J. Microbiol. Biotechnol. (2004), **14**(6), 1310–1317

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Process Strategies to Enhance the Production of 5-Aminolevulinic Acid with Recombinant *E. coli*

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Received: August 26, 2004
Accepted: October 11, 2004

Abstract The extracellular production of 5-aminolevulinic acid (ALA) by recombinant *E. coli* BL21 harboring a fusion gene *hemA* was investigated in a fermenter. For this purpose, the effects of various physiological factors, such as isopropylthio-β-D-galactopyranoside (IPTG) concentrations and the time of induction, on enzyme activity were studied. Optimum concentrations of glycine and succinic acid were found to be 30 mM and 90 mM, respectively. When the cells were permitted to grow for 2 h prior to the addition of 0.1 mM IPTG, the activity of ALA synthase was higher than when IPTG was initially added. A 36-fold increase in the activity was observed with only 0.1 mM IPTG added. The pH of the medium also influenced the ALA synthase activity with the maximal activity occurring at pH 6.5. In recombinant *E. coli* extracts, the repeated addition of glycine and D-glucose increased the production of ALA and the inhibited intracellular ALA dehydratase activity, with up to 32 mM ALA being produced in the cultivation.

Key words: 5-Aminolevulinic acid, aminolevulinic acid synthase, aminolevulinic acid dehydratase, isopropylthio-β-D-galactopyranoside (IPTG), levulinic acid, D-glucose

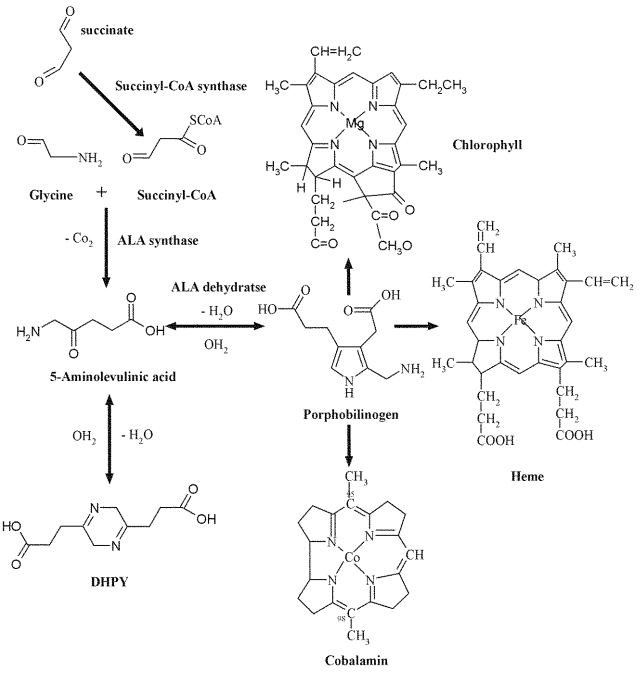

Fig. 1. Alternative ALA biosynthesis pathways and tetrapyrrole compounds such as heme, cobalamin, chlorophyll, DHPY.

5-Aminolevulinic acid or δ-aminolevulinate (5-amino-4-oxopentanoate, ALA) is a derivative of a 5 (δ)-carbon amino acid and present in all living organisms. As shown in Fig. 1, ALA is a precursor of tetrapyrroles such as porphyrin, heme, chlorophyll and vitamin B_{12}, factor F_{430} via porphobilinogen, and it is a key intermediate in regulating tetrapyrrole biosynthesis [2, 14, 19]. Recently, ALA has received wide attention for its potential use as a herbicide [25, 34, 37], insecticide [35], antimicrobial drug [23], and growth regulator for plants [10, 41], and it has also been reported to restore hair growth and prevent hair loss [12] and is a photosensitizer for photodynamic cancer therapy [17, 21, 33].

