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Nitrile-synthesizing enzyme: Gene cloning, overexpression and application for the production of useful compounds

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One of the nitrile-synthesizing enzymes, β-cyano-L-alanine synthase, catalyzes β-cyano-L-alanine (β-CNAla) from potassium cyanide and *O*-acetyl-L-serine or L-cysteine. We have identified this enzyme from *Pseudomonas ovalis* No. 111. In this study, we cloned the β-CNAla synthase gene and expressed it in *Escherichia coli* and *Rhodococcus rhodochrous*. Furthermore, we carried out co-expression of β-CNAla synthase with nitrilase or nitrile hydratases in order to synthesize aspartic acid and asparagine from KCN and *O*-acetyl-L-serine. This strategy can be used for the synthesis of labeled amino acids by using a carbon-labeled KCN as a substrate, resulting in an application for positron emission tomography.

Key Words: cyanide; enzyme; nitrile

Introduction

Cyanide and nitriles are known to be toxic compounds for almost all living organisms. However, some higher plants and microorganisms produce and accumulate them in their living cells. In higher plants, cyanide is produced through the biosynthesis of ethylene (Yip and Yang, 1988), and certain microorganisms also produce cyanide (Knowles, 1976). We have studied the microbial degradation (Hashimoto et al., 2005; Kobayashi et al., 1993; Komeda et al., 1996a; Zhou et al., 2008) and synthesis (Konishi et al., 2006; Nomura et al., 2013) of nitriles not only from a basic standpoint but also from an applied point of view (Herai et al., 2004; Kobayashi and Shimizu, 1998; Komeda et al., 1996b).

It has been reported that microorganisms and plants can degrade cyanides to less toxic compounds through biochemical reactions. Some of these are degradative pathways involving cyanide hydratase, nitrile hydratase, cyanidase, nitrilase, thiocyanate hydrolase, cyanide dioxygenase and cyanase. Others are pathways for the assimilation of cyanide as nitrogen and carbon sources in microorganisms. The enzymes β-cyano-L-alanine (β-CNAla) synthase and γ-cyano-α-aminobutyric acid synthase are involved in this assimilation pathway.

Cyanide-resistant bacteria and plants detoxify cyanide via its conversion to β-CNAla. β-CNAla synthase catalyzes the formation of β-CNAla from potassium cyanide and *O*-acetyl-L-serine or L-cysteine. We have identified β-CNAla synthase from *Pseudomonas ovalis* No. 111 (Kumano et al., 2016). This enzyme catalyzes the synthesis of β-CNAla from potassium phosphate and *O*-acetyl-L-serine.

On the other hand, nitrile-degrading enzymes (i.e., nitrilase and nitrile hydratases) have received much attention in applied fields. Nitrile hydratase (NHase) from *Rhodococcus rhodochrous* J1 has been used for the industrial production of acrylamide (Yamada and Kobayashi, 1996) and nicotinamide (Nagasawa et al., 1988). Nitrilases from *R. rhodochrous* J1 and *R. rhodochrous* K22 act on aromatic nitriles and aliphatic nitriles, respectively. In combination with β-CNAla synthase and each of these

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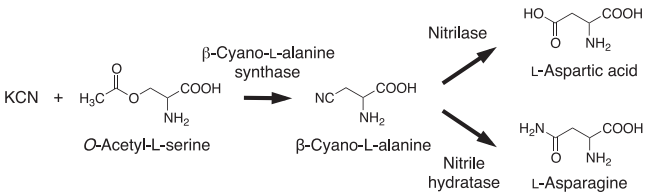

Fig. 1. Enzymatic synthesis of amino acids by β-CNAla synthase and nitrilase or nitrile hydratase.

nitrile-converting enzymes, we have designed the direct production of aspartic acid or asparagine from cyanide as a nitrile substrate (Fig. 1). When carbon-labeled KCN is used as a substrate, this reaction can result in the synthesis of carbon-labeled amino acids used for positron emission tomography (PET), an image diagnostic technique which has attracted considerable attention in the field of medical diagnosis.

In this study, we cloned β-CNAla synthetic gene from *Pseudomonas ovalis* No. 111, and the recombinant β-CNAla synthase protein was overexpressed in *Escherichia coli*. We co-expressed β-CNAla synthase with *R. rhodochrous* K22 nitrilase in *E. coli* or *R. rhodochrous* J1 nitrile hydratase in *R. rhodochrous* ATCC12674, and produced aspartic acid or asparagine using their enzymes in a "one-pot reaction".

