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BIOTECHNOLOGY METHODS

Effective approach to greatly enhancing selective secretion and expression of three cytoplasmic enzymes in *Escherichia coli* through synergistic effect of EDTA and lysozyme

Sen-Lin Liu · Kun Du · Wei-Zhao Chen · Gang Liu · Miao Xing

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Abstract An effective approach to greatly enhancing the selective secretion and expression of recombinant cytoplasmic enzymes in *Escherichia coli* was successfully developed through the synergistic effect of ethylenediaminetetraacetate (EDTA) and lysozyme. The method was applied to two endoglucanases (EGs) and an amylase. The optimal culture conditions of temperature and concentration of isopropyl-β-D-1-thiogalactopyranoside (IPTG) were 23–30 °C and 0.2 mM, respectively, under which the three enzymes could be expressed in active form. Among all the chemicals tested, EDTA was found to be most suitable for enhancing the secretion of EG-I-1A into the medium. Addition of lysozyme alone had little influence on the secretion and expression. In contrast, on the basis of the addition of 5 g EDTA/L at the induction time of 12 h, the simultaneous addition of 0.15 g lysozyme/L further significantly increased the secretion and expression of the three enzymes, demonstrating the synergistic effect of EDTA and lysozyme. The production of EG-I-1A in the culture medium by adding 5 g EDTA/L and 0.15 g lysozyme/L under the optimal culture conditions of 23 °C and 0.2 mM IPTG was over 260-fold higher than that without EDTA and lysozyme under the standard conditions of 37 °C and 1 mM IPTG. In summary, the advantage of this novel cultivation approach for secretion was that not only did it selectively enhance the secretion of the proteins of interest, but also greatly increased the expression of the three enzymes by over 80 %.

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Introduction

Recombinant *Escherichia coli* (*E. coli*) is widely used for the expression of proteins of interest in both biological research and the biotechnology industry, and has a variety of advantages, such as fast growth, simple fermentation, favorable economics, and ease of genetic manipulation [3, 13, 18, 22]. However, in the *E. coli* expression system, most proteins synthesized in the cytoplasm of *E. coli* are not secreted into the culture medium, but remain in the *E. coli* cell, because the cell envelope of this gram-negative bacterium has not only a cytoplasmic but also an outer membrane, which is a barrier to permeability for cytoplasmic proteins [1, 13]. This is especially the case in terms of enzymes, in which the expression products are usually accumulated in *E. coli* cells in the form of inclusion bodies without activity [14, 19, 21]. Therefore, it is of great interest to selectively secrete cytoplasmic enzymes in active form into the medium of recombinant *E. coli* in significant amounts, which may allow the highly efficient purification of the proteins of interest [7].

Some strategies for the permeabilization of the outer membrane of *E. coli* have been developed to enhance the secretion of periplasmic proteins into the medium. These mainly include the employment of *lky*- and *exc*-mutants with a defect in the cell envelope components [9, 10], and the addition of chemicals such as glycine [1, 6]. However, most of these approaches for secretion of periplasmic proteins are non-selective and result in the release of all other periplasmic proteins into the medium, along with the proteins of interest [1]. In contrast, there have been few

reports on the secretion of cytoplasmic proteins into the medium during the cultivation of this gram-negative bacterium.

The approach described in this study for the secretion of cytoplasmic enzymes by the addition of EDTA and lysozyme is a significant improvement on previous chemical permeabilization procedures, and it is selective. More interesting is the finding that the synergistic effect of EDTA and lysozyme could greatly increase the expression of the three enzymes by over 80 %. In this report, we primarily describe the greatly enhanced selective secretion and expression of three cytoplasmic enzymes in *E. coli* through the synergistic effect of EDTA and lysozyme.

Materials and methods

Biological and chemical materials

EDTA was purchased from Amresco (USA). Lysozyme (derived from egg white, potency ≥20,000 U/mg) was purchased from NBC (USA). Carboxymethylcellulose (CMC) and all other chemicals were also from commercial sources and had the highest purity available.

