

Customer Application Note

Rapid Analysis of Recombinant Protein Production During Fermentation Using Dionex ProPac SCX Columns

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Introduction

Production of recombinant proteins using bacterial or mammalian cell culture involves lengthy fermentations that usually undergo very little monitoring of the protein that is expressed. During fermentation, the desired protein may suffer from delayed expression or modifications, yielding undesired variants or aggregates. Real-time monitoring of protein expression is difficult due to the requirement for time-consuming cell harvesting, protein extraction, and purification procedures prior to analysis. On a laboratory scale, the cost and implications of a failed fermentation are insignificant compared to the massive costs involved in industrial scale protein fermentations. Therefore, the development of a laboratory scale, real-time analysis workflow is extremely desirable.

Using strong cation-exchange chromatography, a reproducible method was developed for rapid analysis of recombinant lysostaphin expression in *Escherichia coli*. Lysostaphin is a glycyglycine endopeptidase that is secreted in *Staphylococcus staphylolyticus*. The extracellular enzyme is a zinc metalloprotease that can specifically degrade the peptidoglycan cell wall of staphylococcal strains. As a result, lysostaphin shows great potential as a novel antimicrobial agent for the treatment of blood-borne and biofilm associated infections caused by multidrug resistant staphylococcal strains. Prior characterization of recombinant lysostaphin is essential, however, to establish the homogeneity, stability, and potential immunogenicity of recombinant preparations. In fact, all therapeutic proteins produced by recombinant DNA technology must be thoroughly characterized to satisfy strict regulatory requirements.

Equipment

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Reagents and Standards

Using genomic DNA from *S. staphylolyticus* as a template, the mature lysostaphin ORF from lysostaphin precursor (glycylglycine endopeptidase) sequence (GenBank Accession Number X06121) was amplified using PCR. The gene was firstly cloned in pCR®-Blunt vector using the Zero Blunt® cloning system (Invitrogen Ltd., U.K.) and then subcloned in the pET vector cloning system using pET-28a vector (EMD4Biosciences, U.S.A.).

Recombinant pET vector DNA encoding N-terminal His-tagged recombinant lysostaphin was transformed into *E. coli* BL21(DE3) for protein expression. Transformed *E. coli* BL21 (DE3) was used to inoculate an LB starter culture, supplemented with 50 µg/ml kanamycin, which was incubated overnight at 37 °C and 200 rpm. The LB starter culture was then inoculated into a 1 L flask of LB media, supplemented with 50 µg/ml kanamycin. The inoculated LB media was incubated at 37 °C and 200 rpm until an OD₆₀₀ of 0.6 to 1.0 had been obtained. Once within the correct optical density range, protein expression was induced through the addition of 0.24 µg/mL IPTG and fermentation conditions were altered to 30 °C and 100 rpm.

At the point of induction, 30 mL of cells were aseptically removed from the 1 L fermentation and cell-free lysate was harvested by centrifugation at 4000 × G for 10 min. The resulting cell pellet was resuspended in 0.75 ml of SCX buffer A and sonicated at 14 mA for a total of 1 min using 10 s intervals. The sonicated cells were centrifuged at 24,000 × G for 10 min and 200 µL of the resulting cell-free extract was subjected to SCX analysis. Cell-free extract was harvested and analyzed at regular time points during the fermentation to monitor expression levels and charge heterogeneity of recombinant lysostaphin during expression.

Method

Chromatographic

Conditions:	UltiMate 3000 Titanium System			
Column:	Dionex ProPac SCX-10, 2.0 mm × 250 mm (P/N 063456)			
Mobile Phase:	A: Sodium phosphate (20 mM), pH 7.4 B: Sodium phosphate (20 mM), sodium chloride (1000 mM), pH 7.4			
Flow Rate:	0.2 mL/min			
Injection Volume:	200 µL			
Detection:	Absorbance at 214 and 280 nm			
Gradient:	Time (min)	Flow (mL/min)	% B	% C
	0.00	0.2	0.0	0.0
	0.10	0.2	0.0	0.0
	20.00	0.2	50.0	0.0
	22.90	0.2	50.0	0.0
	23.00	0.2	90.0	0.0
	26.00	0.2	90.0	0.0
	26.10	0.2	0.0	0.0
	37.00	0.2	0.0	0.0

Results

Recombinant lysostaphin is a basic protein that binds well to cation-exchange columns, even at pH 7.4. This trait is beneficial for analysis, as most cellular proteins do not bind to a strong cation-exchange [SCX] column at this pH. When hyperexpressed, recombinant lysostaphin can contribute approximately 50% of the protein content of the cell-free extract. The small percentage of *E. coli* cellular proteins that can bind to the SCX column at this pH are individually present in much smaller amounts than the lysostaphin; therefore, they do not interfere in the analysis. The ability to purify and analyze recombinant lysostaphin directly from cell-free lysate, without any further purification steps, is advantageous as it permits rapid monitoring as the fermentation progresses.

