Proteolysis-PrEP: Activity and Specificity of Chymotrypsin Under Pressure Cycling Condition

Introduction

Pressure Cycling Technology (PCT) has been proven to accelerate enzymatic protein digestion. The positive effect of PCT on trypsin digestion is well established for improved sequence coverage, higher recovery and significantly reduced digestion times [1-5]. Additionally, the enhancing effects of PCT on the activity of other enzymes, including Proteinase K, PNGase F, Glu-C, Lys-C, and lysozyme have been reported [6-12].

Pressure induces protein denaturation, but the pressure-perturbed proteins assume conformational forms that are different from those caused by thermal or chemical treatment [13]. Pressure-induced denaturation of the substrate proteins leads to better enzyme access to previously inaccessible target sites. This, in turn, results in improved and accelerated digestion, as long as the level of pressure that is applied is below the level at which the enzyme itself is denatured and inactivated. In addition, under certain conditions, hydrostatic pressure can have a positive effect on enzyme activity, apparently independent of substrate conformation.

Here we report that PCT can increase specific chymotrypsin activity and accelerate the digestion of model proteins in solution. The goal of this work is to examine the effect of PCT on chymotrypsin activity and to provide the user with the best set of *starting conditions* for pressure-enhanced chymotrypsin digestion.

PCT Sample Preparation System (PCT SPS)

The Pressure Cycling Technology Sample Preparation System (PCT SPS) uses cycles of hydrostatic pressure between ambient and very high levels to control biomolecular interactions and to accelerate certain enzymatic reactions. The PCT SPS uses small, semi-automated bench-top Barocycler instruments, in combination with PCT MicroTubes or FT500-ND PULSE Tubes. The specially designed PCT MicroTubes are single-use sample processing containers designed to hold 50-150 µL, while the FT500-ND PULSE Tubes are suitable for larger sample volumes up to 1.4 mL. Samples in MicroTubes can be pressure cycled in sets of 16 in the Barocycler 2320EXT, to allow batch-mode processing of multiple samples.

Results and Discussion

PCT-Enhanced Chymotrypsin Activity

To assay the effect of pressure on the enzyme, rather than on the substrate proteins, tests were carried out using small synthetic substrates which are unlikely to be directly affected by pressure. Enzymatic cleavage of the substrates leads to an increase in optical density at 405nm (OD405). Increase or decrease in enzyme activity was assayed by comparing the OD405 of treated samples to control samples incubated at ambient pressure (pressure treatment of the substrates alone was also performed to rule out the possibility that the increase in OD405 was due to non-specific breakdown of the chromogenic substrates, data not shown).

Under normal conditions, chymotrypsin cleaves efficiently at phenylalanine, but not at alanine or leucine. To examine the activity of chymotrypsin at pressure, we used a substrate that contains phenylalanine as the only amino acid (N-Succ-L-Phenylalanine-pNA). Chymotrypsin activity at 15, 30 and 45 kpsi was compared to atmospheric pressure (“0 kpsi”). **Figure 1** shows that chymotrypsin activity is significantly increased by PCT, with the greatest increase occurring at 45 kpsi, the highest pressure tested. Furthermore, the reaction rate appears linear during the ~40 minute timeframe, suggesting that chymotrypsin activity remains stable during this incubation at high pressure. Since chymotrypsin is a protein, pressure levels above a certain point may be expected to induce denaturation of the enzyme and lead to reduction or loss of enzymatic activity, however, no pressure-induced decrease in activity was observed, suggesting that Chymotrypsin is an extremely pressure-stable protein.

In order to determine whether the specificity of chymotrypsin was being adversely affected by the high pressure treatment, activity assays were carried out using synthetic substrates that contain amino acids (leucine and alanine) which are not normally cleaved by chymotrypsin. All parameters (substrate concentration, enzyme concentration, buffer, temperature, pressure, etc.) were the same for the assays carried out with the three different substrates (phenylalanine, leucine and alanine). **Figure 2** shows that at all pressures tested, no measurable increase in non-specific chymotrypsin activity was detected, suggesting that high pressure treatment does not cause chymotrypsin to become a more promiscuous protease, at least at these two amino acids. These data, combined with the results in Figure 1 and data presented elsewhere [4], strongly support the conclusion that PCT can significantly improve chymotrypsin activity, without inducing a significant loss of specificity.

