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Application Note

Benzonase® endonuclease for improved primary recovery in an *E. coli*–based process for Fab production

Introduction

For the production of antibody-derived fragments, such as Fab fragments, *E. coli* expression systems are a cost-effective alternative to animal cell culture technologies due to their less expensive cell culture media, faster growth rates, high productivity, and as there is no need for virus filtration.

E. coli systems are classically designed for periplasmic expression, which renders the primary recovery of the product a challenge, since cell harvest, release of the product from the cells, and preparation of the final cell extract for initial purification result in a complex process scheme.

Each processing step for the lysate preparation is potentially associated with a loss of product. The pressure used for cell homogenization and the conditions for cell extraction greatly affect the amount of product that can be recovered from the cells, as well as the size of cellular debris and the extent of host cell impurities. All these factors have a significant effect on the performance of the subsequent clarification and filtration unit operations and on product yield.

Since DNA and RNA account for approximately 25% of the *E. coli* dry cell mass,[1] the disruption of the bacterial cells necessarily leads to enormously high levels of nucleic acids in the lysate solution. This can cause a considerable increase in viscosity of the cell extract and introduces an additional challenge for clarification and filtration steps. Many problems associated with increased lysate turbidity and occurrence of precipitation in cell extracts relate to high nucleic acid levels.

Nucleic acids, with their polyanionic charge characteristics, can naturally bind oppositely charged proteins, resulting in the formation of protein-nucleic acid complexes. These tend to precipitate under unfavorable buffer conditions, such as acidic pH and low salt concentration. Precipitation of DNA and host cell proteins in cell culture broth under acidic conditions has been reported.[2]

The charge-based binding of polyelectrolytes to proteins has been intensively studied and has been proven particularly suitable for protein precipitation.[3] The ability of polyelectrolytes to form non-soluble complexes with proteins has been turned into practical applications and utilized for protein purification.[4]

However, during the processing of *E. coli* extracts, this complex formation can lead to undesired effects. There is a risk that the presence of high levels of high-molecular-weight DNA can cause coprecipitation of the target protein, resulting in a loss of soluble product in the cell extract. This holds true especially for molecules exhibiting a basic isoelectric point, like Fab fragments.

The use of DNA- and RNA-precipitating agents or degrading enzymes in microbial processes could potentially improve this process greatly.

This Application Note describes an investigation into the use of Benzonase® endonuclease during development of a primary recovery process for a recombinant Fab fragment from *E. coli* cells. The process has been designed to prepare a Fab-containing lysate as a suitable starting material for subsequent chromatographic purification employing cation exchange capture. The results clearly indicate that the use of Benzonase® endonuclease was essential in achieving high product yield. Precipitation loss of Fab during the primary recovery sequence was dramatically reduced.

Materials and methods

High-pressure cell homogenization

A pellet of frozen bacterial wet cell paste (wcp) was dissolved in a lysis buffer at approximately 20 °C (25 mM CH_3COONa, 25 mM NaH_2PO_4, 62 mM NaCl, and 5 mM $MgCl_2$ at pH 6.5, 10 mS/cm) supplemented with Benzonase® endonuclease at two different concentrations, as described below. The ratio of wcp (g) to buffer (mL) was 0.15:1.

A homogeneous cell suspension with a final temperature of 14 °C was obtained after mixing for 15 minutes without cooling, then further processed using a high-pressure homogenization system from Constant Systems Ltd. (model TS 1.1). Efficient release of the Fab from the cells was achieved in a single pass using a pressure of 1,500 bar. The temperature of the disruption chamber was kept in the range 4 – 10 °C using an external recirculating cooler.

Benzonase® endonuclease treatment for nucleic acid degradation

Benzonase® endonuclease of grade II purity (> 90 %) for biotechnology purposes from Merck Millipore (Cat. no. 1.01656) with a specific activity of 250 U/µL was used. The enzyme was added to the lysis buffer at the levels 25 U/mL, 2.5 U/mL, and 0 U/mL shortly before use.

After high-pressure homogenization, the crude cell extract was incubated for 1 hour at 10 – 15 °C to allow for the degradation of nucleic acids by Benzonase® endonuclease before the subsequent heat treatment step was started.