Until recently, mass production of ALA has been very difficult due to the numerous steps required for its chemical synthesis [39]. Therefore, ALA is very expensive, because it is synthesized via a relatively complex process. ALA can be biosynthesized through two major pathways [14]. One route is the C_4 pathway, in which the pyridoxal 5-phosphate-dependent enzyme ALA synthase (E.C. 2.3.1.37) catalyzes

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the condensation of succinyl-CoA and glycine to yield ALA. Another route is the C_5 pathway, in which ALA is formed via three enzymatic steps. The biosynthesis of ALA is tightly regulated by feedback inhibition of ALA synthase (in the C_4 pathway) or glutamate-tRNA ligase and glutamyl-$tRNA^{glu}$ reductase (in the C_5 pathway) [14]. In order to accumulate ALA, the inhibition of ALA dehydratase (E.C.4.2.1.24) is essential, because this enzyme converts two molecules of ALA into porphobilinigen (PBG) (Shown in Fig. 1). To enhance the accumulation of ALA in the biological production, levulinic acid (LA) or glucose, competitive inhibitors of ALA dehydratase in the tetrapyrrole biosynthetic pathway [20, 29, 36], should be added to the culture medium. ALA is produced from the inexpensive precursors, including succinic acid and glycine. Succinic acid is converted to succinyl-CoA by the succinyl-CoA synthase (E.C. 6.2.1.5) of the host strain. This compound is then coupled to glycine by heterologous ALA synthase activity, thus forming ALA [24]. The stability of ALA is influenced very much by pH and temperature. ALA is decomposed into 2,5-dicarboxyethyl-3,6-dihydropyrazine (DHPY) and pyrazine (PY) by alkaline pH [4, 13].

In the present study, we had constructed an *E. coli* strain harboring a plasmid carrying the *hemA* gene encoding ALA synthase from *B. japonicum*. Then, we studied the effects of concentrations of succinic acid, glycine, LA, and IPTG on the production of ALA. Also, the effect of pH and time of induction affecting the level of ALA synthase activity and ALA production were investigated. Moreover, methods of D-glucose treatment to affect ALA production were examined.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, Enzymes, and Chemicals

Bradyrhizobium japonicum (ATCC 10324) was cultured at 26°C in broth or on agar plates according to the suggestion of Korean Collection for Type Cultures: Culture medium contained Bacto yeast extract 0.1% (w/v), 55 mM mannitol, 3 mM K_2HPO_4, 0.8 mM $MgSO_4 \cdot H_2O$, 3.4 mM NaCl, 30 mM $FeCl_3 \cdot 6H_2O$. *E. coli* BL21 (Amersham Phamacia Biotech Uppsala, Sweden) was used for cloning and overexpression of the encoding gene. For subcloning and expression, pBluescript SK(+) plasmid (Novagen, San Diego, CA, U.S.A.) and pGEX-3T (Amersham Phamacia Biotech Uppsala, Sweden) were used, respectively. Restriction enzymes and *Pfu* DNA polymerase were purchased from Bioneer Co. (Daejeon, Korea). GSH-Sepharose was purchased from Amersham Pharmacia Biotech. Succinic acid, glycine, D-glucose, IPTG, glutathione (GSH), imidazole, and thrombin were supplied by Sigma Co. (St. Louis, MO, U.S.A.). Amino acid standard and AccQ-tag reagent kit were purchased from Waters Co. (Milford, MA, U.S.A.).

Construction of pGEX-3T-*hemA* Gene

Genomic DNA from *B. japonicum* was purified by using a Promega Wizard Genomic DNA kit (Promega Madison, WI, U.S.A.). The *B. japonicum hemA* gene was amplified, using the polymerase chain reaction (PCR). The *Pfu* DNA polymerase was used instead of *Taq* DNA polymerase and the pBluescript SK(+) plasmid served as the DNA template. Primers were designed, based on the published *B. japonicum hemA* gene sequence [6], and contained an *Eco*RI (GAATTC) restriction site and a *Bam*HI (GGATCC) restriction site at the end of the amplified fragment; forward primer 5'-GGGATCCAATGGATTACAGCCAG-TTCTT-3'; reverse primer 5'-GGGGAATTCCTACTCCG-CCGCCAGCGA-3' (the *Eco*RI, ATG start, and *Bam*HI sites are underlined). The resulting 1.2 kb PCR product was gel isolated, restricted with *Eco*RI and *Bam*HI, and ligated into the pGEX-3T expression vector, which had been restricted with the same two enzymes. *E. coli* BL21 cells were transformed by resulting plasmid pGEX-3T-*hemA*.