The results presented in **Figure 1** show that the highest observed enzyme activity was at 45kpsi, which was the highest pressure tested. These results suggest that the enzyme is quite stable at high pressure and is not significantly denatured even at 45kpsi. To further examine the stability and activity of chymotrypsin after exposure to pressure cycling, the reactions shown in Figure 1 were transferred to a 96-well plate and incubated at ambient pressure for an additional 2.5 hours. The data in **Figure 3** demonstrate that, at ambient pressure, the activity of pressure-treated and non-pressure-

treated chymotrypsin is essentially the same, as indicated by the rate of increase in OD405. These data support the conclusion that the chymotrypsin enzyme is a very pressure-stable protein that can be used for digestion of difficult samples where longer incubation under pressure may be required.

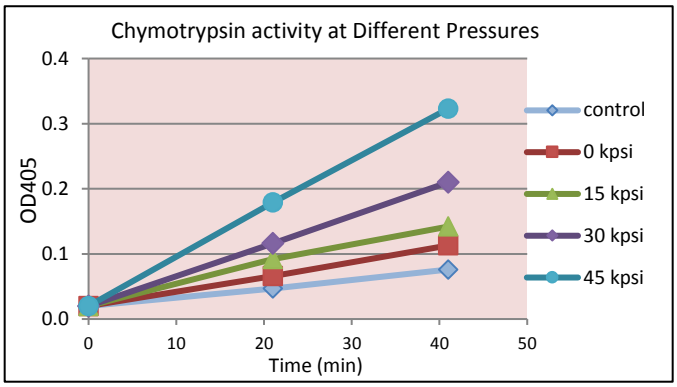

Figure 1. **Pressure cycling significantly accelerates chymotrypsin activity.** The effect of pressure on cleavage at phenylalanine was measured using N-Succ-L-Phe-pNA. OD405 was measured at the start of the reaction, and after 15 and 30 cycles of PCT. The data show that the positive effect of PCT on chymotrypsin activity increases with increasing pressure, up to the highest pressure tested (45 kpsi). The slight difference between the 0kpsi sample and the control may be due to a slight difference in temperature or heat transfer between the incubator where the plate was held and the waterbath where the MicroTubes were held.

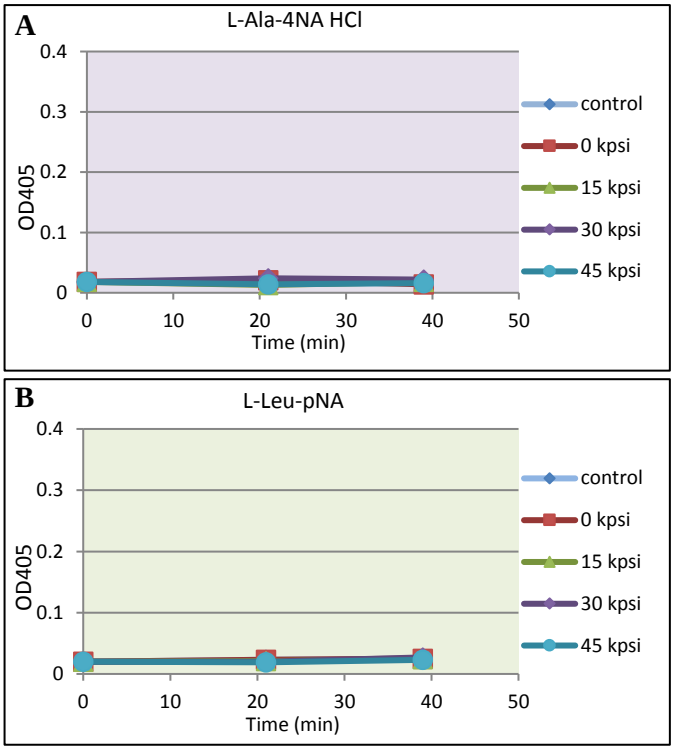

Figure 2. Pressure cycling does not induce non-specific cleavage by Chymotrypsin. Enzyme activity assays were carried out as described in Figure 1. **A**. Effect of pressure on chymotrypsin cleavage at alanine. **B**. Effect of pressure on chymotrypsin cleavage at leucine. To confirm that both these substrates are functional, positive control reactions were carried out with the general protease proteinase K (data not shown). The scale of the graphs is adjusted to match the scale in Figure 1.

