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Calcium Leads to Further Increase in Glycine-Enhanced Extracellular Secretion of Recombinant α-Cyclodextrin Glycosyltransferase in *Escherichia coli*

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Overexpression of recombinant genes in *Escherichia coli* and targeting recombinant proteins to the culture medium are highly desirable for the production of industrial enzymes. However, a major barrier is inadequate secretion of recombinant protein across the two membranes of *E. coli* cells. In the present study, we have attempted to circumvent this secretion problem of the recombinant α-cyclodextrin glycosyltransferase (α-CGTase) from *Paenibacillus macerans* strain JFB05-01. It was found that glycine, as a medium supplement, could enhance the extracellular secretion of recombinant α-CGTase in *E. coli*. In the culture with glycine at the optimal concentration of 150 mM, the α-CGTase activity in the culture medium reached 23.5 U/mL at 40 h of culture, which was 11-fold higher than that of the culture in regular TB medium. A 2.3-fold increase in the maximum extracellular productivity of recombinant α-CGTase was also observed. However, further analysis indicated that glycine supplementation exerted impaired cell growth as demonstrated by reduced cell number and viability, increased cell lysis, and damaged cell morphology, which prevented further improvement in overall enzyme productivity. Significantly, Ca^{2+} could remedy cell growth inhibition induced by glycine, thereby leading to further increase in the glycine-enhanced extracellular secretion of recombinant α-CGTase. In the culture with 150 mM glycine and 20 mM Ca^{2+}, both extracellular activity and maximum productivity of recombinant enzyme were 1.5-fold higher than those in the culture with glycine alone. To the best of our knowledge, this is the first article about the synergistic promoting effects of glycine and Ca^{2+} on the extracellular secretion of a recombinant protein in *E. coli*.

KEYWORDS: Cyclodextrin glycosyltransferase; *Paenibacillus macerans*; extracellular secretion; glycine; *Escherichia coli*; Ca^{2+}

INTRODUCTION

For production of industrial enzymes through genetic engineering, the bacterium *Escherichia coli* remains a very attractive expression system because of its simplicity, safety, and known genetic properties (*1*,*2*). However, overexpression of recombinant genes often results in the formation of inactive protein aggregates (inclusion bodies) in the cytoplasm, which is a significant barrier of gene expression in *E. coli* (*3*, *4*). To avoid the formation of inclusion bodies and recover high yields of correctly folded recombinant proteins, considerable efforts have been made to target the recombinant proteins outside the cytoplasm, mostly to the periplasmic space (*5*, *6*). Noteworthily, it is often observed that, from the periplasm, the recombinant proteins can leak spontaneously to the culture medium. In fact, targeting recombinant proteins to the culture medium presents significant advantages over periplasmic expression, including higher level of expression, lower level of proteolysis, and, most important, simpler large scale recovery and substantial purification by eliminating the need to disrupt cells (*4*). Despite these advantages, extracellular secretion of recombinant proteins has been limited, mainly due to the difficulties in translocating proteins across the two membranes of *E. coli* cells (*3*, *7*).

Various attempts have been made to facilitate extracellular secretion of recombinant proteins in *E. coli*. These included either molecular approaches, such as coexpression of molecular chaperones, manipulation of the various transport pathways, and fusing of the product to a carrier protein that is normally extracellularly secreted (*4*,*8*−*10*), or cultivation strategies, such as the changes of

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culture medium composition, temperature, pH, or aeration, medium supplements, and taking advantage of the growth-coupled effects on membrane components (*2*, *11*, *12*). The strategy using medium supplements, such as lysozyme, magnesium, calcium, EDTA, glycine, and Triton X-100, was frequently employed (*3*, *12*−*14*). Generally, these medium supplements were used individually; only seldom were they evaluated synergistically, although glycine and Triton X-100 had been found to have a synergistic effect on the extracellular secretion of recombinant proteins (*13*, *15*). Here, using an important industrial enzyme, cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19), as a model protein, we have attempted to explore the synergistic effect of glycine and calcium ion on the extracellular secretion of recombinant proteins in *E. coli* and to reveal the underlying mechanisms of the synergistic effect.

CGTase is an extracellular enzyme capable of converting starch or starch derivates into cyclodextrin through an intramolecular transglycosylation reaction (*5*,*6*). Cyclodextrins are cyclic, nonreducing oligo-glucopyranose molecules linked via α(1,4)-glycosidic bonds (*16*). They can form inclusion complexes with various hydrophobic guest molecules (*17*), and thus have wide applications in the industries related to food, pharmaceuticals, agriculture, etc. (*18*−*20*). The secretion of recombinant CGTase into the culture medium of *E. coli* remained a challenging task since this enzyme is usually accumulated in the cytoplasm as biologically inactive inclusion bodies and/or partially in the periplasm as soluble forms (*5*, *6*, *21*). This greatly limited the industrial applications of recombinant CGTase.