Heat treatment and clarification

Cell extracts were heat-treated in batch mode at 60 °C for 1.5 hours, then cell debris and heat-denatured host cell impurities removed by centrifugation (16,000 x g for 15 minutes at 20 °C). The final pH of the clarified lysate was in the range 6.2 – 6.3.

Lysate adjustment/Acidic precipitation and filtration

The clarified lysate was adjusted to pH 4.5 using 1 M hydrochloric acid. After being held at this pH for 1.5 hours (ambient temperature, no stirring), the final adjustment to pH 5.0 was made using 1 M sodium hydroxide. The lysate was incubated for 16 hours at 10 °C without stirring to allow for settling of the precipitate formed under acidic conditions, before final filtration of the supernatant using a filter train consisting of a pre-filter (1.2 µm normal flow depth glass fiber filter) followed by a sterile filter (polyethersulfone, 0.2 µm).

Concentration of Fab lysate

The Fab lysate was concentrated using tangential flow filtration (TFF) performed with Pellicon® XL 50 Ultrafiltration cassettes containing a 10 kD Ultracel® membrane (Merck Millipore Cat. No. PXC 101C50). During TFF, the transmembrane pressure (TMP) was kept at 0.7 – 0.8 bar by using a feed flow rate of approximately 520 L/m^2/h. The feed inlet pressure was 1.4 – 1.6 bar, permeate pressure was 0 bar (open valve), and the retentate set to 0 – 0.2 bar.

Analytical methods

The concentration of Fab fragment in lysate samples was monitored using analytical Protein A affinity chromatography which allowed the Fab to bind via the VH3 heavy chain. To prevent the affinity column from fouling, all samples were 0.2 µm filtered. Lysate samples without Benzonase® endonuclease or with only low levels (2.5 U/mL) were spiked with Benzonase® endonuclease (25 U/mL) and incubated for 15 minutes at room temperature to degrade genomic DNA prior to injection into the column.

Results & Discussion

Primary recovery process scheme

A major focus of this work was the development of a scalable process for the production of a Fab extract from *E. coli* cell paste that would be suitable for subsequent cation exchange capture chromatography.

The final primary recovery process encompassed a high-pressure homogenization step (HPH) for cell lysis, reduction of the nucleic acid level by enzymatic treatment using Benzonase® endonuclease, and a heat treatment step (HT) for the effective precipitation of host cell impurities in Fab lysates from *E. coli* as described by UCB Pharma.[5] A centrifugation step served to clarify the heat-treated lysate by removing precipitated host cell impurities and cellular debris. The sequence also included conditioning of the lysate to an acidic pH to allow the Fab to efficiently bind to the CEX capture resin and to ensure a reasonable binding capacity. Removal of the precipitate formed during acidic pH adjustment was achieved by filtration. As the final step, the lysate was concentrated using ultrafiltration (TFF), then subjected to chromatographic capture (Figure 1).

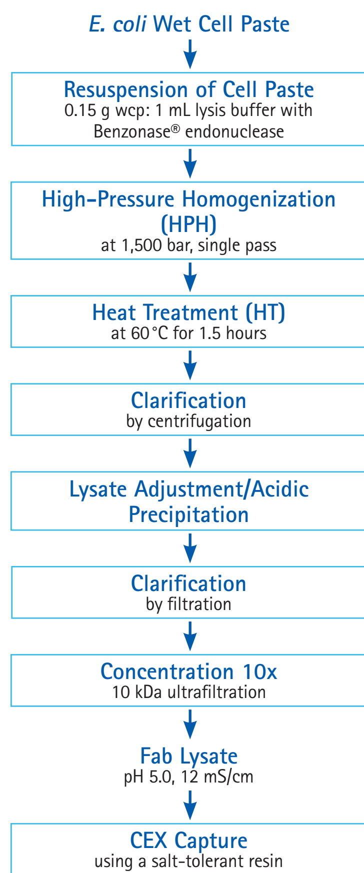

Figure 1. Primary recovery process scheme. Illustrates the sequence of unit operations required for the preparation of a Fab lysate feed stream suitable for the capture of Fab by cation exchange chromatography.

