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# One Hundred Seventy-Fold Increase in Excretion of an FV Fragment-Tumor Necrosis Factor Alpha Fusion Protein (sFV/TNF-α) from *Escherichia coli* Caused by the Synergistic Effects of Glycine and Triton X-100

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**To target tumor necrosis factor alpha (TNF-α) to tumor cells, recombinant DNA techniques were used to construct and express the fused gene $V_K LV_H$–TNF-α, which encodes the secreted form of single-chain fusion protein sFV/TNF-α in *Escherichia coli*. sFV/TNF-α was secreted into the culture medium and purified by affinity chromatography. The production of the fusion protein in the culture medium under the optimal conditions of 30°C and 37 μmol of isopropyl-β-D-thiogalactopyranoside (IPTG) per liter was 16- and 5-fold higher than that under the standard conditions of 37°C and 1 mmol of IPTG per liter. Fusion protein excretion into culture medium with 2% glycine, 1% Triton X-100, or both of these two chemicals was either 14-, 38-, or 170-fold higher, respectively than that without the two chemicals. The final yield of sFV/TNF-α was estimated to be 50 mg/liter. The loss of integrity of the cellular membrane may be a potential mechanism for enhancement of fusion protein production and excretion by treatment with glycine and Triton X-100. This study thus provides a practical, large-scale method for more efficient production of the heterologous fusion protein sFV/TNF-α in *E. coli* by using glycine and Triton X-100.**

The bacterium *Escherichia coli* has become a commonly used system for expression of heterologous recombinant proteins of interest in both biological research and the biotechnology industry. A variety of properties make the *E. coli* expression system attractive, namely, ease of genetic manipulation, efficient transformation, fast growth, simple fermentation, and favorable economics. Many recombinant proteins, including antibodies (15, 24) and single-chain fusion proteins (5, 25), have been successfully expressed in *E. coli*. Two major forms of heterologous proteins are usually expressed in this bacterium, i.e., insoluble and soluble. The former do not contain a signal peptide and are expressed in the cytoplasm and subsequently packaged into highly condensed inclusion bodies (2), while the latter have a signal peptide that is expressed in the cytoplasm and subsequently secreted into the periplasmic compartment. Although a high level of heterologous protein expression in inclusion bodies can be attained, these proteins are insoluble and therefore nonfunctional. A process of protein denaturing followed by a complex renaturing procedure must be conducted to obtain properly refolded functional proteins (3). However, the final yield of these soluble refolded proteins is usually very low, due mainly to protein aggregation resulting from the exposure of hydrophobic peptide regions (10).

An alternative is to express the secretory form of heterologous proteins from the *E. coli* periplasmic fractions (23). Sometimes, however, the secreted heterologous proteins can leak from the periplasm into the culture medium, possibly due to the increased permeability of cellular membranes during long incubation periods (24). Secretion of these proteins into the *E. coli* periplasm is a useful ploy that can lead to the rapid isolation of recombinant proteins for biological evaluation. Its application on an industrial scale is limited by the general unavailability of efficient large-scale methods for the selective release of periplasmic proteins from the cell. Since it is easier to process the heterologous proteins in the culture medium than in the periplasmic fraction, various approaches have been developed to enhance the secretion of heterologous proteins of *E. coli* into culture media. These include (i) optimizing culturing conditions by modifying the temperature (4) or the concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) (3), (ii) coexpression of molecular chaperones (13, 26), (iii) genetic modification of expression vectors (6, 11) or recombinant genes (8), and (iv) addition of chemicals such as glycine (9, 14).