Previously, we had constructed an *E. coli* expression system of the *cgt* gene encoding α-CGTase from *Paenibacillus macerans* strain JFB05-01 (*22*). However, the extracellular production efficiency of recombinant α-CGTase needed to be improved. In the present study, we demonstrated significant enhancement of the extracellular secretion of recombinant α-CGTase by the synergistic effect of glycine and Ca^{2+}. Possible mechanisms are also discussed.

MATERIALS AND METHODS

Materials. The recombinant plasmid *cgt*/pET-20b(+), in which the *cgt* gene encoding the mature α-CGTase from *P. macerans* strain JFB05-01 (CCTCC M203062) was placed downstream of a DNA sequence coding *pelB* signal peptide, and *Escherichia coli* BL21(DE3) harboring plasmid *cgt*/pET-20b(+) were constructed previously (*22*). Peptone and yeast extract powder were obtained from Oxoid Ltd. (Basingstoke, England). Glycine (AR) was purchased from ShangHai Chemical Reagent Ltd. attached to China Medicine Group (ShangHai, China). *O*-Nitrophenyl-*β*-D-galactopyranoside (OPNG), isopropyl *β*-D-1-thiogalactopyranoside (IPTG), and methyl orange were purchased from Beyotime Institute of Biotechnology (Nantong, China). *N*-Phenyl-α-naphthylamine (NPN) was purchased from Aladdin-reagent Ltd. (ShangHai, China). Glucose-6-phosphate, NADP, and NADPH were purchased from Sigma-Aldrich (Milwaukee, WI, USA). All inorganic compounds were of reagent grade or higher quality.

Expression of α-CGTase. A single colony of *E. coli* BL21 (DE3) cells harboring plasmid *cgt*/pET-20b(+) was inoculated into 10 mL of Luria−Bertani medium containing 100 *μ*g/mL ampicillin and grown at 37 °C overnight. The overnight culture (1 mL) was then diluted into 100 mL of terrific broth (TB) containing 100 *μ*g/mL ampicillin in a 500 mL flask and incubated on a rotary shaker (200 rmp) at 30 °C until optical density at 600 nm (OD_{600}) of the culture reached 0.6. IPTG was added to a final concentration of 0.01 mM to induce the expression of the target protein. Protein induction was performed at 25 °C and continued for 90 h. To investigate the effect of glycine or Ca^{2+} on the growth of *E. coli* cells and extracellular secretion of recombinant α-CGTase, glycine and/or Ca^{2+} were (was) added to the culture medium at the beginning of the culture. Samples were withdrawn hourly and analyzed for OD_{600} and enzyme activities.

Cell Fractionation. Cell fractionation was performed as described by Kato et al. (*23*) with some modifications. The extracellular fraction was obtained by centrifugation of the culture broth at 10,000*g* for 20 min at 4 °C. To separate the periplasmic fraction, 1-mL culture was harvested and resuspended in 1 mL of 30 mM Tris-HCl solution (pH 7.0) containing 25% (w/v) sucrose and 1 mM EDTA. The cell suspension was incubated on ice for 2 h and pelleted by centrifugation at 10,000*g* for 20 min at 4 °C. The supernatant was collected as a periplasmic fraction. The residual cells were then resuspended in 1 mL of 50 mM sodium phosphate buffer (pH 6.0), and disrupted by ultrasonication with SONIFER 450 (Branson, Danbury, CT, USA). After centrifugation at 10,000*g* for 20 min at 4 °C, the supernatant and cell debris were collected as a soluble cytoplasmic and insoluble inclusion body fraction, respectively. The cell debris was resuspended in 0.1 mL of sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris, pH 7.6; 100 mM 2-mercaptoethanol; 2% SDS; 5% glycerol; and 0.150 mM bromophenol blue), and boiled for 10 min to solubilize the inclusion bodies.

N-Terminal Amino Acid Sequencing. Isolated inclusion bodies were subjected to automated Edman degradation sequencing by using a 492cLC protein sequencer (Applied Biosystems Inc., Foster City, CA).

Assay of α-CGTase. The α-cyclodextrin forming activity was determined by the methyl orange method as described previously (*24*) with some modifications. The culture or cytoplasmic supernatant (0.1 mL) (appropriately diluted in 50 mM sodium phosphate buffer) was incubated with 0.9 mL of 3% (w/v) soluble starch in 50 mM phosphate buffer (pH 6.0) at 40 °C for 10 min. The reaction was terminated by the addition of 1.0 M HCl (1.0 mL), and then 1.0 mL of 0.1 mM methyl orange in 50 mM phosphate buffer (pH 6.0) was added. After the reaction mixture was incubated at 16 °C for 20 min, the amount of α-cyclodextrin in the mixture was spectrophotometrically determined by measuring the absorbance at 505 nm. At the chosen conditions, the molar extinction coefficient of methyl orange was $\varepsilon = 2.98 \times 10^4$ L/(mol cm). One unit of α-CGTase activity was defined as the amount of enzyme that was able to produce 1 *μ*mol of α-cyclodextrin per min under the above conditions.

