[12] Preparation of Extracts from Prokaryotes

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The preparation of lysates is one of the more critical steps in the purification process, since this step influences the total quantity of the desired protein recovered, the biological activity of the protein, the completeness of its primary sequence by avoidance of proteolytic degradation, its association with other cellular components, and the presence of contaminants that may influence subsequent purification steps.

A number of variables determine the success of a lysis method. The degree of lysis and retention of biological activity may be influenced by strain differences, choice of growth media, whether the cells are processed immediately or frozen, the presence of protease inhibitors, the choice of buffers, resuspension densities, osmolarity of the resuspension buffer, and growth phase at which the cells are harvested. A trial-and-error approach is often required to optimize lysis conditions.

Enzymatic Lysis

Enzymatic lysis methods minimize denaturation, are scale independent, and allow some selectivity in the release of cellular products. The drawbacks to enzymatic methods include the large number of variables that can influence lysis and the addition of substances that may complicate subsequent purification steps.

Enzymatic methods work through digestion of bacterial structural components. In general, enzymatic lysis methods expose the inner, cytoplasmic membrane by degrading the peptidoglycan cell wall with degradative enzymes. The cytoplasmic membrane is breached by solubilization in detergent, osmotic pressure, shear, or by mechanical disruption.

Enzymatic Lysis of Gram-Positive Bacteria

Lysis of gram-positive cells is relatively straightforward, with a few exceptions. For example, lysozyme works poorly on *Staphylococcus aureus;* lysostaphin is used in its place.[1]

[1] J. O. Cohen, ed., "The Staphylococci." Wiley, New York, 1972.

Procedure for Lysing Bacillus subtilis[2]

1. Resuspend cells 50% (w/v)[3] in 50 m*M* Tris–HCl (pH 7.6), 10% sucrose, 1 m*M* dithiothreitol.
2. Add lysozyme to 300 μg/ml, EDTA to 1 m*M*, and Brij 58 to 0.1% final concentration.
3. Incubate on ice for 1 hr.
4. Centrifuge for 30 min at 40,000 *g* to remove cellular debris.

Enzymatic Lysis of Gram-Negative Bacteria

Degradation of the peptidoglycan in gram-negative cells is made more difficult by the presence of an asymmetric lipid bilayer. The outer membrane is external to the peptidoglycan[4] and acts as a permeability barrier to large molecules. Thus, gram-negative bacteria are less susceptible than gram-positive bacteria to lysozyme and detergents.[5] Enzymatic lysis of gram-negative bacteria requires the outer membrane to be permeabilized to expose the peptidoglycan layer to attack.

Permeabilizing Outer Membrane. The permeability barrier posed by the outer membrane arises, in part, from the presence of lipopolysaccharide (LPS) on the outer leaf of the lipid bilayer. The polyanionic LPS molecules have strong lateral interactions in the presence of divalent cations, such as Mg^{2+}, which are postulated to neutralize electrostatic repulsion between the LPS molecules.[6] Substances which permeabilize the membrane and release LPS include chelators of divalent cations (e.g., EDTA), polycationic species, and various small molecules (e.g., Tris).

Tris, often used as a buffer in lysis methods, effectively releases LPS and permeabilizes outer membranes.[7] Treatment of *Staphylococcus typhimurium* with Tris buffer alone (0.1 *M*, pH 7.2) releases 20% of the LPS.[8] Ammonium glycylglycinate exerts a permeabilizing effect similar to Tris, but TES, HEPES, Bicine, and phosphate buffers do not potentiate lysozyme lysis in *Escherichia coli*.[5] The permeabilizing effect of Tris can be enhanced by the addition of EDTA; treatment of *E. coli* cells with EDTA in Tris buffer liberates about one-half of the LPS.[5]

[2] M. A. Penlava and M. Salsa, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5522 (1982).
[3] Throughout this chapter, all concentration percentages are given as weight/volume unless otherwise noted. Cell weights are given as total packed wet weight.
[4] H. Nikaido and M. Vaara, *Microbiol. Rev.* **49**, 1 (1985).
[5] L. Lieve, *Ann. N.Y. Acad. Sci.* **235**, 109 (1974).
[6] M. A. Asbell and R. G. Eagon, *J. Bacteriol.* **92**, 380 (1966).
[7] R. T. Irvin, T. J. MacAlister, and J. W. Costerton, *J. Bacteriol.* **145**, 1397 (1981).
[8] H. Nikaido and M. Vaara, *in* "*Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology" (J. L. Ingrahm, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, eds.), p. 14. Am. Soc. Microbiol., Washington, D.C., 1987.