Recombinant single-chain fusion proteins have increasingly attracted attention in both research and clinical use due to their novel bifunctional activity and small size (5, 25). We have previously reported the construction and expression of single-chain fusion protein FV/TNF-α in inclusion bodies of *E. coli* (29). The fusion protein contains a single-chain FV fragment consisting of an immunoglobulin variable region of the heavy ($V_H$) (12.5 kDa) and light ($V_K$) (12.5 kDa) chains of the B72.3 antibody recognizing the human tumor-associated TAG72 antigen (28) and the tumor necrosis factor alpha (TNF-α) moiety (18 kDa). Previous studies have demonstrated that small antibody fragments such as FV (25 kDa) showed deeper, as well as more homologous, penetration of tumors by the molecule (19) and a higher localization index of tumors versus normal tissues (7) than the large intact antibody molecule (150 kDa). Therefore, this fusion protein (43 kDa) has the potential to efficiently target TNF-α to tumors expressing the TAG72 antigen for induction of active antitumor immune responses. Although the fusion protein retained its bifunctional activity after the process of denaturing and refolding, it still tended to aggregate especially in concentrations used in experimental animal mod-

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el studies. This greatly limited its potential use as an antitumor therapeutic reagent. In this report, we describe the construction and expression of a secreted form of single-chain fusion protein sFV/TNF-α in *E. coli*. We also demonstrate dramatic enhancement of the excretion of this heterologous fusion protein from *E. coli* into culture media by the synergistic effect of glycine and Triton X-100.

## MATERIALS AND METHODS

**Genes, plasmid, *E. coli* strain, antigen, and antibodies.** The genes encoding the $V_H$ and $V_K$ regions were cloned from a cDNA library of antibody B72.3 (28). The cDNA gene of TNF-α and the recombinant TNF-α protein were obtained from R&D Systems (Minneapolis, Minn.). FLAG expression vector pF1 was obtained from International Biotech, Inc. (New Haven, Conn.). This vector contains the OmpA leader sequence, the *tac* promoter, the Lac repressor, the ampicillin resistance gene as a drug selection marker, the transcriptional termination signal region, and the multiple cloning site. *E. coli* K802 was obtained from the American Type Culture Collection, Rockville, Md. Mucin type I-S from bovine submaxillary glands containing a large amount of the TAG72 epitope (28) was obtained from Sigma Chemical Co., St. Louis, Mo. A rabbit anti-TNF-α antibody and a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G antibody were obtained from GIBCO (Burlington, Ontario, Canada).

**Construction and expression of single-chain fusion protein sFV/TNF-α in *E. coli*.** Fused gene $V_KLV_H$–TNF-α, consisting of gene fragments in the sequence $V_K$, linker (L), $V_H$, and TNF-α was constructed in a manner similar to that described previously (29) and inserted into the *Nde*I/*Hin*dIII site in the multiple cloning site of plasmid pF1, which is at the 3′ end of the bacterial OmpA signal sequence, to form expression vector pF1–$V_KLV_H$–TNF-α. The expression vector was then transfected into K802. The transfected bacterial clone selected from L-broth plates with ampicillin (100 μg/ml) was further grown in L-broth medium with 0.4% glucose and 100-μg/ml ampicillin at 37°C overnight in a rotatory shaking (300 rpm) incubator. The bacterial cells were pelleted by centrifugation and resuspended in Terrific broth (TB) containing ampicillin (100 μg/ml) and IPTG (Promega Inc., Madison, Wis.) at different concentrations to an optical density (OD) at 600 nm of 4.0 for induction of fusion protein expression. The expression of the secreted form of fusion protein sFV/TNF-α was induced at 37 or 30°C for 10 h in a rotatory shaking (300 rpm) incubator. To study whether the addition of glycine and Triton X-100 affects the excretion of sFV/TNF-α, the growth media were further supplemented with various amounts of glycine or Triton X-100 as described in the appropriate figure legends. After incubation, the culture media were collected, clarified by centrifugation, and subjected to further characterization in the TAG72-binding enzyme-linked immunosorbent assay (ELISA) or purification by mucin affinity chromatography (28). Bound sFV/TNF-α was eluted from the mucin affinity column with an elution buffer (50-mmol/liter glycine, pH 2.7) and dialyzed against phosphate-buffered saline.