Initial developmental trials were conducted with Benzonase® endonuclease at a level of 25 U/mL, corresponding to the manufacturer´s recommendation. The focus during this stage was on solving the problem of Fab precipitation observed at acidic pH, which would prohibit efficient Fab capture by CEX chromatography. Here, the heat treatment step plays a particularly important role. Our studies showed that this step was essential in preventing massive Fab loss at acidic pH (Figure 2).

While the Fab titer and protein concentration in non-treated lysate dropped drastically with decreasing pH (Figure 2A), the Fab concentration in the heat-treated sample could be maintained at high levels, even at a low pH of 4.5 (Figure 2B). During the incubation at elevated temperature, most of the host cell impurities precipitated (> 80 %), while approximately 80 % of the Fab was recovered from this step. The precipitate could easily be removed during the subsequent clarification. The resulting lower impurity load of the lysate is an important benefit of this primary recovery process and simplifies the complete downstream purification of the Fab considerably.

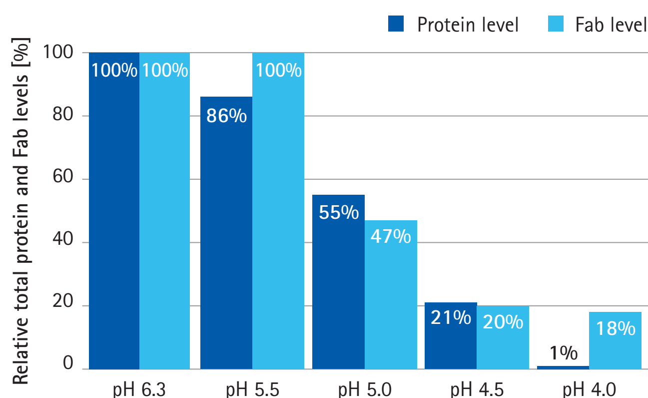

Figure 2A. Fab and total protein levels in an *E. coli* extract without heat treatment during pH adjustment. All percentages relate to the lysate after homogenization.

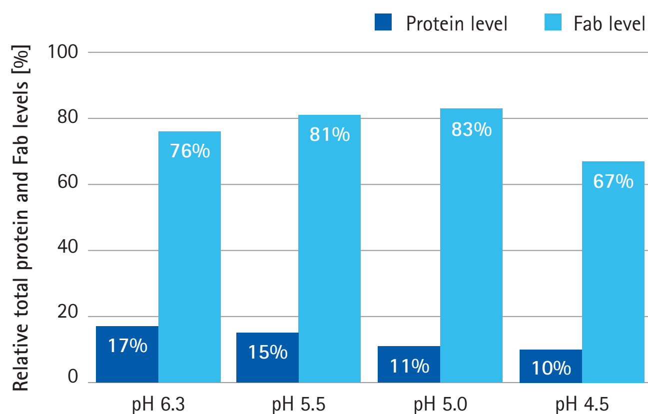

Figure 2B. Fab and total protein levels in a heat-treated *E. coli* extract (60 °C, 1 hour) during pH adjustment. All percentages relate to the lysate after homogenization.

Studies have shown that correctly folded Fab fragments are remarkably stable at elevated temperature, whereas incorrectly folded Fab species or free heavy or light Fab chains precipitate and form aggregates.[6]

The thermal stability of the Fab examined in this work was verified by monitoring the Fab titer and host cell protein (HCP) concentration in cell lysate at 60 °C over an exposure period of 5 hours (Figure 3). An approximate 15% decrease in the Fab titer at high temperature was observed during the first 15 minutes only, after which the Fab titer remained stable. This behavior supports the assumption that, in our work, the Fab fragments lost during the heat step were incorrectly assembled Fab species, not intact Fab.

