

Protein Extraction from Yeast: Comparison of the Covaris Adaptive Focused Acoustics™ (AFA) Process to Conventional Bead Beating and Probe Sonication

OVERVIEW

The efficiency of several mechanical-based lysis and extraction techniques, such as Adaptive Focused Acoustics (AFA), probe sonication, and bead beating from yeast isolates was compared for (i) total protein yield, (ii) preservation of enzymatic activity, (iii) fragmentation of proteins, and (iv) protein bias (i.e., the failure to isolate specific proteins). Protein bias was determined from both the number and relative abundance of proteins separated by SDS PAGE and by two-dimensional gel electrophoresis (2-DE) analysis.

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INTRODUCTION

The significant mechanical strength of the yeast cell wall of *Saccharomyces cerevisiae* makes the recovery of proteins, and especially biologically active proteins, particularly challenging. The techniques often utilized to improve cell lysis employ rigorous mechanical agitation and/or harsh chemicals that can result in both protein denaturation and the loss of protein activity. Alternatively, the use of enzymes (such as lysozyme) to hydrolyze the cell wall may result in the loss of cell wall proteins and the elimination of post-translational protein modifications such as glycosylation [1]. More problematic, lysozyme preparations can be biased towards the recovery of cytoplasmic preparations [2].

Typically, rigorous mechanical methods such as bead beating or probe sonication have been used for hard, difficult cell disruption, however, both of these methods will generate heat. (NOTE: even though the pressure density of bath sonicators is low and the efficiency is poor, they are still utilized. Undesirable heat is still generated as a consequence of the total energy required for cavitation formation of the acoustic process.) There are two intrinsic limitations of these mechanical techniques: 1) they are not highly repeatable which is an important prerequisite for advanced bioanalysis (such as mass spectrometry based pattern recognition) and 2) they lack precise thermal control which can lead to significant denaturation, aggregation, and precipitative

loss of proteins under conditions where the preservation of native conformation and activity are required. In addition, most probably because of the extreme pressures and temperatures generated at the end of the probe in direct contact with the sample, probe sonication has also been shown to cause protein fragmentation [3]. Furthermore, the aerosolization of samples by probe sonication also represents a serious biohazard and laboratory acquired infections have been reported [4,5].

Uncontrolled temperature generated during a conventional lysis and extraction is not desirable. Protein extraction at elevated temperatures risks the cleavage of Asp-Pro bonds, particularly in temperature-sensitive Tris buffers [6]. Excessive heat can also drive the desulfurization of disulfide-bonded cystine and free cysteine and their conversion to dehydroalanine and alanine, respectively [7].

In a significant contrast to uncontrolled heating of both bead beating and probe sonication, the precise control of both mechanical and thermal energy of a Covaris AFA-based extraction protocol enables highly reproducible lysis and extraction. The efficient non-contact isothermal mechanical disruption of cells by AFA leads to a high degree of extraction reproducibility while eliminating temperature fluctuations that can modify or damage proteins. The energy of the AFA process may also be tuned to the desired target.

METHODS AND MATERIALS

Yeast cultures

A mass of 0.35 g of dried active Baker's yeast (ConAgra, Naperville, IL) was hydrated in 40 mL of 80 mM sucrose and incubated for three hours with shaking at 300 rpm at 20°C. The cells were pelleted by centrifugation at 800 x g for one minute, washed in 40 mL H₂O, and pelleted again. Cells were resuspended in 20 mL H₂O and an aliquot was diluted 1:1000 in PBS for cell counting using the Scepter 2.0 Automated Cell Counter (Millipore, Danvers, MA). Halt™ and EDTA protease inhibitors (Thermo Scientific Pierce Biotechnology, Rockford, IL, USA) were added to the second wash and the cells were pelleted by centrifugation at 1000 x g for two minutes. Cells were resuspended to a final concentration of 10⁹ cells/mL.

AFA, bead beating, and probe sonication methods

To minimize chemical effects and isolate the mechanical effects of AFA, probe sonication and bead beating were compared under non-denaturing conditions. Cells were resuspended in Covaris Reagent N and dispensed into multiple milliTUBEs (Covaris, Woburn, MA, USA). AFA was performed in the Covaris M220 focused ultrasonicator. Probe sonication was performed using the Branson 450 Sonifier with stepped microtip (Branson Ultrasonics Corporation, Danbury, CT). AFA and probe sonication were normalized to 75W peak incidence power (PIP) at 10% duty cycle (DC) for 0, 90, or 180 seconds at a set temperature of 4°C.

