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RESEARCH PAPER

Glycine and Triton X-100 Enhanced Secretion of Recombinant α-CGTase Mediated by OmpA Signal Peptide in *Escherichia coli*

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Abstract OmpA signal peptide mediated *cgt* gene from *Paenibacillus macerans* JFB05-01 was cloned and expressed in *E. coli* BL21 (DE3). The effects of glycine and Triton X-100 on extracellular production of α-cyclodextrin glycosyltransferase (α-CGTase) were investigated. When supplemented with Gly or Triton X-100 to the culture media individually, the secreted extracellular enzyme reached 32 or 33 U/mL at 48 h of cultivation, respectively. When supplemented with Gly and Triton X-100 together, the extracellular α-CGTase activity reached 48 U/mL after 48 h cultivation, which was 20-fold of the control group without any additives. Analysis of membrane permeability demonstrated that addition of glycine and Triton X-100 enhanced the permeability of both outer and inner membrane. The potential mechanism of the enhanced protein secretion was discussed.

Keywords: *E. coli*, α-cyclodextrin glycosyltransferase, OmpA signal peptide, extracellular expression

1. Introduction

Cyclodextrin glucosyltransferase (CGTase, EC2.4.1.19) is a member of the α-amylase family of glycosyl hydrolases (family 13) [1]. It can convert starch or starch derivates into cyclodextrin (CD) through a cyclization reaction. In addition to cyclization reaction, CGTase also catalyzes three other reactions: coupling, disproportionation, and starch hydrolysis reactions. Since CDs have a cylindrical shape with a nonpolar cavity, they are able to form an inclusion complex with many hydrophobic molecules, and thus have wide applications in the industries related to food, pharmaceuticals, agriculture, cosmetics, chemical, and environmental protection [2].

CGTases can be produced by a variety of bacteria. However, their yields and productivity were relatively low in wild-type strain. To overcome its low productivity, a great deal of attention has been paid to express CGTases in *E. coli*. However, previous reports showed that the α-CGTase expressed in *E. coli* was usually accumulated in the cytosol as biologically inactive inclusion bodies, which needs to be converted to the active proteins by a refolding process. Thus, its industrial application was limited.

The α-CGTase from *Paenibacillus macerans* is most commonly used for the commercial production of α-cyclodextrin. In a previous study, our group has cloned *P. macerans* α-CGTase and extracellular expressed the enzyme in *E. coli* using PelB as a signal peptide. In addition, the effect of glycine on the secretory expression of recombinant α-CGTase was investigated [3,4]. It was found that glycine could stimulate the extracellular enzyme production, but a high concentration of glycine could accelerate cell lysis. Effects of media additives on extracellular secretion of α-CGTase mediated by PelB signal peptide were investigated as well. The optimal condition to achieve maximal secretion of α-CGTase was the supplementation with 0.03% SDS, 400 mM Na^+, 0.3% glycine, and 10 mM Ca^{2+} together [5].

Several reports have shown that OmpA signal peptide could mediate successful secretion of exogenous recombinant

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protein in *E. coli* [6,7]. In the present study, the effect of OmpA signal peptide on the secretory expression of *P. macerans* α-CGTase in *E. coli* was investigated. In addition, it was reported that glycine and Triton X-100 had a synergistic effect on the extracellular expression of recombinant enzymes in *E. coli* [8,9]. In the present study, the effect of glycine and Triton X-100 on the extracellular production of α-CGTase mediated by OmpA signal peptide was investigated and the potential mechanism was explored.

2. Materials and Methods

2.1. Materials

The recombinant plasmid pT7-7/*cgt* that carries the mature *cgt* gene of *P. macerans* JFB05-01 and expression host *E. coli* BL21 (DE3) were preserved previously by our laboratory. Peptone and yeast powder were obtained from Oxiod Ltd. Glycine (AR) and Triton X-100 were purchased from Shanghai Chemical Reagent Ltd. attached to China Medicine Group (Shanghai, China). N-phenyl-1-naphthylamine (NPN) was purchased from Aladdin-reagent Ltd (Shanghai, China).

