One-Pot Production of L-threo-3-Hydroxyaspartic Acid Using Asparaginase-Deficient *Escherichia coli* Expressing Asparagine Hydroxylase of *Streptomyces coelicolor* A3(2)

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We developed a novel process for efficient synthesis of L-threo-3-hydroxyaspartic acid (L-THA) using microbial hydroxylase and hydrolase. A well-characterized mutant of asparagine hydroxylase (AsnO-D241N) and its homologous enzyme (SCO2693-D246N) were adaptable to the direct hydroxylation of L-aspartic acid; however, the yields were strictly low. Therefore, the highly stable and efficient wild-type asparagine hydroxylases AsnO and SCO2693 were employed to synthesize L-THA. By using these recombinant enzymes, L-THA was obtained by L-asparagine hydroxylation by AsnO followed by amide hydrolysis by asparaginase via 3-hydroxyasparagine. Subsequently, the two-step reaction was adapted to one-pot bioconversion in a test tube. L-THA was obtained in a small amount with a molar yield of 0.076% by using intact *Escherichia coli* expressing the *asnO* gene, and thus, two asparaginase-deficient mutants of *E. coli* were investigated. A remarkably increased L-THA yield of 8.2% was obtained with the asparaginase I-deficient mutant. When the expression level of the *asnO* gene was enhanced by using the T7 promoter in *E. coli* instead of the *lac* promoter, the L-THA yield was significantly increased to 92%. By using a combination of the *E. coli* asparaginase I-deficient mutant and the T7 expression system, a whole-cell reaction in a jar fermentor was conducted, and consequently, L-THA was successfully obtained from L-asparagine with a maximum yield of 96% in less time than with test tube-scale production. These results indicate that asparagine hydroxylation followed by hydrolysis would be applicable to the efficient production of L-THA.

Hydroxylated amino acids occurring in nature possess important physiological functions as bioactive substances. For example, 5-hydroxy-L-tryptophan, which can be extracted from the African plant *Griffonia simplicifolia*, is clinically used for the treatment of depression, insomnia, headache, and obesity, owing to its ability to stimulate serotonin biosynthesis in the human brain (1). *cis*-4-Hydroxy-L-proline, which occurs in leaves of sandalwood, is a potent antitumor drug that prevents the folding of collagen triple helices in cancer cells (2). In addition, (4S)-4-hydroxy-L-isoleucine, which is contained in the seeds of fenugreek, is effective for the treatment of type II diabetes, since it promotes insulin secretion in a blood glucose-dependent manner (3). Moreover, derivatives of these hydroxylated amino acids can also be used in the synthesis of pharmaceuticals.

3-Hydroxyaspartic acid is one such compound, and much attention has been paid to its synthesis and biological properties over the years. 3-Hydroxyaspartic acid contains two asymmetric centers at C-2 and C-3; thus, four stereoisomers are expected: L-threo-3-hydroxyaspartic acid [(2S,3S)-form] (L-THA), L-erythro-3-hydroxyaspartic acid [(2S,3R)-form] (L-EHA), D-threo-3-hydroxyaspartic acid [(2R,3R)-form] (D-THA), and D-erythro-3-hydroxyaspartic acid [(2R,3S)-form] (D-EHA) (see Fig. S1 in the supplemental material). L-THA is especially attractive for chemists and biologists owing to its broad clinical and material utility, including as an antimicrobial agent against various microorganisms (4), as an inhibitor of glutamate transporters (5), and as a functional moiety of polymethacrylamide polymers (6). Its derivatives also display competitive inhibitory activity for glutamate/aspartate transporters (7, 8) and antitumor activity (9).

The synthesis of L-THA has typically relied on chemical methods, e.g., ammonolysis of DL-*cis*-epoxysuccinic acid (10) or hy-

droxylation of L-aspartic acid diesters using oxaziridine or oxodiperoxymolybdenum pyridine hexamethylphosphoric triamide (11). However, these methods are accompanied by the simultaneous formation of undesirable stereoisomers, including some amount of D-THA together with L-THA; thus, complex purification procedures are often required. Optical resolution with diastereomeric salt formation (12) and its modified method (13) were attempted, to obtain optically pure hydroxyaspartic acids; however, highly selective synthesis and purification have not been accomplished. This difficulty in obtaining optically pure hydroxyaspartic acid is due to the apparent lack of chiral selectivity in conventional chemical synthesis.

