Proteolysis-PrEP: Pressure-accelerated Activity of Elastase Enzyme

Introduction

The positive effect of Pressure Cycling Technology (PCT) on trypsin digestion is well established [1-4], and has been shown to result in improved sequence coverage, higher recovery and significantly reduced digestion times. Additionally, the enhancing effect of PCT on the activity of several other enzymes, including Lys-C, Proteinase K, PNGase F, chymotrypsin, Glu-C, thermolysin, and lysozyme has been reported [5-12].

Pressure, heat, and many different chemicals can be used to denature proteins, but the pressure-perturbed proteins assume conformational forms that are different from those caused by thermal or chemical treatments [13]. Pressure-induced denaturation of substrate proteins leads to better access of enzymes to previously inaccessible, or poorly accessible, 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, independent of substrate conformation.

Here we report that protein digestion by elastase enzyme is accelerated when the enzyme reaction is carried out under pressure cycling conditions. The goal of this work is to provide the user with the best set of *starting conditions* for pressure-enhanced Elastase digestion.

PCT Sample Preparation System (PCT SPS)

Pressure Cycling Technology uses hydrostatic pressure cycles to control biomolecular interactions and to accelerate certain enzymatic reactions. The samples in the pressure chamber are exposed to rapid and repeated pressurization from atmosphere (14.7 psi) to levels as high as 45,000psi (45kpsi), or higher in some applications.

The PCT sample preparation system is comprised of a Barocycler instrument, such as the 2320EXT, and high pressure-resistant sample containers such as PCT MicroTubes with MicroCaps. The specially designed PCT MicroTubes are single-use sample processing containers made of a non-reactive Teflon-like material. These tubes are designed to hold 50-150µl of sample. MicroCaps of different lengths are used to displace excess air in MicroTubes. Other sample containers, that are suitable for larger sample volumes up to 1.4ml, are also available. Up to 16 MicroTubes at a time can be pressure cycled in the 2320EXT, to allow batch-mode processing of multiple samples.

Results and Discussion

Effect of pressure cycling on elastase activity

The effect of pressure cycling on the activity of elastase was assayed using a chromogenic substrate. Enzymatic cleavage of the substrate results in color development which is assayed at 405nm. As shown in Figure 1, enzyme activity measured at atmospheric pressure (control) and at 20kpsi is essentially unchanged. At 45kpsi, a slight reduction in activity of elastase was observed over time, which is likely due to pressure-induced protein denaturation. These results suggest that elastase is quite pressure-stable and that pressures above 20 kpsi may be useful for digestion of difficult-to-digest targets, where more extensive denaturation of the target protein may improve enzyme access to cleavage sites.

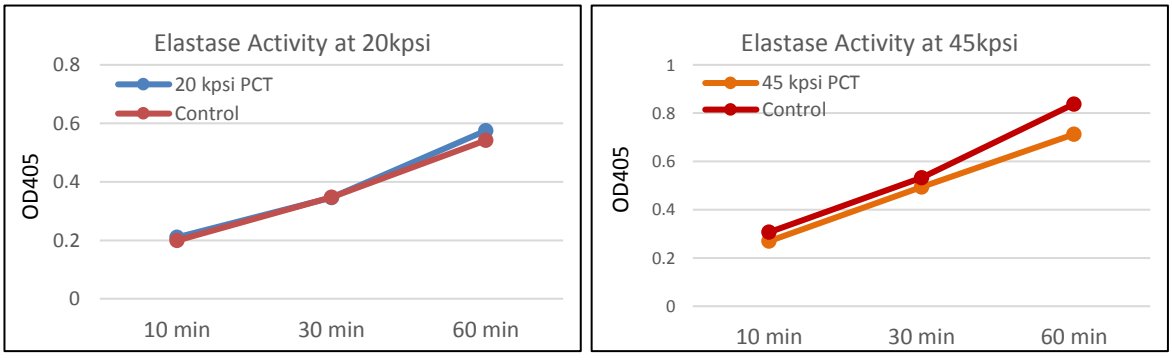

Figure 1. Elastase Activity Assays at 20 and 45kpsi. Unpressurized controls were processed in parallel and are shown on each graph. Pressure cycling at 20kpsi for 1 hour has no negative impact on enzyme activity. Pressure cycling at 45kpsi results in a very slight drop in elastase activity over time.

Pressure cycling-accelerated elastase digestion of model proteins

Elastase digestion of native (disulfide-intact) BSA, myoglobin, and ubiquitin is shown in Figure 2. Panel A shows model protein digestion for 6 hours at ambient pressure using a range of enzyme-to-substrate (E/S) ratios from 1:20 to 1:10,000 (1:10k). Based on these preliminary results, elastase digestion of the model proteins was evaluated using 1:20, 1:50 and 1:100 E/S at ambient pressure (0kpsi) and with pressure cycling at 20 or 45kpsi. The results in panel B demonstrate that pressure cycling at both 20 and 45kpsi improves elastase digestion of native BSA, especially at the lower enzyme-to-substrate ratios. Ubiquitin, a very difficult to digest protein, shows detectable digestion only at 45kpsi. It is likely that 20kpsi is not sufficient pressure to unfold this small protein to allow access of elastase to its target sites in this short time (all digests were carried out for 1 hour). The myoglobin protein appears to be very resistant to elastase digestion; the only condition that results in any noticeable (very slight) digestion of myoglobin, is at 45kpsi with the highest enzyme concentration (E/S 1:20).