2.2. Cloning and expression of CGTase

The *cgt* gene was amplified from recombinant vector pT7-7/*cgt* (lab stock) by PCR using Taq DNA polymerase. The forward primer was: 5'-GATATACATATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCCTCACCCGATACGAG-3' (*Nde* I underlined), which contained OmpA signal sequence gene (NCBI accession number: AAC74043), the reverse primer was 5'-CATCTCGAGAGAATTCGGATTTTGCCAGTCCACCGTC-3' (*EcoR* I underlined). The PCR product was digested by *Nde* I/*EcoR* I and ligated into the similarly restriction-digested expression vector pET-20b (+). The recombinant plasmid was verified by DNA sequencing and named *ompA*-pECGT. In order to compare the extracellular production of α-CGTase with and without OmpA signal sequence, the mature gene of α-CGTase was inserted into the *Nde* I/*EcoR* I digested pET-20b (+) and the recombinant vector was named as pECGT. Both vectors were subsequently transformed into chemically competent *E. coli* BL21 (DE3) cells.

2.3. Cell culture and the supplemental of enhancers

A single colony of *E. coli* BL21 (DE3) cells harboring the recombinant plasmid was inoculated into 10 mL Luria–Bertani (LB) medium containing 100 μg/mL ampicillin and grown at 37°C overnight. The culture was diluted to 4:100 with terrific broth (TB). The cultures occurred in a 500 mL flask containing 100 mL TB medium supplemented with 100 μg/mL ampicillin on a rotary shaker (200 rpm) at 25°C. To investigate the effect of glycine or Triton X-100 on the cell growth and extracellular secretion of the recombinant α-CGTase, different amounts of glycine were added into TB medium of 15 h cultivation and Triton X-100 were added into the culture medium upon inoculation. Aliquots of samples were analyzed for dry cell weight (DCW) and enzyme activities.

2.4. Cellular fractionation

The cell fractionation method was performed as follows: the extracellular fraction was obtained after centrifugation of the culture broth. The fully washed and centrifuged cells from 1 mL of the culture broth were suspended in a fixed volume of 25% (w/v) sucrose solution containing 1 mM EDTA, and treated with ice-cold water. The supernatant was collected as a periplasmic fractionation. The residual cells were then resuspended in 1 mL of 50 mM sodium phosphate buffer and disrupted by ultrasonication with a SONIFIER (Branson, USA) at 4°C for 5 min.

2.5. Assay of α-CGTase activity

The α-CGTase activity was determined by the methyl orange (MO) method as described previously [3].

2.6. Assay of inner and outer membrane permeability

Permeability of the inner membrane was assessed by measuring the access of o-Nitrophenyl-β-D-galactoside (ONPG) to the cytoplasm as described previously [4]. Briefly, ONPG was added to a final concentration of 100 μg/mL into the cell suspension prepared as described above. Substrate cleavage by β-galactosidase was determined by light absorption measurements at 420 nm in a spectrophotometer.

The N-Phenyl-1-naphthylamine (NPN) access assay was performed essentially, as described previously [4]. Briefly, NPN was added to achieve a final concentration of 20 μmol. Fluorescence was measured with slit widths set to 5 nm and excitation and emission wavelengths were set to 350 nm and 428 nm, respectively.

The principle of the assay was as follows: the fluorescence of NPN is weak in the aqueous solution and strong in the hydrophobic environment. Under normal circumstances, when NPN is added into the solution containing normal cell, it is isolated by the outer lipopolysaccharide layer of the outer membrane of the cell and is in aqueous solution, so its fluorescence is weak. However, when the cell membrane becomes incomplete, NPN will combine into the cell membrane and in this hydrophobic environment, the NPN fluorescence becomes strong and NPN fluorescence assay can be used to evaluate the membrane permeability of the cell.

