Protein Release from *Escherichia coli* Cells Permeabilized with Guanidine–HCl and Triton X100

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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 of a permeabilization process using guanidine–HCl and Triton X100. The protein release rate and yield were determined as a function of the guanidine and Triton concentrations. The initial release rate increased monotonically with increasing concentrations of Triton and guanidine whereas the release yield varied in a complex manner. Electron microscopy indicated that the permeabilization process involves a solubilization of the inner membrane and molecular alteration of the outer wall. Some advantages of this process over mechanical disruption include avoiding extensive fragmentation of the cells and retainment of nucleic acids inside the cell structure.

INTRODUCTION

The development of recombinant DNA technology has made it feasible to produce a wide range of valuable protein products in the bacterium *Escherichia coli*. An important factor complicating the recovery process is retention of the product inside the microbial cell. This has necessitated the use 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. These mechanically based protein release methods have several undesirable properties. One problem is that extensive fragmentation of the cells can make the subsequent clarification difficult.[1,2] Adding to the problem of cell fragment removal is the high viscosity often imparted to the solution by the released nucleic acids.[3] A nucleic acid removal step is usually necessary to accomplish one or more of the following: decrease the solution viscosity, avoid potential interference with fractional precipitation and chromatography,[4] and lower the DNA concentration in the final product to an acceptable level. Another undesirable prop-

erty is that the harsh action of mechanical disruption causes the release of nearly all soluble cellular protein. Extensive purification schemes may be required to isolate the product from these extraneous cellular proteins.

Since protein release is the first recovery process, it impacts all downstream steps. Consequently, it would be advantageous to optimize the release step with criteria that keep this relationship in mind. The ideal protein release process would give a selective and rapid release of the product from the cell. Undesired cell components such as nucleic acids and native cell proteins would be retained in the cell. The resulting solution could be readily clarified by filtration or centrifugation because the cells have not been fragmented. Obviously, no protein release step could result in a pure product, but it may be possible to develop a process which approximates the ideal release characteristics to a greater extent than mechanical disruption methods.

One alternative to mechanical disruption is to utilize chemicals which interact with the cell structure in a manner which causes it to become permeable. An excellent review of a wide range of chemicals that can alter the permeability of many microorganisms can be found in Felix.[5] In this article, we examine the potential of guanidine–HCl and Triton X100 to release proteins from *E. coli*. Guanidine–HCl, a chaotropic agent, has been demonstrated to be capable of solubilizing protein from *E. coli* membrane fragments.[6] 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.[7] Consequently, guanidine–HCl and Triton X100, by interacting with the *E. coli* cell structure, could lead to an effective permeabilization method.

MATERIALS AND METHODS

Cell Growth

Escherichia coli K12, strain W3110, was maintained in a 50% glycerol solution at $-15°C$. A portion of the glyc-

erol stock culture was used to inoculate 1000 mL Luria broth (10 g/L bacto tryptone, 5 g/L bacto yeast extract, 0.5 g/L NaCl, pH 7.0) supplemented with 5 g/L glucose. Following overnight growth at 37°C on a rotary shaker (300 rpm), the culture was used as an inoculum for a 14-L fermentation using 10 L defined media (Table I). The fermentation was conducted at 37°C and the pH was maintained at 7.0 by the addition of NH_4OH, which also served as an additional nitrogen source. Cell growth was monitored by periodically withdrawing a sample and measuring the optical density with a Klett–Sumerson photoelectric colorimeter. Late in the exponential growth phase, the fermentation broth was harvested and immediately cooled to 4°C.

Chemical Permeabilization

The cooled fermentation broth was centrifuged at 4°C for 15 min at 6000g. The cell pellet was washed with the treatment buffer (0.1M Tris, pH 7.0) and centrifuged. The resulting cell pellet was resuspended in buffer to give a final cell density of approximately 50 g protein/L. Thirty milliliters of the dense cell suspension was placed in a 1000-mL Erlenmeyer flask. The permeabilization process was initiated by adding 70 mL buffered solution containing guanidine–HCl and/or Triton X100. The solutions were rapidly mixed and agitated at 300 rpm on a rotary shaker which was located in a 4°C walk-in incubator. The reported concentrations of Triton X100, guanidine–HCl, and cells always correspond to the concentrations of the final reaction mixture. For each experiment, a control consisting of 30 mL dense cell suspension and 70 mL buffer was also run.

Sampling Procedure

One-milliliter portions of the reaction mixtures were withdrawn at various times for sample analysis. The samples were centrifuged at 12,000g for 20 min in an Eppendorf 5415 centrifuge. The supernatants were then separated

Table I. Composition of fermentation media.

Media		
glucose	50	g/L
$(NH_4)_2SO_4$	1.5	g/L
KH_2PO_4	1.75	g/L
K_2HPO_4	5.0	g/L
$MgSO_4 \cdot 7H_2O$	4.0	g/L
antifoam	0.1	mL/L
trace salts	1.5	mL/L
Trace salts		
$FeCl_3 \cdot H_2O$	50.0	g/L
$ZnSO_4 \cdot 7H_2O$	8.0	g/L
$CoCl_2 \cdot 6H_2O$	7.0	g/L
$CaCl_2 \cdot 2H_2O$	3.0	g/L
$CuSO_4 \cdot 5H_2O$	8.0	g/L
$MnSO_4 \cdot 5H_2O$	3.8	g/L
H_3BO_3	2.0	g/L
concentrated HCl	100	mL/L

from the pellets and both were kept for analysis. Uncentrifuged samples of the reaction mixture, referred to as "whole-broth" samples, were also taken and kept for analysis.