In contrast to chemical synthesis, biotechnological approaches using enzymes are promising alternatives for the control of chirality, since enzymes are generally regarded as having excellent chemo-, regio-, and stereoselectivity. Thus, biotechnological syn-

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thesis could serve as a model for efficient L-THA synthesis. L-THA has been found in the fermentation broth of *Arthrinium phaeospermum* strain T-53 and *Streptomyces* sp. strain 7540-MC₁ during antibiotic screening (4). Although the chemical and biological properties of L-THA were revealed by the isolation of this amino acid, the efficiency of its production remains unclear. Other methods using L-amino acid transaminase have facilitated the production of L-THA; however, chiral selectivity was not sufficiently achieved (14). Recently, a role for L-asparagine hydroxylase (AsnO) in the biosynthesis of daptomycin-type antibiotics has been reported (15), and the AsnO-D241N mutant, which was engineered by rational design, was able to form L-THA directly from L-aspartic acid (16). However, the productivity and yield of the preparative-scale production of L-THA were not investigated. More recently, another biocatalytic approach, using pyridoxal 5'-phosphate-dependent D-THA dehydratase from *Delftia* sp. strain HT23, was reported (17). This unique D-THA dehydratase was purified and characterized and was shown to have tremendous potential for the optical resolution of DL-THA in the preparation of L-THA. Indeed, this method is advantageous for selective L-THA production compared to currently available methods; however, D-THA dehydratase degrades a small amount of L-THA as well as a large amount of D-THA.

Asparaginase (asparagine amidohydrolase; EC 3.5.1.1) is widely distributed in microorganisms, plants, and animals and plays a significant role in asparagine metabolism. Commonly, the substrate range of the enzyme is relatively restricted, and thus, we sought to screen for an asparaginase that can convert 3-hydroxyasparagine to L-THA and ammonia. Previously, plant asparaginase was purified from seed extracts of *Lupinus polyphyllus*, and the characterization of the enzyme revealed that it catalyzed the formation of L-THA and ammonia to some extent (18); however, it remains unclear whether bacterial asparaginase recognizes 3-hydroxyasparagine as a substrate and, if so, whether it has sufficient activity. Thus, it was hypothesized that bacterial asparaginase can also act on 3-hydroxyasparagine to form L-THA.

The present paper describes the evaluation of the activity and stability of known asparagine hydroxylases and their corresponding rationally engineered enzymes and the optimization of biocatalytic routes for L-THA synthesis. Furthermore, this method was adapted to one-pot bioconversion using genetically engineered *Escherichia coli* with a jar fermentor.

MATERIALS AND METHODS

Chemicals. L-THA was purchased from Tocris Bioscience (Bristol, United Kingdom). DL-THA was obtained from Tokyo Chemical Industry (Tokyo, Japan). Asparaginase II was purchased from ProSpec-Tany TechnoGene (Rehovot, Israel). All other chemicals were of analytical grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). Oligonucleotides were synthesized by FASMAC (Kanagawa, Japan), and their sequences are listed in Table S1 in the supplemental material.

Bacterial strains and culture conditions. *Streptomyces coelicolor* A3(2) (synonym, *Streptomyces violaceoruber* NBRC 15146) and *Bacillus subtilis* NBRC 3134, which is the same strain as PCI-219, which was previously used for antimicrobial assays (4), were obtained from the Biological Resource Center, NITE (Chiba, Japan). *E. coli* JM109 (Nippongene, Tokyo, Japan) was used for gene cloning. *E. coli* strains JW1756 and JW2924 were obtained from the National Bio Resource Project (NBRP) (National Institute of Genetics, Japan) (19) and used for whole-cell reactions together with pUC19-based vectors (TaKaRa Bio, Shiga, Japan). *E. coli* Rosetta2(DE3) and its asparaginase I-deficient mutant strain were

used for gene expression and whole-cell reactions together with pET-21a(+)-based vectors (Novagen, Darmstadt, Germany).