Strains and plasmids

Bacillus akibai III-3A was deposited in the China General Microbiological Culture Collection Center (CGMCC no. 2138), which was used as the source of the endoglucanase gene EG-III-3A, and parent strain. *Bacillus* sp. I-1A was used as the source of the endoglucanase gene EG-I-1A, which was originally isolated in a previous study [12]. *Bacillus subtilis* 32356 was used as the source of the amylase gene.

Plasmid pET-28a(+), which has no N-terminal signal sequence, was used for the expression of the three enzymes and the expression products were usually located in the cytoplasm of *E. coli*. The host strain used in this study was *E. coli* BL21(DE3). Plasmid pET-28a(+) and *E. coli* BL21(DE3) were products from Novagen (now Merck-Millipore) and the *E. coli* growth media were prepared in accordance with the *E. coli* expression system manual.

Construction of the expression plasmid

Deoxyribonucleic acid (DNA) extraction and plasmid DNA preparation were carried out according to the established methods [15]. The coding sequence, without the signal peptide coding sequence, was amplified by polymerase chain reaction (PCR) by using specific primers as shown in Table 1. The PCR product was gel purified, digested with restriction enzymes, and ligated into the corresponding sites of the plasmid pET-28a(+). The gene insert was confirmed by DNA sequencing. The recombinant plasmid was then transformed into *E. coli* BL21(DE3). The transformant that carried the desired gene was directly screened on the kanamycin-containing solid plate.

Expression of recombinant enzymes in *E. coli*

As a typical experiment, the transformant was grown overnight at 37 °C in the Luria–Bertani (LB) medium, supplemented with 25 mg/mL kanamycin. The culture was then incubated in the fresh LB medium (100 mL in 250-mL flask) that contained 25 mg/mL kanamycin to an OD600 of 0.6 at 37 °C. The cells were then induced by IPTG with a final concentration of 0.2 mM and cultured at 23 °C. At the induction time of 12 h, 5 g EDTA/L and 0.15 g lysozyme/L were simultaneously added into the culture medium. The culture was incubated for 22 h of induction and harvested by centrifugation at 14,000×*g* for 10 min at 4 °C. The supernatant of the culture was directly used for the measurement of extracellular activity and SDS-PAGE analysis. In order to determine the intracellular activity, the remaining cells collected by the same centrifugation were washed with 10 mM Tris–HCl buffer, and

Table 1 Sequence of primers

Name	Primer sequence
Primers for cloning of EG-I-1A gene from *Bacillus* sp. I-1A	
EG-I-1A-F	5′-CCGGAATTCGAAGGAAACACTCGTGAAGA-3′ (containing *Eco*RI site)
EG-I-1A-R	5′-CCCAAGCTTTTATTTTTTCGTAGCCTCTTTC-3′ (containing *Hin*dIII site)
Primers for cloning of EG-III-3A gene from *Bacillus akibai* III-3A	
EG-III-3A-F	5′-GATGAATTCGAAGGAAACACTCGTGAAGAC-3′ (containing *Eco*RI site)
EG-III-3A-R	5′-GTTAAGCTTTTATTTTTTCGTAGCCTCTTTC-3′ (containing *Hin*dIII site)
Primers for cloning of amylase gene from *Bacillus subtilis* 32356	
Amylase-F	5′-GTCCATATGGTAAATGGCACGCTGATG-3′ (containing *Nde*I site)
Amylase-R	5′-CGCGGATCCTTATTTCTGAACATAAATGGAGAC-3′ (containing *Bam*HI site)

disrupted by French press [11] in Tris–HCl buffer (pH 8.0) and then subjected to centrifugation at 14,000×*g* for 10 min at 4 °C. The supernatant of clear lysate was used for the measurement of intracellular activity. The total activity was the sum of the extracellular and intracellular activities. Under the optimal culture conditions with the addition of 5 g EDTA/L and 0.15 g lysozyme/L, the extracellular activity reached about 2/3 of total activity.