Mechanical Disruption

Mechanical disruption was accomplished by grinding an *E. coli* cell suspension with 0.1-mm glass beads in a Biospec Products bead beater. The bead beater was operated for 30-s periods separated by 30-s intervals during which time the chamber was kept immersed in the ice bath. The disrupted cell suspension was centrifuged at 13,000g for 30 min.

Electron Microscopy

The electron microscopy procedure consisted of several stages: fixation, dehydration, infiltration, embedding, sectioning/staining, and micrograph development. The procedures are similar to those described by Baic.[8] The first stage, fixation, consisted of adding 0.4 mL 40% glutaraldehyde fixative (50 mL 50% glutaraldehyde, 3.5 mL 0.35M Na_2HPO_4, 9 mL d-H_2O) to 5.0 mL cell suspension (i.e., the gu-HCl–Triton cell reaction mixture). The cells were fixed for 1 h at 4°C followed by 1 h at room temperature. The glutaraldehyde fixed cells were then centrifuged at 13,000g for 15 min. The cell pellet (~0.75 mL) was resuspended in 1.5 mL 50°C 2% agar noble by stirring with a metal rod. The mixture was then spread over a glass slide, allowed to cool, and cut into 1-mm cubes. The agar cubes were washed in phosphate buffer (14.2 g/L Na_2HPO_4, 7.1 g/L KH_2PO_4, pH 7.0) 3 times, each wash being 20 min. The cubes were postfixed with 1% OsO_4 fixative for 3 h at room temperature. The 1% OsO_4 fixative was prepared just prior to fixation by mixing 1 part 4% OsO_4 and 3 parts working buffer. The working buffer consists of 5 mL stock buffer, 13 mL d-H_2O, 7 mL 0.1N HCl, 0.25 mL 1M $CaCl_2$ (pH 6.1). The stock buffer consists of 1.94% sodium acetate–$3H_2O$, 2.04% sodium veronal, and 3.4% NaCl.

The dehydration stage utilizes solutions of increasing ethanol concentrations. Following OsO_4 fixation, the cubes were first washed 3 times for 10 min in d-H_2O. The cubes were then washed in the following series of ethanol solutions: 40% (2 times, 5 min each), 60% (3 times, 10 min), 80% (3 times, 10 min), 95% (3 times, 10 min), and 100% (3 times, 10 min).

Infiltration was initiated by washing the cubes in propylene oxide (3 times, 10 min). The cubes were then infiltrated for 1.5 h with a 1:1 volume ratio of propylene oxide and Epon working mixture [4.2 g Epon-812, 2.3 g Araldite, 8.35 g dodecenylsuccinic anhydride, 30 drops 2,4,6-tri(dimethylaminomethyl)phenol]. Final infiltration is achieved in a 3:1 volume ratio of propylene oxide and Epon working mixture for 4 h.

The cubes were then placed in embedding capsules filled with the Epon working mixture. The capsules were

cured according to the following schedule: 12 h at 35°C, 12 h at 45°C, and 36 h at 60°C.

Silver sections (~600 Å thick) were cut with an ultramicrotome and picked up onto 300-mesh copper grids. The sections were stained for 1 h with saturated uranyl acetate and 10 min with lead citrate. The lead citrate stain was prepared as follows: add 1.33 g lead nitrate and 1.76 g sodium citrate to 30 mL boiled d-H_2O, add 8 mL $1N$ NaOH and enough d-H_2O to bring the volume to 50 mL, and mix until precipitate dissolves.

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 assay sample was 0.2% Triton. In order to determine the amount of unreleased protein from the sample pellets, all samples were treated for 5 min with $1N$ NaOH at 100°C.

DNA was determined by the diphenylamine reaction.[10] Two 45-min extractions at 70°C with $0.5N$ $HClO_4$ were used to release DNA from the sample pellets. Interference from guanidine was accounted for by ensuring that every assay sample was $0.4M$ guanidine.

RNA was determined by the orcinol procedure.[11] Two 15-min extractions at 70°C in $0.5N$ $HClO_4$ were used to release RNA from the sample pellets. Interference from Triton X100 was accounted for by making each sample 1% Triton.

Cell Counts

Escherichia coli cell counts were determined with a Hausser cell counting chamber. The procedure was as follows: dilute the original sample in $0.1M$ Tris, pH 9.0, to give a cell density of 22 kletts, mix 0.1 mL this diluted cell suspension with 0.8 mL $0.1M$ Tris, add 0.1 mL methylene blue staining solution, fill the counting chamber by capillary action, let cells settle for 30 min, and count cells, being sure to adjust focus to get any unsettled cells or cells adhering to the cover slip. The methylene blue staining solution consisted of 1 part methylene blue stock solution and 9 parts $0.1M$ Tris. The methylene blue stock solution was prepared as follows: dissolve 0.3 g methylene blue in 30 mL 95% ethanol, add 100 mL distilled water, and gravity filter through Whatman No. 1 filter paper.

Enzymatic Activity

The enzymes used in these studies were obtained from mechanically disrupted cells. A dense cell suspension (30,000 kletts) in 0.1M Tris, pH 7.0, was mechanically disrupted for 5 min as described previously. The disrupted cell suspension was centrifuged at 13,000g for 30 min. The resulting supernatant was used as the stock enzyme solution.

The stock enzyme solution was used to prepare the control ($0.1M$ Tris) and the gu-HCl–Triton treatments.