Bacterial Expression and Purification of Recombinant ALA Synthase

The pGEX-3T-*hemA* was transformed into *E. coli* BL21 cells according to the manufacturers recommendations (Amersham Pharmacia Biotech). Recombinant *E. coli* ALA synthase was purified as described by Oh *et al*. [32]. Five milliliters of cultures were used to inoculate 50 ml of Luria-Bertani (LB) medium containing 100 μg/ml ampicillin, and it was inoculated at 37°C until OD_{600} reached 0.4–0.6. After the addition of 0.1 mM IPTG, the culture was grown at 25°C for 6 h. The bacteria were then harvested by centrifugation at 10,000 ×*g* for 10 min and washed in 20 mM potassium phosphate buffer, pH 7.5 (buffer A). The cells were repelled, suspended in buffer A, and then sonicated until lysed with an ultrasonic processor (Tokyo, Japan). The lysate was centrifuged at 45,000 ×*g* for 90 min at 4°C. The supernatant was loaded onto GSH-Sepharose affinity column which had been preequilibrated with buffer A. The column was exhaustively washed with buffer A containing 200 mM potassium chloride. The enzyme was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM GSH. The active fractions were pooled and dialyzed against buffer A. Cleavage of the fusion protein was carried out by incubation with an excess amount (a 1:50 enzyme: substrate ratio) of thrombin in buffer A for 1 h at 25°C. The solution was applied to GSH-Sepharose affinity column to remove the N-terminal glutathione S-transferase, and the unbound enzyme fractions were collected and dialyzed against buffer A. The dialyzed purified enzyme was used for the next experiment. Unless otherwise indicated, all purification procedures were performed either at 4°C or on ice.

Fermentation Conditions

All optimization studies were conducted in 250-ml shake flasks containing 50 ml of medium. Recombinant *E. coli*

Table 1. Purification of recombinant *E. coli* 5-aminolevulinic acid synthase.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	72,030	1,474	48.86	100	1
GSH affinity	38,682	32	1,208.81	53	24.74

Purification was carried out as described in Materials and Methods. Enzyme activities were determined using glycine and succinic acid as substrates. Results represent experiments done in triplicate.

strains were grown in LB medium or LB containing glycine (30 mM), and succinic acid (90 mM) as a substrate for ALA. All cultures contained 100 μg/ml ampicillin. The results from optimal shake flask were used to conduct controlled fermentation (KF-2.5 l, KFC, Korea). A 2.5-l fermenter contained 1 l of medium, and it was inoculated with 50 ml of a shake flask culture to provide an initial optical density of approximately 1.0 at 600 nm. The fermenter was operated at 400 rpm, 37°C, air flow rate of 1.5 l/min, and pH 6.5, which was controlled with 5 N HCl and 5 N NaOH. Silicone antifoamer (non-ionic emulsifiers, Sigma, U.S.A.) was occasionally added to remove foam in the culture.

Analyses

Cell growth was monitored, based on the sample's OD_{600} (UV2501 PC spectrophotometer, Shimadzu, Austria). Succinic acid analysis was determined using the method of Cho *et al*. [5]. Succinic acid was analyzed by a high-pressure liquid chromatography (HPLC) equipped with an Atlantis organic acid column (Waters Co, Milford, MA, U.S.A.). Glycine was analyzed by HPLC, using an amino acid analysis column (Waters Co, Milford, MA, U.S.A.) with AccQ-tag reagent kit. The concentrations of ALA and PBG in the culture were measured by the method described by Mauzerall and Granick [26]. Briefly, a solution containing ALA and PBG was diluted with an equal volume of regular Ehrlich's reagent. Using this value together with the rate of ALA and PBG formulation, the specific activity was calculated. All assays were carried out in triplicate.