Assay of Glucose-6-phosphate Dehydrogenase. Glucose-6-phosphate dehydrogenase (G6PD) activity in the extracellular or cytoplasmic fraction was determined as described previously (*25*) with some modifications. The incubation mixture contained 1 mM glucose-6-phosphate, 0.075 mM NADP, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0), and the culture or cytoplasmic supernatant in a volume of 1.0 mL. The increase in absorbance at 340 nm was measured at 25 °C in a spectrophotometer (UV-2450; Shimadzu Co., Kyoto, Japan). At the chosen conditions, the molar extinction coefficient of NADPH was $\varepsilon = 6.22 \times 10^3$ L/(mol cm). One unit of G6PD activity was defined as the amount of enzyme that was able to produce 1 *μ*mol of NADPH per min.

Assay of Outer Membrane Permeability. Outer membrane permeability was measured using the NPN access assay as described previously (*26*). Samples of *E. coli* BL21 (DE3) cells were withdrawn at different growth stages, rinsed once using centrifugation at 3000*g*, and resuspended in 10 mM sodium phosphate buffer (pH 7.4) to an OD_{600} of 0.5. NPN was added to a final concentration of 10 mM into quartz cuvettes containing 2 mL of cell suspension. The sample was mixed by inversion of the cuvette immediately prior to fluorescence monitoring. Fluorescence was measured using a Shimadzu RF-1501 spectrofluorometer (Shimadzu Co., Kyoto, Japan) with slit widths set to 1 mm and excitation and emission wavelengths set to 350 and 420 nm, respectively.

Assay of Inner Membrane Permeability. Permeability of the inner membrane was assessed by measuring the access of ONPG to the cytoplasm essentially as described previously (*27*). Briefly, ONPG was added to a concentration of 100 *μ*g/mL to *E. coli* BL21 (DE3) cells prepared as described above, and substrate cleavage by *β*-galactosidase was monitored by light absorption measurements at 420 nm in a spectrophotometer (UV-2450; Shimadzu Co., Kyoto, Japan).

Cell Morphology. *E. coli* BL21 (DE3) cells were withdrawn at 36 h of culture, rinsed once by centrifugation at 3000*g*. For scanning electron micrography (SEM), cell pellets were fixed in 6% glutaraldehyde and 100 mM sodium phosphate buffer. After overnight fixation at 4 °C, the cell pellets were washed in sodium phosphate buffer, postfixed in 150 mM OsO4 (100 mM sodium phosphate buffer, pH 7.0), then suspended in 150 mM agar, dehydrated in ethyl alcohol, and embedded in Araldite. Samples were sputter-coated with Au/Pd using a vacuum evaporator

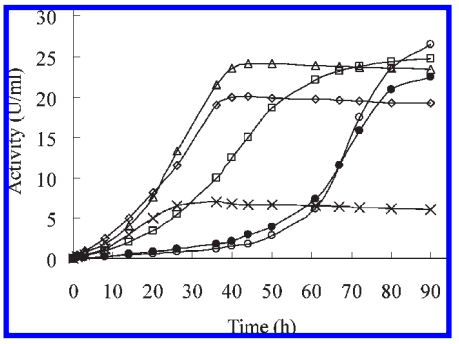

Figure 1. Effects of glycine or Ca^{2+} on extracellular secretion of recombinant α-CGTase. *E. coli* cells were grown in regular TB medium (●) or TB medium supplemented with glycine at 75 mM (□), 150 mM (Δ), 200 mM (◇), 300 mM (×), or Ca^{2+} at 20 mM (○). Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%.

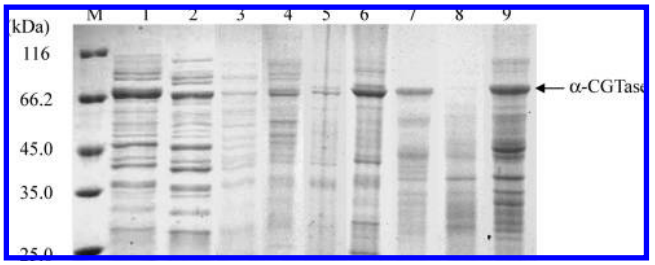

Figure 2. SDS−PAGE analysis of α-CGTase distribution. The distribution was analyzed after 40 h of culture. *E. coli* cells were grown in regular TB medium (lanes 3, 6, and 9) and TB medium supplemented with 150 mM glycine (lanes 2, 5, and 8) or 150 mM glycine−20 mM Ca^{2+} (lanes 1, 4, and 7). Lane M, protein size marker; lanes 1, 2, and 3, extracellular fraction (10 μL); lanes 4, 5, and 6, periplasmic fraction (10 μL); lanes 7, 8, and 9, intracellular insoluble fraction (5 μL).