The following conditions were used for protein expression. *E. coli* strains carrying expression vectors were grown at 37°C for 6 h in 5 ml of Luria-Bertani medium (20) containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol with vigorous shaking. Next, 1 ml of the cell culture was transferred into 100 ml of the same medium, and cells were cultivated until the optical density at 600 nm (OD₆₀₀) reached ~0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added at a final concentration of 0.1 mM. Incubation was carried out for another 16 h at 25°C, and the cells were collected by centrifugation at 5,000 × g for 10 min at 4°C. The cell pellet was stored at -80°C until it was used for enzyme purification or small-scale whole-cell reactions.

For large-scale protein expression, a 2-liter jar fermentor (model BNR-C 2L; B. E. Marubishi, Tokyo, Japan) was used as follows. An *E. coli* seed culture, which was grown in 50 ml of Luria-Bertani medium containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol at 37°C for 6 h, was transferred into 1 liter of Terrific broth (20) containing 50 µg/ml ampicillin, 30 µg/ml chloramphenicol, and 1 mM IPTG, and cultivation was continued until the OD₆₀₀ reached ~8. This incubation was performed at 25°C at 600 rpm with a 1-liter/min aeration and with pH 7 maintained with 7% (vol/vol) NH₄OH. The cells were subsequently harvested by centrifugation at 5,000 × g for 10 min at 4°C, and the cell pellet was stored at -80°C until it was used for preparative-scale production.

For evaluations of antimicrobial activity, *B. subtilis* was inoculated onto solidified minimal medium (21), and cellulose discs containing 15 µl of 500 µg/ml L-THA or L-EHA were placed onto the agar surface, followed by incubation for 16 h at 30°C.

Gene cloning. Chromosomal DNAs of *S. coelicolor* A3(2) and *E. coli* were extracted according to a standard procedure (20), and these DNAs were used as the templates for PCR. The genes for AsnO and SCO2693 of *S. coelicolor* A3(2) were amplified by using the GC-Rich PCR system (Roche, Basel, Switzerland) supplemented with 0.5 M dimethyl sulfoxide. The asparaginase I gene of *E. coli* was amplified with Kod Plus (Toyobo, Osaka, Japan). The following primer pairs were used, as listed in Table S1 in the supplemental material: AsnO-fwd1 and AsnO-rvs1, AsnO-fwd2 and AsnO-rvs2, SCO2693-fwd and SCO2693-rvs, and ansA-fwd and ansA-rvs. PCR was performed with an iCycler system (Bio-Rad Laboratories, Hercules, CA, USA), as follows: 94°C for 2 min and 25 cycles each of 94°C for 15 s, 50°C for 30 s, and 68°C for 1 min. The amplified PCR products were digested with appropriate restriction enzymes and then ligated into the corresponding sites of pUC19 or pET-21a(+).

Site-directed mutagenesis was performed to generate the AsnO-D241N and SCO2693-D246N mutants according to the circular mutagenesis method (20), which introduced specific mutations into their parent vectors, using the following mutagenic primer pairs: D241N-fwd and D241N-rvs, and D246N-fwd and D246N-rvs (see Table S1 in the supplemental material). PCR was performed as follows: 94°C for 2 min and 10 cycles each of 94°C for 15 s, 60°C for 30 s, and 68°C for 7 min. The remaining template was digested with DpnI, and the resulting PCR product was transformed into *E. coli* JM109. After the purification of the plasmids, the D241N mutation for AsnO and the D246N mutation for SCO2693 were verified by DNA sequencing.