Enzyme Assays

The activity of ALA synthase (E.C.2.3.1.37) was measured by the method described by Burnham [3], and the activity of ALA dehydratase was determined by the method described by Mitchell and Jaffe [28]. All assays were carried out in duplicate or triplicate. Proteins were determined by the methods of Lowry *et al*. [22], using bovine serum albumin as a standard.

SDS-PAGE Analysis

Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 10% gel by the method of Laemmli [18] with the molecular weight marker protein (SDS molecular weight standard markers (Bio-Rad Cat# 161-0374)). Gel was stained with Coomassie Blue R-250.

RESULTS

Purification of the Recombinant ALA Synthase in *E. coli*

The results of purification of *B. japonicum* ALA synthase from *E. coli* BL21, containing plasmid pGEX-3T-*hemA*, are shown in Table 1. By GSH-Sepharose affinity chromatography of the crude extract, the GST-fusion protein was purified to an electrophoretic homogeneity (Fig. 2, lane 3). After the cleavage of the fusion protein followed by GSH-Sepharose affinity chromatography, the enzyme on SDS-PAGE appeared as a single band corresponding to a molecular mass of ~44.6 kDa, which was in good agreement with the reported size of *B. japonicum* ALA synthase [6] (Fig. 2, lane 4).

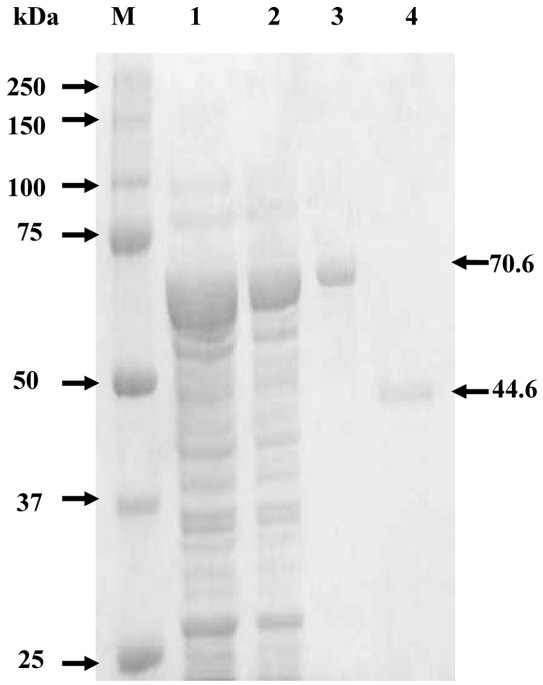

Fig. 2. Electrophoresis of the recombinant *E. coli* ALA synthase under denaturing conditions.

Denaturing SDS-PAGE was carried out on 10% gel, using the method of Laemmli (1970). Coomassie brilliant blue R-250 was used for staining. Lane M, molecular-mass standard markers; lane 1, total protein from recombinant *E. coli* lysates of pGEX-*hemA* vector transformants; lane 3, the purified GST fusion protein by GSH-Sepharose affinity chromatography; lane 4, the purified enzyme by GSH-Sepharose affinity chromatography after thrombin treatment.

Effect of Glycine and Succinic Acid on ALA Production

Glycine and succinic acid are the precursors of ALA biosynthesis. Therefore, the effect of these precursors on ALA formation was examined (data not shown). When added to the mineral medium, at 30 mM glycine dose-dependently improved the growth of *E. coli* up to 30 mM (data not shown). Furthermore, the addition of over 90 mM succinic acid to the medium inhibited the growth of *E. coli* immediately, whereas glycine did not exhibit any effect on bacterial growth (data not shown). Thus, we used the condition under which 30 mM glycine and 90 mM succinic acid were added to the medium for the production of ALA, and up to 32 mM ALA production were produced. These results indicate that the addition of optimal balanced amounts of glycine and succinic acid is important for maximal production of ALA.