**N-terminal amino acid sequencing.** To check whether the OmpA signal sequence was cleaved from the fusion protein, purified sFV/TNF-α was subjected to Edman degradation sequencing (27) by using a 471A sequencer equipped with an MG5 microgradient pump and a Blott cartridge for polyvinylidene difluoride membranes. Data was acquired and analyzed with a 610A data system (Applied Biosystems Inc.).

**ELISA.** The TAG72-binding ELISA was performed to examine the production of sFV/TNF-α in culture media as previously described (29). Briefly, 300 ng of bovine mucin was used to coat each well of microliter plates. The plates were blocked with 5% bovine serum albumin. Fifty-microliter volumes of the IPTG-induced culture media and their twofold dilutions were added to the wells and incubated at 37°C for 1 h. After three washes, plates were incubated with rabbit anti-TNF-α serum (1:500), followed by horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G antibody (1:1,000). After another three washes, the substrates were added to each well for generation of a color reaction. The OD of each well was determined at 415 nm in a Bio-Rad 3550 microplate reader.

**Ultrastructural studies.** For electron microscopy, bacterial pellets were fixed in 6% glutaraldehyde and 0.1-mol/liter sodium phosphate buffer. After overnight fixation at 4°C, the cell pellets were washed in sodium phosphate buffer, postfixed in 1% $OsO_4$ (0.1-mol/liter sodium phosphate buffer, pH 7), and then suspended in 1% agar, dehydrated in ethyl alcohol, and embedded in araldite. Ultrathin sections were stained with alcoholic uranyl acetate and basic lead citrate (22).

**β-Galactosidase assay.** β-Galactosidase activity was measured by estimating the amount of *o*-nitrophenol released from *o*-nitrophenyl-β-D-galactopyranoside at 37°C for 10 min by the method previously described (17). Briefly, 1 ml of the overnight growth of a K802 cell suspension was inoculated into 50 ml of TB medium containing 37-μmol/liter IPTG and incubated at 30°C until the cell density reached an OD of 0.4 at 600 nm. Five milliliters of the cell culture was added with 2% glycine and/or 1% Triton X-100. At different time points, 0.5 ml of the cell culture was collected and 0.5 ml of growth medium containing 100-μg/ml streptomycin was added to stop translation. After centrifugation, 0.8 ml of supernatant was collected and 0.2 ml of 12-mmol/liter *o*-nitrophenyl-β-D-galactopyranoside was added for measurement of extracellular β-galactosidase activity. Reactions were carried out at 37°C for 10 min and stopped by addition of 0.4 ml of 1-mol/liter sodium carbonate. Absorbance at 420 nm was determined in a Bio-Rad 3550 microplate reader.

## RESULTS

**Construction, expression, and purification of fusion protein sFV/TNF-α.** The strategy used to construct the fused gene $V_KLV_H$–TNF-α was similar to that previously described (29). Its entire nucleotide sequence was verified by the dideoxynucleotide sequencing method. Since expression of the fused gene was under control of the *tac* promoter, fusion protein sFV/TNF-α would be produced through derepression by addition of the inducer IPTG. K802 cells harboring expression vector pF1–$V_KLV_H$–TNF-α were grown at 30°C in TB medium containing ampicillin (100 μg/ml) and IPTG (37 μmol/liter) for 10 h. Culture media were collected and clarified by centrifugation. The cell extract was also prepared from the periplasmic fraction of cell pellets (23). Interestingly, most of the TAG72-binding activity (more than 90%) was found in culture media while only a little was detected in the periplasmic fraction, as measured in the TAG72-binding ELISA (data not shown), indicating that most of the secreted sFV/TNF-α was excreted into the culture media after a long incubation period of 10 h. The fusion protein was then purified from culture media by mucin affinity chromatography. To check its purity, the purified sFV/TNF-α was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The fusion protein displayed a single band of 43 kDa under nonreducing and reducing conditions (data not shown), indicating that sFV/TNF-α is a homologous single-chain protein. Furthermore, the amino acid sequence at the N terminus of the purified fusion protein was determined to be $NH_2$-Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ala, the same as the N-terminal sequence of $V_K$ (28), indicating that the OmpA signal sequence had been correctly removed from the fusion protein during the secretion process.

