PBI Pressure BioSciences Inc.

Disaggregation of Proinsulin Inclusion Bodies by High Pressure

Introduction

Protein expression in *E. coli* is an efficient and commonly used method to generate large quantities of protein for research or therapeutic applications. Unfortunately, proteins expressed at high levels in *E. coli* are often packaged into inclusion bodies (IBs). These tightly-packed structures have the advantage of being composed of almost pure expressed protein, but the serious disadvantage that the protein is so tightly aggregated that high concentrations of chaotropes or detergents are required to extract soluble protein from the aggregates. These solubilization reagents must then be diluted or removed by buffer exchange, so that the extracted protein can be refolded into its native, functional conformation.

High hydrostatic pressure has shown promise as a means of disaggregating and solubilizing protein aggregates using relatively mild buffer conditions [1-4]. By disaggregating IBs without the high levels of denaturants required under conventional conditions, subsequent protein refolding can be improved.

Here we report that high hydrostatic pressure can be used to efficiently disaggregate proinsulin inclusion bodies in order to extract soluble proinsulin protein. This disaggregation can be carried out in mild buffer conditions at ambient temperature in as little as 5 minutes at 45kpsi.

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

Results and Discussion

Pressure disaggregation of proinsulin inclusion bodies.

Rapid solubilization of IBs can be achieved at elevated pressure. **Figure 1** shows the effect of incubation at 45,000psi (45kpsi) on the solubilization of proinsulin IBs in 50mM CAPS buffer, in the absence of any detergents or chaotropes. As indicated by protein quantification and SDS-PAGE, 5 minutes at 45kpsi is enough to solubilize proinsulin IBs in buffer alone. Incubation for 2 hours at 45kpsi did not significantly increase the yield, suggesting that 5 mins is enough for maximal solubilization of this protein in this buffer.

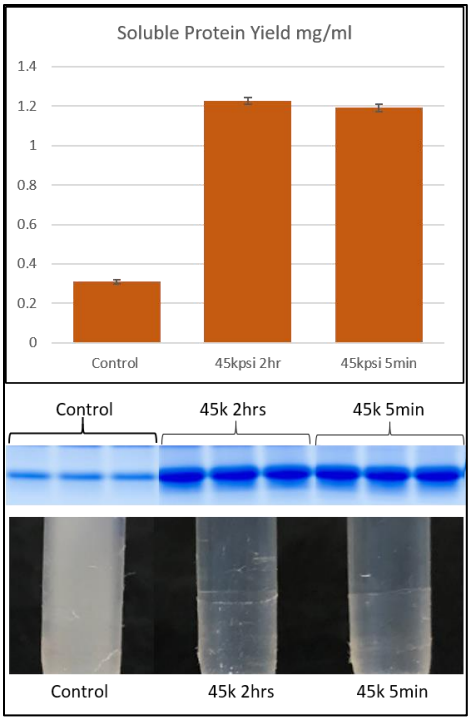

Figure 1. Proinsulin Inclusion Bodies are rapidly solubilized at 45kpsi. IBs were suspended in 50mM CAPS pH 10.5 at ~2mg/ml and treated at 45kpsi for 5 mins or 2 hours at room temp. Controls were incubated for 3.5 hours at ambient pressure. After incubation, all samples were centrifuged (10,000g 5 mins) to pellet intact IBs. The solubilized protein in the supernatant was quantified by Bradford assay (top panel, average of triplicates ± std dev), and visualized by SDS-PAGE (middle panel). The bottom panel shows the appearance of the samples before centrifugation. Note that the control sample is visibly cloudy because it is a suspension of intact IBs, while the pressure-treated samples are clear, as expected for a protein solution.

IB solubilization is usually carried out in high concentrations of chaotropes, such as urea. We compared proinsulin IB solubilization at high pressure in CAPS buffer alone, to solubilization in urea without pressure. **Figure 2** shows that in samples incubated at

PBI
14 Norfolk Avenue South Easton, MA 02375
TEL 508-230-1828 • FAX 508-580-1829
www.pressurebiosciences.com

ambient pressure, a high concentration of urea is required to dissolve IBs and release soluble proinsulin, while little, if any, urea is necessary for solubilization when the IBs are treated at high pressure.