3. Results and Discussion

3.1. Cloning and expression of *P. macerans* α-CGTase mediated by OmpA signal peptide

The gene of *P. macerans* α-CGTase mediated by OmpA signal peptide was cloned and expressed in *E. coli* BL21 (DE3). The recombinant cell was cultivated in TB medium supplemented with 100 μg/mL ampicillin at 25ºC. The growth of *E. coli* cells and the activity of α-CGTase in the supernatant were monitored. As shown in Fig. 1, it has a significant lag phase in the extracellular production of α-CGTase. After 80 h of cultivation at 25ºC, α-CGTase activity in the supernatant reached 40 U/mL, which is about 2-fold of that by PelB signal peptide [3]. When the mature CGTase was expressed without mediation of the signal peptide, the expressed protein mainly existed as inclusion bodies in the cytosol and no detectable enzymatic activity was observed in the break supernatant and in the culture medium.

The reason why OmpA mediated CGTase secretion is more efficient than that of PelB is not clear. In fact, even though many researchers have attempted to find the general rule between the target protein and their matched signal sequences [6], currently, it is somewhat difficult to select a proper signal sequence for a given recombinant protein to guarantee its successful secretion without trial-and-error.

3.2. Effects of glycine on extracellular production of α-CGTase

In a previous study, it was found that glycine could stimulate the extracellular secretion of α-CGTase mediated by PelB signal peptide and significantly shorten the fermentation period. In the present study, the effect of glycine on the production of α-CGTase mediated by OmpA was also investigated. Different concentration of glycine was added into TB medium of 15 h cultivation. The cell growth and extracellular enzyme production upon glycine supplementation were analyzed (Fig. 2). The results showed that extracellular α-CGTase production with different concentration of glycine were all increased after 48 h cultivation when compared with that from the control group. However, when glycine concentration was 1% or more, *E. coli* cell growth was profoundly inhibited, the maximum bacterial biomass was only 72% of that from the control group; in addition, cell lysis was also accelerated. Among the concentration of glycine tested, the optimum concentration appeared to be 0.7% (w/v). In this condition, the extracellular enzyme was 32 U/mL by 48 h of cultivation and the productivity reached 0.66 U/mL/h, which was 12.4 times that of the control group.

The mechanism for enhanced secretion of recombinant

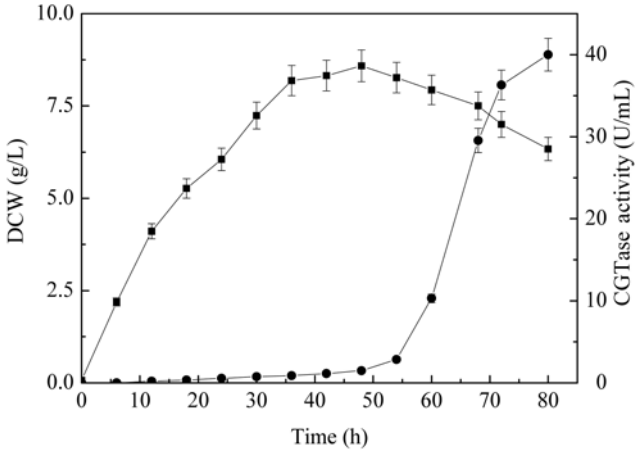

Fig. 1. Time courses of cell growth (■) and extracellular α-CGTase activity (●) by recombinant *E. coli*. *Error bars* correspond to the standard deviation of three independent determinations.