Disruption of the asparaginase gene. An asparaginase I-deficient mutant of *E. coli* Rosetta2(DE3) was engineered by replacing the asparaginase I gene with a kanamycin resistance gene cassette using the λ Red homologous recombination system (22). The deletion cassette for the asparaginase I gene was amplified by PCR using FRT-PKG-gb2-neo-FRT (Gene Bridges, Heidelberg, Germany) as a template, and primer pair ansA-kanr-fwd and ansA-kanr-rvs was used. The PCR program was as follows: 94°C for 2 min and 25 cycles each of 94°C for 20 s, 60°C for 15 s, and 68°C for 2 min. *E. coli* Rosetta2(DE3) was transformed with the cassette and incubated on Luria-Bertani agar containing 30 µg/ml kanamycin. The resulting asparaginase I-deficient mutant (*ΔansA*) was selected and used as a host for the following experiments.

Enzyme purification. Frozen and thawed cell pellets were suspended in 5 ml of binding buffer (20 mM potassium phosphate, 50 mM imidazole, and 500 mM NaCl [pH 7.0]), and cells were then disrupted with an ultrasonic homogenizer (model UD-201; Tomy Seiko, Tokyo, Japan). After centrifugation at $20,000 \times g$ for 30 min at 4°C, the supernatant was loaded onto an ÄKTAprime Plus (GE Healthcare, Little Chalfont, United Kingdom) liquid chromatography system equipped with a HisTrap HP column (GE Healthcare). Unbound protein was removed by washing with binding buffer, and His₆-tagged protein was then eluted with elution buffer (20 mM potassium phosphate, 500 mM imidazole, and 500 mM NaCl [pH 7.0]), followed by desalting and buffer exchange into 100 mM potassium phosphate buffer (pH 6.5) with a PD-10 column (GE Healthcare).

Enzyme assay. Purified AsnO and SCO2693 were used for L-asparagine hydroxylation, and AsnO-D241N and SCO2693-D246N were used for L-aspartic acid hydroxylation. The reaction mixture consisted of 5 mM the substrate, 10 mM 2-oxoglutarate, 1 mM L-ascorbic acid, 0.5 mM FeSO₄, and 100 mM potassium phosphate buffer (pH 6.5), and the enzyme reaction was initiated by adding 0.03 mg of each enzyme (preincubated for the assessment of stability at 25°C for up to 24 h) at 25°C for 1 h in a total volume of 0.3 ml. Asparaginase I or II was used for the hydrolysis of 3-hydroxyasparagine to form L-THA and ammonia. The reaction mixture consisted of 4 mM 3-hydroxyasparagine, 100 mM potassium phosphate buffer (pH 7.0), and 0.05 mg asparaginase I or II in a total volume of 0.5 ml and was incubated at 30°C for 1 h.

L-THA synthesis by *E. coli* whole cells. The reaction mixture for test tube-scale synthesis of L-THA was composed of 100 mM potassium phosphate buffer (pH 6.5), 50 mM L-asparagine, 100 mM 2-oxoglutarate, 1 mM L-ascorbate, 0.5 mM FeSO₄, and *E. coli* (OD₆₀₀ = 30) in a total volume of 5 ml, and the reaction was performed at 25°C for 72 h with vigorous shaking. In this reaction, *E. coli* strain W3110 (wild type), JW1756 (asparaginase I-deficient mutant), or JW2924 (asparaginase II-deficient mutant), each carrying pUC19/AsnO (pUAsnO), was used as a biocatalyst. For enhanced production of L-THA, the same reaction mixture composition and conditions were used, except that the *E. coli* strain used was wild-type Rosetta2(DE3) or the *ΔansA* strain carrying pET-21a(+)/AsnO (pEAsnO).

The reaction mixture for preparative-scale production of L-THA was composed of 50 mM L-asparagine, 100 mM 2-oxoglutarate, 0.5 mM FeSO₄, and *E. coli* (OD₆₀₀ = 30) in a total volume of 200 ml. The *ΔansA* strain carrying pEAsnO was employed, and the reaction was performed in a jar fermentor by agitation at 600 rpm with an aeration rate of 1 liter/min at 25°C. During the bioconversion, the pH of the reaction mixture was maintained at 6.5 with 10% (vol/vol) H₂SO₄ by an automatic pH-stat instrument (B. E. Marubishi).