Endoglucanase and amylase assays

CMC was used as the substrate for the assay of the EG activity. The reaction mixture contained 1 mL 1 % (w/v) CMC solvated in Tris–HCl buffer (0.05 M, pH 8.0) and 0.1 mL appropriately diluted enzyme solution. The enzymatic reaction was carried out for 20 min in a 40 °C water bath, then 2 mL of 3,5-dinitrosalicylic acid (DNS) was added into the reaction mixture and incubated in boiling water for 10 min. The reducing glucose released in the enzymatic reaction was then determined by recording the absorbance at 540 nm. One unit of EG activity was defined as the amount of enzyme that produced 1 mg of product per hour.

To assay the amylase activity, the reaction mixture contained 1 mL 1 % (w/v) starch solvated in Tris–HCl buffer (0.05 M, pH 6.0) and 0.1 mL appropriately diluted enzyme solution. The enzymatic reaction was performed for 30 min in a 60 °C water bath, then 1 mL DNS was added into the reaction mixture and incubated in boiling water for 10 min. Distilled water (2 mL) was added and the reducing glucose was determined by the same method as for EG. One unit of the amylase activity was defined as the amount of enzyme that produced 1 mg of product per hour.

Protein electrophoresis

SDS-PAGE was basically performed according to the method described by Sambrook et al. [15]. The concentration of the separation gel was selected as 12.5 %. The prestained protein molecular weight marker was used and protein was stained with Coomassie Brilliant Blue R-250.

Results

Effect of culture conditions on the expression and secretion

EG-I-1A was expressed in recombinant *E. coli*. However, only extremely low EG activity (≤0.1 U/mL) was detected in the culture medium under the standard conditions of 37 °C and 1 mM IPTG, which was similar to previous reports [8, 20].

The effect of the culture temperature on the expression of EG-I-1A was explored at three different temperatures (23, 30, and 37 °C) and the total EG activity was measured. As is evident in Fig. 1, under the standard conditions of 37 °C, the total EG activity was very low, which indicated the formation of inclusion bodies without activity. However, at the optimum culture temperature of 23–30 °C, the recombinant enzymes could be successfully expressed in active form.

To determine the optimal concentration of IPTG, the expression of EG-I-1A was induced at an OD600 of 0.6 by different concentrations of IPTG and then incubated at 23 °C. As depicted in Fig. 2, the optimal concentration of IPTG for the induction of enzyme production was about 0.2 mM. At this concentration, the activity was about two

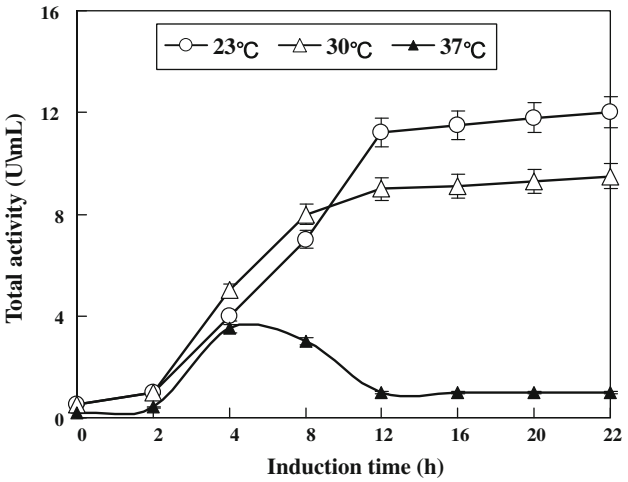

Fig. 1 Effect of temperature on the expression of EG-I-1A. The expression of EG-I-1A in *E. coli* was induced by 1 mM IPTG and incubated at different temperatures

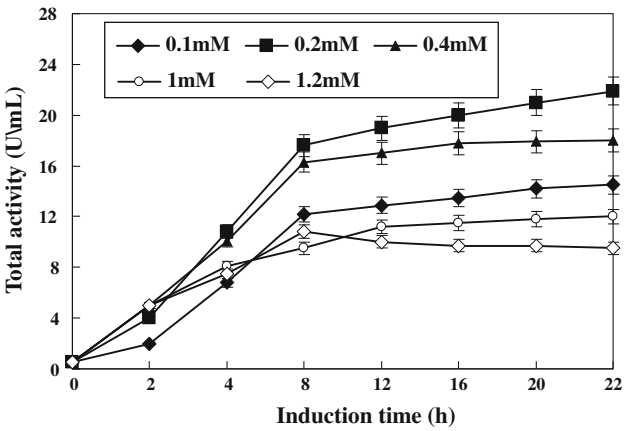

Fig. 2 Effect of IPTG concentration on the expression of EG-I-1A. The expression of EG-I-1A in *E. coli* was induced by different concentrations of the inducer IPTG and incubated at the optimal temperature of 23 °C