Digestion of Cell Wall. Egg-white lysozyme, which hydrolyzes *N*-acetylmuramide linkages, is most often used to degrade bacterial cell walls. Occasionally, T4 lysozyme will be used on *E. coli*. T4 lysozyme is more *E. coli* specific than the egg white lysozyme, but it is difficult to obtain.[9]

The activity of lysozyme is influenced by the pH and ionic strength. Hen egg white lysozyme has been shown to be active over a wide pH range (pH 4 to 10), but is most commonly used in the pH range of 6.7 to 8.6.[10] Lysozyme requires an ionic strength of at least 0.01 for 50% of maximum activity regardless of the pH. However, the upper range of ionic strength at which lysozyme retains 50% of maximum activity decreases with increasing pH. At pH 6.2, this upper limit occurs at 0.14, decreases to 0.09 at pH 8.0, and to 0.07 at pH 9.2. Lysozyme has close to optimum activity over a wide pH range at an ionic strength of 0.05.[11]

Gentle Enzymatic Lysis Method for Escherichia coli.[12,13] All manipulations should be performed in a cold room at 4° using prechilled equipment. The cells used in the described procedure were grown in rich medium, harvested at midlogarithmic growth, resuspended in Tris–sucrose buffer (50%), and poured into liquid N_2 with a circular motion to give cell clumps the consistency of popcorn.

Solutions

Tris–sucrose buffer: 50 m*M* Tris–HCl (pH 7.5), 10% sucrose
Lysis solution: 0.3 *M* spermidine–HCl, 2 *M* NaCl, 10% sucrose; adjust to pH 7.5

Procedure

1. Warm 750 ml Tris–sucrose buffer + 75 ml lysis solution (825 ml total volume) to 37° in a 2-liter beaker.
2. Weigh out 600 g of frozen, resuspended *E. coli* cells that have been at −20° for at least 24 hr (300 g cells total).
3. Quickly add frozen cells to Tris–sucrose–lysis solution prewarmed to 37° while stirring with an overhead stirrer. The temperature quickly drops to around 0°.
4. Adjust to pH 7.5 with 2 *M* Tris base. Monitor pH using narrow-range pH stick.
5. Once a thawed homogeneous mixture is achieved, add 300 mg lyso-

[9] M. Tsugita, M. Inouye, E. Terzaghi, and G. Streisinger, *J. Biol. Chem.* **243**, 391 (1968).
[10] P. Jolles and J. Jolles, *Mol. Cell. Biochem.* **63**, 165 (1984).
[11] R. C. Davies, A. Neuberger, and B. M. Wilson, *Biochim. Biophys. Acta* **178**, 294 (1969).
[12] W. Wickner and A. Kornberg, *J. Biol. Chem.* **249**, 6244 (1974).
[13] C. S. McHenry, *J. Biol. Chem.* **257**, 2657 (1982).

zyme dissolved in a minimal amount of Tris–sucrose buffer, rapidly mixing to disperse the lysozyme. *Caution:* It is important that all ice crystals have melted at this step to achieve efficient lysis.

6. Pour the resulting suspension immediately into centrifuge bottles and place on ice for 1 hr.
7. Then, swirl centrifuge bottles in a large 37° water bath for 4 min, gently inverting every 30 sec.
8. Place immediately on ice.
9. Centrifuge at 23,000 *g* for 1 hr at 4°.
10. Decant and save supernatant.

The resulting supernatant should contain 20 mg protein/ml if lysis is complete. If cells are difficult to lyse, a more vigorous lysis can be obtained by adding EDTA to the Tris–sucrose solution (5 to 10 m*M* final concentration) or by increasing the heating step for up to 15 min.

