Real-time insights into pressure-mediated protein refolding:

Reversible inactivation of alkaline phosphatase at high pressure.

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ABSTRACT

Objectives: Alkaline phosphatase (ALP) is reversibly inactivated at high temperature and activity may be restored upon return to physiological temperature. However, high pressure has been shown to irreversibly inactivate this enzyme. The synergistic effects of elevated temperature and pressure on ALP inactivation were investigated using high pressure optical spectroscopy (HiPOS).

Methods: HiPOS enabled the real-time analysis of ALP activity at pressures ranging from 0-3.1 kilobar and at temperatures ranging from 20-55°C. The rate of conversion of the chromogenic substrate p-nitrophenyl phosphate (pNPP) was quantified over time course at each pressure and temperature using an automated fiber optics-based spectrophotometer coupled to a high pressure optical cell. Activity is expressed in a slope.

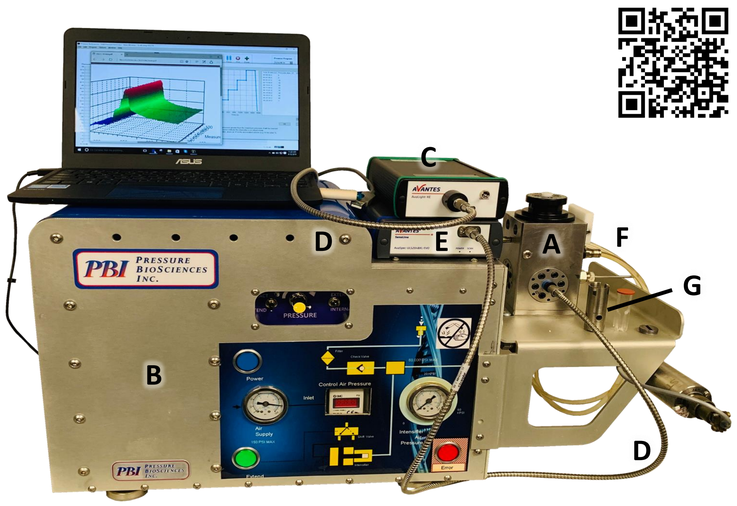

FIGURE 1. Components of the fiber optics-based HiPOS system. **(A)** the high pressure optical cell interfaces with **(B)** the programmable PBI HUB440 pressure generator. **(C)** The xenon light source is coupled to the optical cell via **(D)** fiber optics, which in turn, is coupled to the **(E)** AvaSpec spectrophotometer. **(F)** Operating temperature in the optical cell is controlled by a peripheral circulating water bath. **(G)** shows cuvette holder, semi-micro volume cuvette, and silicon seal.

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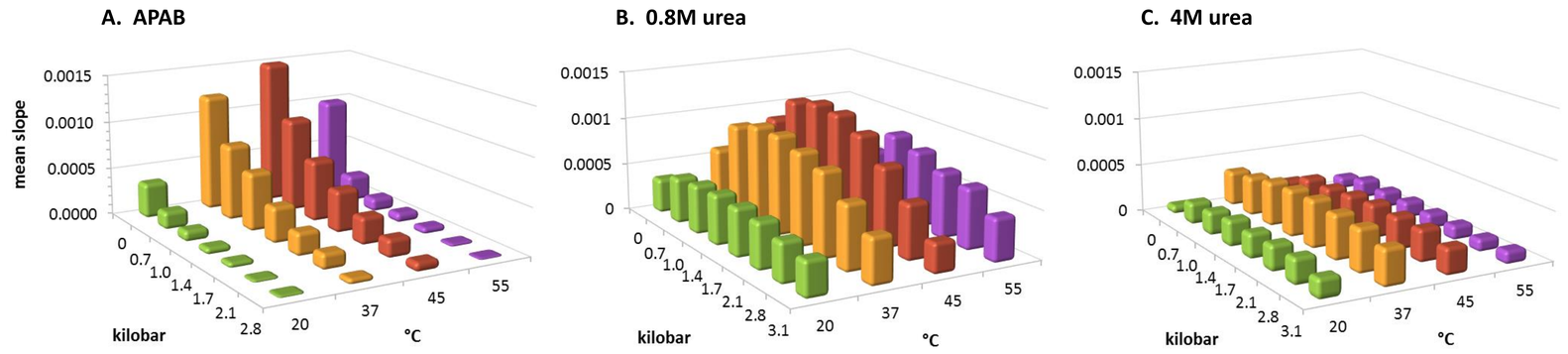

FIGURE 2. Synergistic effects of elevated pressure and temperature on ALP activity in three different buffers. **(A)** Abcam phosphatase assay buffer (APAB). **(B)** 100 mM ammonium bicarbonate pH 8.0 containing 0.8M urea and emulating the conditions of trypsin digestion. **(C)** 100 mM ammonium bicarbonate pH 8.0 containing 4M urea and emulating the conditions of Lys-C digestion.

Results: At atmospheric pressure, the rate of substrate conversion increased linearly from 20°C to 45°C. In phosphatase assay buffer, ALP was inactivated at 2.8 kilobar over the temperature range tested. At 20°C and 37°C, inactivation at 3.1 kilobar was reversible with 99% and 73%, respectively, of the enzyme's initial activity being restored upon return to atmospheric pressure. Following 3.1 kilobar at 55°C for ten minutes, less 30% of initial enzyme activity was recovered upon return to atmospheric pressure.

FIGURE 3. PBI High pressure cell used for HiPOS. Cuvette holder shown in middle. Specialized versions of the cell and sample holder are available for SAXS applications.

Conclusions: HiPOS provided real-time analysis of ALP activity at elevated pressure and temperature. At low temperatures, ALP can be reversibly inactivated at high pressure with nearly complete restoration of activity upon return to atmospheric pressure. The observed loss of recoverable activity at 55°C upon return to atmosphere pressure indicates misfolding of the protein. Reverse pressure gradients at optimal temperature could potentially guide slower, more controlled refolding and result in an improved recovery of functional proteins.

SUMMARY

- HiPOS enables real-time analysis of enzymes at high pressure for rapid optimization of reaction conditions.
- HiPOS is a useful tool for monitoring protein unfolding and refolding where proper refolding restores activity.
- ALP activity persists in 4M urea, the conditions typically used for lysyl endopeptidase (Lys-C) digestion of proteins, allowing the opportunity for spurious dephosphorylation of protein analytes. ALP is inactivated at the same elevated pressure and temperature at which Lys-C activity is accelerated.
- ALP activity persists in 0.8M urea, the conditions typically used trypsin digestion of proteins. ALP activity is decreased at the same pressure at which trypsin digestion is enhanced.