Separation, Recovery, and Purification in Biotechnology

Recent Advances and Mathematical Modeling

Edited by
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Separation, Recovery, and Purification in Biotechnology

Recent Advances and Mathematical Modeling

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FOREWORD

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PREFACE

ONE OF THE MOST DIFFICULT and challenging problems facing large-scale biotechnology today is to find and develop appropriate recovery, separation, and purification processes. The area of large-scale bioseparations is one to which biologists, physical biochemists, and particularly biochemical engineers have important contributions to make. Some of the most recent advances and developments that have already started to find practical applications are

- membrane separations, including the use of membrane bioreactors and liquid emulsion membranes;
- continuous or semicontinuous chromatographic separations, including the use of a number of affinity methods and monoclonal antibodies;
- two-phase extraction processes such as aqueous systems and the use of reverse micelles;
- precipitation techniques;
- electrically driven separation processes;
- methods of product secretion, cell permeation, disruption, and selective enzymatic lysis of microbial cells for intracellular product release;
- product solubilization and renaturation of proteins or polysaccharides present in inclusion bodies or granules.

This book covers several of the emerging areas of separations in biotechnology and is not intended to be a comprehensive handbook. It includes recent advances and latest developments in techniques and operations used for bioproduct recovery in biotechnology and applied to fermentation systems as well as mathematical analysis and modeling of such operations. The topics have been arranged in three sections beginning with product release from the cell and recovery from the bioreactor. This section is followed by one on broader separation and concentration processes, and the final section is on purification operations. The operations covered in these last two sections can be used at a number of different stages in the downstream process.

A crucial question remaining is how to design a flowsheet or product recovery operation sequence. Three main points to keep in mind are (1) integrating recovery with the fermentation system, (2) integrating the different separation and purification stages to design the optimum sequence, and (3) assessing the possibility of a continuous operation.

Revised versions of papers presented in the symposium upon which this book is based as well as papers presented in other sessions that were relevant

to the topic have been included in this volume. In addition, we have included a few keynote chapters on areas we felt had not been well covered at the meeting.

We gratefully acknowledge the assistance of many reviewers who helped us with critical and constructive comments on the original manuscripts. We would also like to acknowledge the support and well-organized help of the staff at the ACS Books Department.

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CONTENTS

PRODUCT RELEASE AND RECOVERY

1

Protein Release from Chemically Permeabilized *Escherichia coli*

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An important factor complicating the recovery of recombinant proteins from Escherichia coli is their intracellular location. An alternative to the commonly used method of releasing these proteins by mechanical disruption is to chemically permeabilize the cells. The objective of this research was to characterize the protein release kinetics and mechanism of a permeabilization process using guanidine-HCl and Triton-X100. The protein release kinetics were determined as a function of the guanidine, Triton, and cell concentrations. Some of the advantages over mechanical disruption include avoidance of extensive fragmentation of the cells and retention of the nucleic acids inside the cell structure.

The recent development of recombinant DNA technology has made it feasible to produce interferon, human growth hormone, insulin, and other proteins in the bacterium Escherichia coli. An important factor complicating the recovery process is the retention of the protein product inside the microbial cell. This has necessitated the development of processes capable of releasing protein from E. coli. Protein release on an industrial scale is commonly achieved by mechanically breaking the cell in a high pressure homogenizer or a ball mill. Disruption in a high pressure homogenizer is caused by pressure gradients established when a pressurized cell suspension is forced through a narrow orifice, whereas with a ball mill, disruption is caused by shear forces generated by grinding the cells with abrasive particles (1).

These mechanically based protein release methods have several undesirable properties. One problem is that extensive fragmentation of the cells makes the subsequent centrifugation difficult (2,3). Adding to the problem of cell fragment removal is the high viscosity imparted to the solution by the released nucleic acids (4). A nucleic acid removal step is necessary to decrease the solution viscosity and avoid potential interference with fractional precipitation and chromatography (5). Another undesirable property is that the harsh action of mechanical disruption causes the release

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of nearly all the soluble cellular protein. Extensive purification schemes are required to isolate the product from these extraneous cellular proteins.