Bead beating was performed in the FastPrep™ using tubes prefilled with Lysis Matrix B silica beads (MP Biomedicals, Solon, OH, USA) for 0, 90, or 180 seconds at a set temperature of 4°C.

All samples were processed in 1 mL volumes.

Total protein and phosphatase assays

Protein concentrations were determined using the Quickstart™ Bradford Reagent (BioRad, Hercules, CA). Phosphatase activity was measured with the Total Phosphatase Assay Kit (G-Biosciences, St. Louis, MO, USA). Samples were supplemented with 10 mM MgCl₂ prior to the activity assay.

Two-dimensional gel electrophoresis

Prior to IEF, each sample was buffer-exchanged into Covaris Reagent TP using Amicon UltraFREE 0.5 mL centrifugal filtration devices with 3,000 Da MWCO (Millipore, Danvers, MA). Protein disulfides were reduced with 5 mM tributylphosphine and alkylated with 10 mM acrylamide. Reduction and alkylation were performed directly in the filtration device as previously described [1]. Protein assay was performed on the retentates and the samples were normalized to protein mass. Non-linear immobilized pH gradients pH 3-10 were each hydrated with 200 uL of sample and isoelectric focusing (IEF) was performed in a Protean i12™ IEF instrument (BioRad, Hercules, CA). Second dimension PAGE was performed in Criterion™ 8-16% Tris-HCl. Gels were stained with SYPRO Ruby™ fluorescent stain (Invitrogen, Carlsbad, CA) for image analysis or colloidal Coomassie stain to guide manual spot excision for LC-MS.

RESULTS AND DISCUSSION

The temperature of samples processed with probe sonication and bead beating increased significantly during the course of processing. At maximum power, probe sonicated samples reached 30°C in 90 seconds and 43°C in 180 seconds. When normalized to 75W, probe sonicated temperature reached 19°C in 90 seconds and 27°C in 180 seconds. At minimum speed, bead beating processed sample temperature reached 21°C in 90 seconds and 29°C in 180 seconds. At maximum speed, bead beating processed sample temperature reached 64°C in 90 seconds and 83°C in 180 seconds (**Figure 1A**). In comparison, the temperature of the AFA-treated samples increased less than 4°C over 180 seconds at 75W.

Probe sonication yielded approximately 20% more total protein than AFA and approximately 26% more protein than bead beating at minimum speed. At maximum speed, total proteins from bead beating were greatly reduced (**Figure 1B**). When extracting under non-denaturing conditions, AFA samples yielded 20% more phosphatase activity than probe sonication or bead beating at minimum speed. At maximum speed, enzyme activity was completely eradicated in BB samples (**Figure 1C**) indicating significant protein damage due to frictional heat.

FIGURE 1. COMPARISON OF AFA, PROBE SONICATION, AND BEAD BEATING

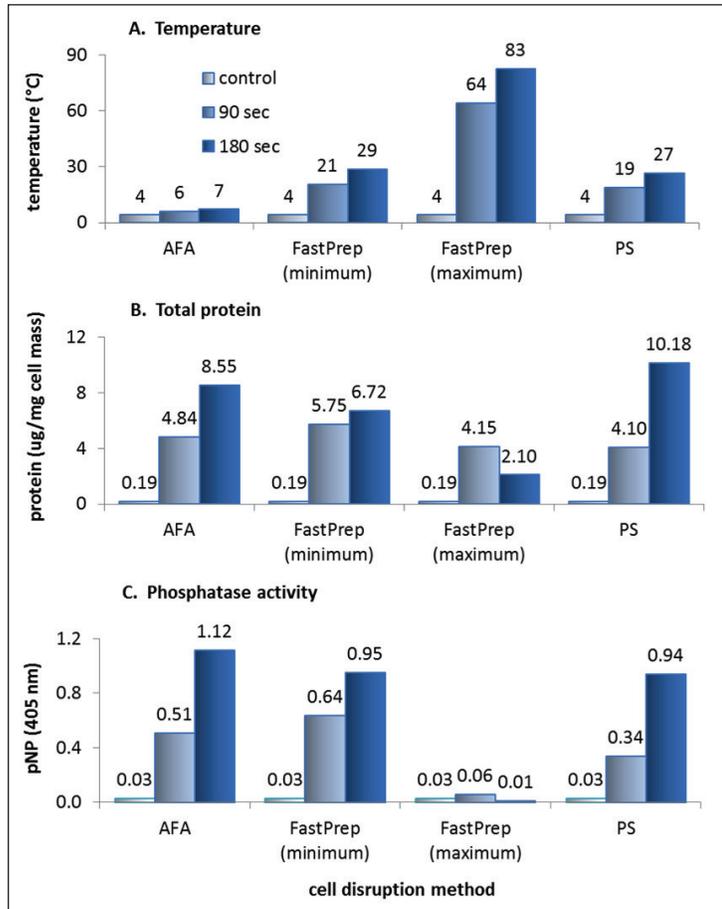


Figure 1. (A) Temperature during AFA, PS and BB at minimum or maximum speed, (B) total protein yields by each method, and (C) residual phosphatase activity. Yeast cells were suspended in Covaris Reagent N. AFA preserved 20% more enzyme activity than probe sonication and bead beating. Activity was completely eradicated using Bbead beating at maximum speed.