**Influence of culture conditions on expression of sFV/TNF-α.** To better determine the optimal temperature for expression of the fusion protein, we measured the TAG72-binding reactivity of culture medium obtained from incubation at three different temperatures (25, 30, and 37°C) in the TAG72-binding ELISA. We found that the optimal temperature for production of the fusion protein was 30°C. At this temperature, the TAG72-binding reactivity of the culture medium was 16 and 4 times greater than that of culture media incubated at 37 and 25°C, respectively. To determine the optimal concentration of IPTG for expression of the fusion protein, we measured the TAG72-binding reactivity of culture media incubated with different concentrations of IPTG compared to that of culture medium incubated under standard culturing conditions (37°C, 1-mmol/liter IPTG). As shown in Fig. 1A, the optimal concentration of IPTG for induction of fusion protein production was 37 μmol/liter (1/27 of 1 mmol/liter). At this concentration, the TAG72-binding reactivity of the culture medium was five times as high as that of the medium incubated under the standard culturing conditions (1-mmol/liter IPTG). Therefore, it appears that the optimal culture conditions for production of the fusion protein include a temperature of 30°C and an IPTG concentration of 37 μmol/liter.

**Synergistic enhancement of fusion protein excretion by glycine and Triton X-100.** Since the fusion protein expressed in *E. coli* was secreted into extracellular media, chemicals such as glycine and Triton X-100, which influence the permeability or integrity of the cell wall, may affect the excretion of the fusion protein into culture media. To test this assumption, strain K802 bacteria harboring expression vector pF1–$V_KLV_H$–TNF-α were grown at 30°C in culture media containing 37-μmol/liter IPTG,

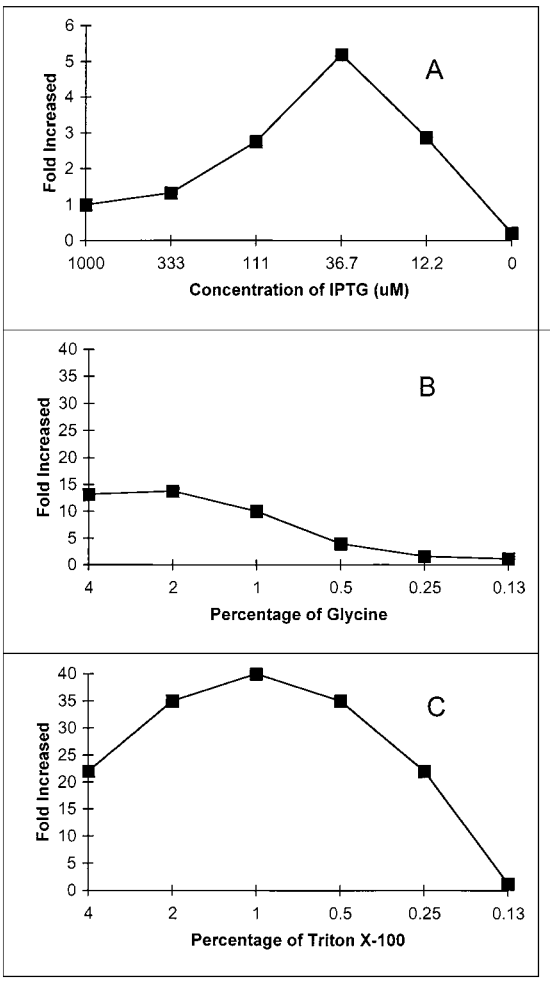


FIG. 1. Effects of different chemicals on the production and excretion of sFV/TNF-α. (A) The TAG72-binding reactivities of culture media containing different concentrations of the inducer IPTG were measured in the TAG72-binding ELISA and compared to that of culture medium containing 1-mmol/liter IPTG as a baseline. (B) The TAG72-binding reactivities of IPTG (37 μmol/liter)-induced culture media containing different concentrations of glycine were measured and compared to that of IPTG-induced culture medium without glycine as a baseline. (C) The TAG72-binding reactivities of IPTG (37 μmol/liter)-induced culture media containing different concentrations of Triton X-100 were measured and compared to that of IPTG (37 μmol/liter)-induced culture medium without Triton X-100.

