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Thymol-triggered lysis of *Escherichia coli* expressing *Lactobacillus* phage LL-H muramidase

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Effective disruption of *Escherichia coli* cells is achieved by the intracellularly accumulated recombinant murein hydrolase (*Lactobacillus* bacteriophage LL-H muramidase) after the addition of 5 mM thymol. Thymol destroys the integrity and electric potential of the cytoplasmic membrane, and as a consequence the muramidase can access and hydrolyze the cell wall murein leading to cell lysis. Lysis occurred within 5 min after the addition of thymol and seemed to be efficient at high culture densities. This lysis method does not require cell harvesting or addition of other cell wall weakening substances or exogenous enzymes. As a cell disruption method, thymol-triggered lysis is as efficient as sonication in the presence of 1% Triton. Furthermore, thymol did not interfere with the purification steps of Mur by expanded bed adsorption chromatography (EBA), suggesting that the lysis method presented here is well suited for large-scale production and purification of intracellular proteins of *E. coli*.

Keywords: murein; cell wall hydrolase; phage lysin; thymol-triggered lysis

Introduction

The cytoplasmic (inner) membrane, the cell wall and the outer membrane (in the case of Gram-negative bacteria) are responsible for the shape and rigidity of bacteria. These barriers have to be broken when purifying intracellular proteins. Mechanical (eg, French press, sonication, bead-mill), or enzymatic (eg, hen's egg lysozyme) disruption methods usually require cell harvesting and resuspension into an appropriate buffer [3]. Some modern purification technologies like expanded bed adsorption [1] permit the use of unclarified homogenates and even untreated cultures as a feed. Cell harvesting can therefore be regarded as an extra step in the protein purification processes. Lysis of the bacterial cells directly in the culture media would mean savings in time, energy, and costs during the downstream purification processes for intracellular proteins.

Compared to mechanical disruption or addition of lytic proteins, intracellular expression of genes encoding lysis proteins (eg, membrane spanning toxic proteins or cell wall hydrolases) is a more economical and better targeted lysis method [4]. Membrane-spanning proteins trigger localized bacterial autolysis by permeabilizing the cytoplasmic membrane [12]. However, the stability of cloned membrane-spanning encoding genes may be poor [4,13]. This function usually depends on the physiological state of the bacteria (membrane lipid composition, growth temperature and culture density) [18]. Therefore efficient lysis by such proteins may be obtained only for low culture densities [8]. The bacteriophage-encoded cell wall murein hydrolyzing enzymes (generally called endolysins or lysins) are not very toxic for the host bacterium, because they are unable to penetrate the cytoplasmic membrane. Accumulation of lysin inside the cell increases the cell's sensitivity to lysis by chemicals (like EDTA or Tris) or physiological conditions (eg, freeze-thawing or osmotic shock) that affect the integrity of the cytoplasmic membrane [4,19]. Chloroform treatment causes very effective lysis of bacteria that have a high intracellular content of lysin [19]. As a toxic material, chloroform is not convenient for large culture volumes, and it may severely disturb the subsequent protein purification steps [17].

The autolytic machinery of *E. coli* is not very efficient [4]. By overexpression of cloned phage lysin, the amount of murein hydrolyzing activity inside *E. coli* can be increased without lysing the cell. Association with the energized cytoplasmic membrane [5,9] may be one mechanism that supresses the murein-degrading activity of bacterial autolysins and phage lysins. Díaz *et al* [6] have suggested that breakage of the lysin's membrane association, rather than transportation of lysin protein through the cytoplasmic membrane, is involved in lysis triggered by phage-encoded membrane-spanning lysis proteins, holins. This view is further supported by the observation that autolysis of Gram-positive bacteria, like *Bacillus subtilis*, can be induced with energy poisons (azide, cyanide) that reduce the electric potential of the cytoplasmic membrane [11]. Alcohols are also capable of inducing small disruptions in biological membranes leading to changes in the properties of membrane-bound proteins, the ion-binding properties of proteins of membranes and changes in ion permeability. The aromatic alcohol thymol (5-methyl-2-(1-methylethyl)phenol) induces those changes at relatively low concentrations [14].

In this study, we wanted to develop for the industrially important bacterium *E. coli* a cell lysis system, which should be functional at high culture densities and scaleable to high culture volumes. In order to raise the amount of the

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cell wall hydrolyzing activity inside the bacterial cell, we used the induced expression of the *Lactobacillus* phage LL-H cell wall hydrolase gene *mur* [16,17]. The cloned gene *mur* can be relatively stably maintained in the *E. coli* host, as Mur is inefficient on the *E. coli* cell wall. We wanted to ensure that the presented lysis method for the *E. coli* system has no side effects for the downstream chromatographic purification of intracellular proteins. Therefore we demonstrated an experimental cultivation of the *E.coli* strain expressing the recombinant phage LL-H muramidase, and purification of Mur by EBA [17].