Effects of method on protein integrity

SDS PAGE clearly indicated protein fragmentation in samples

processed with probe sonication and bead beating than AFA processed samples. From image analysis of integrated band intensity, it was determined that 41% and 48% of the total protein was smaller than 15 kDa in probe sonication and bead beating samples respectively. In contrast, only 35% of the AFA proteins were of a molecular mass less than 15 kDa (**Figure 2**).

FIGURE 2. PROTEIN FRAGMENTATION

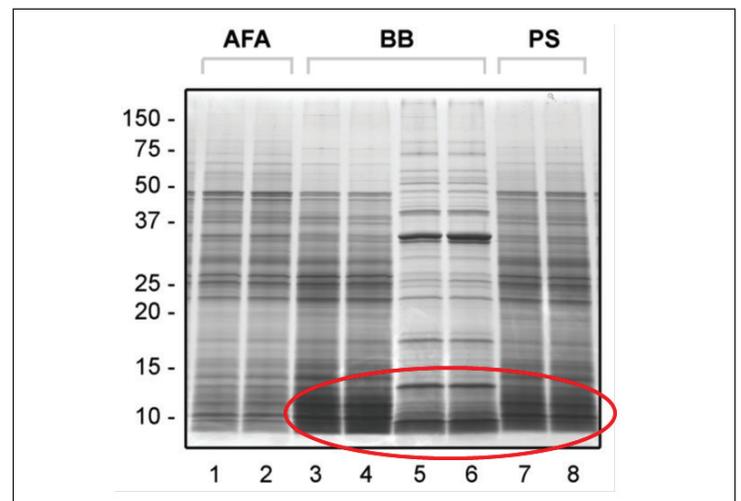


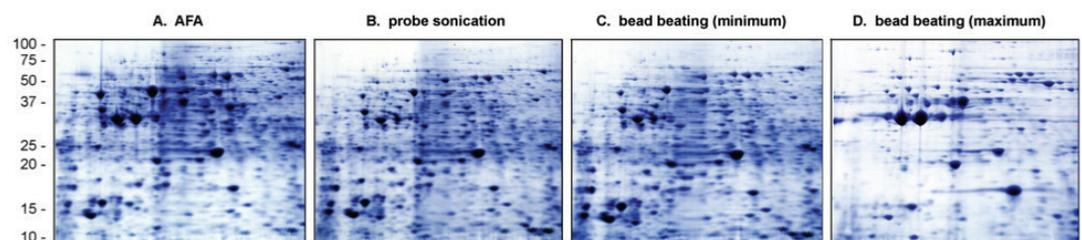
Figure 2. Duplicate lanes on 8-16% SDS PAGE lanes showing protein fragmentation (circled) resulting from probe sonication (7-8) and bead beating at minimum (3-4) or maximum (5-6) speed. Gel demonstrates the effect of overprocessing samples. Sample loads were normalized to 3 X 10⁶ yeast cells.

Two-dimensional gel electrophoresis

2-DE showed a decreasing in high molecular mass proteins in PB and BB samples compared to AFA samples. Bead beating at maximum speed lead to the significant loss in the number of proteins spots, exemplifying a severe protein bias resulting when samples are over processed (**Figure 3**)

FIGURE 3. COMPARATIVE 2-DE OF YEAST CELL LYSATES

Figure 3. 2-DE comparing yeast cell lysates from AFA, PS, and BB. Bead beating was performed at minimum speed for 90 seconds (BB min) or maximum speed for 180 seconds (BB max) to illustrate the range of protein products recovered. Sample loads were normalized to 180 ug total protein.



CONCLUSION

Probe sonication and bead beating, due to their inherent lack of control over energy and thermal events, damage proteins. The precise energy and thermal control of Covaris AFA allows for an isothermal and reproducible protein extraction from yeast cells making it an ideal mechanical extraction technology for applications where native conformation and biological activity need to be preserved and for applications where reproducible pre-analytical sample preparation is beneficial.

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PART NUMBER: M020003 REV A | EDITION DECEMBER 2014

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