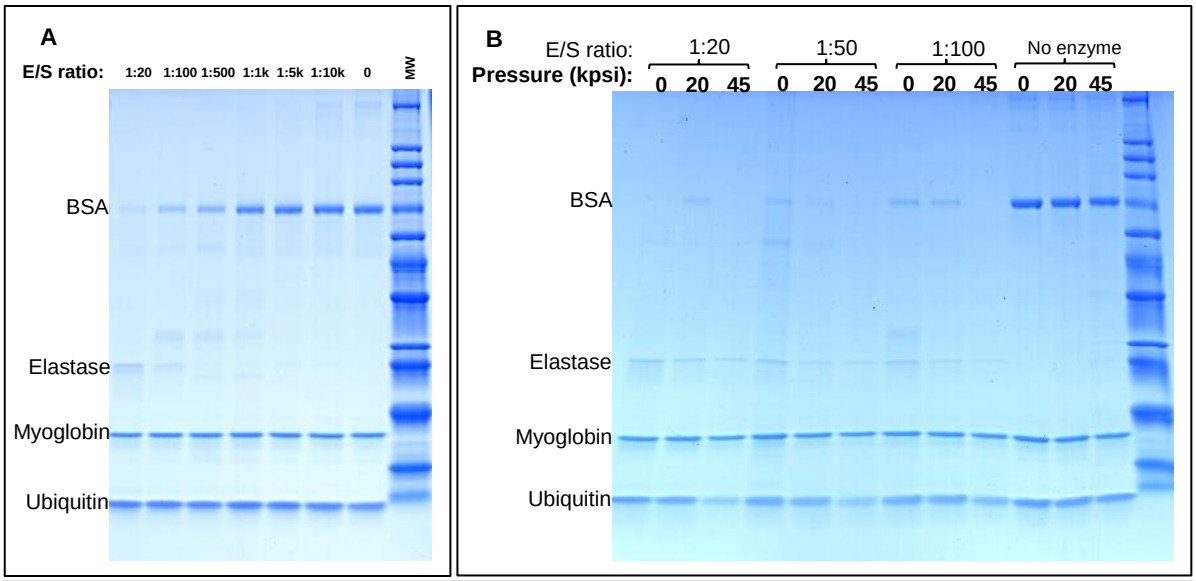

Figure 2. Elastase Model Protein Digestion. **A**. Digestion at ambient pressure for 6 hours using a range of enzyme-to-substrate (E/S) ratios from 1:20 to 1:10,000 (1:10k), demonstrates slow digestion of BSA by the enzyme and essentially no detectable digestion of both myoglobin and ubiquitin, even at high E/S ratio (1:20). Control incubated without enzyme (0) is also shown. **B**. Digestion of the model proteins with 1:20, 1:50 and 1:100 E/S at ambient pressure (0kpsi) and with pressure cycling at 20 or 45kpsi. In some samples, the elastase enzyme is visible on the gels as a band at ~25kDa.

Conclusion

Here we examined the effect of hydrostatic pressure on Elastase enzyme activity and digestion of model proteins, in order to determine the best starting conditions for PCT-accelerated digestion with this enzyme. These suggested conditions 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.

Table1. Suggested Starting Conditions for PCT-enhanced Elastase Digestion Protocol Optimization

Pressure	20-45 kpsi
Cycle profile	50 sec at high pressure/ 10 sec at ambient, per cycle.
Temperature	Ambient temperature (~20-25^0 C) to 37C. At higher or lower temperatures, additional optimization of PCT conditions may be required.

Materials and Methods

Elastase enzyme (Cat# E1250) and colorimetric substrate (N-succinyl-ala-ala-pro-leu p-nitroanilide) (Cat# S8511) for activity assays were purchased from Sigma. Activity assays were carried out at the indicated pressure in 100mM HEPES pH 7.5, 500mM NaCl, 10% DMSO at ambient temperature (~25^0 C). Pressure cycling was performed using one minute cycling parameters (55 seconds at high pressure and 5 seconds at ambient pressure, per cycle). To measure OD405 at each timepoint, an aliquot of reaction mix was transferred from a PCT MicroTube to a 96-well plate.

Elastase enzyme for model protein digests was purchased from Promega (cat# V189A). Model proteins (BSA, equine myoglobin, bovine ubiquitin) were purchased from Sigma. Protein disulfide bonds were not reduced prior to digestion. Protein digests were carried out in PCT MicroTubes in 50mM Tris at pH 9.4 at 37^0 C. Pressure cycling was performed in the Barocycler 2320EXT using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure, per cycle).

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