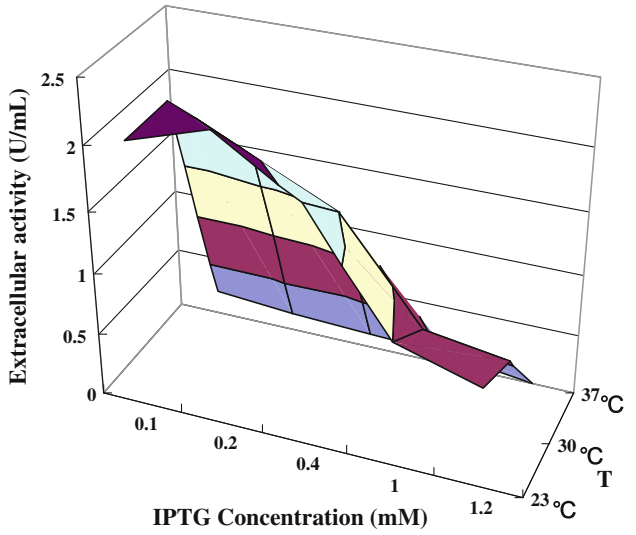

Fig. 3 Effect of temperature and IPTG concentration on the secretion of EG-I-1A. The expression of EG-I-1A in *E. coli* was induced by IPTG with different concentrations and incubated at different temperatures and the extracellular activity was measured

times as high as that under standard conditions (1 mM IPTG).

In addition, as shown in Fig. 3, it was found that secretion might be influenced by the optimization of culture conditions, and the extracellular activity of EG-I-1A in the culture supernatant of recombinant *E. coli* under the optimal conditions of 23 °C and 0.2 mM IPTG (2.3 U/mL) was over 23-fold higher than that under the standard conditions of 37 °C and 1 mM IPTG.

Effect of the addition of different chemicals on the secretion

It has been reported that recombinant proteins could be extracted from inclusion bodies located in the cytoplasm of *E. coli* through treatment with chemicals, such as EDTA, Triton-100, and guanidine–HCl [5], which may affect the secretion of proteins into the culture medium. Therefore, we attempted to develop some strategies, including the addition of chemicals, such as EDTA, ethylene glycol tetraacetic acid (EGTA), glycine, guanidine–HCl, and Triton-100, to the medium at the induction time of 12 h under optimal culture conditions of 23 °C and 0.2 mM IPTG, to enhance the secretion of EG-I-1A into the medium of recombinant *E. coli*. From the data listed in Table 2, it was clear that all of the mentioned chemicals had significant influence on the secretion. Among all the chemicals tested, EDTA was the most suitable for the enhancement of secretion and used for further investigation. The optimal final concentration of EDTA was about 5 g/L, at which the extracellular activity was seven-fold higher than that without EDTA.

Synergistic enhancement of selective secretion by EDTA and lysozyme

EDTA has been demonstrated to be particularly effective at permeabilizing the outer membrane of *E. coli* [4], whereas lysozyme influences the structure of the peptidoglycan layer in the bacterial cell wall. A combination of EDTA and lysozyme may have a synergistic effect on the secretion of recombinant enzymes. To test this assumption, we added 5 g EDTA/L, 0.15 g lysozyme/L, and 5 g EDTA/L and 0.15 g lysozyme/L, separately, to the culture medium at the induction time of 12 h and assessed the effect on the secretion of two EGs (EG-I-1A, EG-III-3A) and an amylase. As shown in Fig. 4, the addition of lysozyme alone had no significant influence on the secretion for the three enzymes examined. In contrast, the simultaneous addition of 5 g EDTA/L and 15 g lysozyme/L at the induction time of 12 h further significantly increased the extracellular activities of the three enzymes. This finding demonstrated the synergistic effect of EDTA and lysozyme on the enhancement of the secretion of enzymes into the medium.