One alternative to mechanical disruption is to treat the cells with membrane active compounds that can permeabilize the cell to protein without causing extensive breakage of the cell. The objective of this research was to study the protein release kinetics and mechanism of a permeabilization process using guanidine-HCl and Triton-X100. Guanidine-HCl, a chaotropic agent, has been demonstrated to be capable of solubilizing protein from E. coli membrane fragments (6). Presumably, this occurs via guanidine's interaction with water which allows hydrophobic groups to become thermodynamically more stable in an aqueous phase (7). Triton-X100, a nonionic detergent that has a high binding affinity for hydrophobic species, is very effective in binding to and solubilizing phospholipids from E. coli inner membrane and outer wall fragments (8).

Methods

Cell preparation. Escherichia coli K12, strain W3110, was grown in a 14 liter fermenter at 37°C, pH 7.0 using defined media. Additional nitrogen was supplied by NH_4OH which was automatically fed to control the pH. The fermentation broth was harvested in the late exponential phase and cooled to 4°C. The cells were immediately centrifuged at 4°C and washed with buffer (.1M Tris, pH 7.0). Following a second centrifugation, the cells were resuspended in buffer to give a dense cell suspension ($\sim$50 g protein/l).

Cell permeabilization. The permeabilization process was started by adding 30 ml of the cell suspension to 70 ml of a buffered solution containing guanidine-HCl and/or Triton X100. The reported concentrations of Triton, guanidine, and cells always correspond to the concentrations after mixing these solutions. The mixture was shaken at 200 rpm in a 4°C incubator. Samples were withdrawn at various times and were immediately centrifuged. The supernatant was assayed to determined the release of the various cell components. Analysis of the pellet was done to perform a mass balance.

Analysis of cell components. Protein was determined with the Bradford dye binding assay using bovine serum albumin as standard (9). Interference by Triton X100 was accounted for by ensuring that every sample had .2% Triton. In order to determine the amount of unreleased protein from the sample pellets, all samples were treated for 5 minutes with 1N NaOH at 100°C.

DNA was determined by the diphenylamine reaction (10). Two 45 minute extractions at 70°C with .5N $HClO_4$ were used to release DNA from the sample pellets. Interference from guanidine was accounted for by making each sample .4M guanidine.

RNA was determined by the orcinol procedure (11). Two 15 minute extractions at 70°C in .5N $HClO_4$ were used to release RNA from the sample pellets. Interference from Triton X100 was accounted for by making each sample 1% Triton.

Results and Discussion

Figure 1 shows the protein, DNA, and RNA release profiles obtained when E. coli cells are mechanically disrupted with .1 mm glass beads. The cell concentration profile, normalized to the initial cell concentration, was obtained with a bacterial counting chamber. The decrease in the cell concentration indicates that extensive fragmentation of the cells is occurring. A nearly mirror image release of DNA, RNA, and protein results as cellular components spill out into the extracellular fluid. The maximum protein release, 70%, is probably indicative of a significant amount of cellular protein being associated with the membrane and wall fragments.

A similar characterization for cells treated with 2M guanidine and 2% Triton is shown in Figure 2. The protein release, based on total cellular protein, levels off at ~35%. RNA is released to a lesser extent (~15%) and very little DNA (~5%) is released from the cells. The constant cell concentration indicates that the release is not the result of cell fragmentation.

From these results, three major differences between chemical permeabilization and mechanical disruption can be identified. First, the release occurs by fundamentally different mechanisms. With mechanical disruption the cells are essentially torn apart, whereas with chemical treatment the cell structure is still present but has been altered to allow release of intracellular components. Second, there is a nearly complete preferential release of protein over DNA. Third, there is a partial selective release of protein over RNA. This selectivity may result from a molecular sieving mechanism. The average protein molecular weight is 40,000 whereas the cellular DNA has a molecular weight of 2.5×10^8 (12). The molecular weight distribution of RNA; 18% is 25,000, 27% is 500,000, and 55% is 1,000,000 is such that most of the RNA is also significantly larger than proteins (12).

These differences suggest several advantages of the chemical permeabilization method. First, avoiding cell breakage should simplify the cell removal step. Second, retention of the nucleic acids inside the cell should eliminate the need for a nucleic acid precipitation step. Another advantage is that the permeabilization process also kills the cells thereby eliminating the need for the federally mandated cell killing step.