Effect of IPTG Induction on ALA Production

We studied the effects of two factors, including the concentration of IPTG and the time of IPTG addition, on the activity of ALA synthase in recombinant *E. coli*. For the study on the time of IPTG addition, the concentration of IPTG that had resulted in the greatest ALA synthase activity was used. As shown in Table 2, the ALA synthase reached maximum activity at 10 h of culture in fermenter. In all cases, the activity of ALA synthase was measured after 10 h of cell growth. Because the level of ALA synthase activity was influenced by the pH of the medium, we examined the effects of different pHs on the ALA synthase levels. A marked effect of pH of the medium on ALA synthase levels was observed with an optimum medium pH of 6.5 (Table 3). As for the IPTG concentration, the activity of ALA synthase in the absence of IPTG was 2.4 U/mg, but the ALA synthase activity increased 36-fold when only 0.1 mM IPTG was initially added. The time of IPTG addition had a significant effect on ALA synthase activity: IPTG was added to the culture medium in the middle of the exponential growth phase in order to investigate the effects of IPTG induction. When the cells were permitted to grow for 2 h prior to the addition of 0.1 mM IPTG, the activity of ALA synthase was higher than that when IPTG was added initially.

Table 2. The effects of IPTG concentration and its addition time on the ALA synthase from recombinant *E. coli* in a fermenter.

Treatment group	Initial IPTG (mM)	Addition time of IPTG (h)	ALA synthase (U/mg protein)
1	0.05	0.0	16.0
2	0.05	1.0	24.4
3	0.05	2.0	36.0
4	0.10	0.0	23.8
5	0.10	1.0	67.0
6	0.10	2.0	86.0
7	0.25	0.0	52.4
8	0.25	1.0	58.2
9	0.25	2.0	72.0

Table 3. The activities of ALA synthase and ALA dehydratase in relation with the culture pH.[a]

pH	ALA synthase[b] (U/mg protein)	ALA dehydratase[c] (U/mg protein)	Maximum ALA production[d] (mM)
5.5	39.42	7.82	14.0 mM
6.0	72.78	2.95	24.0 mM
6.5	82.60	3.62	32.0 mM
7.0	68.60	3.70	24.5 mM
7.5	28.64	6.23	20.0 mM
8.0	24.50	9.60	12.6 mM

[a]Cells were grown at 37°C on LB medium in a fermenter containing 100 μg of ampicillin per ml for 12 h.
[b]One unit of enzyme activity was defined as the amount of enzyme to produce 1 μmol of ALA in 1 h at 37°C.
[c]One unit of enzyme activity was defined as the amount of enzyme to produce 1 μmol of PBG in 1 h at 37°C.
[d]Data from Fig. 3.
[e]Data express averages of the duplicates.

Effect of Culture pH on ALA Production

The intracellular balance of both ALA synthase and ALA dehydratase activities plays an important role in the extracellular ALA production. Therefore, the activities of ALA synthase and ALA dehydratase at various pHs of culture broth were examined. As shown in Table 3, the activity of ALA synthase was significantly enhanced by pH controlling, while the activity of ALA dehydratase was