Isolation of L-THA. After the reaction, cells were removed by centrifugation, the supernatant was harvested and then treated with activated charcoal, and the resulting solution was passed through a Dowex 50 column (50 ml; H⁺ form). The active fractions containing L-THA were collected after elution with 2% (vol/vol) ammonium hydroxide. Subsequently, the concentrated solution containing L-THA was chilled in an ice bath, and L-THA was crystallized by the addition of cold ethanol. After filtration and drying, a grayish solid of L-THA was obtained.

Analytical instruments. Amino acids were determined as their N^α-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide derivatives (23) by high-performance liquid chromatography (HPLC) using a Hitachi High-Technologies L-7000 series system (Tokyo, Japan) equipped with a Cosmosil 5C₁₈ AR-II column (4.6-mm inner diameter by 150-mm length; Nacalai Tesque, Kyoto, Japan). HPLC conditions were as follows: a column oven temperature of 40°C, mobile phase A consisting of 50 mM KH₂PO₄ buffer (pH 2.7)-CH₃OH-CH₃CN (18:1:1, vol/vol/vol), mobile phase B consisting of 50 mM KH₂PO₄ buffer (pH 2.7)-CH₃OH-CH₃CN (12:1:7, vol/vol/vol), and a detection wavelength of 340 nm. Linear gradient elution was employed, with a flow rate of 1 ml/min and a gradient program of 70% mobile phase A–30% mobile phase B (0 min) to 20% mobile phase A–80% mobile phase B (12 min).

FIG 1 Scheme of L-THA synthesis through biocatalytic hydroxylation.

RESULTS

Gene expression and enzyme purification. After purification by Ni²⁺ affinity and gel filtration chromatographies, the apparent molecular masses of the enzymes were confirmed by SDS-PAGE analysis, which showed that all enzymes were expressed in a soluble form and purified to apparent homogeneity (see Fig. S2 in the supplemental material).

Synthesis of L-THA by biocatalytic reactions. Two biocatalytic routes were investigated, as shown in Fig. 1: (i) direct hydroxylation of L-aspartic acid by AsnO-D241N or its homologous mutant enzyme SCO2693-D246N and (ii) a two-step reaction with the combination of L-asparagine hydroxylase and asparaginase that consists of L-asparagine hydroxylation by AsnO or SCO2693 and subsequent amide hydrolysis of 3-hydroxyasparagine by asparaginase to form L-THA. Although Strieker et al. predicted the substrate of SCO2693 to be L-asparagine based on homology modeling, experimental evidence was not provided (15). Each reaction product was confirmed by HPLC analysis. The reaction product of SCO2693 from L-asparagine was detected as a peak at 6.87 min (Fig. 2a, peak 3). This retention time was identical to that observed with AsnO, indicating that SCO2693 hydroxylated L-asparagine at C-3, similarly to AsnO (15). Subsequently, the reaction product 3-hydroxyasparagine was hydrolyzed by either asparaginase I or II, leading to the disappearance of the peak at 6.87 min and the appearance of a peak with a retention time of 6.37 min (Fig. 2b, peak 2). This peak corresponded to that of authentic L-THA, indicating that both asparaginases I and II of *E. coli* were able to hydrolyze 3-hydroxyasparagine as well as L-asparagine. Furthermore, SCO2693-D246N showed slight L-aspartic acid-specific hydroxylation, since the same retention time of L-THA was observed (Fig. 2c, peak 2), similar to that produced by AsnO-D241N (16).

Enzyme activity and stability. Similar hydroxylation profiles were obtained for AsnO and SCO2693 and for AsnO-D241N and SCO2693-D246N; however, their catalytic activity and stability have not been investigated. Thus, we compared the stabilities at 25°C for various preincubation times prior to the initial velocity of these enzymes for their efficiency in producing 3-hydroxyasparagine (wild-type enzymes) or L-THA (mutant enzymes). The half-life of AsnO-D241N was calculated to be 27 min; in contrast,

