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Enhanced secretion of recombinant α-cyclodextrin glucosyltransferase from *E. coli* by medium additives

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ABSTRACT

The purpose of this study was to investigate the effect of medium additives on the secretion of recombinant α-cyclodextrin glucosyltransferase (α-CGTase) into the culture media of *Escherichia coli*. It is found that supplementation of the *E. coli* culture with SDS, glycine, Ca^{2+} or Na^{+}, individually, facilitated the secretion of α-CGTase. Orthogonal experiment showed that 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. Under this condition, extracellular enzyme activity reached 12.89 U/ml, which is 15 times higher than that of the culture without any additives. Further analysis showed that the permeability, fluidity and phosphatidylglycerol content of the *E. coli* cell membrane under the optimized condition were significantly increased in comparison to those under the control condition. These might be the potential mechanisms for the increased secretion of α-CGTase from the periplasmic compartment into the culture medium.

1. Introduction

Cyclodextrin glucosyltransferase (CGTase, EC2.4.1.19), an important member of glycosyl hydrolase family, catalyses the formation of cyclodextrins (CDs) [1] from starch and related carbohydrates. CDs have a cylindrical shape, which is hydrophobic inside and hydrophilic outside. The most common types of CDs are α-, β- and γ-CDs consisting of 6, 7 and 8 glucose units linked by α-1,4-glycosidic, respectively. CDs are able to form inclusion bodies with many hydrophobic molecules and change their physical and chemical properties, which makes CDs attractive for various applications in many different fields, e.g. food, medicine, agriculture, cosmetics, chemical and environmental protection [2,3]. However, due to the high cost of production, application of CDs has been restricted. In order to reduce the cost, significantly improving the low yields of CGTase during the process of production has been expected [4].

CGTase is an extracellular enzyme identified in a number of bacteria, mainly *Bacillus* [4]. To overcome the low yield of CGTase produced by wild strains, studies have been carried out to genetically engineer a CGTase gene in *Escherichia coli* for its overexpression [5]. Previously, our laboratory cloned CGTase gene from *Paenibacillus macerans* strain JFB05-01 and expressed it in *E. coli* [6]. The expression vector, pET-20b(+)/cgt, contains a periplasmic PelB secretory signal peptide. During the process of bacterial cultivation, it is observed that most of the PelB-fused CGTase was accumulated in the periplasmic compartment and small amount of CGTase was non-specifically secreted to the culture medium. This "leaky" phenomenon was also reported in studies on the expression and secretion of other recombinant proteins [7].

The microbial cell membrane is highly selective for the translocation of intracellular and extracellular materials. Alterations of cell membrane permeability normally change the rates of absorbing raw materials and exporting the metabolites, which is one of the important approaches for artificial control of microbial metabolisms. Alteration of the membrane permeability can be achieved by multiple strategies, e.g. genetic engineering [8] or utilization of media additives [9]. Chemical additives are currently being used for multiple purposes. For example, Liu et al. [10] found that 0.05–0.1% (v/v) Tween-80 could improve extracellular thermophilic protease secretion by *Bacillus stearothermophilus* and a maximal 12.7% enhancement was achieved by addition of 0.1% Tween-80.

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Table 1
Results and analysis of orthogonal experiment.

No.	SDS (%)	Na^+ (mM)	Glycine (%)	Ca^{2+} (mM)	α-CGTase activity (U/ml)
1	0.01	400	0.3	2.5	9.37
2	0.01	500	0.75	5	6.03
3	0.01	600	0.1	10	6.03
4	0.02	400	0.75	10	10.6
5	0.02	500	0.1	2.5	6.64
6	0.02	600	0.3	5	9.38
7	0.03	400	0.1	5	9.08
8	0.03	500	0.3	10	12.12
9	0.03	600	0.75	2.5	8.47
K_1	21.43	29.05	30.87	24.48	
K_2	26.62	24.79	25.1	23.88	
K_3	29.67	23.88	21.75	28.75	
k_1	7.14	9.68	10.29	8.16	
k_2	8.87	8.26	8.37	7.96	
k_3	9.89	7.96	7.25	9.53	
Error *R*	2.75	1.72	3.04	1.62	

Na^+ and Ca^{2+} were added at the beginning of cultivation, glycine and SDS were added after cultivation of 16 and 20 h, respectively.

To achieve high production of recombinant α-CGTase from *P. macerans*, in our laboratory, the effect of SDS, Triton X-100, Tween-80, Tween-20, cetyltrimethyl ammonium bromide (CTAB), pronine, glycine, sucrose, sorbitol, Na^+, Mg^{2+} or Ca^{2+} on the secretion of CGTase was investigated. It was found that, among all these chemicals, SDS, Na^+, glycine and Ca^{2+} could improve extracellular production of the enzyme. In the present study, the effect of these four chemical additives as well as their interaction on the extracellular secretion of α-CTGase was investigated in detail. In addition, the underlying mechanisms for the enhanced enzyme secretion were exploited.