as well as glycine or Triton X-100 at different concentrations, for 10 h. The culture media were collected and clarified by centrifugation, and their TAG72-binding reactivity was measured by the TAG72-binding ELISA. As shown in Fig. 1B, glycine treatment significantly increased the production and excretion of fusion protein into culture media. The enhanced production and excretion were found to be proportional to the glycine concentration present in the medium. The optimal concentration of glycine was 2%. The TAG72-binding reactivity was 14-fold higher in culture media containing 2% glycine than in media without glycine. As shown in Fig. 1C, the treatment of Triton X-100 also significantly increased the production and excretion of the fusion protein into culture media as measured by the TAG72-binding ELISA. The optimal concentration of Triton X-100 was 1%. The TAG72-binding reactivity of culture media containing 1% Triton X-100 was 38-fold higher than that of media without Triton X-100. Therefore, the optimal concentrations of glycine and Triton X-100 with respect to enhanced production and excretion of the fusion protein were 2 and 1%, respectively. To study whether the combined use of these two chemicals had any synergistic effect on fusion protein production and secretion, bacteria harboring the expression vector were grown in TB media containing 37-μmol/liter IPTG, as well as 2% glycine and 1% Triton X-100. Interestingly, this dramatically increased the production and excretion of the fusion protein to 170-fold higher than that of culture media without glycine and Triton X-100 (Fig. 2). This finding demonstrates that glycine and Triton X-100 synergistically enhance the production and excretion of the fusion protein into culture media. The final yield of secreted sFV/TNF-α was estimated to be 50 mg/liter.

**The study of potential mechanism.** β-Galactosidase is an autologous *E. coli* protein present in the cytoplasm. To check whether other *E. coli* autologous proteins, such as β-galactosidase, were leaked into culture media because of the treatment with glycine and Triton X-100, we measured its activity in culture media containing 2% glycine and 1% Triton X-100 at different time points of incubation. As shown in Fig. 3A, glycine treatment alone or the combined use of glycine and Triton X-100 significantly increased the excretion of β-galactosidase into culture media. However, treatment with Triton X-100 alone did not affect its excretion. This suggests that treatment with these two chemicals, especially glycine, may cause membrane alterations. To confirm this, we conducted paired experiments with and without addition of $MgCl_2$ to culture media, since it can stabilize and maintain the permeability of cellular membranes (18, 21). As shown in Fig. 4, treatment with glycine and Triton X-100 increased the TAG72-binding reactivity of culture media in the TAG72-binding ELISA, indicating enhancement of fusion protein production and excretion by these two chemicals. Addition of 40-mmol/liter $MgCl_2$ to culture medium containing 2% glycine or 1% Triton X-100 was able to neutralize the enhancement of the production and excretion of the fusion protein by glycine and Triton X-100. As shown in Fig. 3B, addition of 40-mmol/liter $MgCl_2$ to culture medium containing 2% glycine or 2% glycine–1% Triton X-100 was also able to block the leakage of β-galactosidase into the culture medium. To check whether there was any morphological