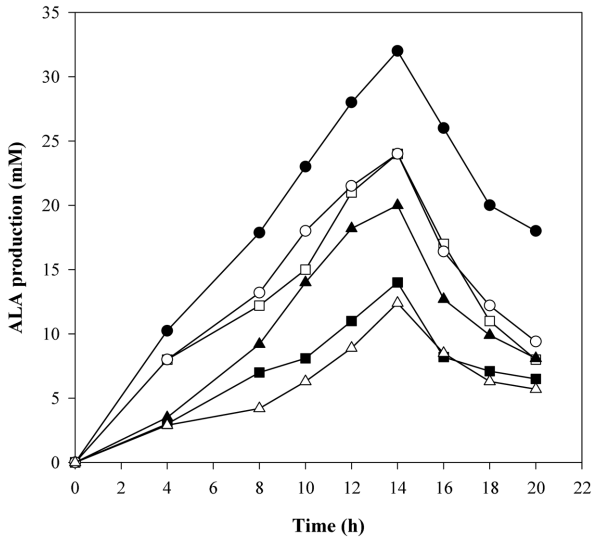

Fig. 3. Effects of various pHs on ALA production in a jar fermenter. The pH range was pH 5.5–pH 8.0. For measuring ALA, the recombinant *E. coli* was incubated in a jar fermenter for 20 h. Symbols: pH 5.5 (△), pH 6.0 (○), pH 6.5 (●), pH 7.0 (□), pH 7.5 (▲), pH 8.0 (■). Data express averages of triplicates.

inhibited: At pH 6.5, ALA synthase levels were quite high, however, ALA dehydratase was moderately inhibited. The extracellular ALA production was significantly enhanced when pH was maintained at pH 6.5, compared with those at lower and higher pH values. The maximum ALA production of 32 mM was observed at pH 6.5. Thus, strict maintenance of pH at 6.5 appears to be important for extracellular production of ALA by this culture system. The influence of culture pH on the growth of recombinant *E. coli* and ALA production was investigated in a 2.5-l fermenter, where the culture pH was maintainced at 5.5– 8.0 during the cultivation (Fig. 3). The enzyme profiles during the cultivation at various pHs showed almost similar tendency.

Based on these observations, we concluded that the high activity of ALA synthase and moderate inhibition of ALA dehydratase were necessary for maximum ALA production. pH 6.5 of the culture broth in this culture system seems to indirectly establish an appropriate balance of the enzyme activities for ALA production in the cells.

Effect of LA and D-Glucose on the Production of ALA

The addition of D-glucose (5 g, 10 g, 15 g, 20 g) at the initial stage of culture significantly affected the growth of cells, resulting in a decrease in ALA production (Fig. 4). The cell growth was slightly retarded at the initial period of fermentation, when LA and D-glucose were added (data not shown). To further enhance ALA production, 30 mM each of LA and D-glucose were additionally fed to the fermenter after 10 h of culture (Fig. 5). The growth of cells recovered, when D-glucose was added at the mid-log or late-log phase of culture. The amount of PBG was drastically decreased, when D-glucose was added to the medium, i.e., the PBG concentration decreased from 9.4 mM to 7.6 mM (data not shown). Therefore, based on the results obtained from the full design experiments, a fermentation was carried out, using recombinant *E. coli* in LB medium with 90 mM (10 g/l) succinic acid, 30 mM (2.25 g/l) glycine, and no D-glucose added initially. An additional 30 mM (2.25 g/l) glycine was included to resupply at 10 h, and the fermentation was terminated at 20 h. The greatest ALA synthase activity obtained was 87 U/mg protein, and the final ALA concentration was 32 mM (Fig. 6).

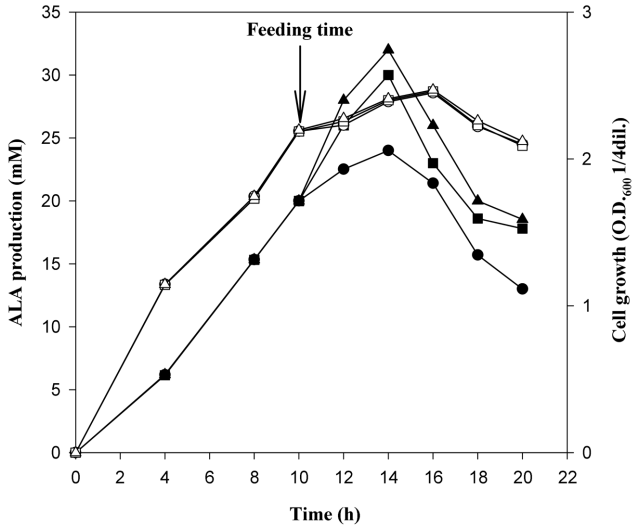

Fig. 5. Production of ALA in recombinant *E. coli*.
Production of ALA: cont (●), 30 mM LA feeding (■), 30 mM D-glucose feeding (▲). Cell growth: cont (○), 30 mM LA feeding (□), 30 mM D-glucose feeding (△). Arrow: LA and D-glucose feeding time (10 h).