Materials and methods

Bacterial strains and DNA techniques

The DNA sequence of the lysin region of phage LL-H genome has been previously published (GenBank accession number M96254) [16]. The gene *mur* was amplified by polymerase chain reaction (PCR) using Dynazyme PCR Polymerase Kit (Finnzymes Oy, Espoo, Finland) (30 cycles of denaturation [92°C, 1 min], annealing [46°C, 1 min] and extension [72°C, 2 min], 3 mM Mg^{2+} concentration) and primers annealing upstream and downstream of the gene *mur*. Phage LL-H DNA was used as a template for the reaction. By modifying the upstream primer sequence (GGCGGTCGGAGCTCAAAAAAGAAAGAAGAAAGA AAATGTCAAG), we created recognition sites for the restriction enzyme *Sac*I and replaced the ribosome-binding site of the gene *mur* by the ribosome-binding site of the phage LL-H holin gene *ORF107* (designated as ORF-2 by Vasala *et al* [16]) to improve the rate of *mur*-expression. The downstream primer (CTTGGCCGTCGACA GCTGCACTC) was designed to contain a recognition site for the restriction enzyme *Sal*I. The resulting PCR product (purified with QIAquick PCR Purification Kit, Qiagen, Hilden, Germany) was cut with the appropriate restriction enzymes, and ligated into the pET21c+-vector (Novagen, Madison, WI, USA) linearized with *Sac*I and *Sal*I using standard protocols. In the resulting plasmid pET21[mur], the gene *mur* was located downstream of the T7 promoter. This plasmid was transformed into the *E. coli* strain BL21(DE3) (Novagen). That strain, when treated with IPTG, produces T7 RNA polymerase needed for expression of the genes cloned under control of the T7 promoter [15]. For comparative analysis of the cell lysis properties, strain BL21(DE3)pLysE (Novagen) constitutively expressing low amounts of the phage T7 lysin and transformed with the plasmid pET21c+, was used.

Bacterial cultivation, induction of mur-expression, and lysis triggering

Cultures originating from single colonies were grown in Luria Broth (LB) supplemented with ampicillin (Sigma, St Louis, MO, USA, 25 μg ml^{-1}) at 37°C in shake flasks (three times 1-liter volumes for Mur-production, otherwise 20–100 ml volumes). Induction of the expression of the cloned genes was performed by addition of 0.5 mM IPTG (technical grade, Calbiochem, San Diego, CA, USA) at the desired culture density. At 2.5 h after the induction, the culture pH was adjusted to 6.0, and the contributions of chloroform (Merck, 2% v/v), potassium cyanide (Sigma, 10 mM), sodium azide (Sigma, 75 mM) and thymol (Merck, 1 and 5 mM) for bacterial lysis were studied. Thymol was added as 0.5 M stock solution in ethanol. For purification of Mur by expanded-bed adsorption, the bacterial cells were disrupted with 5 mM thymol (for 30 min) and 1% Triton X-100 (Merck, Whitehouse Station, NJ, USA, further 30 min) after 4 h IPTG induction at an OD_{600} of 0.7.

Estimating the efficiency of bacterial lysis

The rate of lysis was followed by measuring the change of culture turbidity by a double beam spectrophotometer (Beckman Coulter, Fullerton, CA, USA, Model 25) at 600 nm. The efficiency of the release of intracellular proteins by 5 mM thymol-treatment (30 min, followed by 30 min incubation in the presence of 1% Triton X-100) was estimated by measuring the Mur activity [16,17] and ß-galactosidase activity [13] released into the medium. As a reference method, sonication in the presence of 1% Triton X-100 (MSE Soniprep 150 apparatus, Sanyo Electric, Osaka, Japan, 25 μm amplitude, three bursts of 20 s, 0.5 ml culture volumes) was performed. Prior to enzyme analysis, intact cells and cell debris were removed by centrifugation ($10\,000 \times g$, 10 min at 4°C).

Purification of Mur by expanded-bed adsorption chromatography

Purification of Mur was performed essentially as previously described [17]. A STREAMLINE™ C50 column (Pharmacia Biotech, Uppsala, Sweden) packed with 300 ml of STREAMLINE™ SP cation exchange resin was used. Prior to loading the mixture on the purification column, conductivity and pH of the bacterial culture (3 litres) lysed by 5 mM thymol and 1% Triton X-100 were adjusted to correspond to 50 mM sodium phosphate, pH 5.6, 0.15 M NaCl. The sample feed, wash, and elution (by 50 mM sodium phosphate, pH 6.7, 0.35 M NaCl) were performed as previously described [17].