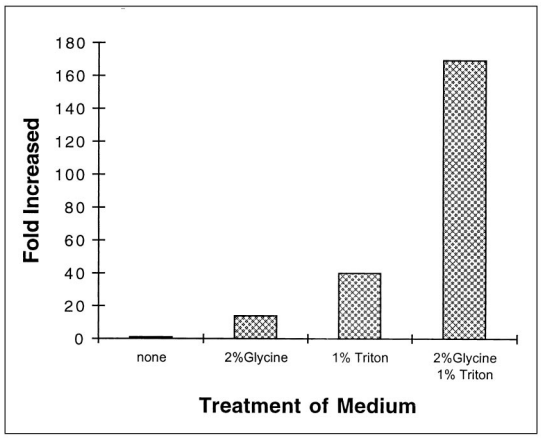


FIG. 2. Synergistic enhancement of sFV/TNF-α production and excretion by glycine and Triton X-100. The TAG72-binding reactivity of IPTG (37 μmol/liter)-induced culture media containing both 2% glycine and 1% Triton X-100 was measured in the TAG72-binding ELISA and compared to that of IPTG (37 μmol/liter)-induced culture medium without glycine and Triton X-100 as a baseline.

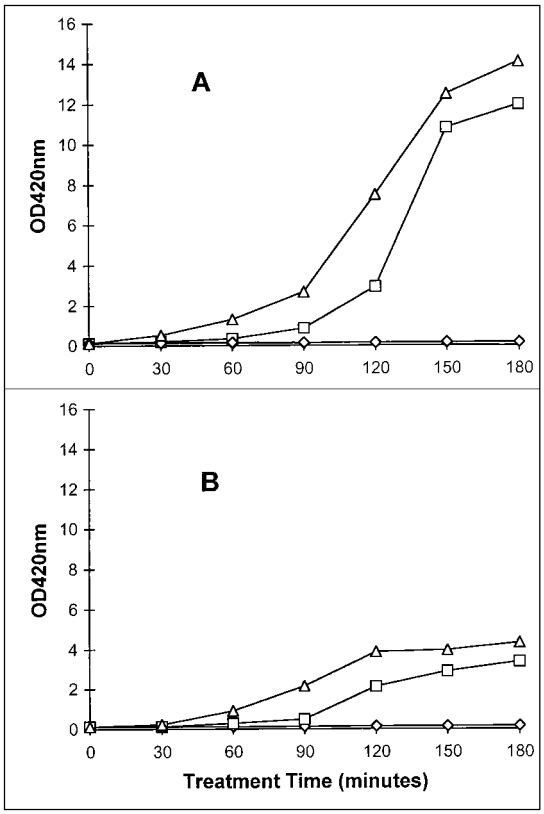


FIG. 3. β-Galactosidase assay. (A) The β-galactosidase activities of IPTG (37 μmol/liter)-induced culture media containing 2% glycine (□), 1% Triton X-100 (◇), and 2% glycine–1% Triton X-100 (△) were measured at different time points of incubation. In this assay, culture medium without glycine and Triton X-100 (◇) was used as a control. (B) The β-galactosidase activities of IPTG (37 μmol/liter)-induced culture media containing 2% glycine–40-mmol/liter $MgCl_2$ (□), 2% glycine–1% Triton X-100–40-mmol/liter $MgCl_2$ (△), and 40 mmol/liter $MgCl_2$ (◇) were measured at different time points of incubation. In this assay, culture medium without glycine and Triton X-100 (◇) was used as a control.

alteration of bacterial cells after treatment with glycine and Triton X-100, electron microscopy was carried out on ultrathin sections of untreated, as well as treated, *E. coli* cells. As shown in Fig. 5, the outermost layer of the cell wall was focally disrupted by treatment with glycine and Triton X-100. Similar morphological changes were also seen in cells treated with glycine or Triton X-100 alone (data not shown).