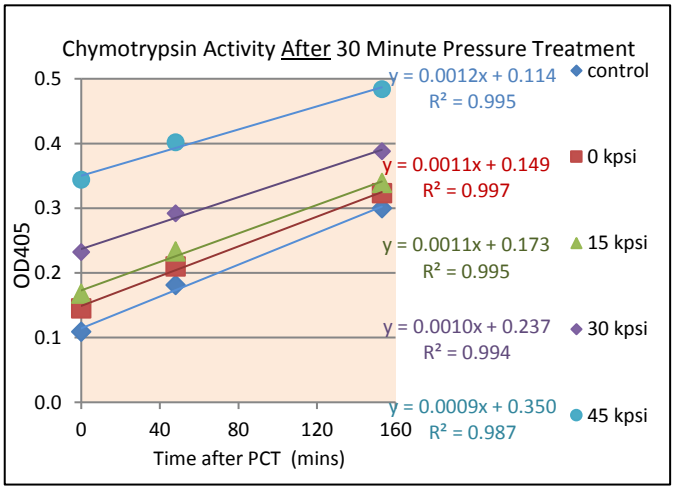

Figure 3. Chymotrypsin remains active after 30 minutes of pressure cycling. Enzyme activity at atmospheric pressure was assayed using the same samples shown in Figure 1. After 30 minutes of pressure cycling, the samples were transferred to a 96-well plate and placed in a 34^0 C incubator. OD405 was measured over the course of 2.5 hours. The linear increase in OD405, and the equation of each line are shown. As can be seen from these equations, the slopes of the lines decrease only slightly in samples treated at high pressure for 30 cycles (from .0011 at 0 kpsi to .0009 at 45 kpsi), indicating that only a slight reduction in enzyme activity occurred during the pressure cycling.

PCT-Enhanced Chymotrypsin Digestion of Model Proteins

PCT-enhanced protein digestion was tested using model proteins. Based on the results shown in Figure 1, reactions were carried out at 35^0 C with or without pressure cycling at 45 kpsi. Protein digestion was visualized by SDS-PAGE as the loss of the intact parent protein bands. As can be seen in Figure 4A, Phosphorylase B and Transferrin are both more effectively digested with pressure cycling, compared to control reactions carried out at atmospheric pressure. The more difficult to digest phosphorylase B protein is still present after 90 minutes in the control digest, and is still faintly visible even after overnight digestion, but appears to be completely digested after 90 minutes of pressure cycling. The transferrin protein (2 bands) demonstrate significant digestion within 90 minutes when reactions are carried at 45 kpsi, while control reactions incubated at 35^0 C overnight with shaking show little, if any, detectable digestion of this difficult-to-digest

protein. β-gal is digested within 90 minutes, with or without PCT, demonstrating that the effect of pressure on digestion efficiency may be substrate-protein specific, and may not be readily apparent with some easy-to-digest model proteins. These results agree well with the colorimetric activity assays and confirm that Chymotrypsin activity is significantly accelerated by pressure cycling at 45 kpsi. Further, these results demonstrate that under these conditions, more extensive digestion can be achieved in one hour with PCT than in an overnight digest at atmospheric pressure.

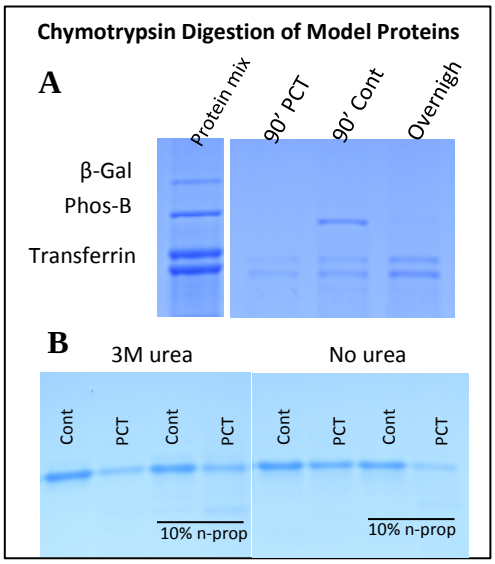

Figure 4. Model protein digests under pressure. A. Transferrin, Phosphorylase B, and β-galactosidase digestion with and without pressure cycling at 35^0 C. Pressure cycling (PCT) was performed for 90 cycles at 45 kpsi (90 mins total). Control digests were incubated at atmospheric pressure for 90 mins or overnight with shaking. Protein digestion is seen as a reduction in the intensity of the intact protein bands (note that transferrin runs as a doublet). **B**. Effect of urea and N-propanol on myoglobin digested with and without pressure cycling. PCT conditions as above.

The effect of several other reagents on chymotrypsin digestion, with and without PCT, were evaluated. As shown in Figure 4B, pressure-accelerated digestion can be further enhanced by adding urea (3M) or N-propanol (10%) to the reactions. Note that combining 3M urea with 10% N-propanol results in poorer digestion compared to either reagent alone, likely due to denaturation of the enzyme. This loss of activity was not observed when the urea was diluted to 0.8M (data not shown), indicating that pressure-accelerated digests can be carried out in solutions containing both urea and N-propanol, as long as the concentration of the denaturants is not too high.