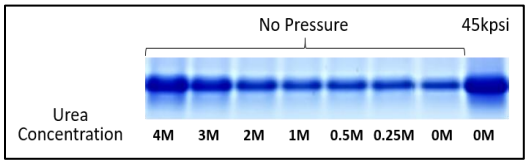

Figure 2. Disaggregation of IBs and release of soluble protein. Effect of urea concentration vs pressure. IBs were suspended at ~1mg/ml, in 50mM CAPS pH10.5 with the indicated concentration of urea and incubated at ambient pressure for 10 mins or at 45kpsi for 5 mins. All samples were centrifuged to pellet intact IBs. The solubilized protein in the supernatant was visualized by SDS-PAGE.

In the absence of chaotropes, proinsulin IB disaggregation can be increased by incubating the suspension at high pH. **Figure 3** shows the effect of CAPS buffer pH on IB disaggregation and solubilization of proinsulin. The results demonstrate that incubation for 5 mins at 45kpsi significantly increases protein yield at all pH levels tested.

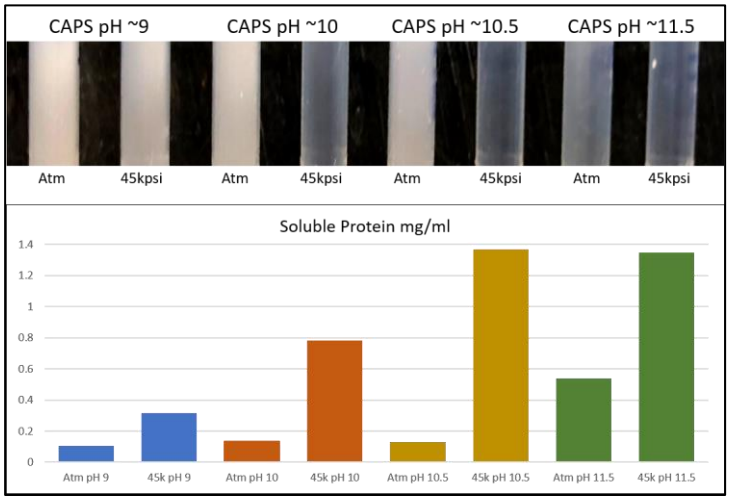

Figure 3. Disaggregation of IBs and release of soluble protein. Effect pH and pressure. IBs were suspended at ~2mg/ml in 50mM CAPS adjusted to the indicated pH and incubated at ambient pressure for 10 mins or at 45kpsi for 5 mins. All samples were centrifuged to pellet intact IBs. The solubilized protein in the supernatant was quantified by Bradford assay. The top panel shows the appearance of the samples prior to centrifugation.

Previously it has been shown that disaggregation of IBs by pressure is less sensitive to protein concentration than chemical-based methods [3], allowing for efficient disaggregation of relatively concentrated samples. **Figure 4** shows the relationship between starting IB concentration and soluble protein yield in samples incubated at 45kpsi for 5 mins in 50mM CAPS pH 10.5 (no urea). Note that the starting IB concentration is an approximate value and may be a slight overestimate due to carryover of some bacterial components into the IB preparation. This is supported by the presence in the IB prep of a soluble 16kDa protein (by SDS-PAGE), that is washed away from the rest of the inclusion bodies prior to disaggregation (data not shown), and the presence of non-proinsulin peaks observed by RP-HPLC (Figure 5). The results in Figure 4 support the conclusion that in the concentration range tested (~1-20mg/ml IBs), solubilization of proinsulin by pressure is equally efficient at both high and low protein concentrations.