## DISCUSSION

Culturing conditions can influence the production of heterologous proteins in *E. coli*. For example, Chalmers et al. reported that increased production of soluble and total epidermal growth factor resulted from a decrease in the incubation temperature of *E. coli* harboring the recombinant gene encoding epidermal growth factor (4). In another study, it was found that a decrease in the culturing temperature was also associated with increased production of single-chain FV molecules in *E. coli* (23). Our results described herein are consistent with these previous reports. The optimal temperature for production of the fusion protein is 30°C. The production and excretion of sFV/TNF-α are 16-fold higher at 30°C than at the standard temperature (37°C). Since the fused gene encoding the secreted form of fusion protein sFV/TNF-α is polycistronically regulated by the *tac* promoter in the expression vector, its expression can be induced by adding IPTG to the culture medium. The study of soluble β-lactamase expression by Bowden and Georgiou (1) showed that lowering the IPTG concentration increased the yield of soluble sFV by slowing the rate of synthesis and preventing aggregation of folding intermediates. In the present study, we found that the concentration of IPTG for optimal production of the fusion protein is 37 μmol/liter. The production and excretion of sFV/TNF-α at this concentration are fourfold higher than at the standard IPTG concentration of 1 mmol/liter.

Glycine has been found to be able to induce morphological alterations of *E. coli*, such as swelling and elongation, by virtue of the fact that it was incorporated into precursors of peptidoglycan. This results in the disruption of peptidoglycan crosslinkages and cell membrane integrity (12). Dramatic enhancement of the secretion of heterologous proteins of *E. coli* into culture media caused by glycine has already been reported (9, 14). In the present study, addition of 2% glycine drastically increased the production and excretion of heterologous sFV/TNF-α (14-fold). This suggests that glycine may have two kinds of influences, namely, a stimulatory effect on fusion protein production and a fusion protein release effect. In addition, the production and excretion of autologous β-galactosidase also significantly increased, suggesting that the increased permeability or the induced bacteriolysis may be one of the potential mechanisms for enhancement of protein excretion into culture media by glycine. To prove this, we used $MgCl_2$, which is a membrane stabilizer (18, 21), to see whether it affects the excretion of the fusion protein and β-galactosidase caused by glycine. Our experiments showed that the addition of $MgCl_2$ blocked the glycine-mediated enhancement of fusion protein and β-galactosidase excretion, indicating that $MgCl_2$ was able to stabilize the membrane and prevent bacteriolysis.

Detergents are commonly used to disrupt lipid membrane structures for extraction of membrane proteins (20). At low

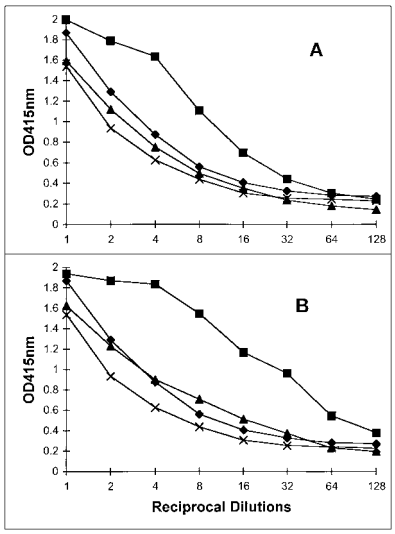


FIG. 4. Effects of $MgCl_2$ on the excretion of sFV/TNF-α. The TAG72-binding reactivities of IPTG (37 μmol/liter)-induced culture media containing different chemicals were measured in the TAG72-binding ELISA. (A) Culture media containing 2% glycine (■), 2% glycine–40-mmol/liter $MgCl_2$ (◆), and 40 mmol/liter $MgCl_2$ (▲) and their twofold dilutions were added to wells of mucin-coated microtiter plates. In this assay, culture medium without glycine or $MgCl_2$ (×) was used as a control. (B) Culture media containing 1% Triton X-100 (■), 1% Triton X-100–40-mmol/liter $MgCl_2$ (◆), and 40-mmol/liter $MgCl_2$ (▲) and their twofold dilutions were added to wells of mucin-coated microtiter plates. In this assay, culture medium without Triton X-100 or $MgCl_2$ (×) was used as a control.