(Ewards, Milano, Italy) and examined using a scanning electron microscope (Hitachi S-4700, Tokyo, Japan) at 10 kV accelerating voltage.

Miscellaneous Methods. Cell density was determined by measuring OD_{600} of the culture using a spectrophotometer (UV-2450; Shimadzu Co., Kyoto, Japan) after an appropriate dilution. Colony-forming unit (CFU) per milliliter of culture per OD_{600} was estimated by plating suitably diluted samples of a culture and counting colonies which appeared after 12 h of incubation at 37 °C. The extracellular productivity of recombinant α-CGTase at each culture time point was the ratio of the α-CGTase activity in the culture medium to the culture time. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE, 10% gel) was performed under denaturing conditions with a Mini-PROTEAN II electrophoresis unit (Bio-Rad Laboratories, Richmond, CA, USA). Protein bands were visualized by staining with 0.25% Coomassie Brilliant Blue R-250.

RESULTS

Effect of Glycine and Ca^{2+} on Extracellular Secretion of α-CGTase. When *E. coli* cells were cultured in regular TB medium, very few recombinant α-CGTases were secreted into the culture medium within the first 40 h (**Figures** 1 and 2). SDS−PAGE analysis of α-CGTase distribution showed that many recombinant enzymes were accumulated in the periplasmic space, and a few insoluble inclusion bodies were formed at 40 h of culture (**Figure** 2). Furthermore, the N-terminal amino acid sequence of inclusion body proteins was determined to mainly be Met-Lys-Tyr-Leu-Leu, the same as that of the *pelB* signal peptide, suggesting that most inclusion bodies were formed in the cytoplasm. In addition, no α-CGTase activity was detected in the soluble cytoplasmic fraction. After the lag phase of extracellular secretion, the activity of α-CGTase in the culture medium increased gradually and reached 22.5 U/mL at 90 h of culture (**Figure** 1), suggesting that extracellular secretion of the recombinant enzyme was limited to the stationary or decline phase of cell growth. Because of the long lag time, the overall extracellular productivity of recombinant α-CGTase was relatively low. The maximum value of extracellular productivity was only 0.26 U/mL/h at 80 h of culture.

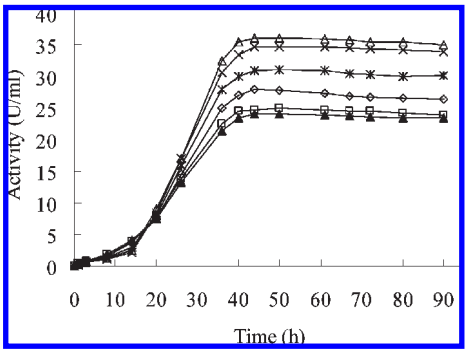

Figure 3. Synergistic effect of glycine and Ca^{2+} on extracellular secretion of recombinant α-CGTase. *E. coli* cells were grown in TB medium supplemented with 150 mM glycine (▲), 150 mM glycine−5 mM Ca^{2+} (◇), 150 mM glycine−10 mM Ca^{2+} (*), 150 mM glycine−20 mM Ca^{2+} (Δ), 150 mM glycine−30 mM Ca^{2+} (×), and 150 mM glycine−20 mM Ca^{2+}−20 mM EDTA (□). Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%.

When *E. coli* cells were cultured in TB medium supplemented with 75−200 mM glycine, the enhancement in the extracellular secretion of recombinant α-CGTase was obviously observed (**Figures** 1 and 2). At the optimal glycine concentration of 150 mM, the α-CGTase activity in the culture medium reached 23.5 U/mL at 40 h of culture, which was 11-fold higher than that of the culture in regular TB medium at the same culture time. At 36 h of culture, the extracellular productivity of recombinant α-CGTase reached the maximum value of approximately 0.60 U/mL/h, which was 2.3-fold higher than that of the culture in regular TB medium at 80 h of culture. Furthermore, SDS−PAGE analysis showed that, at 40 h of culture, no appreciable recombinant enzyme was accumulated in the periplasmic space, and almost no insoluble inclusion body was formed (**Figure** 2), suggesting successful translocation of this protein across the cell membranes. It is worthy of note that, at the glycine concentrations of above 200 mM, the enhancing effects of glycine on the extracellular activity and productivity of recombinant enzyme were significantly reduced (**Figure** 1), although neither recombinant enzyme was accumulated in the periplasmic space, nor was inclusion body formed at 40 h of culture (data not shown).

When *E. coli* cells were cultured in TB medium supplemented with 20 mM Ca^{2+}, no significant change in the extracellular secretion of recombinant α-CGTase was observed (**Figure** 1). This phenomenon was also apparent at other Ca^{2+} concentrations ranging from 5 to 30 mM (data not shown).