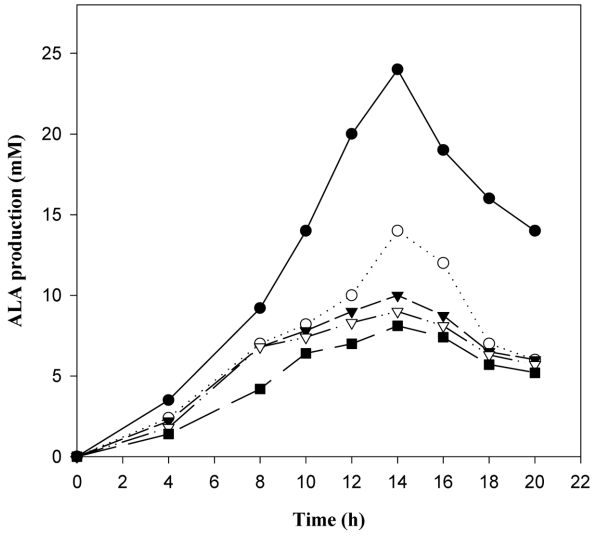

Fig. 4. Effects of initial addition of D-glucose on ALA production in a jar fermenter.
Symbols: control (LB) (●), D-glucose addition: 5 g (○), 10 g (▼), 15 g (▽), 20 g (■). Data express averages of triplicates.

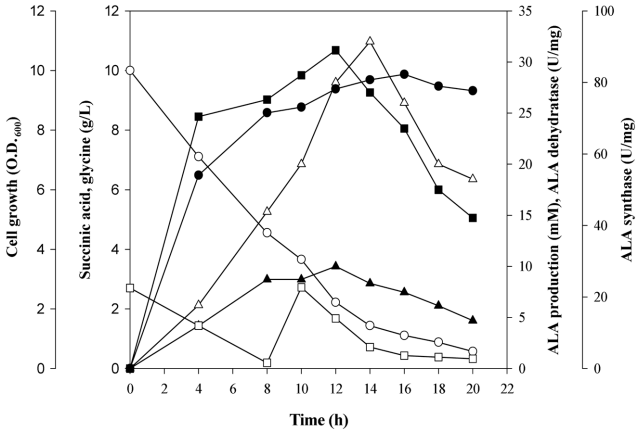

Fig. 6. Fermentation of recombinant *E. coli* BL21, using LB medium with 10 g/l succinic acid, 2.25 g/l glycine, no glucose, and 0.1 mM IPTG added after 2 h. An additional 2.25 g/l of glycine and 5.4 g/l of glucose were added at 10 h.
Symbols: (●) O.D, (○) succinic acid, (▲) ALA dehydratase, (△) ALA, (■) ALA synthase, (□) glycine.

DISCUSSION

In the present study, both genetic and physiological factors affecting the expression of *B. japonicum hemA* in *E. coli* BL21 and the production of ALA were studied. The ALA synthase gene of *B. japonicum* was cloned in 1987 and identified as *hemA*, which encodes a 409-amino acid protein with a molecular weight of 44,599 [27]. Several other ALA synthase genes have also been cloned and characterized in detail [9, 11].

