requires more energy input to change its performance, which might be related to the enzyme conformation or to the capability of HPH to cause reversible modification. Enzymes with no quaternary structure that possess good thermal resistance could be resistant to HPH processing [4,33]. This means that homogenization is not adequate to improve the activity of this enzyme; since the protease was resistant to treatment, the HPH cannot change its performance.

As communicated in this study, some authors observed no modifications on the activity of enzymes treated with HPH. Tribst et al., evaluated the HPH effect on activity and stability of one commercial protease from *Bacillus subtillis* in the range of 0-2,000 bar at pH 7.5. The authors observed no increased in the activity measured at 55 and 70°C [4]. Liu et al., studied the effect of HPH microfluidization on the activity, stability, and conformation of trypsin from porcine pancreas and observed that the enzyme activity was not affected by 3 passes of HPH treatment in the range of 80-160 MPa [3]. Studying a protease from *Rhizomucor miehei*, Leite Júnior et al., observed that this enzyme was resistant to homogenization, obtaining a slight increase of 6% in its proteolytic activity only after 3 cycles at 190 MPa [33].

On the other hand, some studies showed an improvement or a reduction on the activity of enzymes treated under HPH. Liu et al., described a 90% reduction on papain activity treated with HPH microfluidization at 180 MPa, pH 6. The protease presented different degrees of reduction on the enzymatic activity after treatment in the range of 120-180 MPa [34].

Leite Júnior et al., studied the effect of HPH under range of pressures between 0 and 190 MPa on commercial porcine pepsin and observed that milk-clotting activity was improved after 150 MPa treatment and 60 days of storage, 15% higher than the control samples. For the proteolytic activity, a continuous decline was observed for all samples, reaching approximately 85% after 14 days. After 30 and 60 days of storage, the proteolytic activity for all samples showed a similar diminution behavior [2].

According to the literature and studies presented for HPH treatment on the behavior of proteases, it can be observed that the results are relative to the source of the protease, temperature, pH, time, pressure applied, etc. The application of homogenization in different types of enzymes has been studied demonstrating that the effect of homogenization varies for each kind of enzyme (bacterial, fungal, plant, animal) and can also be affected by the treatment conditions [4,30-32].

UV- absorption spectra analysis of untreated and HPH processed protease

According to Liu et al., alterations on the UV-absorption spectra after HPH treatment can indicate that the process was able to promote changes on the protein molecule structure [3]. Figure 4 shows the results for UV-absorption spectra analysis at different pH values. Absorption was noted in the interval of 230-240 nm and a slight absorption peak at the interval of 270-280 nm for the distinct pH values. In the spectrum of proteins and peptides, absorption between 220 and 240 nm is due to the presence of peptide bonds and carboxyl groups. Other materials, as impurities in solvents, can also exhibit absorption at this wavelength range. The peak at 280 nm is caused by the absorbance of aromatic amino acids, as tryptophan, tyrosine and, to a small extent, cystine [35,36].

There was a normalization of the curves behavior. The protease treated at pH 7 and 9 showed similar UV-absorption curves indicating that the treatment was not able to change the enzyme structure in a detectable way using this measurement. HPH treated protease (50-200 MPa) at pH 4 presented different UV-absorption curve profiles when compared to the control sample (untreated) and to the sample without application of pressure (0 MPa) (Figure 3A). However, a behavior pattern was observed for all samples treated at pH 4. The semi-purified protease from *B. licheniformis* LBA 46 was unstable at pH 4 and the HPH treatment was not able to increase the activity or stability of the enzyme.

In general, no relation was observed between UV-absorption spectra and protease activity, for the control or the homogenized samples at different pH values.

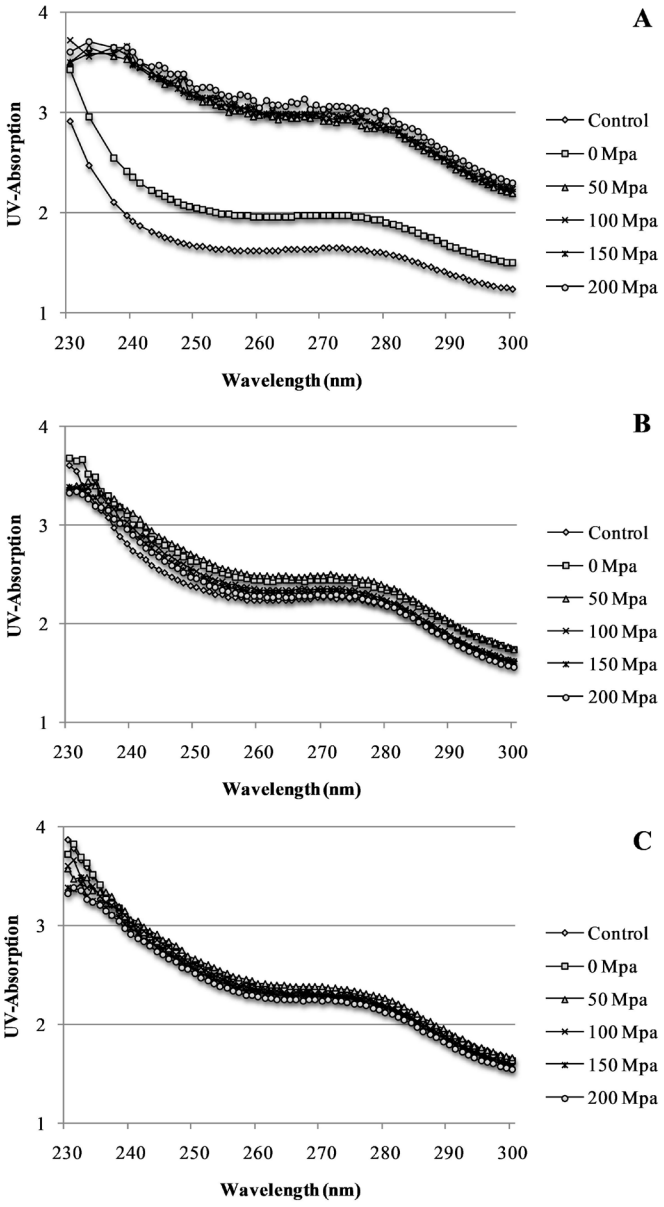

Figure 4: UV-absorption spectra between 230 and 300 nm of treated and untreated semi-purified protease at pH 4 (A), 7 (B) and 9 (C).

Conclusion

Treatment under high pressure is classified within emerging technologies and has been used in the treatment of different molecules. Due to the energy employed in the process, the pressure can modify different balances involved in the reaction mechanisms, thus explaining its ability to modify various types of chemical bonds in biological molecules such as proteins. The HPH treatment (50-200 MPa) of the protease from *B. licheniformis* LBA 46 in different pH values was not able to improve its activity, on the contrary; at pH 7 and 9, the treatment decreased the enzyme activity at 60°C. The treatment at pH 4 and 9 did not change the activity and stability of the enzyme at 40°C and 90°C, which means that homogenization is not adequate to improve the activity of this enzyme. The protease was resistant to treatment showing activities very similar to the untreated control sample. The HPH treatment cannot change the enzyme's performance.

Conflicts of Interest

All authors declare no conflicts of interest in this article.

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