PBI Pressure BioSciences Inc.

Pressure-enhanced Disaggregation and Solubilization of Aggregated β-casein Protein

Introduction

Disaggregation and solubilization of protein aggregates in mild reagents is challenging. Most disaggregation protocols call for protein denaturation in harsh reagents such as detergents, concentrated guanidine-HCl, or 8M urea. Pressure can also be used to denature proteins, and high hydrostatic pressure has shown promise as a means of solubilizing and/or refolding insoluble aggregates due to its effects on electrostatic and hydrophobic interactions – two key components of aggregate formation [2-3]. Disaggregation by pressure works in manner similar to chemical disaggregation, with one significant advantage; pressure-disaggregated proteins do not require extensive clean-up to remove the high concentrations of denaturing chemicals required by conventional methods.

Here we report that solubilization of aggregated β-Casein can be enhanced when carried out under high pressure, even in the absence of strong chaotropes. The goal of this work is to provide the user with the best set of *starting conditions* for pressure-enhanced solubilization of β-Casein or similar aggregated proteins.

PCT Sample Preparation System (PCT SPS)

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 treated in the 2320EXT, to allow batch-mode processing of multiple samples.

Results and Discussion

β-Casein, a major component of casein micelles in milk, is well known to precipitate at low pH in the presence of calcium. For solubilization studies, β-Casein was aggregated and precipitated as described below. Various disaggregation reagents were initially tested at atmospheric pressure. The extent of aggregate solubilization was assayed by measuring soluble protein concentration. High urea concentrations, up to 8M, were required to solubilize 60-80% of the precipitated casein protein. In 3M urea, only ~50% of total protein was recovered in the soluble fraction. Other reagents, such as 10% acetonitrile were even less effective. Little, if any soluble protein was recovered when samples were incubated in water at ambient pressure.

High pressure disaggregation of casein aggregates is shown in Figure 1. The aggregated protein was solubilized in water without addition of any chaotropes or detergents under various pressure conditions. Disaggregation was assessed by measuring soluble protein concentration in supernatants after centrifugation to pellet any residual aggregates. At ambient pressure (Atm), less than 35% of the protein is recovered in the soluble fraction after 3 hours in water. Conversely, after just one minute at high pressure, ~80% of the protein can be found in the soluble supernatant.

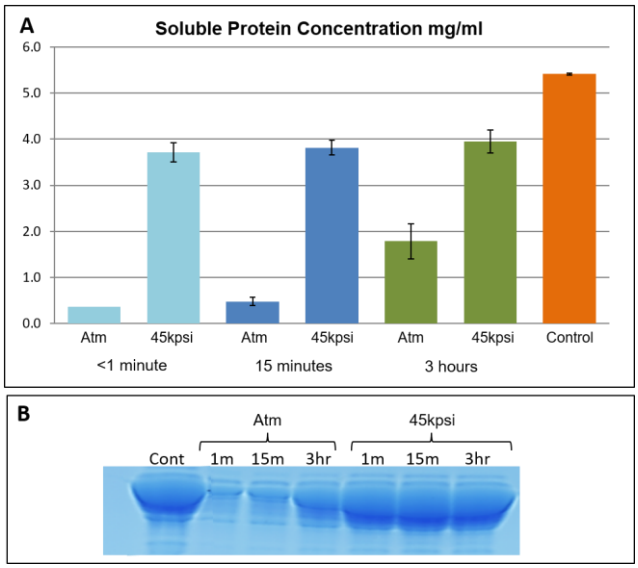

Figure 1. Influence of time at high pressure on β-Casein aggregate solubilization. Protein aggregates were incubated at room temperature in water, either at ambient pressure (Atm) or high pressure (45kpsi). Samples were centrifuged to pellet insoluble material. Soluble protein in supernatants was quantified by by BCA assay (**A**) and visualized on SDS-PAGE (**B**). For comparison, the starting protein concentration (before aggregation) is also shown (control). This represents the theoretical maximum yield. At ambient pressure, only about 30% of the aggregated protein is solubilized after 3 hours. High pressure treatment for less than 1 minute was sufficient to solubilize ~80% of the aggregated protein, and longer incubation (up to 3 hours) did not further increase yield.