Figure 2 showed that ~ 35% of the total cellular protein is released upon treating the cells with 2M guanidine and 2% Triton. A more complete description of the effect of varying the guanidine and Triton concentrations on the final amount of protein released is shown in Figure 3. Two sets of extractions were conducted: one consisted of using 2% Triton with a range of guanidine concentrations, the other consisted of using 2M guanidine with a range of Triton concentrations. These results indicate that the guanidine-HCl concentration is the more sensitive parameter. Manipulation of the guanidine concentration in the presence of 2% Triton lead to release yields that ranged from 6% to 60% whereas varying the Triton concentration from 0% to 8% in the presence of 2M guanidine only changed the yield from 25% to 40%.

The time profiles of the 2M/2%, 2M, and 2% treatments, shown in Figure 4, indicate a synergistic effect between guanidine and Triton. The protein release profile of the 2M/2% treatment is not simply the

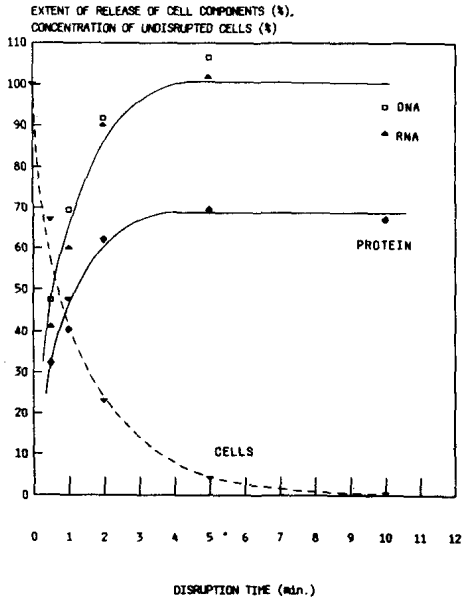

Figure 1. Extent of cell breakage and release of cellular protein, DNA, and RNA during mechanical disruption with .1 mm glass beads.

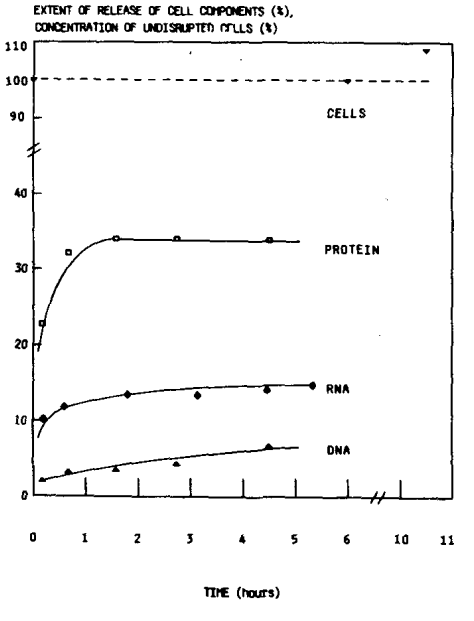

Figure 2. Release of cellular protein, DNA, and RNA during treatment with 2M guanidine HCl and 2% Triton X100.

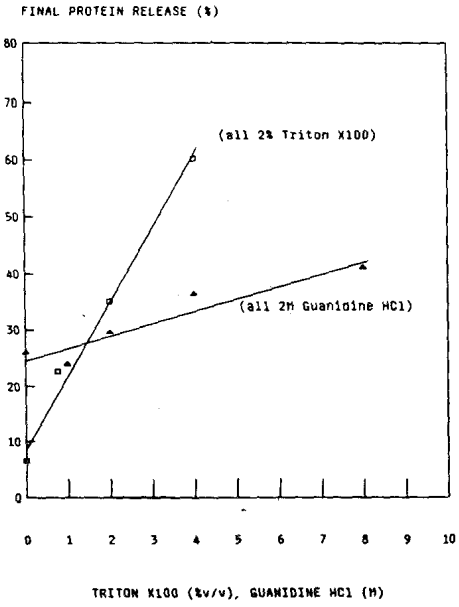

Figure 3. Effect of Triton X100 and guanidine HCl on the protein release yield.