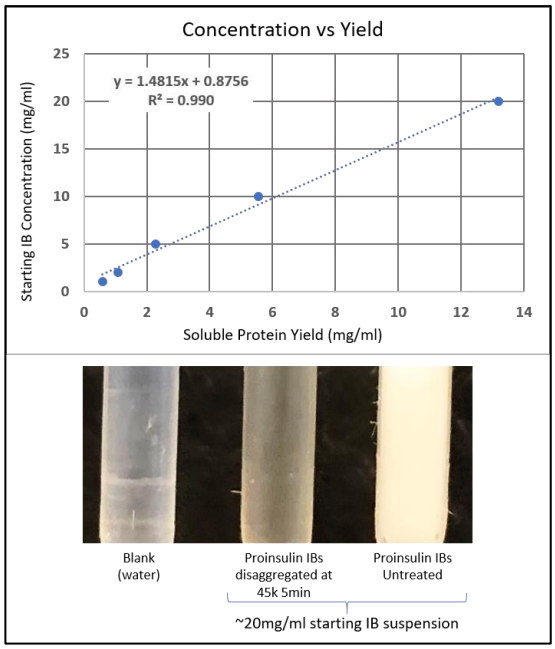

Figure 4. Effect of total concentration on disaggregation of IBs and release of soluble protein. IBs were suspended at ~1-20 mg/ml in 50mM CAPS pH 10.5 and incubated at 45kpsi for 5 mins. The solubilized protein in the supernatant was quantified by Bradford assay. Starting IB concentration was plotted against soluble protein concentration after centrifugation. The linear relationship (R^2=0.99) indicates that disaggregation of IBs is as efficient at high concentration as at lower concentrations, within the range tested. The panel on the bottom shows the appearance of the 20mg/ml IB sample with and without pressure treatment, prior to centrifugation. The disaggregated proinsulin solution is transparent and yellowish, the slightly cloudy appearance is due to the MicroTube (a tube with just water is shown for comparison).

We compared soluble proteins extracted from samples treated with and without pressure, to assess recovery of IB-derived proinsulin and other soluble proteins that are present in the whole inclusion body preparation. The results, shown in **Figure 5**, demonstrate that soluble proinsulin protein recovered from pressure-treated IBs is present as a mixture of unfolded and partially-folded forms. Since proinsulin does not form disulfide bonds when expressed in E. coli and packaged into inclusion bodies, the presence of disulfide-containing proinsulin suggest that some protein oxidation had occurred during either storage or processing of the IBs.