Four treatments were investigated: $0.1M$ gu-HCl/0.5% Triton X100, $0.2M$/0.5%, $1M$/0.5%, and $2M$/0.5%. The treatments consisted of adding a portion of the enzyme stock solution to a solution containing the appropriate guanidine and Triton concentrations. The control was prepared by adding the same portion of the enzyme stock solution to the buffer. Both were maintained at 4°C for specific periods of time. The treatment and control solutions were then assayed for the enzymatic activity of glucose-6-phosphate,[12] fumarase,[13] acid phosphatase,[14] isocitrate dehydrogenase,[13] and alkaline phosphatase.[14] The substrate concentration in all studies was sufficiently high to saturate the enzyme. Therefore, the rate of enzymatic activity was constant and did not initially vary with time as the substrate was consumed.

RESULTS AND DISCUSSION

Assessment of Protein Release Kinetics

As described in the Methods section, the permeabilization process was initiated by adding a buffered solution containing guanidine–HCl and Triton X100 to a buffered cell suspension. Figures 1 and 2 show the supernatant, pellet, and whole-broth protein concentrations as a function of time for a treatment consisting of $2M$ guanidine and 2% Triton (henceforth, such a treatment will be designated
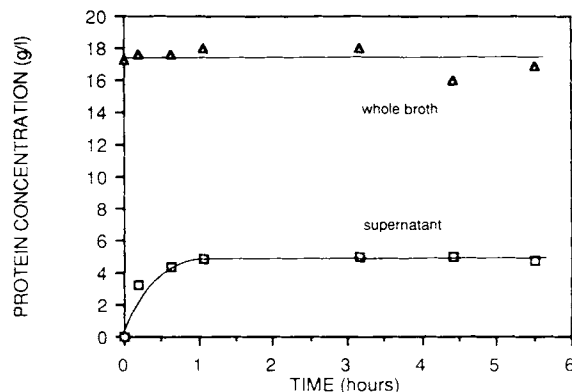

Figure 1. Whole-broth and supernatant protein concentration profiles during treatment with $2M$ guanidine–HCl and 2% Triton X100.
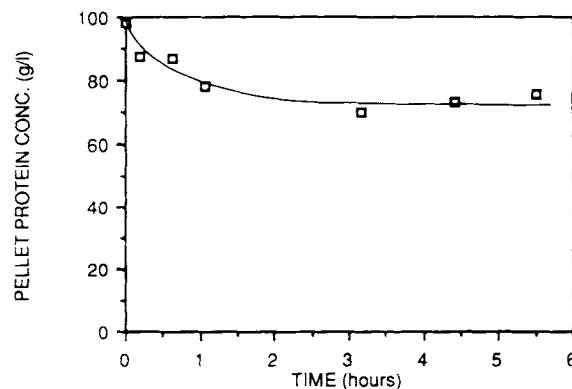

Figure 2. Pellet protein concentration profiles during treatment with $2M$ guanidine–HCl and 2% Triton X100.

$2M/2\%$). For this treatment, the supernatant protein concentration rose to a final value of 5 g/L within approximately 1 h. The protein concentration of the pellet decreased from nearly 100 g/L (based on pellet volume) to approximately 70 g/L and the whole broth was constant at 17.3 g/L. It should be noted that cells exposed only to the treatment buffer do not experience any protein release.

The protein data were normalized to a form that is more informative and amenable to making comparisons to other treatments. The normalization procedure involved dividing the supernatant protein concentration by the whole-broth protein concentration. In this way, the protein release is expressed as a percentage toward complete protein release with 100% being the maximum attainable release. With this method of presenting the data, it is seen that treatment with $2M$ guanidine and 2% Triton leads to release of $\approx 28\%$ of the cellular protein (Fig. 3).

Assessment of the supernatant, pellet, and whole-broth protein concentrations provides a means to check the reliability of the data because a mass balance can be performed. The sum of the protein contents of the supernatant and pellet divided by the total solution volume should equal the value obtained from a protein assay on the whole solution. As seen in Figure 4, the mass balance closes to within $\approx 10\%$.

Since the protein release results are in terms of total protein, these data provide no information about the origi-

Figure 3. Normalized protein release profiles for $2M/2\%$ treatment.

Figure 4. Comparison of whole-broth protein determination to that obtained by summing supernatant and pellet protein contents.

nal location of the protein. The protein found in the supernatant at a given time is protein that has been released from the cells, but it is not known how much of the protein is from the outer membrane, inner membrane, cytoplasm, or periplasm. Two-dimensional electrophoresis gels (not shown) indicate that the protein released is similar to the overall cellular protein pool. Proteins that span the entire spectrum of isoelectric points and molecular weights are released. This is a clear indication that intracellular and membranous proteins are being released by these treatments.

Effect of Varying the Gu–HCl and Triton X100 Concentrations

A large number of different treatments were studied to evaluate the effect of guanidine and Triton X100 on the protein release process. Various combinations of Triton concentrations up to 2% and guanidine concentrations up to $4.0M$ were examined. Protein release profiles from more than 35 treatments were evaluated. A summary of the protein release yields, i.e., the final protein release value, is shown in Figure 5. Note that the guanidine and Triton concentrations are plotted on a logarithm scale. Although it is impossible to show a zero concentration on a logarithm scale, it should be noted that the back and left edges of this figure correspond closely to the release obtained when only guanidine or only Triton were used, respectively.

Figure 5 can be viewed as consisting of several regions with distinct characteristics. The left edge consists of treatments using essentially only Triton. Triton X100 at concentrations up to 2% is incapable of causing any appreciable protein release. The back edge corresponds to treatments using essentially only guanidine. At guanidine concentrations greater than $\approx 1.0M$, substantial protein release is observed even in the absence of Triton. Protein release time profiles for treatments using different concentrations of guanidine in the absence of Triton are shown in Figure 6. At concentrations below $0.25M$, no observable protein release occurs. There appears to be a critical gu-HCl concentration above which substantial protein release is observed. A gradual protein release up to 20% is observed with $2M$ guanidine and a very rapid release is observed with $4M$.