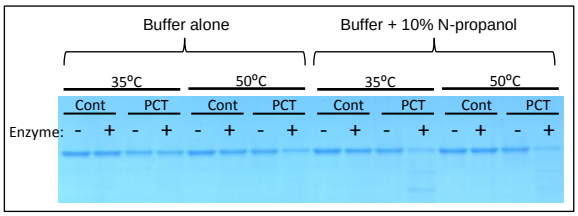

Figure 5. Effect of reaction temperature on model protein digests under pressure. Myoglobin digestion with and without pressure cycling at 35^0 C and 50^0 C. Pressure cycling (PCT) was performed for 90 cycles at 45 kpsi (90 mins total). Control digests were incubated at atmospheric pressure for 90 mins. Protein digestion is seen as a reduction in the intensity of the intact protein band. No non-specific loss of protein was observed in samples treated with pressure in the absence of enzyme.

The effect of reaction temperature on pressure-enhanced digestion was also examined. Model protein digests, carried out at 35^0 C and at 50^0 C with or without pressure cycling, demonstrated a synergistic effect of heat and pressure [Figure 5]. Increasing the reaction temperature to 50^0 C resulted in significantly accelerated digestion in the pressure-treated samples. Addition of 10% N-propanol further accelerated the reaction.

Conclusions

Here we demonstrate that PCT can be used to accelerate chymotrypsin activity in a pressure-dependent manner. Furthermore, we show that PCT improves chymotrypsin digestion of model proteins that are not efficiently digested under normal atmospheric pressure. Our data presented here and elsewhere [4] suggest that pressure cycling does not adversely affect the specificity of chymotrypsin while significantly accelerating the enzyme activity.

Based on the results presented here, the following suggested conditions for PCT-accelerated chymotrypsin digestion can be used "as-is" or as a starting point for further optimization by individual users to generate optimized pressure-enhanced protocols for their specific needs and sample types.

Table 1. Suggested Starting Conditions for PCT-enhanced Chymotrypsin Digestion Optimization

Pressure	45 kpsi
Cycle profile	50 sec at high pressure/ 10 sec at ambient, per cycle
Temperature	35^0-50^{0}C
Cycles	60-90 (more or fewer may be required for complete digestion of mixed protein samples)
Denaturants	10% N-propanol *or* up to 3M urea

Materials and Methods

Sequencing grade chymotrypsin for model protein digests was purchased from Promega. Model proteins, chymotrypsin for enzyme assays, and chromogenic substrates (N-Succ-L-Phe-pNA, L-Leu-pNA and L-Ala-4NA HCl), were purchased from Sigma. PCT was performed either in a 2320EXT Barocycler or in an SW16 pressure chamber attached to a HUB440 Barocycler. Both units were equipped with built-in electric heating.

Chymotrypsin activity was measured at 34^{0}C in 50mM ammonium bicarbonate. Chromogenic substrates were diluted to 60mM in DMSO and used at a final concentration of 1.6 mM. Control reactions were incubated in a 96-well plate. All other reactions were carried out using 150µl of reaction per MicroTube. Pressure cycling was performed at the indicated pressure using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure, per cycle). Samples labeled "0kpsi" were incubated in a 34^{0}C waterbath at atmospheric pressure (14.7psi) in PCT MicroTubes. Optical density at 405nm (OD405) was measured after 15 and 30 cycles.

Model protein digestion was carried out in 100mM tris-HCl pH8 with 10mM $CaCl_2$ at the indicated temperature using a 1:50 or 1:100 enzyme-to-substrate ratio. B-galactosidase, phosphorylase B and Holo-transferrin were reduced with 6mM DTT and alkylated with 10mM IAA using standard protocols. Native myoglobin was dissolved in buffer and used without further processing. All reactions (including controls) were carried out in PCT MicroTubes (50µl per reaction). Pressure cycling was performed at 45kpsi, using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure per cycle) for 90 cycles. Control reactions were incubated for 1.5 hours at the indicated temperature. Overnight digests were incubated at 35^{0}C with shaking on a Thermomixer (Eppendorf). Reactions were stopped by addition of 2x Laemmli buffer supplemented with 50mM DTT and stored frozen. SDS-PAGE was carried out using Criterion gels (Bio-Rad). Proteins were visualized by staining with coomasie blue (Bloo Moose Staining Solution, Kerafast). Protein digestion was assayed by SDS-PAGE as the disappearance of the intact parent protein bands.

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