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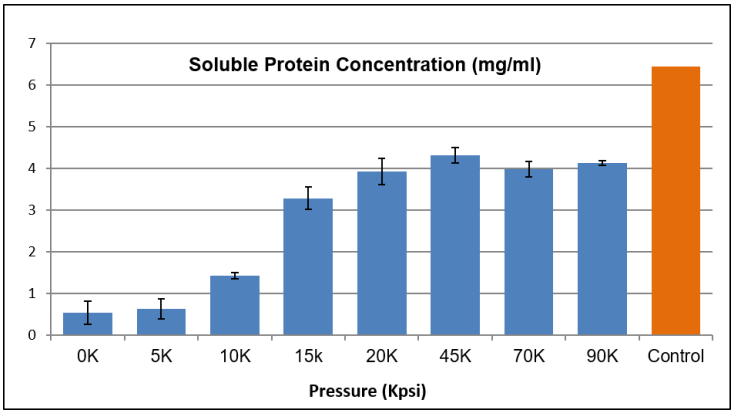

Figure 2. Effect of pressure level on β-Casein disaggregation. Aggregated protein was treated in water at room temperature at the indicated pressure, for 1 minute. Soluble protein was quantified by BCA Assay. At pressures up to 15Kpsi pressure level is proportional to protein yield. At pressures above 15kpsi, maximum protein yield is reached, and no additional benefit of higher pressure levels is observed. For comparison, the starting protein concentration (before aggregation) is also shown (control). This represents the theoretical maximum yield.

Multiple pressure levels were tested to determine the optimum pressure range for aggregate solubilization. The data presented in **Figure 2** suggest that a one-minute incubation at 0-15kpsi, results in an increase in protein yield with increasing pressure. At pressures above ~15kpsi, yield is maximal and is not affected by increasing pressure. We conclude that maximal β-Casein solubilization in water, can be achieved in ~1 minute at 15-20kpsi.

Conclusion

Here we demonstrate the benefit of hydrostatic pressure for protein aggregate solubilization. Data generated with aggregated β-Casein suggest that aggregate solubilization at high pressure is a very rapid process that can significantly improve soluble protein yields even in very mild reagents. Based on the data presented here, the best starting conditions for pressure-enhanced solubilization are summarized in Table 1. Since different proteins and aggregates will require individually optimized disaggregation conditions, these suggested conditions should be used as a starting point for further optimization by individual users to generate optimized pressure-enhanced protocols for specific applications and sample types.

Table1. Suggested Starting Conditions for Pressure-enhanced Solubilization

Pressure	15-45kpsi (higher or lower pressures may be beneficial in some applications)
Cycle profile	Static pressure. Time at pressure may vary depending on target protein, buffer/reagent composition, and temperature.
Temperature	Room Temperature. At higher or lower temperatures, additional optimization of pressure conditions and/or buffer components may be required depending on target protein.

Materials and Methods

β-Casein was purchased from Sigma Aldrich (cat # C-6905) and dissolved in 100mM Tris (pH 7.5) to a final concentration of 5mg/ml. Aggregation was induced by heating for 30 minutes at 42ºC in the presence of 10mM $CaCl_2$, followed by a 10min incubation at room temperature with 10mM HCl. The sample was then centrifuged for 5min at 12,000g to pellet the aggregates. All pellets were re-suspended in dH_2O and incubated at room temperature for 10min before pressure treatment [4].

Pressure Disaggregation

100µl of aggregated protein in water was transferred to PCT-MicroTubes (all samples in triplicate). Samples were treated at room temperature either at ambient pressure or at high hydrostatic pressure (not cycled pressure). Pressure incubations, up to 45 kpsi, were carried out in a 2320EXT Barocycler (Barocycler mode), and higher pressures in a HUB 880 Explorer (Pump Mode). The treated samples were centrifuged to pellet any residual undissolved protein. Soluble protein in the supernatant was quantified by Bradford and/or BCA Assay. All data are presented as an average of n=3 ± standard deviation. Control samples (5mg/ml stock before aggregation) are also shown for comparison. For SDS-PAGE, samples were denatured and reduced with DTT in Laemmli buffer prior to gel electrophoresis [1].

References

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