Addition of Freeze–Thaw Step.[14] A freeze–thaw step is added to some procedures after the incubation with lysozyme; the freeze–thaw procedure is more effective on spheroplasts than whole cells.

For the procedure described below, cells were grown to late-logarithmic phase in rich medium, harvested, and resuspended 50% in 50 m*M* Tris–HCl (pH 8.0), 10 m*M* EDTA, 10% sucrose. Resuspended cells were frozen in a dry ice/ethanol bath and stored at −70°.

Procedure

1. Thaw 300 g (600 ml) frozen cells on ice.
2. Transfer to centrifuge tubes and adjust to 140 m*M* NaCl, 1 m*M* dithiothreitol, and 0.4 mg/ml lysozyme.
3. Place mixture on ice for 45 min, mixing gently every 15 min.
4. Quick-freeze cells in a dry ice–ethanol bath.
5. Thaw frozen cells in a 37° bath with gentle mixing.
6. Repeat freeze–thaw four times.
7. Centrifuge lysate at 23,000 *g* for 2 hr to remove cell debris.

Simple Vigorous Lysis Method for Lysing Escherichia coli.[15] The first two enzymatic lysis methods are relatively gentle because of the addition of sucrose to stabilize the spheroplasts against osmotic shock. This next method uses osmotic shock to help lyse the cells.

Procedure

1. Thaw 300 g of cell paste and resuspend in 300 ml lysis buffer [50 m*M* Tris (pH 7.5), 200 m*M* NaCl, 5% glycerol (v/v), 1 m*M* dithiothreitol, 1 m*M* phenylmethanesulfonyl fluoride (PMSF)].

[14] E. R. Wood and S. W. Matson, *J. Biol. Chem.* **263**, 15270 (1988).
[15] C. I. Pao, T. E. Lee, Y. D. Liao, and C. W. Wu, *J. Biol. Chem.* **263**, 10295 (1988).

2. Add lysozyme to 300 μg/ml and incubate at 4° for 1 hr.
3. Add $MgCl_2$ to 10 mM and treat with DNase I at 4° for 30 min.
4. Remove cell debris by centrifuging at 15,000 g for 1 hr.

Notes on Enzymatic Lysis Procedures

1. Test the degree of spheroplast formation quickly and easily during the course of the lysis by adding 0.1 ml of the lysis mixture to 2 ml distilled water and measuring turbidity at OD_{590} in a spectrophotometer.[16]

2. Occasionally, inclusion of detergent in the lysis procedure increases the yield of some enzymes.[17] The polyoxyethylene detergents [e.g., Brij 58, Nonidet P-40 (NP-40), and Triton X-100 at ca. 0.5%] are most commonly used in lysis methods because they are nonionic and relatively nondenaturing.[18]

3. Some enzymes are sensitive to freezing. In these cases it may be best to resuspend the cell paste immediately upon harvesting with cold buffer, and use the more vigorous lysis procedures.

Mechanical Lysis Methods

Mechanical lysis methods have several advantages over enzymatic lysis techniques: (1) Mechanical lysis methods do not require the addition of chemicals; (2) strain differences are minimized; and (3) they are economical for large-scale preparations. However, mechanical force is translated into heat which must be carefully controlled, and foaming must be controlled to prevent surface denaturation and oxidation. Mechanical methods, if too rigorous, may form fine cellular debris which may hinder subsequent processing. Mechanical methods release DNA which often must be removed.

Mechanical lysis methods fall into two broad categories: Agitation with abrasives and liquid shear methods.

Agitation with Abrasives

The bead mills represent the most common example of agitation with abrasives. The Dyno-Mill (manufactured by Willy A. Bachofen AG, Switzerland) is a widely used apparatus. The Dyno-Mill is a horizontal chamber filled to 80–85% with lead-free glass grinding beads. Inside the chamber, a shaft fitted with disks imparts a rotation to the glass beads. The glass beads rupture the cells by a combination of high shear and

[16] M. H. Malamy and B. L. Horecker, *Biochemistry* **3**, 1889 (1964).
[17] G. N. Godson and R. L. Sinsheimer, *Biochim. Biophys. Acta* **149**, 476 (1967).
[18] M. D. Womack, D. A. Kendall, and R. C. MacDonald, *Biochim. Biophys. Acta* **733**, 210 (1983).

impact with the cells. The suspension of broken cells exits the machine but the beads are retained. Thermal control is maintained by pumping coolant through the chamber jacket.