2. Materials and methods

2.1. Materials

The recombinant plasmid *cgt*/pET-20b(+) carrying the mature *cgt* gene of *P. macerans* JFB05-01 and *E. coli* BL21(DE3) harboring plasmid *cgt*/pET-20b(+) were constructed previously by our laboratory. Peptone and yeast powder were obtained from Oxiod Ltd. Glycine (AR) and SDS were purchased from Shanghai Chemical Reagent Ltd. attached to China Medicine Group (Shanghai, China). o-Nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Beyotime Institute of Biotechnology (Nantong, China). N-phenyl-1-naphthylamine (NPN) was purchased from Aladdin-reagent Ltd. (Shanghai, China). Glucose-6-phosphate, NADP, NADPH, phosphatidylethanolamine and phosphatidylglycerol were purchased from Sigma–Aldrich (Milwaukee, WI, USA). All inorganic compounds were of reagent grade or higher quality.

2.2. Cultivation condition

A single colony of *E. coli* BL21 (DE3) cells harboring plasmid *cgt*/pET-20b(+) was inoculated into 10 ml Luria–Bertani (LB) medium containing 100 μg/ml ampicillin in a 25 ml flask and grown at 37 °C. The overnight culture was diluted (1:25) in 100 ml of terrific broth (TB) medium in a 500 ml flask and then incubated on a rotary shaker (200 r/min) at 25 °C. To investigate the effect of chemical additives on the growth of *E. coli* or extracellular secretion of recombinant α-CGTase, chemical additives were supplemented to the culture medium in the beginning of culture unless otherwise stated. At certain time intervals, samples were collected and analyzed for OD_{600} and enzyme activities. Each value represents the mean of three independent measurements and varied from the mean by not more than 10%.

2.3. Orthogonal experiment

Based on the individual experiment of SDS, Na^+, glycine and Ca^{2+}, an $L_9(3^4)$ orthogonal experiment was designed by using these four chemicals as parameters. The level of experimental factors and orthogonal experimental designs were summarized in Table 1.

2.4. Cell fractionation

Culture supernatants were obtained by centrifugation at 10,000 × *g* for 5 min at 4 °C and the supernatant was used as an extracellular fraction. To separate the periplasmic fraction, the bacterial pellets from 1 ml broth culture were washed twice by 30 mM Tris–HCl buffer (pH 7.0) and then completely resuspended in the same buffer containing 25% (w/v) sucrose and 1 mM EDTA. After incubation on ice for 2 h, the bacterial suspension was centrifuged at 10,000 × *g* for 5 min at 4 °C and the supernatant was used as a periplasmic fraction.

2.5. Assay of α-CGTase

The α-cyclodextrin forming activity was determined by the methyl orange (MO) method as described previously [11] with slight modifications. Briefly, 0.1 ml of the culture supernatant (appropriately diluted in 50 mM 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 1.0 M HCl (1.0 ml) followed by addition of 1.0 ml MO (0.1 mM) in 50 mM phosphate buffer (pH 6.0). 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. One unit of α-CGTase activity was defined as the amount of enzyme that was able to produce 1 mol of α-cyclodextrin in every minute under the test conditions described above.

2.6. Assay of glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase was assayed as described previously [12] with some modifications. The total volume of the reaction mixture was 1.0 ml containing 1 mM glucose-6-phosphate, 0.075 mM NADP, 10 mM $MgCl_2$, 50 mM Tris–HCl (pH8.0) and appropriate culture supernatant. The production of NADPH was determined by measuring absorbance at 340 nm using a spectrophotometer at 25 °C. One unit of glucose-6-phosphate dehydrogenase (G6PDH) activity was defined as the amount of enzyme that was able to produce 1 μmol NADHP per minute under the test conditions described above.

2.7. Assay of outer and inner membrane permeability

Outer membrane permeability was determined by using N-phenyl-1-naphthylamine access assay as described previously [13]. Briefly, *E. coli* cells were collected at different time points, centrifuged at 3000 × *g* and suspended in 10 mM sodium phosphate buffer (pH 7.4) to an OD_{600} of 0.5. NPN was added with a concentration of 10 μM into quartz cuvettes containing 2 ml of cell suspension. The sample was mixed by inverting the cuvette immediately prior to fluorescence monitoring. Fluorescence was measured using a 650-60 spectrofluorometer with slit widths set to 5 mm and excitation and emission wavelengths set to 350 and 428 nm, respectively.

Permeability of the inner membrane was assessed by measuring the access of o-nitrophenyl-β-D-galactoside to the cytoplasm as described previously [14]. Briefly, ONPG was added with a concentration of 100 μg/ml to the *E. coli* BL21 (DE3) suspension as described above. Substrate cleavage by β-galactosidase was determined by measuring OD_{420} using a spectrophotometer.

2.8. Analysis of phospholipids in the cell membrane

The phospholipids were extracted as described previously [15] with slight modifications. Briefly, 30 ml of broth culture was centrifuged at 10,000 × *g* for 5 min and pellets were resuspended in 30 ml of 50 mM sodium phosphate buffer (pH 6.0). The sample was disrupted by ultrasonication with a SONFER. Two volumes of chloroform/methanol (2/1) were added and vortexed for 30 min. Two phases were separated by low-speed centrifugation (2500 × *g*). The lower phase was collected and mixed with 1/4 volume of methanol/water (1/1) solution. After centrifugation, the chloroform phase containing phospholipids was evaporated under vacuum in a rotary evaporator till the volume was below 2–3 ml. The remaining solution was dried under a nitrogen stream and redissolved in 1 ml chloroform. Phospholipids are