Materials and Methods

Enzymes and chemicals. Restriction endonucleases and other DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) and Toyobo Co., Ltd. (Osaka, Japan). Lysyl-endopeptidase, isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-1-galactopyranoside were obtained from Wako Pure Chemicals (Tokyo, Japan). All other chemicals were of the highest purity commercially available.

Bacterial strains and plasmids. *Pseudomonas ovalis* No. 111 was used for a source of the β-CNAla synthase gene. *Rhodococcus rhodochrous* ATCC12674 and *E. coli* JM109 were the host for pUC and pBluescript plasmids. pCRII vector (included in the Original TA cloning kit) was purchased from Invitrogen (USA).

Cultivation of Pseudomonas ovalis No. 111. *P. ovalis* No. 111 was collected from an agar slant and inoculated into the subculture. The subculture was carried out at 37°C overnight in a medium consisting of 5 g peptone, 5 g meat extract and 2 g NaCl/l tap water (pH 7.0). The subculture was then inoculated into a medium consisting of 5 g glycerol, 1 g yeast extract, 1 g $(NH_4)_2SO_4$, 1 g $K_2HPO_4 \cdot 7H_2O$ and 10 g L-serine/l and cultured overnight at 37°C.

Preparation and isolation of internal peptides and sequencing of their amino acids. The purified enzyme was subjected to SDS-PAGE, and then transferred to the membrane. The electro-blotted membrane was treated with 0.5% polyvinylpyrrolidone-40/100 mM acetic acid at 37°C for 30 min, and digested with lysyl-endopeptidase in 5% acetonitrile/50 mM Tris-HCl (pH 9.3). The digested peptides were subjected to HPLC on a Cosmosil 5C18-AR (C-18, 4.6 × 250 mm) at a flow rate of 1 ml/min. The sample was eluted with a liner gradient of acetonitrile (0–60%, v/v) in the presence of 0.1% trifluoroacetic acid, and fractions containing each peptide were collected. Twelve internal peptides were sequenced by automated Edman degradation.

Cloning of the structural gene for β-CNAla synthase. The peptide fragments obtained by digestion of the purified enzyme with lysyl-endopeptidase (Wako Pure Chemicals) were sequenced as VLK, IEGRNPGYSVK, IQGIGAGFVPK, NLDLSMVDRVEQVTDDESK, YFMPSQFDNPANPAIHEK, ILSVAVEPVSXP, XTGPEIWND, KXGPEIWNDTDGAVDVLVAG, AIVVILPDDGERYLXAMLFXXR and AIVVILPDDGERYL. The *N*-terminal partial amino acid sequence was SRIFADNAHSIGNTPLVQINRIAPRGVTIL (Kumano et al., 2016). For PCR, we synthesized oligonucleotide primers that corresponded to the determined sequences. Oligonucleotide primers were synthesized based on the amino acid sequences of the *N*-terminus and the internal fragment generated with lysyl-endopeptidase. The sense primer was designed as 5′-GTGTCGACT(A/C/T)TT(C/T)GCIGA(C/T)AA(C/T)GC(A/C/G/T)C-3′, and the antisense primer was designed as 5′-TCTAGAGCTCC(A/G)TC(A/C/G/T)GT(A/G)TC(A/G)TC(A/G)TTCCA(A/G/T)AT(C/T)TC-3′. A nucleotide fragment of the partial structural gene of the *P. ovalis* β-CNAla synthase was amplified by PCR with these primers. The amplified 700-bp fragment was directly ligated with pCRII vector to yield pCP10, the nucleotide sequence of which was found to be derived from the determined amino acid sequence. With the 700-bp fragment which was labeled with [α-^{32}P]dCTP using a Multiprime DNA labeling system (Amersham) as a probe, we carried out Southern hybridization against the *Bcl*I-digested genomic DNA of *P. ovalis* No. 111 with the following modification. Hybridization was carried out at a higher stringency, using a buffer containing 40% (v/v) formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate), and 0.1% (m/v) SDS at 42°C for 12 h. A single, approximately 3-kb, fragment was detected. This fragment was recovered and ligated with T4 DNA ligase to linear pBluescript KS$^+$ successively treated with *Bam*HI and bacterial alkaline phosphatase. By transformation, the ligated mixture was introduced into *E. coli* JM109, and ampicillin-resistant transformants were selected on the 2 × YT agar plate containing 100 μg/ml ampicillin. This *Bcl*I fragment containing the partial gene of β-CNAla synthase was then screened by colony hybridization, resulting in the isolation of the plasmid DNA (pCP20) from positive clones.