The right edge of Figure 5 represents another region with distinct properties. In this region, where the guanidine concentration is greater than $2M$, significant protein release is observed. The guanidine concentration is sufficiently high to induce protein release, and addition of Triton does not substantially affect the final yield. However, these data do not give an indication of the rate at which protein is released. Figure 7 shows the protein release time profiles for $2M$, 2%, and 2M/2% treatments. Although the final yield of the 2M/2% treatment is higher than the $2M$ treatment, the more pronounced effect of adding Triton is an enhancement of the release rate. Although Triton itself is not capable of releasing more than
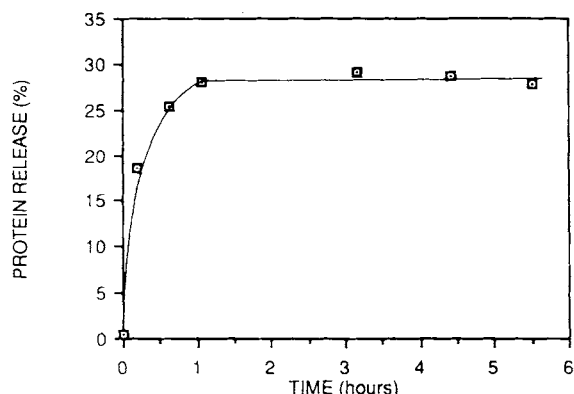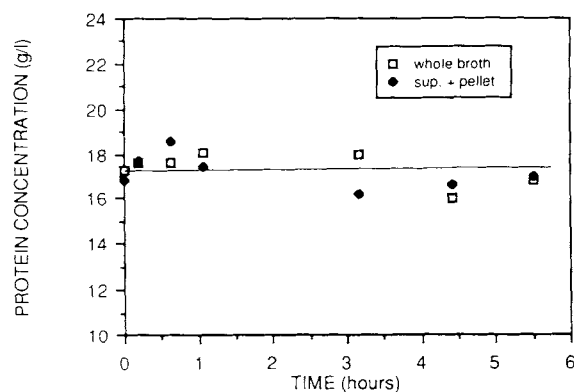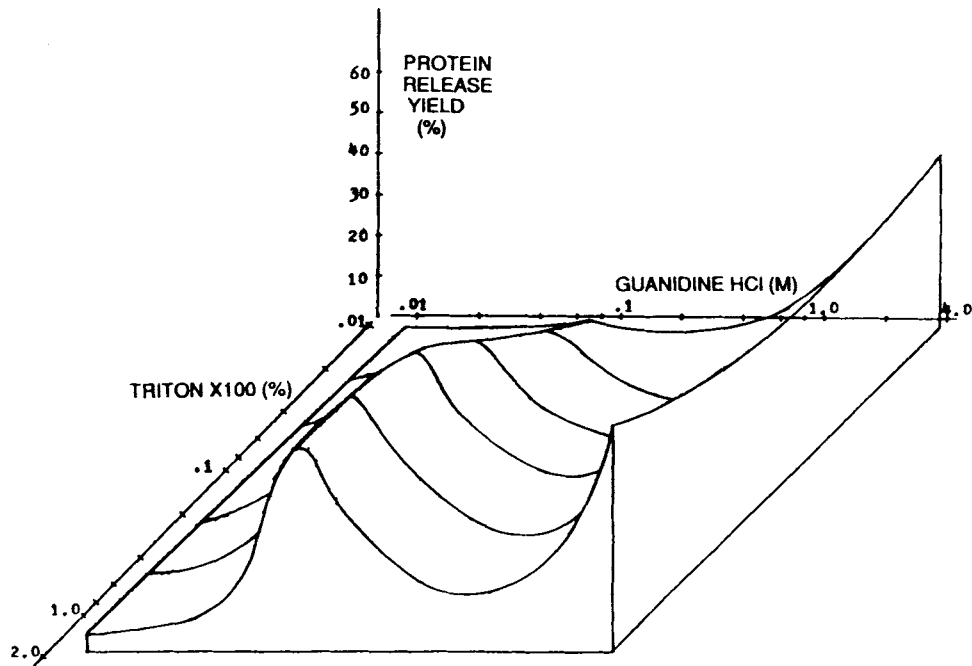
HETTWER AND WANG: PROTEIN RELEASE FROM *ESCHERICHIA COLI* CELLS   889

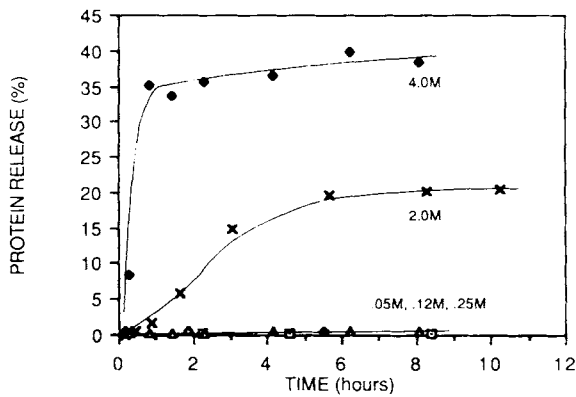**Figure 5.** Summary of effect of guanidine–HCl and Triton X100 on protein release yield.