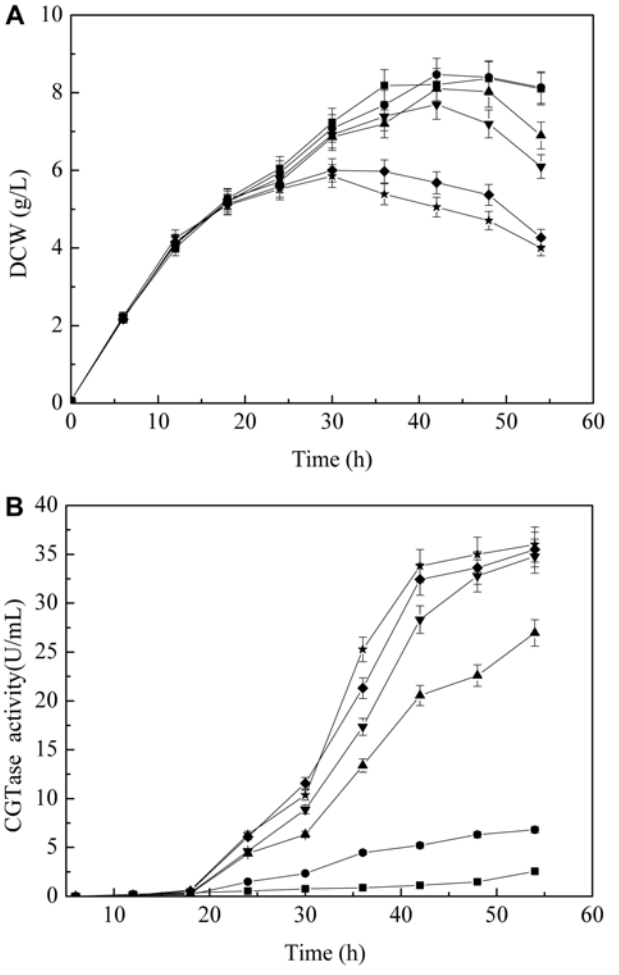

Fig. 2. The effects of glycine on *E. coli* cell growth (A) and extracellular α-CGTase production in *E. coli* (B) cultured in TB medium with 0 (■), 0.2% (●) (w/v), 0.5% (▲), 0.7% (▼), 1% (◆), or 1.2% (★) glycine, respectively. *Error bars* correspond to the standard deviation of three independent determinations.

enzymes from *E. coli* by glycine has been considered to be glycine-induced modification of peptidoglycan structure in the cell wall. It has been reported that glycine could be incorporated into the peptidoglycan instead of alanine and led to a more loosely cross-linked peptidoglycan [10]. In the present study, the enhanced CGTase secretion by glycine may have another reason: glycine is the most abundant amino acid in the α-CGTase (11.2%) and it was reported that the addition of the amino acid, which was most abundant in a protein into the culture media could promote the secretory expression of the target protein [11]. For example, the addition of 0.5% (w/v) alanine, which was the most abundant amino acid in glucose isomerase, increased the expression by 24%, and extracellular secretion of the protein enhanced by 18% [11]. The reason for this phenomenon is not clear, it might be that more target protein could be synthesized and then secreted upon amino acid supplementation.

The optimized concentration of glycine in this study was 0.7% (w/v). It was higher than that of the previous report, which was 0.3% [5]. The optimal amount of glycine supplementation may be dependent on the culture medium, host strain, the cultivation temperature, and the manner of additive supplementation. In addition, it seems like that the enhancement secretion of α-CGTase in the present study was not due primarily to cell lysis and osmolarity effect due to the fact that, under optimum conditions, the cell growth was not obviously inhibited (Fig. 2) and the outer membrane permeability was enhanced greatly (Fig. 6) when compared to the control group.

3.3. Effects of triton X-100 on extracellular production of α-CGTase

It was reported that Triton X-100 could inhibit the synthesis of membrane phospholipids, thus affect the integrity of the cell membrane and promote the extracellular translocation of recombinant enzyme. At different concentrations, Triton X-100 was added into the medium at the beginning of cultivation. Its effect on cell growth and extracellular production of α-CGTase were shown in Figs. 3A and 3B, respectively. It was observed that Triton X-100 had almost no inhibition on cell growth in its growing stage, but its inhibition became obvious since the stable phase started. The higher the concentration of Triton X-100 used, the greater the inhibition obtained. As for the enzyme secretion, the addition of Triton X-100 enhanced the activity of extracellular α-CGTase. The optimum concentration was found to be 0.2% (v/v). In the supplementation condition, the extracellular α-CGTase activity reached 33 U/mL by 48 h of cultivation and the productivity was 0.69 U/mL/h, which was 12.8 times that of the control group.