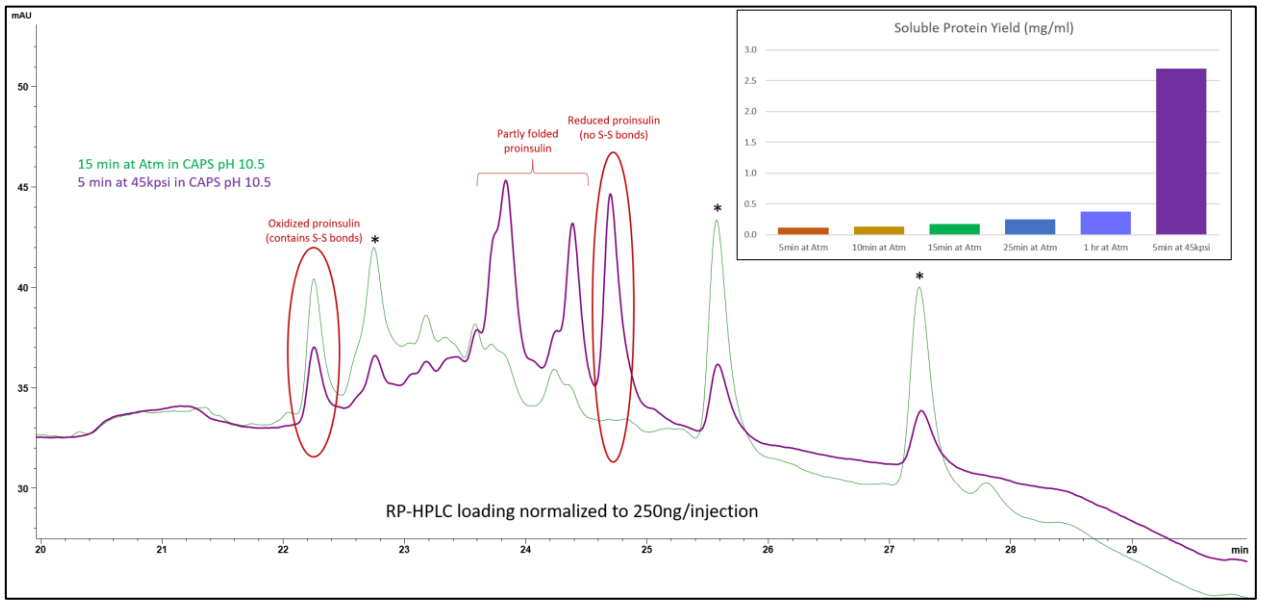

Figure 5. Visualization of solubilized proinsulin by RP-HPLC. Aliquots of IB suspension (~4mg/ml in 50mM CAPS) were allowed to incubate at ambient pressure for 5 min to 1 hour. One aliquot was solubilized at 45kpsi for 5 mins. All samples were centrifuged at 10,000g for 5 mins and the soluble proteins in the supernatants were quantified by Bradford assay (inset) and separated by reverse-phase HPLC (only the 45kpsi 5min and Atm 15min samples are shown for clarity). The sample incubated briefly without pressure, contains little IB-derived proinsulin. The sample solubilized at 45kpsi contains primarily proinsulin derived from IBs. Some oxidation of proinsulin during storage and/or processing is evident from the presence of oxidized proinsulin in both samples. Putative E. coli-derived proteins are indicated by asterisks (*) and are enriched in the non-pressure treated sample. Note that RP-HPLC loading was normalized to 250ng protein per injection.

Conclusions

Here we examined the benefit of brief hydrostatic pressure treatment for disaggregation of proinsulin inclusion bodies to generate soluble proinsulin protein. The results presented in Figures 1-4 demonstrate that proinsulin inclusion bodies can be efficiently disaggregated in as little as 5 mins at 45kpsi, in mild buffer such as 50mM CAPS pH 10.5 without addition of urea or other chaotropes or detergents. The results in Figure 5 show that the solubilized proinsulin is present in multiple folded forms and indicate that additional refolding steps may be necessary to generate fully refolded proinsulin from the solubilized protein. Optimization of pressure-assisted protein refolding will be described in upcoming Application Notes.

Materials and Methods

Human proinsulin was expressed in E. coli and purified as inclusion bodies (IB). The raw IB suspension in 40% glycerol (approximately 29.5mg/ml proinsulin) was stored frozen in aliquots. For IB disaggregation, aliquots were thawed and centrifuged at 10,000g for 5 min. The glycerol-containing supernatants were discarded and the IB pellets were suspended in the indicated buffer. In some experiments the IB pellets were subjected to additional washes with CAPS buffer to reduce carry-over of E. coli-derived proteins. The wash supernatants were discarded and the washed IB pellets were suspended at ~2mg/ml (based on a 29.5 mg/ml staring concentration, unless indicated otherwise) in 50mM CAPS. CAPS pH 10.5 was used for all processing, unless indicated otherwise. The washed IB suspensions were transferred into PCT MicroTubes and processed at the indicated pressure (atmosphere or 45kpsi) for the indicated time, at

ambient temperature. All high-pressure incubations were carried out at constant (“static”) pressure in a Barocycler 2320EXT, running in Barocycler Mode.

Disaggregated samples were centrifuged at 10,000g for 5 min to pellet residual intact IBs, prior to Bradford assay or SDS-PAGE. The 9kDa proinsulin protein in the soluble fraction was visualized by SDS-PAGE using AnyKD gels (Bio-Rad). Samples were reduced with DTT prior to electrophoresis. Reverse phase HPLC was run on a YMC Pro C18, 150x3mm, 120A with standard reverse-phase mobile phase (A- Water with 0.1% TFA, B- Acetonitrile with 0.1% TFA) and a 45-minute gradient from 2-60% B. Samples were diluted to a final concentration of 0.05mg/ml in mobile phase A. All samples were normalized to 250ng per HPLC run.

References

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2. Kim, Y.S., Randolph, T.W., Seefeldt, M.B., and Carpenter, J.F. 2006. High pressure studies on protein aggregates and amyloid fibrils. Methods Enzymol. 413:237-253.
3. St. John, R. J., Carpenter, J. F., & Randolph, T. W. (1999). High pressure fosters protein refolding from aggregates at high concentrations. Proceedings of the National Academy of Sciences of the United States of America, 96(23), 13029–13033.
4. Pressure-enhanced solubilization of β-casein protein aggregates. PBI Application Note. In preparation.