Very interestingly, when *E. coli* cells were cultured in TB medium supplemented with both glycine and Ca^{2+}, further increase in the glycine-enhanced extracellular secretion of recombinant α-CGTase was also obviously observed (**Figures** 2 and 3). As shown in **Figure** 3, the optimal Ca^{2+} concentration was 20 mM. In the culture with 150 mM glycine and 20 mM Ca^{2+}, the α-CGTase activity in the culture medium reached 35.5 U/mL at 40 h of culture, which was approximately 1.5-fold higher than that in the culture with 150 mM glycine alone at the same

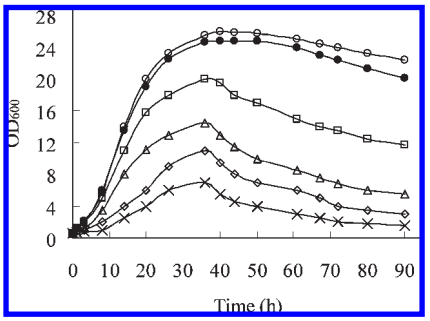

Figure 4. Effects of glycine or Ca^{2+} on cell density. *E. coli* cells were grown in regular TB medium (●) or TB medium supplemented with glycine at 75 mM (□), 150 mM (Δ), 200 mM (◇), 300 mM (×), or Ca^{2+} at 20 mM (○). Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%.

culture time. At 36 h of culture, the extracellular productivity of recombinant α-CGTase reached the maximum value of approximately 0.90 U/mL/h, which was also 1.5-fold higher than that in the culture with 150 mM glycine alone. Furthermore, SDS−PAGE analysis showed very little periplasmic accumulation and inclusion body formation of recombinant enzyme at 40 h of culture (**Figure** 2), suggesting almost adequate secretion of this protein across the cell membranes.

To confirm that the above further increase in the glycine-enhanced extracellular secretion was exerted by Ca^{2+}, 20 mM EDTA was added to the TB medium supplemented with 150 mM glycine and 20 mM Ca^{2+}. We found that the extracellular secretion of recombinant α-CGTase indeed returned to almost the same level as that in the culture with glycine alone (**Figure** 3).

Effect of Glycine and Ca^{2+} on Cell Growth. Although glycine stimulated the extracellular secretion of recombinant α-CGTase, it was found to inhibit the growth of *E. coli* cells in a dose-dependent manner. As shown in **Figure** 4, in the culture with glycine, the OD_{600} value at each culture time point was significantly lower than that of the culture in regular TB medium, indicating a reduction in total cell number. For example, in the culture with 75 mM, 150 mM, or 300 mM glycine, the OD_{600} value at 36 h of culture decreased by approximately 20%, 42%, or 72%, respectively. In contrast, in the culture with 20 mM Ca^{2+}, the OD_{600} value at each time point was slightly higher than that of the culture in regular TB medium (**Figure** 4). Furthermore, Ca^{2+} at other concentrations ranging from 5 to 30 mM also resulted in slightly increased OD_{600} values (data not shown).

Subsequently, the effects of glycine or Ca^{2+} on cell viability and possible cell lysis were analyzed. CFU was used as an indicator of viable cell number. In the culture with 150 mM glycine, CFU per milliliter of culture per OD_{600} was relatively low (**Figure** 5). At 36 h of culture, it was 45% lower than that of the culture in regular TB medium, indicating that glycine had a significantly negative effect on cell viability. G6PD is a cytoplasmic protein, and the level of this enzyme in the culture medium is expected to be very low unless substantial cell lysis has occurred (*25*). Thus, G6PD activity in the culture medium can be used as an indicator of cell lysis. It was found that, in the culture without glycine and Ca^{2+}, G6PD activity in the culture medium was very low (**Figure** 5), while G6PD activity in the cytoplasmic fraction reached 2.23 U/mL at 36 h of culture, indicating almost no cell lysis. In the culture with 150 mM glycine, G6PD activity in the culture medium increased gradually and reached 0.28 U/mL at 36 h of culture (**Figure** 5), which was approximately 23% of G6PD activity in the cytoplasmic fraction (1.23 U/mL), indicating that 150 mM glycine supplementation caused obvious cell lysis. However, in

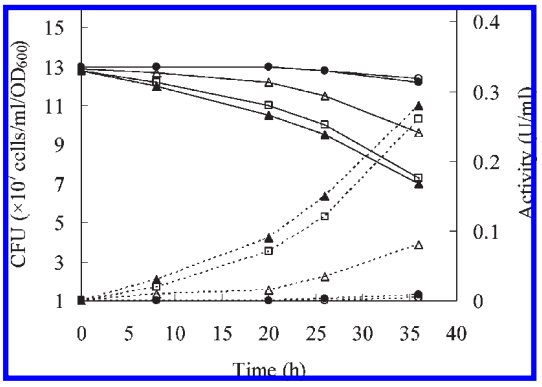

Figure 5. Effects of glycine and/or Ca^{2+} on cell viability and cell lysis. *E. coli* cells were grown in regular TB medium (●) or TB medium supplemented with 150 mM glycine (▲), 20 mM Ca^{2+} (○), 150 mM glycine −20 mM Ca^{2+} (Δ), or 150 mM glycine−20 mM Ca^{2+}−20 mM EDTA (□). Time-courses of CFU per milliliter of culture per OD_{600} (solid line) and G6PD activity in the culture medium (dashed line) were analyzed. Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%.