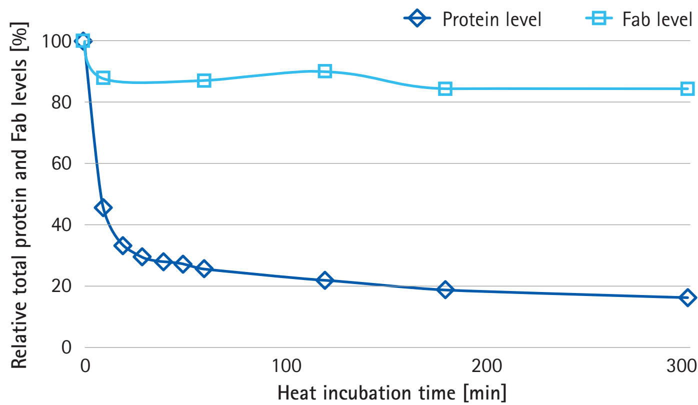

Figure 3. Total protein and Fab fragment concentrations during heat treatment (60 °C).

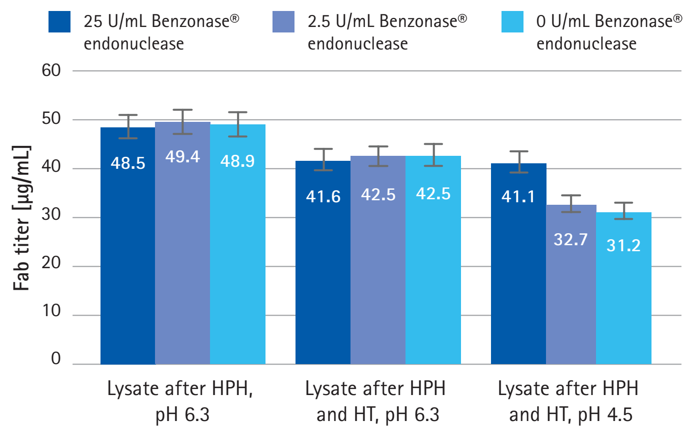

Figure 4. Feasibility study at different Benzonase® endonuclease levels (on 25 mL scale). Data show the titer of recombinant Fab in lysate samples after HPH, HPH+HT, and HPH+HT+ adjustment to acidic pH.

Effect of Benzonase® endonuclease on primary recovery of Fab

The primary recovery scheme described in Figure 1 was successfully applied to process *E. coli* cell paste at different lab scales (ranging from 0.1 kg up to 3 kg wcp) with comparable results. Table 1 summarizes the results from a lab-scale trial (approximately 0.1 kg wcp) including the final TFF step. Processing of the cell extract using lysis buffer with a standard Benzonase® endonuclease level of 25 U/mL delivered good results in terms of Fab yield. All clarification and filtration steps were performed routinely without any difficulties.

Sample	Volume [mL]	Fab titer [µg/mL]	Fab mass [mg]
Crude lysate after HPH	721	52	37.8
Crude lysate after HT	721	42	30.3
Clarified lysate after pH adjustment	649	36	23.3
Fab yield before TFF = 62%			
TFF feed material	605.0	36	21.7
TFF concentrate	65.5	299	19.6
TFF wash	10.2	77	0.8
TFF permeate	561.0	0	0.0
TFF recovery = 94%			

Table 1. Fab mass balance for the entire primary recovery sequence. 25 U/mL Benzonase® endonuclease in lysis buffer, 0.1 kg wcp processed.

With the aim of lowering cost, a smaller amount of Benzonase® endonuclease was considered, but only on the condition that the overall performance of the process would not be compromised. The feasibility of lysate preparation with a tenfold reduced amount and without any Benzonase® endonuclease addition was tested in a parallel experiment on a reduced scale (25 mL total volume). A stock solution of *E. coli* cell paste suspended in lysis buffer was split into three aliquots to which Benzonase® endonuclease was added at the specified concentrations. The three cell suspensions were homogenized and heat-treated under standard conditions, then the crude extracts clarified by centrifugation and subjected to pH adjustment, including being held at pH 4.5 for 30 minutes. Samples were taken from the individual steps and the Fab titer was assayed (Figure 4).

The data illustrate that under the conditions tested, the release of recombinant Fab from the cells was not affected by Benzonase® endonuclease. Almost the same concentration of soluble Fab in the homogenized cell suspensions was retained independently of the level of

Benzonase® endonuclease added to the lysis buffer. This holds also true for the exposure of lysates to elevated temperatures. Fab recovery after heat treatment was approximately 85% regardless of whether Benzonase® endonuclease was present or not.