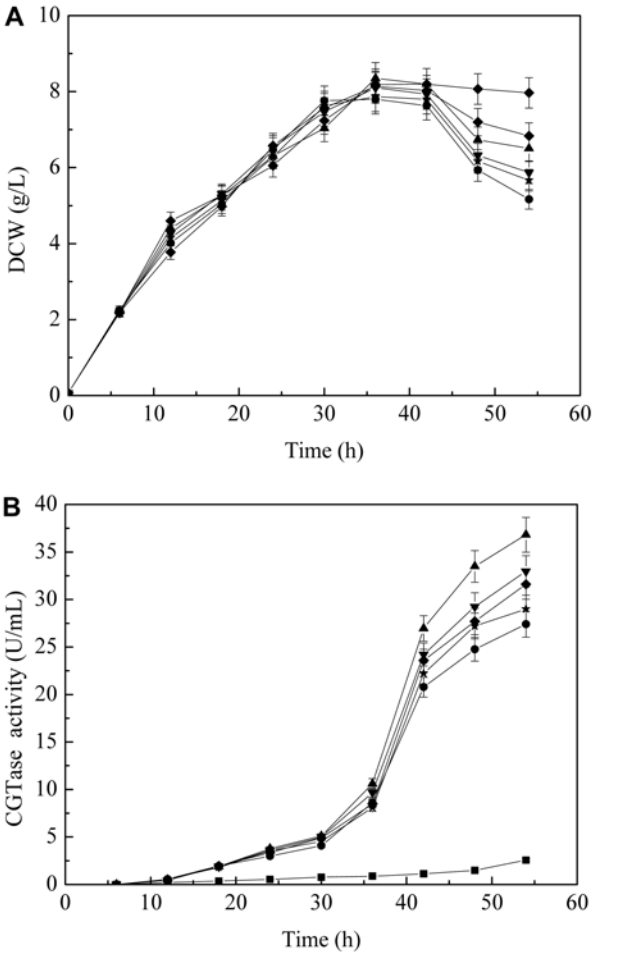

Fig. 3. The effects of Triton X-100 on *E. coli* cell growth (A) and extracellular α-CGTase production in *E. coli* (B) cultured in TB medium with 0 (■), 0.1% (◆) (v/v), 0.2% (▲), 0.5% (▼), 0.7% (★), or 1% (●) Triton X-100, respectively. *Error bars* correspond to the standard deviation of three independent determinations.

3.4. The effects of glycine and triton X-100 on the secretion and expression of α-CGTase

In order to investigate the potential interaction effect between glycine and Triton X-100 on the secretion of α-CGTase, a two-factor and three-level fractional factorial design was employed based on the results of above single-factor analysis. As shown in Fig. 4, the optimal conditions to achieve maximal extracellular production of α-CGTase was supplementation of the culture with 0.5% glycine and 0.5% Triton X-100 together. Comparing the results of 0.7% of glycine/0.2% of Triton X-100 with those of 0.5% of glycine/0.5% of Triton X-100, both the cell growth and extracellular enzyme activity in the former were slightly lower than that of the latter. Under optimal conditions, the extracellular α-CGTase activity reached 48 U/mL after 48 h cultivation; this enzyme productivity was 19 times higher