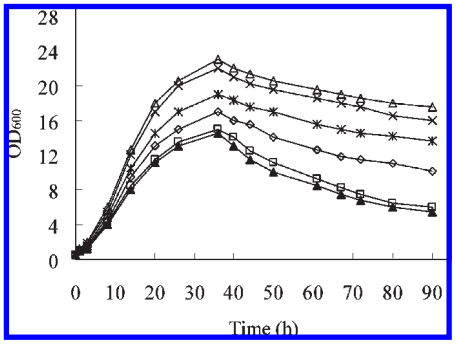

Figure 6. Effects of glycine and Ca^{2+} on cell density. *E. coli* cells were grown in TB medium supplemented with 150 mM glycine (▲), 150 mM glycine−5 mM Ca^{2+} (◇), 150 mM glycine−10 mM Ca^{2+} (*), 150 mM glycine−20 mM Ca^{2+} (Δ), 150 mM glycine−30 mM Ca^{2+} (×), or 150 mM glycine−20 mM Ca^{2+}−20 mM EDTA (□). Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%.

the culture with 20 mM Ca^{2+}, almost no change in cell viability and cell lysis was observed as compared with the culture in regular TB medium (**Figure** 5). Furthermore, 5−30 mM Ca^{2+} had similar effects on cell viability and cell lysis (data not shown).

Although Ca^{2+} did not appear to obviously promote cell growth in the culture without glycine, it was found to significantly remedy cell growth inhibition induced by glycine (**Figures** 5 and 6). In the culture with 150 mM glycine and 20 mM Ca^{2+}, both the OD_{600} value and the CFU value at each culture time point were higher than those in the culture with 150 mM glycine alone, and close to those in the culture without glycine (**Figures** 5 and 6), indicating increases of cell number and viability. At 36 h of culture, OD_{600} and CFU values were approximately 1.6- and 1.4-fold higher, respectively, than those in the culture with 150 mM glycine alone. Similarly, in the culture with 150 mM glycine and 20 mM Ca^{2+}, G6PD activity in the culture medium at each culture time point was lower than that in the culture with 150 mM glycine alone (**Figure** 5). At 36 h of culture, G6PD activity in the culture medium (0.08 U/mL) had an approximately 71% reduction as compared with that in the culture with 150 mM glycine alone. Furthermore, there was only 4% of G6PD activity in the cytoplasmic fraction (2.01 U/mL), indicating no obvious cell lysis.

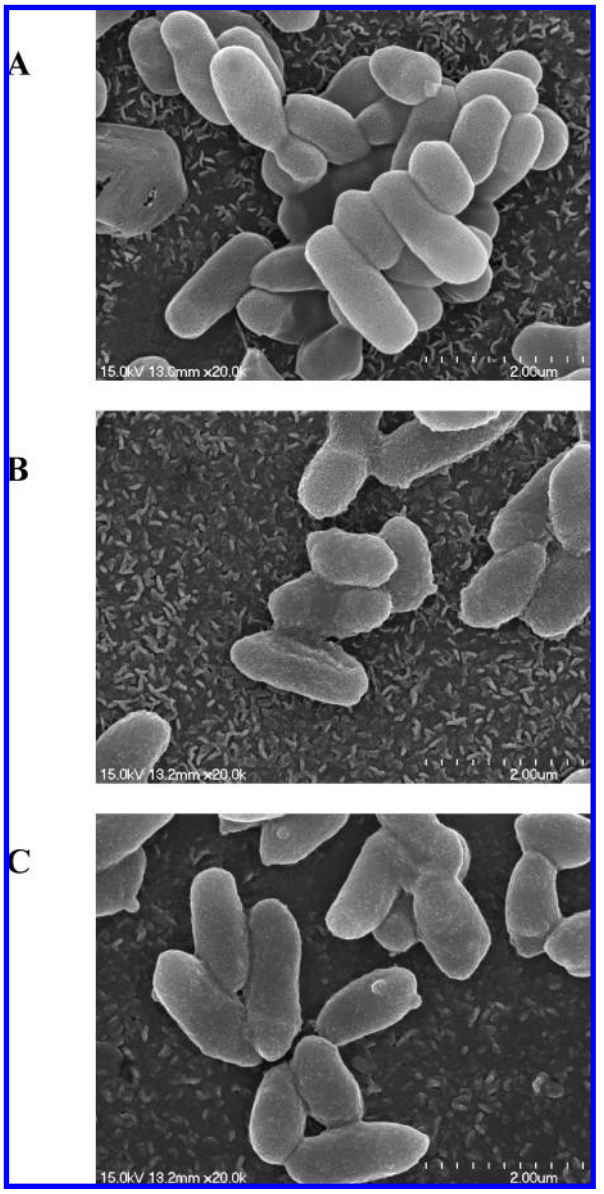

Figure 7. Effects of glycine and/or Ca^{2+} on cell morphology. *E. coli* cells were imaged by scanning electron microcopies when grown for 36 h in regular TB medium (**A**), TB medium supplemented with 150 mM glycine (**B**), or 150 mM glycine−20 mM Ca^{2+} (**C**).