Results

In order to estimate the efficiency of the intracellularly accumulated protein Mur and the autolytic system of *E. coli* for cell lysis, we treated bacteria with chloroform, which is a potent agent to trigger lysis of bacterial strains containing an increased amount of cell wall hydrolase activities [7,19]. Induced expression of the cloned *Lactobacillus* phage LL-H gene *mur* in the plasmid pET21[mur] did not retard the growth of bacteria (results not shown), but after the addition of chloroform (2% v/v final concentration), a rapid decrease in absorbance was observed (Figure 1). The autolysin activity in the cloning host strain BL21(DE3), or the amount of the phage T7 lysin in the commercially available strain BL21(DE3)pLysE were too low for rapid lysis after chloroform treatment (Figure 1). These strains contained the cloning vector pET21c+, and were treated with 0.5 mM IPTG as was the *mur*-expressing strain pET21 [mur].

Triggering the lysis of muramidase-expressing E. coli cells

Destruction of the membrane energy potential, followed by the activation of the membrane-associated cell wall

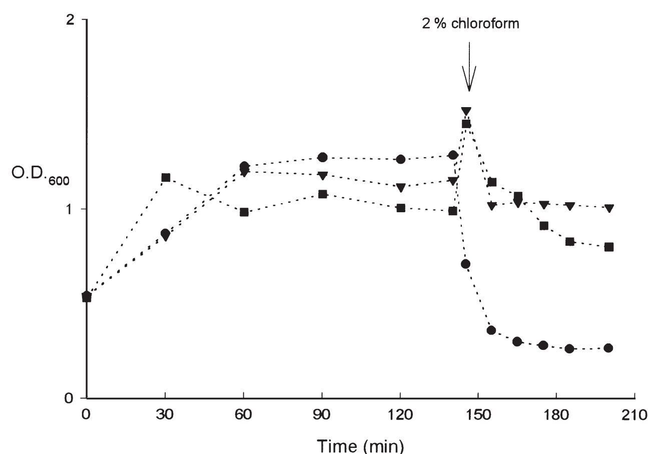

Figure 1 Intracellular accumulation of the cell wall hydrolase Mur makes *E. coli* cells sensitive to chloroform-triggered lysis. 0.5 mM IPTG was added at time point 0, the addition of chloroform (2% v/v) was performed at 150 min. – –●– – Mur; – –▼– – control; – –■– – T7 lysin.

hydrolyzing enzymes, and their release into the cell wall murein layer is supposed to be a major mechanism causing lysis of bacteria infected by bacteriophages [6,20]. For damaging the bacterial membranes, we compared the efficiency of chloroform (2% v/v), the energy poisons cyanide and azide (10 mM and 75 mM, respectively) and thymol (1 and 5 mM) as lysis triggering agents for the *mur*-expressing cells. Thymol induced a rapid decrease in culture turbidity at a concentration of 5 mM (Figure 2). A slower decrease was observed at 1 mM thymol. Formation of filamentous aggregates, similar to those resulting in coexpression of the phage LL-H lysin and holin genes (*mur* and *ORF107*) in *E. coli,* were produced [17]. This suggests membrane damage, leakage of Mur into the periplasm and the start of cell wall murein hydrolysis. The addition of thymol to the control strains BL21(DE3) or BL21(DE3)pLysE decreased the turbidity only slightly (results not shown), indicating the necessity of adequate amounts of intracellular cell wall hydrolase activity. The addition of the nonionic detergent Triton X-100 (1% v/v) to the thymol-treated *E. coli* culture improved the solubilization of Mur, but also increased the turbidity of the culture (results not shown). At temperatures lower than 20°C, clarification of the culture was observed. The energy poisons cyanide (10 mM) and azide (75 mM), which efficiently trigger lysis of *Bacillus subtilis*, did not trigger lysis of the *mur*-expressing *E. coli*. Addition of the non-ionic detergent Triton X-100 led to lysis of azide-containing sample suggesting that the bacterial outer membrane is not permeable to azide.