To further investigate the influence of adding EDTA and lysozyme on the secretion, the time-course study of the extracellular activity and SDS-PAGE analysis of samples of the culture supernatant of recombinant *E. coli* were carried out by using EG-I-1A as the model (Fig. 5). As illustrated in Fig. 5, prior to the addition of EDTA and lysozyme, low extracellular activity and low secreted

Table 2 Effect of the addition of different chemicals on the secretion

Chemicals	Extracellular activity (U/mL)					
	0	2 g/L	5 g/L	10 g/L	15 g/L	20 g/L
EDTA	2.3 ± 0.1	10.8 ± 0.5	17.4 ± 0.9	12.5 ± 0.6	7.1 ± 0.4	5.4 ± 0.3
EGTA	2.3 ± 0.1	2.4 ± 0.1	3.1 ± 0.2	3.9 ± 0.2	4.2 ± 0.2	3.6 ± 0.2
Glycine	2.3 ± 0.1	2.4 ± 0.1	3.2 ± 0.2	4.1 ± 0.2	4.6 ± 0.2	5.1 ± 0.3
Guanidine–HCl	2.3 ± 0.1	4.9 ± 0.3	10.3 ± 0.5	9.3 ± 0.5	8.7 ± 0.4	7.3 ± 0.4
Triton-100	2.3 ± 0.1	5.0 ± 0.3	9.6 ± 0.5	9.2 ± 0.5	9.1 ± 0.5	7.6 ± 0.4

The expression of EG-I-1A in *E. coli* was carried out under the optimal culture conditions of 23 °C and 0.2 mM IPTG. Different chemicals were respectively added at the induction time of 12 h. The extracellular EG activity was measured after 22 h of induction

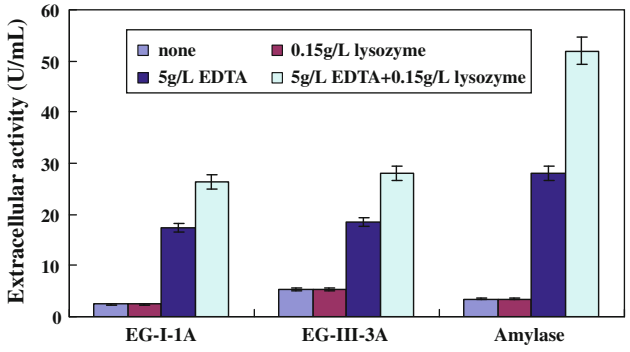

Fig. 4 Synergistic enhancement of the secretion of three enzymes by addition of EDTA and lysozyme. The expression of two EGs (EG-I-1A, EG-III-3A) and an amylase in *E. coli* was separately performed under optimal culture conditions of 23 °C and 0.2 mM IPTG. The extracellular activity with the addition of both 5 g EDTA/L and 0.15 g lysozyme/L was measured and compared to that without EDTA and/or lysozyme

protein level were detected. In contrast, after the addition of 5 g EDTA/L and 0.15 g lysozyme/L at the induction time of 12 h, the extracellular activity and the secreted protein level sharply increased, which led to an 11-fold increase in secretion. These results demonstrated the dramatic enhancement of the secretion of recombinant enzyme into the medium. Interestingly, as shown in Fig. 5b, the protein band of interest in the SDS-PAGE was very bright and the concentration of other foreign proteins in the culture supernatant of the recombinant *E. coli* was much lower, which demonstrated that this approach that greatly enhanced secretion was selective, promoting the purification of the protein of interest.

Synergistic enhancement of expression by EDTA and lysozyme

The effect of adding EDTA and/or lysozyme on the total enzyme activity was determined by experiments performed on the three enzymes mentioned above. In Fig. 6, it can be observed that the addition of 5 g EDTA/L increased the total activities of EG-I-1A, EG-III-3A, and amylase by 45, 40, and 30 %, respectively, whereas addition of lysozyme alone had little influence on the expression of the three recombinant enzymes. In contrast, when 5 g EDTA/L and 0.15 g lysozyme/L were added together at the induction time of 12 h, it can be observed that there was over 80 % increase in total activity for the three enzymes, which demonstrated the synergistic enhancement of the expression of recombinant enzymes by EDTA and lysozyme.