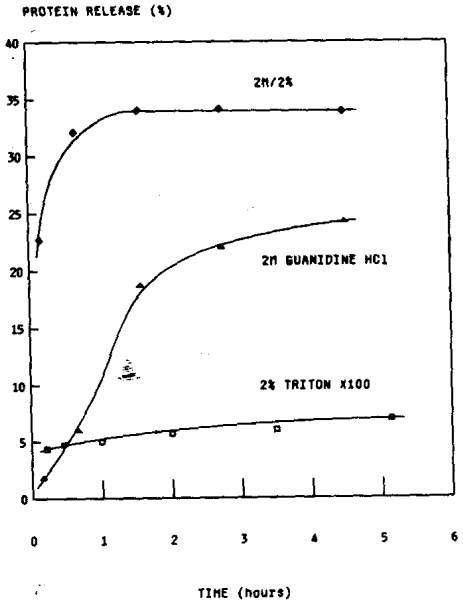

Figure 4. Synergistic effect on the protein release profile between guanidine HCl and Triton X100.

addition of the profiles obtained when 2M guanidine and 2% Triton are used individually. The acceleration of the rate of protein release by Triton may be related to the ability of Triton to solubilize lipid membranes. One would anticipate that the combination of 2M guanidine and 2% Triton alters the E. coli inner membrane and outer wall to a greater extent than either individual treatment, thereby producing a more permeable cell.

The effect of varying the cell concentration on the protein release profile of 2M/2% treatments is shown in Figure 5. The cell concentrations are expressed in terms of the protein concentration of the extraction solution. Although no significant effect was observed on the release profile, the release yield decreased by a factor of two upon increasing the cell concentration from 3.6 g/l to 43.3 g/l. The exact nature of the reason for the decreased yield at high cell concentrations is not known. However, depletion of the guanidine and/or Triton during the process is not occurring, as evidenced by the fact that treating cells a second time with fresh guanidine and Triton does not induce additional release (data not shown). If depletion of the guanidine and/or Triton caused the protein release to cease, one would expect that a second treatment would cause further release of protein from the partially affected or as yet unaffected cells.

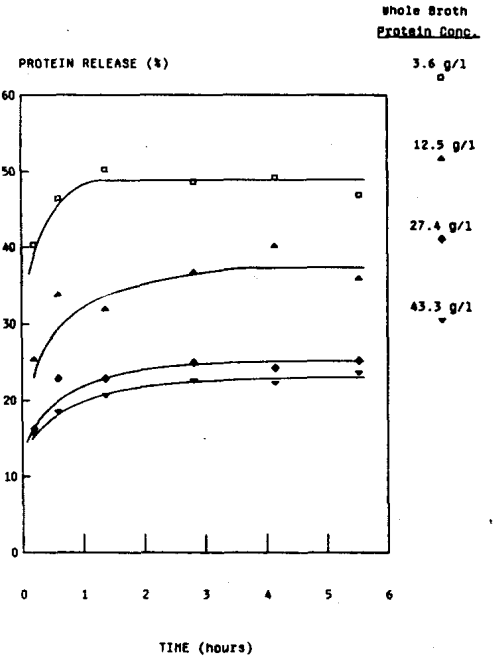

Figure 5. Effect of cell concentration on the protein release profile.

Conclusions

Exposure of E. coli to guanidine-HCl and Triton-X100 induces the release of cellular proteins. The release rate and yield were found to be dependent on the guanidine, Triton, and cell concentrations. Higher concentrations of guanidine and Triton and lower cell concentrations gave greater release rates and yields. Guanidine alone is capable of releasing a significant amount of protein. Triton releases a very low level of protein but substantially increases the rate of release when used in conjunction with guanidine.

The mechanism of the release, a permeabilization of the cell, is fundamentally different from mechanical disruption which involves extensive fragmentation of the cells. The avoidance of extensive cell breakage should simplify the cell removal step and retention of the nucleic acids inside the cell should eliminate the need for a nucleic acid precipitation step. Furthermore, since the treatment kills the cells, a separate cell killing step may be unnecessary.

Acknowledgment

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