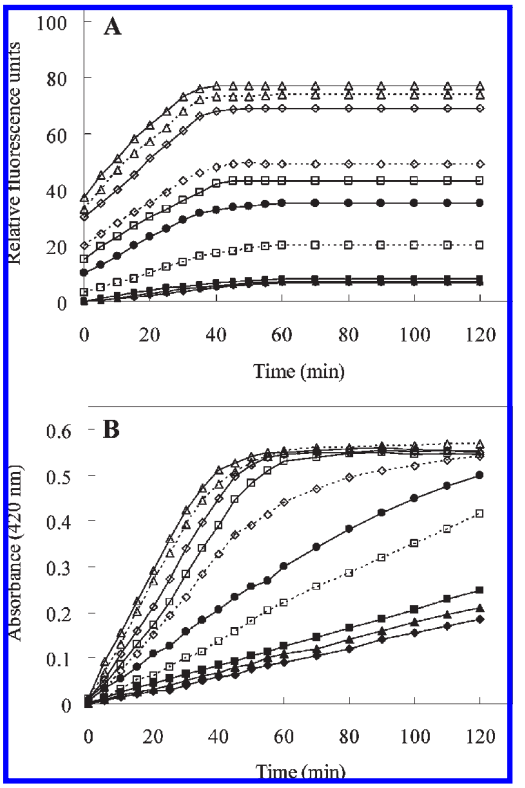

Figure 8. Effects of glycine and/or Ca^{2+} on the permeabilities of outer (**A**) and inner (**B**) membranes. After *E. coli* cells had been grown in regular TB medium for 12 h (■), 24 h (♦), 36 h (▲), and 60 h (●) and TB medium supplemented with 150 mM glycine (solid line) or 150 mM glycine−20 mM Ca^{2+} (dashed line) for 12 h (□), 24 h (◇), and 36 h (Δ), they were harvested, rinsed, and resuspended in 10 mM phosphate buffer (pH 7.4) to an OD_{600} of 0.5. NPN or ONPG was added to the cell suspension. NPN fluorescence and the absorbance at 420 nm were monitored for 120 min. Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%.

To further confirm that Ca^{2+} remedied cell growth inhibition induced by glycine, 20 mM EDTA was added to the TB medium supplemented with 150 mM glycine and 20 mM Ca^{2+}. It was observed that the OD_{600} value, CFU value, and G6PD activity in the culture medium indeed returned to almost the same levels as those in the culture with 150 mM glycine alone (**Figures** 5 and 6).

In the meantime, the effects of glycine or Ca^{2+} on the morphology of *E. coli* cells were evaluated using SEM. *E. coli* cells in the culture without glycine exhibited a regular shape and a smooth surface (**Figure** 7**A**). However, glycine supplementation caused the collapse and irregular shape of the cell granules giving a rough surface (**Figure** 7**B**), whereas Ca^{2+} partially counteracted the destructive effect of glycine on cell morphology (**Figure** 7**C**).

Effect of Glycine and Ca^{2+} on Cell Membrane Permeability. The hydrophobic fluorescent probe NPN was used as an indicator of outer membrane integrity. NPN has a low fluorescence quantum yield in aqueous solution but fluoresces strongly in the hydrophobic environment of a biological membrane. Normally, NPN is excluded from *E. coli* by the lipopolysaccharide layer of the outer membrane but can enter at points where membrane integrity is compromised (*28*). Thus, fluorescence value and its increased rate indicated the outer membrane permeability. Permeability of the inner membrane was evaluated using the β-galactosidase substrate ONPG as a probe. When ONPG passes the inner membrane, it can be cleaved by β-galactosidase localized within the cytoplasm, resulting in the appearance of yellow color. Thus, absorbency at 420 nm and its increased rate indicated inner membrane permeability.

As shown in **Figure** 8, in the culture without glycine, both outer and inner membranes of *E. coli* cells had very low permeabilities during the first 36 h of culture. Upon further culture, the membrane permeabilities gradually increased, which might be the main reason for the increase in extracellular secretion of recombinant α-CGTase at the later stage of culture (**Figure** 1). In the culture with 150 mM glycine, the outer and inner membranes of *E. coli* cells had high permeabilities soon after the culture started. Furthermore, the glycine-induced increases in the membrane permeabilities were dose-dependent (data not shown). Although, in the culture with 150 mM glycine and 20 mM Ca^{2+}, the outer and inner membranes of *E. coli* cells had relatively low permeabilities during the first 24 h of culture, their permeabilities kept on increasing and reached almost the same levels as those in the culture with 150 mM glycine alone at 36 h of culture.