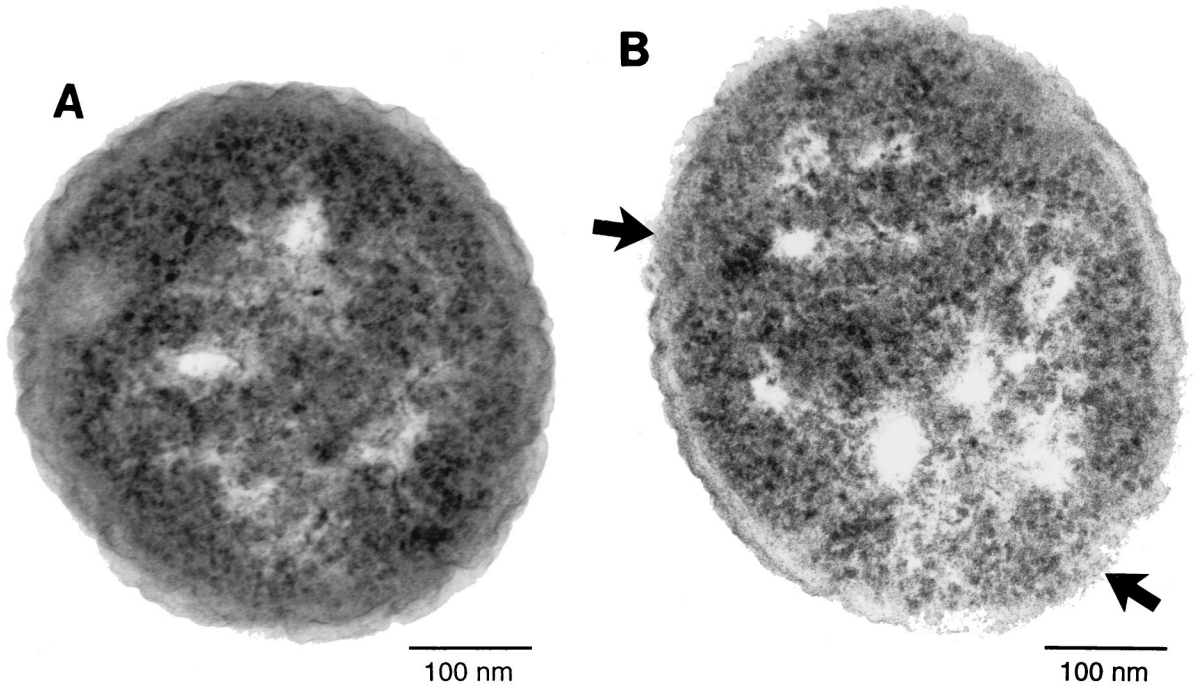


FIG. 5. Electron micrographs of K802 cells harboring expression vector pF1–$V_K LV_H$–TNF-α. Cell envelopes of untreated cells (A) and cells treated with 2% glycine and 1% Triton X-100 (B) are shown. Note the focally disrupted outermost cell wall (arrows).

concentrations, detergents partition into the lipid bilayer without causing solubilization. However, at high concentrations, they saturate the membrane lipid and become transformed into free detergent-lipid mixed micelles, leading to loss of bacterial outer membrane integrity (16). In the present study, we found that treatment of *E. coli* with 1% Triton X-100 increased fusion protein production and excretion 38-fold. However, this did not affect the excretion of *E. coli* β-galactosidase. A possible explanation for this is that Triton X-100 exerts its major effect on the outer membrane by causing loss of membrane integrity and has only a minor effect by increasing the permeability of inner membranes so that the leakage of the inner membrane caused by Triton X-100 treatment may not be sufficient to release the large (118-kDa) β-galactosidase molecule, which is almost three times as large as fusion protein sFV/TNF-α (43 kDa). Our electron microscopy studies further confirmed that the outermost layer of cell walls was disintegrating as a result of glycine and Triton X-100 treatment.

More interesting is the finding that when glycine and Triton X-100 were applied in a combination, the production and excretion of fusion protein sFV/TNF-α by *E. coli* increased 170-fold. It appears that the combined use of 2% glycine and 1% Triton X-100 may be advantageous for practical application in the production of large amounts of our fusion protein sFV/TNF-α. This cultivation method could be used for large-scale production of other heterologous proteins expressed in *E. coli*.

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