Discussion

Culture conditions, such as temperature and concentration of IPTG, can influence the expression of heterologous proteins in recombinant *E. coli*. Sawyer et al. [16] reported that a decrease in the culture temperature was associated with an increased production of the soluble antitumor sFV in *E. coli*. A study on β-lactamase expression by Bowden and Georgiou [2] showed that lowering the IPTG concentration increased the yield of soluble sFV by slowing the rate of synthesis and preventing aggregation of folding intermediates. The results described in this study are in accordance with these previous reports. Under the standard conditions of 37 °C and 1 mM IPTG, the total activity of EG-I-1A is very low, probably due to the formation of inclusion bodies without activity [19, 21]. In contrast, at the optimal temperature of 23–30 °C, the recombinant enzyme could be expressed in active form. Additionally, it has been reported that secreted heterologous proteins could leak from the periplasm into the medium, possibly owing to the increased permeability of cellular membranes during long incubation periods [17]. In the present study, the secretion level of EG-I-1A in the medium of recombinant *E. coli* under the optimal conditions of 23 °C and 0.2 mM

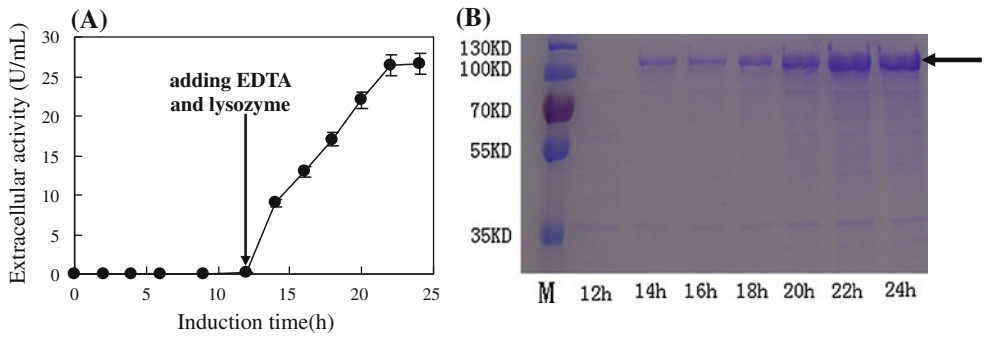

Fig. 5 **a** Time-course of extracellular activity of EG-I-1A produced by recombinant *E. coli*. 5 g EDTA/L and 0.15 g lysozyme/L added together at the induction time of 12 h. **b** SDS-PAGE analysis of samples of the culture supernatant of recombinant *E. coli*. *Lanes 1–7* samples of culture supernatants 12, 14, 16, 18, 20, 22, and 24 h after induction by IPTG, containing about 1, 51, 73, 96, 124, 150, and 151 mg total protein/L, respectively; *M* molecular weight markers. Five grams of EDTA/L and 0.15 g lysozyme/L were added together at the induction time of 12 h. The protein of interest is marked by the *arrow*

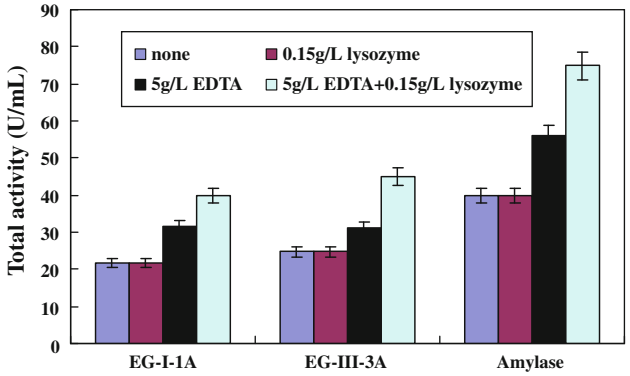

Fig. 6 Synergistic enhancement of the expression of the three enzymes by EDTA and lysozyme. The expression of two EGs (EG-I-1A, EG-III-3A) and an amylase in recombinant *E. coli* was separately performed under optimal culture conditions of 23 °C and 0.2 mM IPTG. The total activity with the addition of 5 g EDTA/L and 0.15 g lysozyme/L together was measured and compared to that without EDTA and/or lysozyme