Factors which influence the efficiency of lysis include rate of agitation, cell concentration, concentration of glass beads, diameter of the beads, residence time in the chamber, and temperature. All these factors may need to be determined empirically.

Starting Conditions for Lysing Escherichia coli in Dyno-Mill Model KDL[19]

1. Resuspend harvested cells at 30–60% in ice-cold buffer and maintain at 0–5°.
2. Glass or stainless steel grinding chambers are available, but the 0.6-liter stainless steel chamber is preferred for bacteria because it has better thermal conductance than the glass chamber. Load the chamber to 0.5 liter (80% of the chamber volume) with 0.2-mm-diameter lead-free glass beads.
3. Pump the cell suspension through the machine at a rate of 4 to 6 liters/hr. This will give a residence time in the chamber of around 1 to 2 min.
4. Rotational speed of the disks is measured at their tips, and a tip speed of 10 m/sec should give a good agitation of the beads.
5. Refrigerate the jacket coolant to −20°. Because of the heat generated by the mechanical action of the beads, it is important that the cell suspension mixture be held as cold as possible.

These initial conditions should give an effluent temperature of less than 10° and lysis of 65–85% of the cells. Because small particles may affect purification, multiple passes and long residence times should be avoided.

Liquid Shear Lysis

The most common devices using liquid shear to lyse bacteria are the Manton–Gaulin press and the French press. Presses lyse cells by pressurizing the cell suspension and suddenly releasing the pressure. The release of pressure creates a liquid shear capable of lysing the cells. Since the efficiency of lysis is highly dependent on the operating pressure,[20] high pressures should be utilized to minimize the number of passes required. Manton–Gaulin presses typically operate with feedstream pressures of

[19] S. Goldberg, Glen Mills, Inc., personal communication.
[20] E. Bjurstrom, *Chem. Eng.* **92,** 126 (1985).

only 6000–8000 psi. Multiple passes are generally required to achieve an adequate lysis.[20] French presses are capable of operating at higher pressures, although for *E. coli* and *S. typhimurium* the pressures used are frequently in the range of 7000 to 10,000 psi.

High operating pressures requires measures to compensate for a rise in operating temperatures; a pressure differential of 1000 psi raises the temperature by 1.5°.[20] In addition to controlling temperature, care should be taken to avoid inactivating proteins by foaming.

In contrast to the bead mill, the efficiency of cell lysis in homogenizers is independent of cell suspension concentration. A typical resuspension density of 50% is used.[20]

Sonication. Sonication lyses cells by liquid shear and cavitation. Sonication remains a popular technique for lysing small quantities of cells, but is of limited value for cell quantities in the 50-g to 1-kg range because of the difficulty in maintaining low temperatures. The problem of controlling temperature during sonication is addressed by using pulses of 30 to 45 sec in duration, with pauses to reestablish a low temperature in the sonicate. Sometimes lysozyme is added to sonication mixtures in order to minimize exposure of the sample and increase the efficiency of the lysis.

Nucleic Acid Removal

The first enzymatic lysis method given for *E. coli* yields a lysate free of the bulk of chromosomal DNA. This is due to a very gently lysis where holes are created in the membrane large enough for large proteins to escape, but small enough to contain the spermidine-condensed nucleoid. Other lysis methods described in this chapter cause release of nucleic acids. These must often be removed, either because of viscosity problems, interference with assays, or due to their serving as a polyelectrolyte that interferes with subsequent chromatographic steps. Often, RNA and DNA are removed by treatment with RNases and DNases. Alternatively, nucleic acids can be removed by phase partitioning[21] or by treatment of extracts with polyethyleneimine.[22]

[21] R. Kula, *in* "Protein Purification: Micro to Macro" (R. Burgess, ed.), p. 99. Alan R. Liss, New York, 1987.

[22] R. Burgess and J. Jendrisak, *Biochemistry* **14**, 4634 (1975).