FIG 2 HPLC chromatograms of the reaction mixture after *in vitro* biocatalytic reactions. (a) 3-Hydroxyasparagine synthesis from L-asparagine using AsnO or SCO2693; (b) L-THA synthesis from 3-hydroxyasparagine by asparaginase I or II; (c) L-THA synthesis by AsnO-D241N or SCO2693-D246N; (d) amino acid standards. Each peak is as follows: 1, D-THA; 2, L-THA; 3, 3-hydroxyasparagine; 4, L-asparagine; 5, L-EHA; 6, L-aspartic acid. AU, arbitrary units.

that of AsnO was calculated to be >24 h. Although AsnO-D241N hydroxylated L-aspartic acid, its stability immediately decreased at 25°C compared to that of AsnO. A similar instability was observed in the cases of SCO2693 and SCO2693-D246N, whose half-lives were calculated to be 32 min and <1 min, respectively. In contrast, only AsnO retained higher catalytic activity and stability up to 5 h of incubation, and $>50\%$ activity remained at 24 h. The specific activities of the wild-type AsnO and SCO2693 enzymes were $2 \times$

10^{-1} and $7 \times 10^{-2} \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively. Comparison of AsnO and SCO2693 revealed that AsnO displayed much higher L-asparagine hydroxylation activity and stability. Hence, we concluded that AsnO appeared to be a suitable enzyme for further production of L-THA, and it was used for the following experiments.

Effect of asparaginase disruption on L-THA synthesis by whole-cell bioconversion. AsnO was expected to be advantageous for the production of L-THA; thus, bioconversion of L-asparagine to L-THA at the test tube scale was attempted by using intact *E. coli* W3110 carrying pUAsnO, in which the reactions of exogenous AsnO and endogenous asparaginases were coupled. However, only 1.9×10^{-4} mmol of L-THA was obtained from 2.5×10^{-1} mmol of L-asparagine with a molar yield of 0.076% (Table 1). One possibility for this low yield was the presence of endogenous *E. coli* asparaginases. Thus, comparative analysis with asparaginase-deficient mutant strains JW1756 and JW2924 as host strains was carried out under the same reaction conditions. The L-THA yield was increased in both asparaginase-deficient mutants (Table 1). In particular, in JW1756, 2.1×10^{-2} mmol of L-THA was obtained from 2.5×10^{-1} mmol of L-asparagine with a molar yield of 8.2%, indicating that asparaginase I-deficient *E. coli* was preferable for L-THA production.

Enhancement of the expression level of the *asnO* gene and disruption of the *ansA* gene. Although asparaginase I-deficient *E. coli* expressing the *asnO* gene was effective for the production of L-THA, the level of productivity remained low, likely due to insufficient L-asparagine hydroxylation. To overcome this low level of productivity, we attempted to improve the expression level of the *asnO* gene, which was remarkably low under the control of the *lac* promoter in *E. coli* JW1756. Some of the more commonly used strong promoters in *E. coli* include the *lacUV5* promoter, which is a modified *E. coli lac* promoter; the *E. coli trp* promoter, a synthetic *trc* promoter; and the T7 promoter derived from bacteriophage T7. Among them, T7 is recognized as the strongest promoter; thus, the *asnO* gene was placed under the control of the T7 promoter of the pET-21a(+) vector instead of the *lac* promoter of the pUC19 vector. Furthermore, the ΔansA strain derived from *E. coli* Rosetta2(DE3) was constructed and used as the host strain. As a consequence, the expression level of the *asnO* gene in *E. coli* Rosetta2(DE3) carrying pEAsnO was significantly increased, as shown by SDS-PAGE analysis (Fig. 3).

In test tube-scale production, the L-THA yield was also significantly increased with the T7 promoter expression system in spite of the use of the same conditions as those described above. A total of 1.9×10^{-1} mmol of L-THA was obtained from 2.5×10^{-1} mmol of L-asparagine in a 72-h reaction with a molar yield of 76% by using *E. coli* Rosetta2(DE3) carrying pEAsnO (Fig. 4a). Similarly to the comparative analysis (Table 1), asparaginase I gene disruption was also beneficial for the production of L-THA, since 2.3×10^{-1} mmol of L-THA was obtained with a molar yield of 92% in the case of the ΔansA strain (Fig. 4b).