In this work, the enzyme appeared as a single band corresponding to a molecular mass of ~44.6 kDa on SDS-PAGE, in good agreement with the reported size of *B. japonicum* ALA synthase [6]. The physiological factors such as growth substrate and medium pH had an enormous effect on the activity of ALA synthase: The effect of pH on ALA production was observed by controlling the pH from 5.5 to 8.0. When the culture pH was adjusted to 7.0, recombinant *E. coli* did not grow well (data not shown). This was found to be abnormal in the cultivation of recombinant *E. coli*. When pH was controlled not in the fermenter, cells grew very well, and the culture pH was maintained in a range of 8.2 and 8.6 during the cultivation. Up to 12 mM ALA was produced at the end of the exponential growth phase, gradually decreasing thereafter, because of enhanced intracellular ALA dehydratase activity. Thus, accurate control of pH at 6.5 is important for the extracellular ALA production by this culture system.

Biosynthesis of ALA proceeds from succinic acid and glycine through a combined action of the host enzymes, succinyl-CoA synthesis, and ALA synthesis [19, 24]. Therefore, the addition of two precursors was investigated in relation with the production of ALA. The addition of excess amount of glycine suppressed cell growth both by itself and by ammonia produced during glycine metabolism [1].

For the pGEX plasmid, induction with IPTG is necessary for the expression of foreign proteins. We found low ALA synthase activity in the absence of IPTG. While an earlier limited study of IPTG induction of *hemA* in *E. coli* indicated no effect of IPTG concentration [24], studies with other recombinant proteins also showed that 0.05–0.1 mM IPTG concentration provided maximal expression without deleteriously influencing metabolism [7, 16]. The high concentration of IPTG might cause the greatest metabolic load [8]. Klotsky and Schwartz [15] reported that the plasmid copy number was dependent on the growth substrate used. Generally, D-glucose is used as a carbon growth factor of microorganism. However, in this study, when D-glucose was initially added, inhibition of the cell growth and no expression of ALA synthase were detected. These may imply that D-glucose regulates the cAMP concentration, resulting in the inhibition of cell growth. Another possible explanation may be that the *B. japonicum hemA* gene itself may contain the regulatory sequences affecting the expression of ALA synthase in *E. coli* [31]. Neidle and Kaplan [31] found the consensus sequence of the binding of the transcriptional regulators Fnr and FixK upstream of the *hemA* gene.

The production of ALA is catalyzed by ALA synthase, and ALA is metabolized to PBG by ALA dehydratase (PBG synthase). ALA dehydratase can be inhibited by analogues of either substrate or product. The most widely used substrate analogue is levulinic acid (LA). Nandi and Shemin [29] reported that LA is a competitive inhibitor of ALA dehydratase which catalyzes condensation of two ALA molecules to form porphobilinogen, a monopyrrole. Beale [2] showed that when an autotrophically grown culture of *Chlorella* was treated with LA, ALA was excreted into the medium, and Sasaki *et al*. [36] showed that when LA was repeatedly added together with glycine to the culture medium of photosynthetic bacteria, *Rhodobacter sphaeroides*, ALA production was significantly enhanced: The ALA production increased linearly for 8 h and gradually increased thereafter for 12 h. In the present study, however, the addition of LA or D-glucose at the initial stage of culture significantly affected the growth of cells, and the ALA production was decreased (6 mM and 8 mM, respectively). The growth of cells recovered, when LA and D-glucose were added at the mid-log or late-log phase of culture, and maximum production of ALA (30 mM and 32 mM) was observed with the addition of LA or D-glucose at the late-log phase of cultures. These results indicate that the production of ALA in recombinant strains proceeds in parallel to the growth of bacteria. Notably, we achieved the highest level of ALA production (32 mM) in recombinant *E. coli*. This value is two times greater than the extracellular production of ALA with *Rhodobacter spheaeroides*, which was reported by Sasaki *et al*. [37], and it is also significantly higher than that reported previously in our laboratory (20 mM) [6]. Using more cells in an immobilized system, the level could be further enhanced. We have already devised an ALA purification process, using a chromatography column (Ion-exchange resin and HPLC), therefore, we expect to supply economically produced ALA in the near future.

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