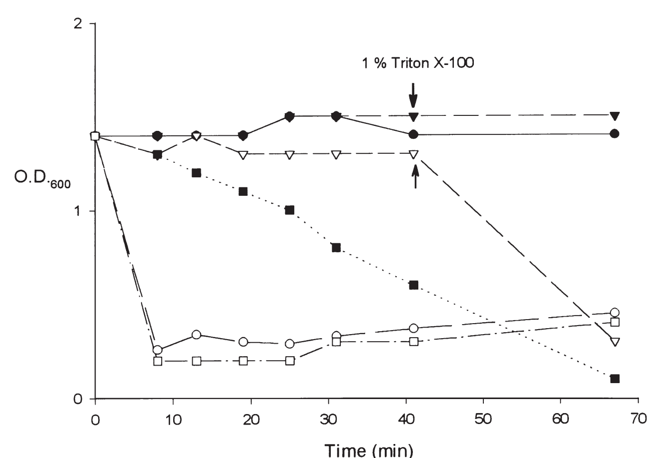

Figure 2 Triggering the lysis of *mur*-expressing *E. coli* cells with compounds that affect the integrity or energy state of the cytoplasmic membrane. Expression of the cloned phage LL-H gene *mur* in the strain BL21(DE3) was induced with 0.5 mM IPTG at cell density 1.0 (measured at 600 nm). Two hours later (time point 0 min), the lysis triggering agent was added. At 40 min, 1% Triton was added to the samples containing cyanide and azide (indicated by arrows). —●— Control; —○— 2% chloroform; – –▼– – 10 mM cyanide; —▽— 75 mM azide; – –■– – 1 mM thymol; – –□– – 5 mM thymol.

To estimate the efficiency of thymol-triggered bacterial lysis at different cell densities, we measured the levels of Mur and β-galactosidase activities released into the medium (Table 1). As a control treatment, thorough sonication (two times 1 min) in the presence of 1% Triton X-100 was employed. Enzyme activities were measured from the supernatant after removal of the cell debris by centrifugation (10 000 × g, 10 min). Equal levels of β-galactosidase activities were released into the medium by sonication (control) and thymol treatment. On the other hand, higher levels of Mur-activity could be released into the medium by thymol treatment suggesting that Mur retains its activity better after this treatment. It should be noted that the detergent Triton X-100 (1% final concentration) was used for both treatments in order to solubilize membrane-bound proteins.

Protein purification from the E. coli cell lysate

In order to detect possible influences of added thymol on the chromatographic purification of intracellular proteins, we performed thymol-triggered lysis for the *mur*-expressing *E. coli* culture and purification of Mur by EBA using the cation exchange resin STREAMLINE^{SP} as described by Vasala *et al* [17]. The purity of the eluted Mur was as high as when sonication or coexpression of the phage LL-H holin gene (*ORF107*) was used as a lysis method [17]. Extremely stable bed expansion was achieved, and no Mur-activity was detected in the flow-through (results not shown).

Table 1 Efficiency of thymol-triggered lysis of *mur*-expressing *E. coli* at different cell densities. Release of intracellular proteins from the bacterial cells expressing phage LL-H lysin was obtained with 5 mM thymol or sonication (both in the presence of 1% Triton X-100). The values given are proportional to the enzyme activities obtained by lysing the cells by sonication

Culture density at time point of IPTG-induction (OD_{600})	Relative activity			
	β-galactosidase		Muramidase (Mur)	
	Sonication	Thymol	Sonication	Thymol
0.4	100%	95%	100%	145%
0.9	100%	99%	100%	131%
1.8	100%	104%	100%	157%
2.1	100%	106%	100%	181%

Discussion

Autolysin activity in *E. coli* is too low for thymol-triggered lysis. Nor was there enough murein-degrading activity present even in the commercial strain BL21(DE3)pLysE, which constitutively produces small amounts of phage T7 lysin (Figure 1). Mur is relatively inefficient on the purified *E. coli* cell wall murein [16]. This can be regarded, however, as a benefit in the following respects: (1) *mur*-expressing clones can be relatively stably maintained, especially when cultivating bacteria in a minimal medium (unpublished results); and (2) high amounts of the intracellularly accumulated Mur are tolerated by *E. coli* (up to 5% of the total soluble proteins). Sufficient accumulation of murein-degrading enzyme activity for thymol-triggered lysis can be achieved by induced expression of the cloned phage LL-H lysin gene *mur*.