A clear difference could be seen for lysates at acidic pH. While the Fab titer remained unchanged in the presence of 25 U/mL Benzonase® endonuclease, a distinct Fab loss of approximately 20% was observed in cell extracts containing only 2.5 U/mL or no Benzonase® endonuclease at all.

Although we did not explicitly measure lysate viscosity, we experienced that the crude cell extract prepared without Benzonase® endonuclease showed poor fluid properties. While the centrifugation of the lysate prepared with a reduced level of 2.5 U/mL Benzonase® endonuclease performed as usual under standard conditions, the solid/liquid phase separation of the lysate produced without any nucleic acid degradation was clearly impaired. During filtration of the supernatant phase recovered from centrifugation, the filter had to be exchanged several times due to rapid blocking. This was most probably caused by insufficient removal of cell debris and solid matter during centrifugation due to the higher viscosity and because of the high level of undigested nucleic acids. Based on this observation, the idea to run the process without any Benzonase® endonuclease was dismissed.

For direct comparison, we ran a complete sequence of the primary recovery process on a lab scale, processing approximately 0.1 kg wet cell paste using lysis buffer with a tenfold-reduced Benzonase® endonuclease level of 2.5 U/mL (Table 2).

Sample	Volume [mL]	Fab titer [µg/mL]	Fab mass [mg]
Crude lysate after HPH	740	59	43.9
Crude lysate after HT	740	51	37.6
Clarified lysate after pH adjustment	666	24	15.8
Fab yield before TFF = 36%			
TFF feed material	547.0	24	13.1
TFF concentrate	43.3	244	10.6
TFF wash	9.6	76	0.7
TFF permeate	498.0	0	0.0
TFF recovery = 86%			

Table 2. Fab mass balance for the entire primary recovery sequence using reduced Benzonase® endonuclease level (2.5 U/mL in lysis buffer).

As already indicated by the feasibility test on the 25 mL scale, lowering of the nuclease level caused a significant drop in the overall Fab yield before the final TFF step. While under standard conditions using 25 U/mL Benzonase® endonuclease, a Fab yield of 62% was achieved after acidic pH adjustment, the amount of Fab obtained with 2.5 U/mL Benzonase® endonuclease fell to 36%.

The flow behavior of both lysates during the final ultrafiltration was very similar (flux data not shown). However, Fab recovery from TFF was lower by 8% at the reduced Benzonase® endonuclease level of 2.5 U/mL, most probably caused by the reappearance of precipitate formation at the end of the concentration step. This additional drop resulted in an overall Fab yield of only 26% from crude lysate after HPH to TFF pool (concentrate plus wash) for the reduced nuclease level. By using the recommended level of 25 U/mL Benzonase® endonuclease, Fab recovery dramatically increased to 54%.

Conclusions

This work shows a new way of using Benzonase® endonuclease to improve microbial processes. It is demonstrated for microbial cell extracts containing a high level of genomic nucleic acids and a recombinant protein that the use of Benzonase® endonuclease opens up a way to prevent precipitation and concomitant product loss at acidic pH.

This study provides an example that the benefits of Benzonase® endonuclease go beyond the commonly known nucleic acid degradation effects of viscosity reduction and DNA removal. More detailed investigations of key Benzonase® endonuclease process parameters are ongoing.

References

1. Neelam Shahab et al. (1996). Growth rate control of protein and nucleic acid content in Streptomyces coelicolor A3(2) and Escherichia coli B/r. Microbiology, 142, 1927-1935
2. Nripen Singh et al. (2013). Clarification of recombinant proteins from high cell density mammalian cell culture systems using new improved depth filters. Biotechnol. Bioeng., 10, 1964–1972
3. A. Kumar et al., “Precipitation of Proteins”, in Isolation and purification of proteins, R. Hatti-Kaula and B. Mattiasson, Eds. (CRC Press, 2003)
4. Robert L. Fahrner et al. (2008). Polyelectrolyte precipitation and purification of proteins. US patent application 20080193981 A1
5. Mariangela Spitali et al. (2013). Protein purification. US patent application 20130184439 A1
6. A.N.C. Weir et al. (1994). Process for obtaining antibodies utilizing heat treatment. US patent 5665866

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