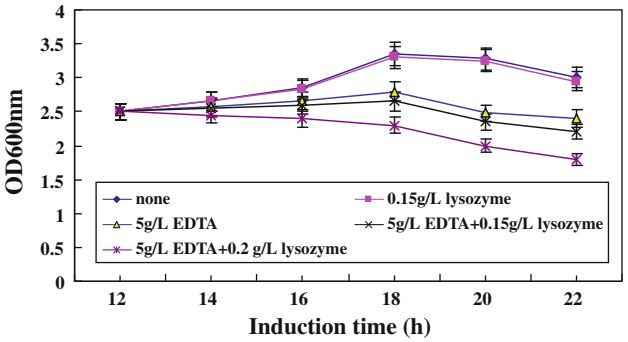

Fig. 7 Effect of the addition of EDTA and/or lysozyme on the growth of recombinant *E. coli*. Recombinant *E. coli* was incubated under optimal culture conditions of 23 °C and 0.2 mM IPTG. Additives were added at the induction time of 12 h. OD600 nm was measured with samples at regular intervals

IPTG is over 23-fold higher than that under the standard conditions of 37 °C and 1 mM IPTG.

EDTA has been found to be able to efficiently extract proteins from inclusion bodies located in the cytoplasm of *E. coli* [5]. In the present study, interestingly, EDTA significantly enhanced the secretion of active enzymes from the cytoplasm of *E. coli* into the medium during the cultivation of this gram-negative bacterium. Among all the chemicals examined, EDTA was found to be the most suitable one for the enhancement of secretion. At the optimal EDTA concentration of 5 g/L, the secretion of EG-I-1A is seven-fold higher than that without EDTA, whereas the addition of lysozyme alone has little influence on the secretion for the three enzymes examined. In contrast, on the basis of the addition of 5 g EDTA/L at the induction time of 12 h, the addition of 0.15 g lysozyme/L could further increase the extracellular activities of the three enzymes, demonstrating the existence of the synergistic effect of EDTA and lysozyme. These results indicate that EDTA may improve the permeability of the outer membrane of *E. coli*, which leads to the permeation of lysozyme through the outer membrane to act on the peptidoglycan layer in *E. coli*. Similarly, the enhanced secretion of the three enzymes from the cytoplasm of *E. coli* into the medium may be partially explained by the improvement of permeability of both cytoplasmic and outer membranes through the synergistic effect of EDTA and lysozyme. On the other hand, EDTA and lysozyme are conventionally used for the extraction of proteins in *E. coli* by the disruption of the cells. As is evident in Fig. 7, the addition of lysozyme alone has little influence on the growth of recombinant *E. coli*, whereas the addition of 5 g EDTA/L and 0.15 g lysozyme/L, or 5 g EDTA/L alone slightly inhibits the growth of this bacterium. Therefore, improved permeability and induced bacteriolysis might partly account for the enhancement of protein secretion into the culture medium by the addition of EDTA and lysozyme.

Interestingly, this approach for secretion of cytoplasmic enzymes selectively enhances the secretion of the protein of interest and significantly lowers the concentration of other foreign proteins in the culture supernatant of recombinant *E. coli*, leading to a more efficient purification of the protein of interest. This is unlike former methods in which, along with the proteins of interest, all of the other periplasmic proteins in *E. coli* are also released into the medium through permeabilization of outer membranes [1, 9, 10]. More interesting is the finding that the addition of 5 g EDTA/L and 0.15 g lysozyme/L could greatly increase the expression of the three enzymes by over 80 %. In conclusion, this study has thus provided a practical, large-scale approach to the more efficient production of recombinant enzymes in *E. coli* by the addition of EDTA and lysozyme.

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