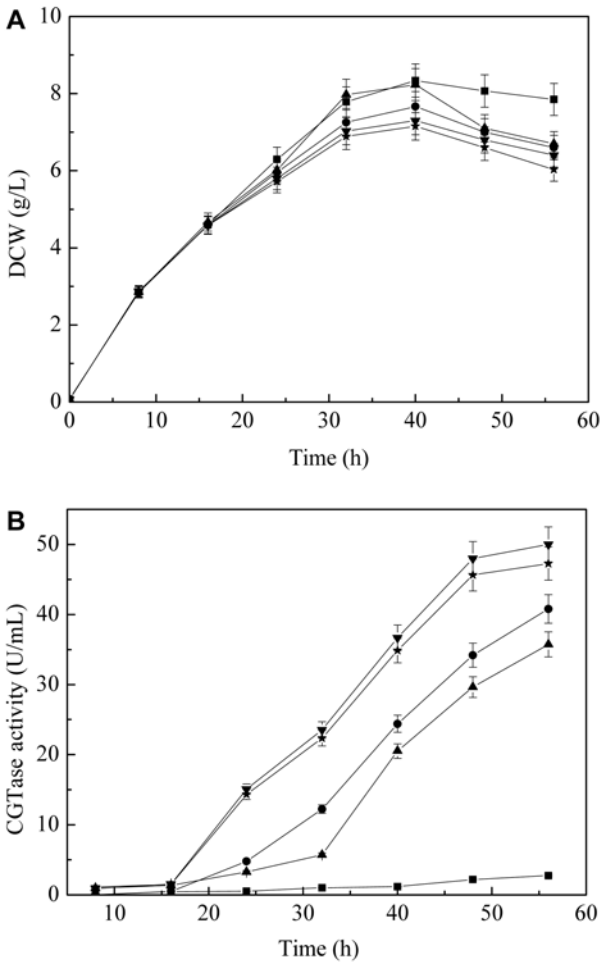

Fig. 4. The effects of glycine or (and) Triton X-100 on *E. coli* cell growth (A) and extracellular α-CGTase production by *E. coli* (B) cultured in TB medium with no additive (■), 0.7% glycine (●), 0.2% Triton X-100 (▲), 0.5% glycine and 0.5% Triton X-100 (▼), 0.7% glycine and 0.2% Triton X-100 (★). *Error bars* correspond to the standard deviation of three independent determinations.

than that of the control group without any additives and 95 times higher than that by cultured *Paenibacillus macerans* JFB05-01. The enhancement efficiency observed in our study is similar to that of ZZ–EGFP [8] and lower than that of sFV/TNF-α [8], in which, the enzyme productivity was 170-fold higher than that without additives.

In addition, the α-CGTase from the culture of above optimum condition (0.5% glycine and 0.5% Triton X-100) and the control without any additive were purified through anion-exchange column (Q-Sepharose) and hydrophobic column (phenyl-Superose), respectively. The specific activity of both purified enzymes were almost identical, 195 U/mg for the former and 200 U/mg for the latter, indicating that the addition of glycine and Triton X-100 in the culture media had no significant effect on the folding of the enzyme. The enhanced yield of enzyme in the culture supernatant with additive was due to the increased protein secretion.

3.5. Cellular distribution of α-CGTase in *E. coli* cells under different culture conditions

Cellular localization of α-CGTase was analyzed by SDS-PAGE. Considering the dynamics of protein synthesis and trans-membrane location, cells from different stages, one was in the late logarithmic phase and another was at stable phase, were chosen for the analysis. Each cellular fraction was prepared according to the method described in Materials and Methods and the results were shown in Fig. 5.

As shown in Fig. 5, in the culture without any additives, the α-CGTase mediated by OmpA signal peptide existed in all the fractions of the cell, cytoplasm, periplasm and extracellular, which was similar to that of α-CGTase mediated by PelB signal peptide [3]. In addition, compared to the extracellular part, there was more intracellular soluble enzyme in the cells of both late logarithmic phase and stable phase. However, when in the culture with media additives, there were decreases in the parts of the intracellular soluble enzyme and greatly enhanced extracellular enzyme, either in late logarithmic phase or stable phase. These phenomena were most obviously observed in the culture supplemented with both glycine and Triton X-100, when compared to the individual addition of glycine or Triton X-100. The distribution of α-CGTase observed from SDS-PAGE is in agreement with that from enzyme assay results. In addition, it was noted that inclusion bodies were found under all culture conditions.