Figure 6. Protein release profiles for treatments using different concentrations of guanidine–HCl in absence of Triton X100. Squares, open diamonds, and triangles correspond to the $0.05M$, $0.12M$, and $0.25M$ guanidine treatments, respectively.
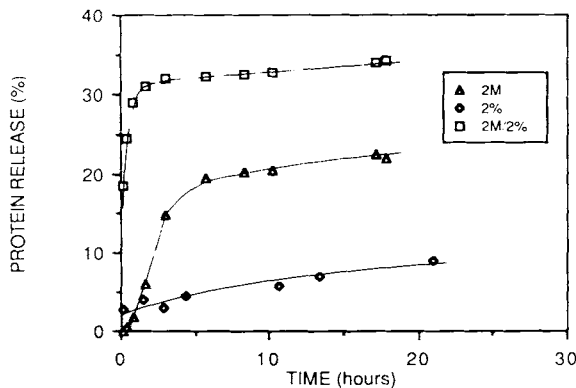
$\approx$ 10% of the cell protein, it interacts with cells exposed to $2M$ guanidine in a manner which significantly increases the protein release rate.

The most dramatic effects on the protein release yield and rate are observed in the central region of Figure 5. A very pronounced synergistic effect between guanidine and Triton occurs for treatments in this region. As an example, the protein release time profiles of the peak treatment of the front face of Figure 5, which corresponds to $\approx$ $0.12M$ guanidine and 2% Triton, is shown in Figure 8. Also shown in Figure 8 are the profiles obtained when $0.12M$ guanidine and 2% Triton are used individually. Treatment with either $0.12M$ guanidine or 2% Triton releases very little protein, yet ~50% of the cell protein is released upon exposure to both chemicals.

The central region of Figure 5 will be discussed in greater detail by examining two sets of treatments. One set
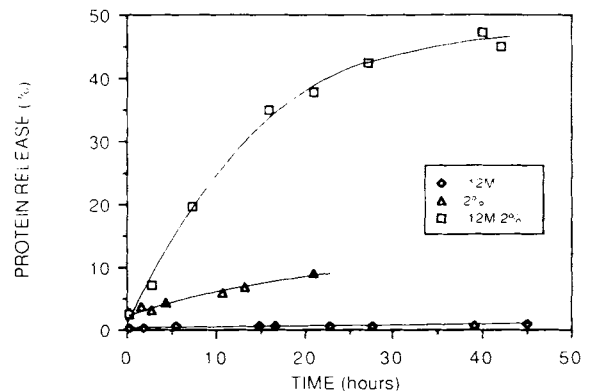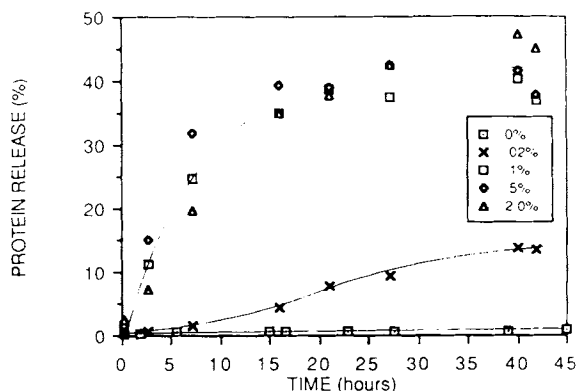

Figure 7. Synergistic effect between $2M$ guanidine–HCl and 2% Triton.

Figure 8. Synergistic effect between $0.12M$ guanidine–HCl and 2% Triton.
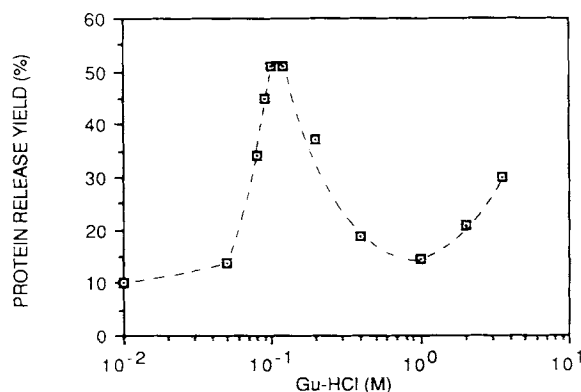
Figure 9. Protein release profiles for treatments using Triton concentrations between 0 and 2% and guanidine concentration of 0.12M.

consists of using 0.12M guanidine and various Triton concentrations. These treatments define the central ridge of Figure 4. The second set of treatments, which consists of 0.5% Triton and various guanidine concentrations, defines the wave-like behavior of the front face of Figure 5.

The protein release time profiles for treatments consisting of 0.12M guanidine and Triton concentrations between 0 and 2% are shown in Figure 9. At concentration of 0.12M guanidine alone resulted in an imperceptible level of protein release. Addition of a small amount of Triton, namely 0.02%, resulted in a slow release of protein that attained a value of 12% within 36 h. A dramatic increase in the protein release is observed when the Triton concentration is increased to 0.1%. Subsequent increases to 0.5 and 2% result in marginally different protein release profiles. These data define the central ridge of Figure 5 as rising rapidly between 0 and 0.1% Triton to a plateau value which remains constant as the Triton concentration is increased to 2%.

The most startling feature of Figure 5 is the wave-like dependence of the protein release yield on guanidine. Figure 10 shows the protein release yields obtained for treatments consisting of 0.5% Triton and guanidine concentrations from 0.01M to 3.5M. As the guanidine concentration is increased from 0.01M to 0.1M, a steady increase in the protein release yield is observed. A maximum value

of ≈ 50% is observed for concentrations near 0.1M–0.12M. As the guanidine concentration is increased further, the yield drastically declines to a minimum near 1.0M before rising again when very high guanidine concentrations such as 3.5M are used.