TABLE 1 Comparison of L-THA yields using different *E. coli* strains

Host/vector	Mean L-THA concn (mM) $\pm$ SD	Yield (mol%)
W3110/pUAsnO	0.038 ± 0.0074	0.076
JW1756/pUAsnO	4.1 ± 0.68	8.2
JW2924/pUAsnO	0.069 ± 0.016	0.13

FIG 3 Comparison of expression levels of the AsnO protein between *lac* and T7 promoters by SDS-PAGE analysis. Lane 1, molecular mass standards; lanes 2 and 3, *lac* expression system using the pUC19 vector; lanes 4 and 5, T7 expression system using the pET-21a(+) vector. Lanes 2 and 4 are soluble fractions and lanes 3 and 5 are insoluble fractions of *E. coli* cell extracts. Arrows indicate AsnO.

Gram-scale preparation with a jar fermentor. Based on the results of the test tube-scale investigations, the *E. coli* Rosetta2(DE3) Δ *ansA* strain carrying pEAsnO was deemed suitable for efficient L-THA synthesis. Subsequently, preparative-scale production of L-THA with a jar fermentor was carried out. After a 28-h reaction, 9.6 mmol of L-THA was obtained from 10 mmol of L-asparagine with the use of the Δ *ansA* strain carrying pEAsnO as a biocatalyst, with a molar yield of 96% (Fig. 5). From this result, it was apparent that the reaction was accelerated and completed in less time than the test tube-scale investigation (28 h versus 72 h).

Antimicrobial assay. Another property of L-THA is its antimicrobial activity (4), and thus, growth inhibition of *B. subtilis* was confirmed by placing the paper disc containing isolated L-THA from the reaction mixture onto culture plates. As shown in Fig. 6, clear inhibition zones around authentic and isolated L-THA were observed.

DISCUSSION

In this study, we have demonstrated biocatalytic L-THA synthesis from L-asparagine as the starting material through L-asparagine hydroxylation and subsequent 3-hydroxyasparagine hydrolysis

FIG 5 Production of L-THA using the Rosetta2(DE3) Δ *ansA* strain carrying pEAsnO with a jar fermentor. Symbols: circles, L-asparagine; squares, 3-hydroxyasparagine; triangles, L-THA. The data given are the means of data from three independent experiments, and error bars show the standard deviations.

(Fig. 1). In addition, this method was applicable to a one-pot bioconversion using intact *E. coli* cells. Compared to the known methods for the synthesis of L-THA, this process is simple and efficient, because >90% of L-asparagine was converted to L-THA without the formation of any stereoisomers. In addition, L-THA crystals were obtained from the reaction mixture by purification via cation-exchange chromatography and chilling after the addition of a poor solvent.

To date, many different methods for L-THA synthesis have received much attention. According to previous studies (15, 16), AsnO-D241N was used for L-aspartic acid hydroxylation; however, in our experiments, the specific activity for L-aspartic acid and the stability of the mutant were significantly low compared to those of wild-type AsnO. Strieker et al. also described the homologous enzymes SCO2693 (57% amino acid sequence identity with AsnO) and SCO2693-D246N (15, 16), which corresponds to AsnO-D241N; however, this mutation was apparently detrimental to both the activity and stability of SCO2693, similar to what we observed with AsnO-D241N. As direct hydroxylation is a simple one-step process, it was concluded that the mutant enzymes were difficult to employ for efficient L-THA production, and an alternative method was explored.

FIG 4 Effects of disruption of the asparaginase I gene on L-THA productivity. (a) Wild-type Rosetta2(DE3) carrying pEAsnO; (b) Rosetta2(DE3) Δ *ansA* strain carrying pEAsnO. Symbols: circles, L-asparagine; squares, 3-hydroxyasparagine; triangles, L-THA. The data given are the means of data from three independent experiments, and error bars show the standard deviations.

FIG 6 Representative plate assay showing antibacterial activity against *B. subtilis*. 1, authentic L-THA (500 μ g/ml); 2, isolated L-THA (500 μ g/ml); 3, authentic L-EHA (500 μ g/ml); 4, H₂O (negative control).