3.6. Potential mechanism of the interaction effect between glycine and triton X-100

To study the potential mechanism of the interaction effect between glycine and Triton X-100, the permeability of the outer cell membrane, with and without additives, were analyzed. Fig. 6A shows that when glycine and Triton X-100 were both added into the culture, the outer membrane permeability of treatment group tripled the membrane permeability (330.1%) compared to that of the control group, which was much higher than that of the individual addition of glycine (186.8%) or Triton X-100 (130%). Usually, the enhanced outer membrane permeability would result in an increase of the protein translocation from periplasm into the culture media; however, it was observed that the recombinant enzyme accumulated in the periplasm in the culture with both glycine and Triton X-100 was not less than that of the individual addition of glycine or Triton X-100 (Fig. 5A), while the soluble protein in the cellular part was much less.

Further analysis of inner membrane showed that, with the addition of both glycine (0.5%) and Triton X-100 (0.5%), the permeability of inner membrane was 9-fold

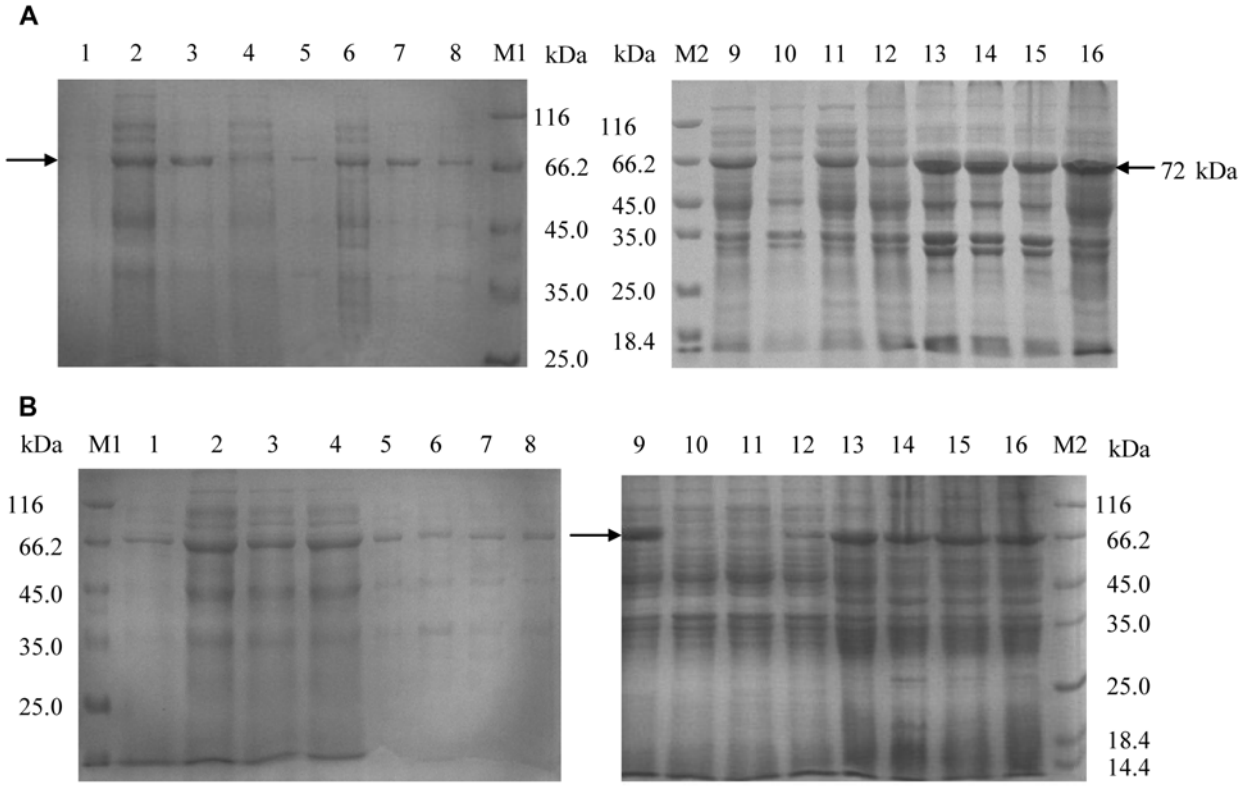

Fig. 5. SDS-PAGE analysis of cellular localization of α-CGTase in the cells grown for 32 h (A) and 48 h (B) respectively. M: molecular marker; 1, 2, 3, 4: supernatant fraction; 5, 6, 7, 8: periplasmic fraction; 9, 10, 11, 12: intracellular soluble fraction; 13, 14, 15, 16: intracellular insoluble fraction. Lanes 1, 5, 9 and 13, without additives; lanes 3, 7, 11, and 15, with 0.7% glycine; lanes 4, 8, 12 and 16, with 0.2% Triton X-100; lanes 2, 6, 10, and 14, with 0.5% glycine and 0.5% Triton X-100.

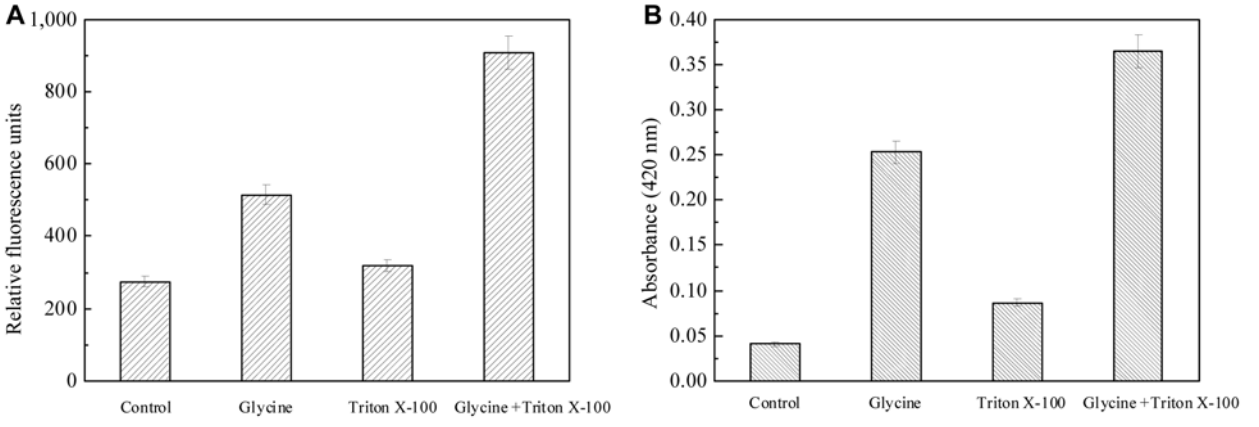