Although the protein release yield exhibits a complex dependence on the guanidine concentration, the protein release rate increases monotonically with increasing guanidine. Figure 11 shows the initial protein release rates normalized to the initial release rate of the 3.5M/0.5% treatment. From these data, the initial rate of protein release is observed to increase with increasing guanidine. The nature of the complex yield dependence and monotonically increasing rate dependence will be discussed in a future paper.

Comparison between Mechanical Disruption and Chemical Permeabilization

As described previously, two of the problems inherent to mechanical disruption are the release of nucleic acids and extensive cell fragmentation. A characterization of protein release by mechanical disruption is shown in Figure 12. The percentage of release of protein, DNA, and RNA are shown as a function of disruption time. Also shown is the
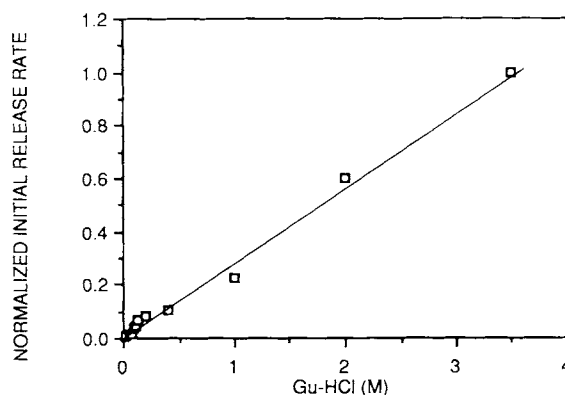
Figure 11. Initial protein release rates of treatments using guanidine–HCl concentrations between 0.01M and 3.5M and Triton concentration of 0.5%. Rates normalized to 3.5M/0.5% treatment.

Figure 10. Semilog plot of influence of guanidine–HCl on protein release yield of treatments using 0.5% Triton X100.
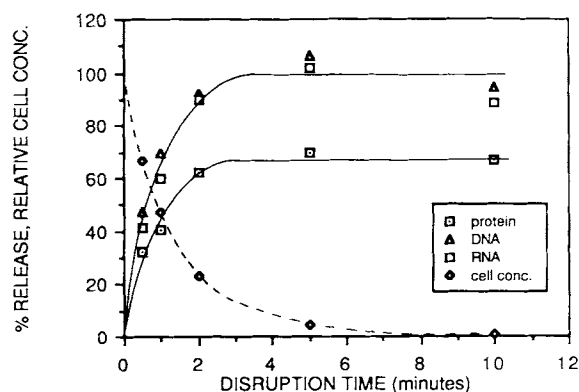
Figure 12. Characterization of protein release by mechanical disruption.

normalized cell concentration as determined with a bacterial counting chamber. The decrease in cell concentration with increasing disruption time indicates that extensive fragmentation of the cells is occurring during the process. A nearly mirror image release of protein, DNA, and RNA results as cellular components spill out into the extracellular fluid. The maximum protein release, $\approx$ 70%, is indicative of a significant amount of cellular protein being associated with the membrane and wall fragments.

A similar comparison for cells treated with $2M$ guanidine and 2% Triton is shown in Figure 13. For this study, the protein release leveled off at $\approx$ 35%. RNA is released to a lesser extent ($\approx$ 15%), and very little DNA ($\approx$ 5%) is released from the cells. Furthermore, the cell concentration remains constant during the treatment.

From these data, two major differences between chemical permeabilization and mechanical disruption can be identified. First, there is a selective release of protein over nucleic acid. The retention of DNA inside the cell may be a result of DNA's high molecular weight. This may benefit the purification process by eliminating or simplifying the nucleic acid removal step.

The second major difference between chemical permeabilization and mechanical disruption is that the release occurs by fundamentally different mechanisms. With mechanical disruption the cells are essentially torn apart, whereas with chemical permeabilization the cell structure as observed with a light microscope is still present.

Clearly, the chemical treatment has altered the cell structure in some manner to enable intracellular components to be released. An indication of the extent of cell structure alteration during the chemical treatment can be obtained by examining the cells with transmission electron microscopy. Figure 14 is an electron micrograph of a cell prior to chemical treatment. The cell structure can be resolved into five distinct layers. The outer wall is a triple layered structure, of which the outer two layers correspond to the outer membrane. The third line corresponds to the peptidoglycan layer. Just interior to the outer wall is the periplasmic space. The inner membrane is also resolved into two lines representing the two leaflets of the bilayer. The inner membrane is best resolved along the right edge of the cell in Figure 14.
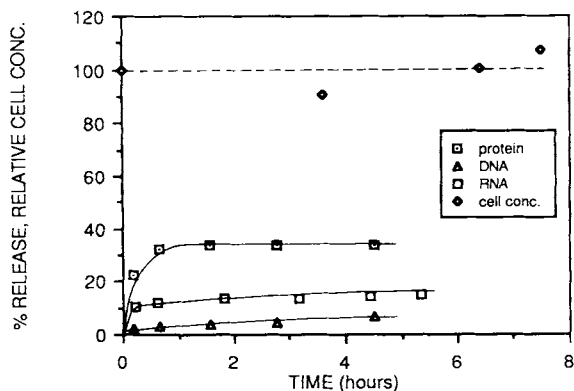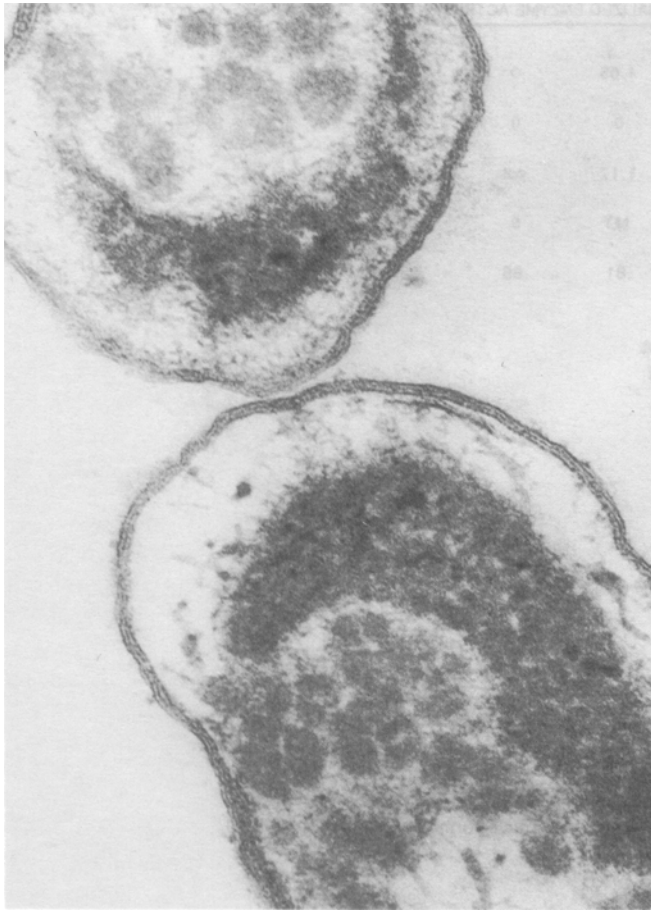