Autolysis is not generally used as a cell disruption method for *E. coli*, as efficient mechanical protocols for cell disruption are available. Due to the thin cell wall and thus lower amount of autolytic enzymes (compared to Gram-positive bacteria), the study of mechanisms that trigger the lysis of *E. coli* has not received much attention. Lysis of the cells with a high concentration of accumulated phage lysin can be obtained by treatment with chloroform (Figure 1) or toluene, by change of osmolarity, or by the addition of cell wall weakening compounds like EDTA [4]. In the present study, we were able to trigger lysis by addition of thymol and lyse the bacteria directly to the culture medium. Thymol is aromatic, aseptic and generally regarded as a non-toxic substance. Its induced lesions into the bacterial cytoplasmic membrane and formation of filamentous aggregates mimic the influence of coexpression of phage LL-H lysin and holin genes (genes *mur* and *ORF107*). As distinct from *Bacillus subtilis*, azide and cyanide did not induce lysis of *mur*-expressing *E. coli*. When supplemented with 1% detergent (Triton X-100), 75 mM azide triggered lysis suggesting that its penetration through the outer membrane or cell wall of *E. coli* occurs inefficiently. Thymol, as a more hydrophobic compound, may permeabilize the outer membrane and cell wall efficiently.

Small damages in the cytoplasmic membrane followed by rapid reduction of its energy potential are involved in bacterial lysis induced by membrane channel-forming proteins like bacteriocins [2] and bacteriophage holin proteins [20]. Cell wall hydrolase activity may be required for the final lysis, as cell wall degradation products are observed after lysis by small bacteriophages (like ϕX174) that do not encode their own lysin [12]. Unfortunately, as the membrane-spanning proteins are toxic for the host bacterium, the stability of the genes encoding them may be poor [13,19,20]. The membrane-spanning step of lytic proteins like colicins requires high membrane energy, which is present in actively growing cells [2,10]. Lysis by phage holin is also affected by the membrane composition, membrane fluidity and inhibitory heat shock proteins [18]. As a consequence, lysis triggering by holin genes may be efficient only for low culture densities. Also the holin/lysin system of phage LL-H cloned into *E. coli* proved to be sensitive to the culture growth state [17]. For large-scale production of recombinant proteins, high bacterial culture densities are preferred. The thymol-triggered lysis system described in this study is insensitive to the culture growth state (if enough cell wall hydrolase activity is present in cells), and thus may be applicable for lysis of large volume *E. coli* cultures with high cell densities. The time point after induction of the lysis gene's expression when the amount of the intracellularly accumulated cell wall hydrolase is sufficient for thymol-triggered lysis can be rapidly determined by treating small (a few millilitres) samples with thymol. Thymol-triggered lysis may be used for purification processes of other intracellular components, for example polysaccharides. This disruption method may not be, however, very applicable for purification of nucleic acids (eg, plasmids). In typical purification methods for nucleic acids, the bacterial cell walls are first digested with an added enzyme (typically hen's egg lysozyme) in an osmotically buffered solution in order to prevent too early lysis and the consequent release of nuclease activity from the broken cells. Rapid lysis is thereafter obtained by addition of a detergent (typically sodium dodecyl sulphate) that disrupts the membranes and denaturates the proteins. On the contrary, the addition of thymol first disrupts the bacterial membranes, after which gradual (although rapid) digestion of the cell wall murein by the intracellular murein hydrolase (Mur) occurs.

For mechanical disruption, bacteria are usually harvested by ultrafiltration and/or centrifugation. Such concentrating steps may require 2–3 hours, and the mechanical disruption 1 or 2 more hours. Concentrated samples are difficult to handle because nucleic acid released from the broken cells increases the viscosity. Also the risk of proteolytic degradation of proteins increases. Therefore mechanical disruption is often performed at low temperature in the presence of (expensive and poisonous) protease inhibitors. By the thymol-triggered lysis method, the purification process can be started very soon (immediately in the case of a stirred-tank adsorption system) after the addition of thymol. Short processing time may significantly reduce the risk of degradation of the proteins being purified. Both the lysis and the purification (eg, by EBA) can be performed at room temperature. However, the cell wall hydrolase Mur is active over a wide temperature range (10–60°C). Consequently, the operating temperature for thymol-triggered lysis can be optimized in respect to maximal stability of the proteins being purified. Thymol treatment was at least as efficient as a cell disruption method as sonication (Table 1). Unlike chloroform, thymol did not reduce expansion of the adsorbent bed during the sample feeding in EBA. Nor was the adsorption of the protein Mur to the cation exchange resin STREAMLINE^{SP} disturbed by the presence of thymol.

Conclusions

Expression level of the cloned *Lactobacillus* phage LL-H lysin gene *mur* sufficient for efficient lysis of *E. coli* could be obtained with IPTG even at relatively high culture densities. After intracellular accumulation of Mur, rapid and efficient lysis of *E. coli* could be triggered by the addition of 5 mM thymol. Thymol, at 5 mM, did not interfere with downstream purification of the intracellular proteins by

expanded bed adsorption. The presented thymol-triggered lysis method is therefore extremely suitable for the lysis of *E. coli* cells in the culture medium and for large-scale industrial purification processes of heterologous proteins produced in *E. coli*.

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