DISCUSSION

The targeting of recombinant proteins for secretion to the culture medium of *E. coli* presents significant advantages. However, translocating the recombinant protein outside of the cell requires overcoming two membrane barriers, which remains a formidable task (*29*). Here, we found that, when *E. coli* cells were cultured in regular TB medium, although the *pelB* signal peptide enabled the translocation of α-CGTase to the periplasic space of *E. coli* cells through s sec-dependent secretion pathway, the secretions of this protein across the inner and outer membranes were inadequate as demonstrated by periplasmic accumulation and insoluble inclusion body formation of the recombinant enzyme. Thus, we have attempted to circumvent the secretion problem of recombinant α-CGTase in *E. coli*.

Glycine, a commonly used medium supplement, was found to efficiently enhance the extracellular secretion of recombinant α-CGTase. Such enhancement has also been reported by other investigators (*13*−*15*, *30*, *31*). A potential mechanism for this enhancement was generally considered to be the increased cell membrane permeabilities (*13*), which could promote the translocation of the recombinant protein across the two membranes of *E. coli* cells. The increase in membrane permeabilities might be induced by the modification of peptidoglycan that resulted from the incorporation of glycine into the nucleotide-activated peptidoglycan precursors (*32*). In the present study, our quantitative analysis confirmed the role of glycine in increasing cell membrane permeabilities. Additionally, cell lysis might also contribute to the extracellular secretion of the recombinant enzyme. However, it was generally undesirable for extracellular recombinant protein production mainly due to more contaminants from the intracellular compartment (*29*, *33*). However, we found that glycine supplementation exerted significantly negative effects on cell growth as demonstrated by reduced cell number and viability, increased cell lysis, and damaged cell morphology, which resulted in less enzyme producers and shorter production runs. Thus, although the increases in cell membrane permeabilities could improve the extracellular secretion of recombinant enzyme, cell growth inhibition prevented a further increase in overall extracellular productivity of the recombinant enzyme.

To overcome the above dilemma, it was desirable to promote healthy cell growth under the premise of maintaining sufficient membrane permeabilities. In search of a solution, we found that calcium was reported to protect bacterial cells (*34*−*36*) through several mechanisms: (i) calcium appears to have a structural role in stabilizing the lipopolysaccharide monolayer of the outer membrane (*34*, *37*) and helping maintain the organization of the cell envelope (*38*, *39*); (ii) calcium plays important roles in the cell cycle and cell division (*34*, *37*). In particular, calcium may help maintain a proper level of a key cell division protein, FtsZ, whose level is reduced in cell wall-impaired *E. coli* cells, thereby promoting the growth of abnormal cells (*40*), and (iii) calcium can suppress peptidoglycan degradation induced by peptidoglycan hydrolases and is often used to control the autolytic process of *E. coli* cells (*39*, *41*). Thus, we speculated that calcium might be able to partially counteract the negative effect of glycine on cell growth.

To test the above possibility, we investigated the effects of Ca^{2+} on the growth of *E. coli* cells in TB medium rich in glycine. Indeed, Ca^{2+} notably increased cell number and viability, and protected cell morphology while effectively suppressing cell lysis. However, an important question arose as to whether Ca^{2+} could counteract the glycine-enhanced extracellular secretion of recombinant enzyme since it has been reported that divalent cations, such as Ca^{2+} and Mg^{2+}, limited membrane permeabilization induced by permeabilizers (*27*). In fact, our results did show that the permeabilities of both outer and inner membranes were decreased by Ca^{2+} during the first 24 h. Nevertheless, they were still higher than these in the culture without glycine. Furthermore, at 36 h of culture, membrane permeabilities increased to levels comparable to those in the culture with glycine alone. Thus, Ca^{2+} did hardly influence the total translocation rates of recombinant α-CGTase across the cell membranes. As a result, Ca^{2+} facilitated healthy cell growth under the premise of maintaining sufficient membrane permeabilities, thereby leading to further increase in the glycine-enhanced extracellular secretion of recombinant α-CGTase. Importantly, in the culture with glycine and Ca^{2+}, the low level of G6PD in the culture medium indicated that cell lysis was almost not responsible for the extracellular secretion of the recombinant enzyme.

In summary, our study demonstrated that glycine and Ca^{2+} markedly enhanced the extracellular secretion of recombinant α-CGTase and enzyme extracellular productivity. To the best of our knowledge, this is the first article about the synergistic promoting effects of glycine and Ca^{2+} on the extracellular secretion of a recombinant protein in *E. coli*. This approach could be used for extracellular secretion of other heterogeneous proteins in *E. coli*.

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