E. coli cultures of 500 to 1000 mL can yield from 50 to over 200 mg of protein, depending on how well the protein is expressed during fermentation. PAGE analysis of cell-free lysate harvested during fermentation demonstrates that high levels of recombinant lysostaphin are present from four hours after induction of protein expression (Figure 1). At this level of protein expression, harvesting small volumes of culture provides sufficient material for analysis. As little as 1 mL of culture can provide enough material for rapid analysis; however, if subsequent analyses are to be performed, then a greater sample concentration will be necessary.

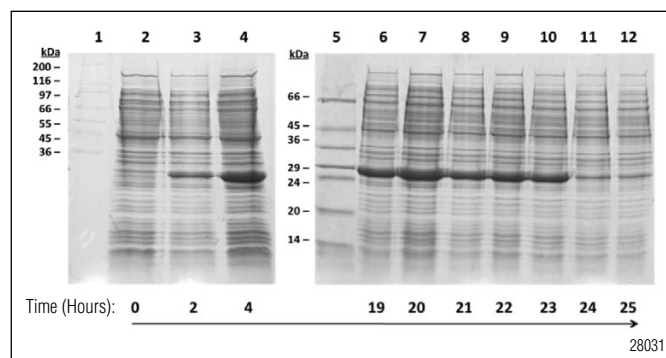


Figure 1. SDS-PAGE analysis of cell-free lysate harvested from an *E. coli* BL21 fermentation. Hyperexpression of recombinant lysostaphin was evident between 4 and 23 h post-induction. Recombinant lysostaphin has a molecular weight of 29.3 kDa. Lanes 1 and 5 contain protein molecular weight markers.

From the PAGE analysis of cell lysates harvested during the fermentation process, it can be seen that expression of recombinant lysostaphin started soon after induction of protein expression. Twenty-four hours after induction of protein expression, recombinant lysostaphin expression ceased and expressed protein was removed by cellular degradative processes. Recombinant lysostaphin has a molecular weight of 29.3 kDa and this was detected by PAGE analysis, which revealed the presence of a 29 kDa protein band throughout the fermentation. However, PAGE analysis does not show how recombinant lysostaphin alters during the fermentation, yielding different charge variants. These protein variants can be observed as they occur during fermentation by analyzing the cell lysate by cation-exchange chromatography using ProPac SCX-10 columns (Figure 2).

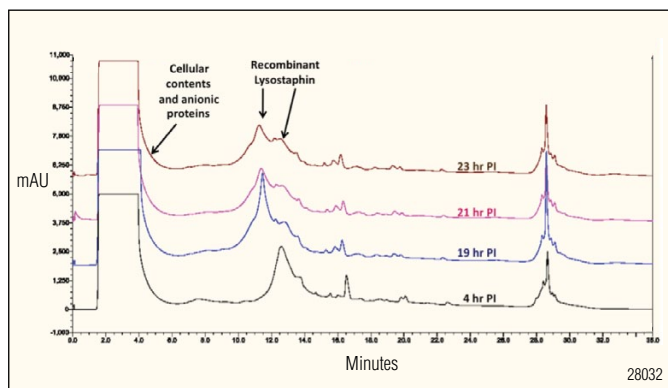


Figure 2. Comparison of SCX separations of recombinant lysostaphin from cell lysate. Chromatograms obtained by analysis of cell lysate at 4, 19, 21, and 23 h post-induction are shown.

Fermentation analysis revealed that recombinant protein expression can yield several protein variants, and that the abundance of each variant can alter dramatically over time. SCX analysis of cell lysate harvested between 4 and 19 h post-induction revealed protein isoforms displaying major differences in charge, which emphasizes the importance of real-time monitoring during fermentation. Although the protein variants differ in their charge, all of the fractions collected exhibited the expected enzymatic activity. In addition, each of the observed peaks showed no differences when analyzed by PAGE, reversed-phase HPLC, and size-exclusion chromatography. Therefore, recombinant lysostaphin produced in *E. coli* appears to display charge heterogeneity, which does not seem to be attributable to aggregation or significant alterations in molecular weight.

Conclusion

The recognition of structural variants is critical in the production of monoclonal antibodies and other therapeutic proteins. Structural variations have been previously identified using Dionex ProPac columns with a high-resolution, inert HPLC system.^{1,2} The work shown here, however, is an example of how such a system can be used for real-time monitoring of recombinant protein production. Charge heterogeneity of other cationic recombinant proteins can also be studied by direct rapid analysis of cell-free lysate during fermentation. It is possible that chromatographic analysis time can be reduced further, with a slight loss in resolution, by using a rapid analysis monolithic ion exchange column such as the ProSwift® SCX-1S 1 × 50 mm column (P/N 071977).

Reference

1. Dionex Corporation, *Analysis of Monoclonal Antibody Heterogeneity by Cation-Exchange Chromatography: Separation of C-Terminal Lysine Variants*. Application Note 127, LPN 1047, 2009, Sunnyvale, CA.
2. Weitzhandler, M.; Farnan, D.; Horvath, J.; Rohrer, J. S.; Slingsby, R. W.; Avdalovic, N.; Pohl, C. Protein Variant Separations by Cation-Exchange Chromatography on Tentacle-Type Polymeric Stationary Phases. *J. Chromatogr., A* **1998**, *828*, 365–372.

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