Figure 14. Electron micrograph of *E. coli* cell prior to chemical treatment (107,000×).

Figure 15 is an electron micrograph of cells exposed for 4 h to $2M$ guanidine and 2% Triton. The inner membrane has been completely solubilized during the treatment. The triple-layered structure that remains corresponds to the outer wall. Since an intact outer wall is only permeable to small compounds, guanidine and Triton must obviously have affected the outer wall to some extent in order to allow release of proteins. However, this interaction appears to be at the molecular level rather than at the macroscopic level as evidenced by the absence of any large gaps or tears in the outer wall. Furthermore, there are no instances where a large portion of either the outer membrane or the peptidoglycan layer has been removed. The outer wall is either clearly resolved into three lines or the wall appears blurred, which can result from imperfect sectioning of the cells. The interaction at the molecular level may involve solubilization of a portion of the outer-wall lipids and/or proteins.

Comparison of Figures 14 and 15 also indicates substantial alteration of the intracellular material. In the untreated cell, the intracellular material has a homogeneous granular appearance. The $2M/2\%$ treatment has led to the formation of distinct dense regions. The nature of these intracellular alterations and its role in determining the protein release rate and yield will be discussed in a later paper.
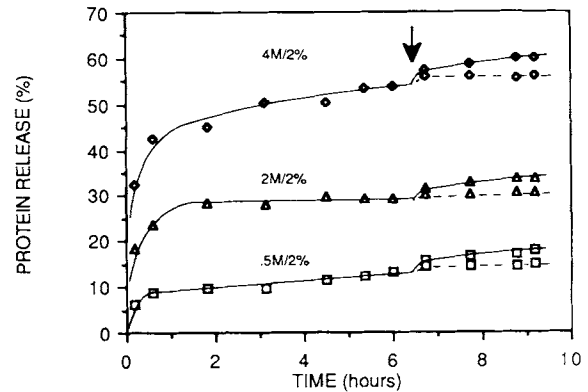

Figure 13. Characterization of protein release by $2M/2\%$ treatment.

Figure 15. Electron micrograph of cells treated with $2M$ guanidine–HCl and 2% Triton ($142,000\times$).

Figure 16. Protein release profiles of cells exposed to two successive treatments.

Effect of Repeated Treatments

One major limitation for some of the treatments is the incomplete extent to which the cell protein is released. From a processing standpoint, it is imperative that the yield be maximized. One possible approach to improving the yield would be to expose the cells to a second treatment. The results of using two successive exposures to a number of treatments is shown in Figure 16. The arrow indicates the time at which a portion of the cells were centrifuged and resuspended in a solution containing guanidine and Triton at the same concentrations as the first treatment. The dashed line represents the cumulative protein release resulting when a portion of the cells is resuspended in buffer and the solid line is the cumulative release of the two exposures to guanidine and Triton. For these treatments as well as others not shown, no significant protein release was observed during the second treatment.

At this point, very little investigation has been done of other means of increasing the yield. For example, it may be advantageous to expose the cells to two different treatments. Alternatively, yield improvements may be observed if other cell disruption techniques are utilized in concert with exposure to guanidine and Triton. A few possibilities include first freeze thawing the cells, adding EDTA, which

is believed to bind to membrane-stabilizing cations, or utilizing other permeabilizing chemicals.