Fig. 6. Cell membrane permeability of *E. coli* cells after 24 h of culture. A: outer membrane; B: inner membrane. *Error bars* correspond to the standard deviation of three independent determinations.

that of the control group without any additives, which was much higher than the individual effect of glycine (6-fold) and Triton X-100 (2-fold) compared to the control group (Fig. 6B). In general, protein translocation across the inner membrane was considered to be through the Sec dependent pathway, and not through non-specific leakage, however, it seems like that, in the present study, the enhanced inner membrane permeability also accelerated the enzyme trans-inner-membrane location and decreased the soluble CGTase accumulation in the cellular part, which eventually resulted in the enhanced yield of CGTase in the culture media. Even though the detailed mechanism for this process is not clear, the effect of the inner membrane on the enhanced protein secretion can not be ignored and additional

experiment is needed to further explore this issue.

4. Conclusion

In the present study, OmpA signal peptide mediated α-CGTase from *P. macerans* was cloned and expressed in *E. coli*. The addition of glycine and Triton X-100 into the culture medium had a strong promotional effect on extracellular production of α-CGTase and could greatly enhance both inner and outer cell membrane permeability. The extracellular α-CGTase activity reached 48 U/mL after 48 h cultivation with media supplementation of 0.5% glycine and 0.5% Triton X-100, which was 20-fold that of the control group without any additives and 95 times higher than that by cultured *P. macerans*.

Acknowledgments

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