We speculated that an alternative method for L-THA production would be possible if the terminal amide moiety of 3-hydroxyasparagine can be hydrolyzed by a hydrolase. Hence, we focused on asparaginase, which is known to catalyze the degradation of L-asparagine to L-aspartic acid and ammonia; however, bacterial asparaginase is commonly highly specific for L-asparagine, and little is known about its catalytic activity for 3-hydroxyasparagine. An evaluation of asparaginases I and II of *E. coli* was then carried out, and both enzymes had the capacity for hydrolyzing 3-hydroxyasparagine to L-THA and ammonia *in vitro* (Fig. 2b). To our knowledge, this is the first example of bacterial asparaginase acting on 3-hydroxyasparagine. Based on these findings, the two-step reaction for L-THA synthesis was experimentally confirmed.

Toward the efficient production of L-THA from L-asparagine, a one-pot reaction for L-asparagine hydroxylation followed by amide hydrolysis was reconstructed in *E. coli* cells. Obviously, the higher hydrolytic activity of asparaginase than of AsnO would result in an acceleration of unfavorable L-asparagine degradation in advance of L-asparagine hydroxylation. The resulting L-aspartic acid would be subsequently deamidated by endogenous aspartase (EC 4.3.1.1), and the formed fumarate may flow straight into the tricarboxylic acid cycle. This degradation route appeared to decrease the overall L-THA yield; therefore, the effect of deleting asparaginases I and II in *E. coli* on the L-THA yield was investigated. Initially, the effects of asparaginase I and II gene disruptions were compared. As expected, L-asparagine degradation was reduced by using asparaginase I- and II-deficient *E. coli* strains, and in particular, the deletion of asparaginase I was more favorable for the L-THA yield (Table 1). This effect may be attributed to the differential regulation of asparaginase activity caused by its distinctive expression mechanism. In asparaginase I-deficient *E. coli*, asparaginase II remained therein, but its activity might have been significantly attenuated, as the expression of the asparaginase II gene has been shown to be suppressed in *E. coli* under aerobic conditions (24). In asparaginase II-deficient *E. coli*, however, asparaginase I was retained, and its activity was not depressed, since this enzyme is constitutively expressed in *E. coli* (25), which led to a lower L-THA yield than that with asparaginase I-deficient *E. coli*. These results suggest that a relatively weak asparaginase activity

rather than a complete disappearance of asparaginase activity would be an important factor for higher-yield L-THA production.

Next, the T7 promoter was used instead of the *lac* promoter to enhance the expression level of the *asnO* gene, and the L-THA yield was also improved by using an asparaginase I-deficient mutant of *E. coli* expressing the *asnO* gene (Fig. 4). As a result, decreased asparaginase activity and increased AsnO activity in *E. coli* appear to be effective for high-yield production of L-THA, since the enhancement of AsnO activity would increase the metabolic flow of L-asparagine to preferable hydroxylation rather than degradation into fumarate.

According to these results, we carried out gram-scale preparation of L-THA under larger-volume conditions with a jar fermentor. L-THA was successfully obtained in a slightly higher yield than with the test tube-scale preparation. Notably, the overall reaction for the conversion of L-asparagine to L-THA was accomplished in a shorter period (Fig. 5). This could probably be attributed to the increased O₂ supplied by the jar fermentor with vigorous agitation and bubbling, since dissolved O₂ is an essential substrate for the L-asparagine hydroxylation reaction. After crystallization, antibacterial activity against *B. subtilis* (Fig. 6) was found to be consistent with data from previous reports (4).

In conclusion, we have developed an efficient process for the production of L-THA from L-asparagine using an asparaginase I-deficient mutant of *E. coli* expressing the genes encoding exogenous AsnO and endogenous asparaginase II. By using this strain as a biocatalyst, >90% of L-asparagine was converted to L-THA with a jar fermentor in a 28-h reaction. The one-pot system that we have described here is a promising approach for the practical production of L-THA.

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