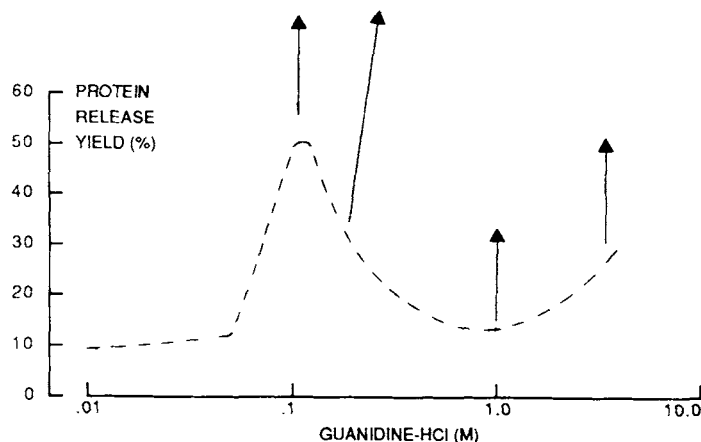## Effect of Guanidine and Triton X100 on Product Activity

Another important processing consideration is the effect of the chemical permeabilizing process on product activity. Although the influence of exposure to guanidine and Triton is obviously product dependent, a general indication of the effect of the chemical treatment was obtained by evaluating how the activities of five *E. coli* enzymes were altered by exposure to varying levels of guanidine and Triton. Figure 17 shows the effect of exposing five enzymes to four treatments: $0.1M/.5\%$, $0.2M/.5\%$, $1M/.5\%$, and $2M/.5\%$. The activities were assessed after 24 h exposure to $0.1M/.5\%$ and $0.2M/.5\%$ and after 6 h exposure to $1M/.5\%$ and $2M/.5\%$. These times correspond to the approximate treatment times needed to achieve the maximum protein release yield. Also shown for reference are the protein release yields observed for these treatments. The enzyme activities are displayed relative to the activity observed in a buffered solution which did not contain guanidine or Triton. From this data, it is clear that the $0.1M/.5\%$ and $0.2M/.5\%$ treatments can be considered to be mild, although some adverse affect on two of the enzymes was detected. These treatments would probably not cause adverse effects on the product. In contrast, treatment with high concentrations of guanidine such as $1M$ or $2M$ significantly decreases the activity of these enzymes. Guanidine's adverse effect is probably related to its well-known ability to denature proteins.[15-18]

The full implications of the data of Figure 17 depends on whether the adverse effects are irreversible. It is quite apparent that proteins will be adversely affected by treatments which utilize high guanidine concentrations. However, in a number of cases, protein denaturation is reversible and enzyme activity can be recovered upon removal of the denaturant.[19-21] This is also possible for nonenzyme protein products. However, if a particular protein product's activity cannot be recovered, a milder treatment such as $0.1M/.5\%$ may be appropriate.

Figure 17. Effect of various treatments on activity of five *E. coli* enzymes. ND indicates this study was not done and ppt indicates precipitate formed upon adding assay reagents and guanidine–Triton solution.

CONCLUSIONS

Guanidine–HCl and Triton X100 have been shown to be capable of permeabilizing *E. coli* to protein. Variation of the guanidine and Triton concentrations was found to lead to a spectrum of protein release processes with widely different kinetic properties. These kinetic properties have been mapped out for guanidine concentrations up to $4M$ and Triton concentrations up to 2%. Transmission electron microscopy revealed that the permeabilization mechanism involves a solubilization of the inner membrane. The outer-wall alteration apparently occurs at the molecular level because extensive disruption of the outer membrane or peptidoglycan layer was not observed. Essentially, treatment with guanidine and Triton resulted in unfragmented cells whose structures are characterized by an outer wall which acts as the final barrier to transport.

Two different regions in the concentration space which give substantial protein release have been identified. In one region, characterized by high guanidine concentrations, the process is dominated by the action of guanidine. Guanidine alone at concentrations greater than $2M$ is capable of altering the cell structure sufficiently to induce substantial protein release. In this region, Triton's primary effect is an enhancement of the protein release rate, possibly due to its effectiveness in solubilizing the inner membrane. A second region which results in significant amounts of protein release consists of guanidine concentrations near $0.1M$ and Triton concentrations between 0.5 and 2%. Although high protein release yields are observed in this region, the protein release rate is low. Approximately 15 h is required to achieve high protein release yields in this region as opposed to approximately 1 h in the region characterized by guanidine concentrations greater than $2M$. These two regions also differ in that a substantial synergistic effect between Triton and guanidine is observed in the second region. Although $0.1M$ guanidine and 0.5% Triton are individually incapable of inducing an appreciable amount of protein release, when used together, protein release yields near 50% are obtained.

Chemical treatment with guanidine and Triton was shown to have certain advantages and disadvantages with respect to mechanical disruption. One advantage is avoidance of cell fragmentation. The *E. coli* cells still retain their gross morphological characteristics after treatment, and consequently the solution can be readily clarified. Another advantage which was demonstrated for treatments with high gu-HCl concentrations is retention of the nucleic acids inside the cell structure. This can help the purification process by eliminating or simplifying the nucleic acid removal step. There are also the obvious advantages of replacing mechanical disruption devices with a simple batch operation which employs only a stirred vessel. Some disadvantages include the necessity to remove the chemicals

from the final product, any irreversible loss of product activity caused by the chemicals, and suboptimum yields. Furthermore, the treatment would not be applicable for recombinant products which form inclusion bodies unless the guanidine concentration is high enough to solubilize the product. The net balance between the advantages and disadvantages would depend on the protein product of interest.

Probably the most striking result of this research was the complex dependence of the release yield on the guanidine concentration for treatments that used Triton concentrations between 0.5 and 2%. At very low guanidine concentrations, such as $0.01M$, the release is negligible. As the gu-HCl concentration is increased to ~$0.05M$, a substantial amount of cell protein is released. At $0.1M$ gu-HCl, the release yield approaches 50–60%. Beyond $0.12M$ gu-HCl, the yield drastically declines until a minimum is obtained near $1M$ gu-HCl. As the gu-HCl concentration is increased even further, the yield begins to increase again. Although the protein release yield varies in this complex wave-like manner, the protein release rate varies monotonically with increasing gu-HCl. The phenomena which lead to these results have been investigated by Hettwer[22] and will be described in a forthcoming paper.

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