The nucleotide sequence of the approximately 3-kb fragment of pCP20 was determined (Fig. S1). However, this plasmid was found not to contain the full-length β-CNAla synthase gene. In order to obtain the full-length gene, we carried out Southern hybridization against the restriction endonuclease-digested genomic DNA using the 680-bp *Eco*RI-*Bcl*I fragment from pCP20 as a probe and detected a single hybridized 2.2-kb *Bss*HII-*Eco*RI fragment. This fragment was blunted and cloned into the *Eco*RV site of pBluescript KS$^+$, and the resultant plasmid was designated pCP30.

Phylogenetic analysis of β-CNAla synthase. Alignment

was performed using Clustal W and the phylogenetic tree was displayed by FigTree v1.4.2.

Protein sequences were retrieved from the NCBI database. The accession number of each protein is as follows: *Bacillus stearothermophilus* (BAC55275.1), *Bacillus thuringiensis* (WP_050842386.1), *Enterococcus gallinarum* EG2 (EEV32406.1), *Pseudomonas fluorescens* (WP_046046084.1), *Pseudomonas ovalis* No. 111 (LC122269), *Salmonella Typhimurium* (gi6980381), *Erwinia herbicola* (WP_003848872.1), *Serratia* sp. ATCC 39006 (ESN63909.1), *Hafnia alvei* (WP_025800770.1), *Methylobacterium radiotolerans* (WP_012320945.1), *Methylobacterium* sp. (ZP_10356649.1), *Achromobacter insuavis* (EGP46495.1), *Sagittula stellate* (ZP_01743962.1), *Mesorhizobium opportunistum* (WP_013895618.1), *Roseovarius nubinhibens* (ZP_00959725.1), *Peptoclostridium difficile* (YP_001088167.1), *Escherichia coli* (ZP_06649856.1), *Photorhabdus luminescens* (WP_011145699.1), *Pseudomonas putida* (WP_014592110.1), *Burkholderia pseudomallei* (CAH36513.1), *Glycine max* (M) (XP_003534555.1), *Arabidopsis thaliana* (M) (NP_191703.1), *Solanum tuberosum* (M) (Q76MX2.1), *Zea mays* (M) (ADG60236.1), *Arabidopsis thaliana* (C) (NP_193224.1), *Glycine max* (C) (AAL66291.1), *Solanum tuberosum* (C) (O81154.1), *Zea mays* (C) (NP_001105469.2), *Apis mellifera* (C) (NP_001035353.1), *Culex quinquefasciatus* (C) (XP_001863044.1), *Tetranychus urticae* (C) (KF981737.1), *Homo sapiens* (IM) (NP_000062.1), *Podospora anserina* (C) (CAP68365.1), *Sclerotinia sclerotiorum* (C) (EDN93051.1), *Aspergillus fumigatus* (M) (XP_748124.1), *Trichoderma reesei* (M) (EGR50915.1), *Rhizopus delemar* (M) (EIE81016.1), *Debaryomyces hansenii* (M) (XP_461064.1), and *Schizosaccharomyces pombe* (M) (NP_595332.1).

Cloning of the structural gene for β-CNAla synthase. To overproduce the β-CNAla synthase in *E. coli*, we improved the sequence upstream from the ATG codon by PCR with pCP30 as a template using the two following primers: Sense primer, 5′-TAGAATTCGTAAGGAGGAAATAAGCCATGAGCCGTATTTTTGCTG-3′; antisense primer, 5′-CATTCTGCAGCATAACGGATCCTGAATCCCG-3′. The sense primer contained the *Eco*RI recognition site, a ribosome-binding site, and a TAA stop codon (shown in 1st, 2nd, and 3rd underlines, respectively) in frame with the *lac*Z gene in pUC18 and 19 nucleotides of the β-CNAla synthase gene starting with the ATG start codon (double underlined). The antisense primer contained 31 nucleotides of the gene and a *Pst*I recognition site (underlined). The amplified 1.1-kb region was inserted between the *Eco*RI and *Pst*I sites of pUC18, resulting in the construction of plasmid pCP40E, where the β-CNAla synthase gene was under the control of the *lac* promoter.

Preparation of cell-free extracts from E. coli transformants. *E. coli* JM109 cells harboring plasmid pCP40E were cultured in 100 mL liquid LB medium containing 100 μg/ml ampicillin, and grown at 37°C with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). The cells were cultured for a further 7 or 12 h and harvested. Three milliliters of 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) was added. The cells were disrupted with a sonifier. The lysate was centrifuged at 20,600 × *g* at 4°C for 10 min.

Purification of the recombinant β-CNAla synthase from E. coli transformants. The recombinant β-CNAla synthase was purified through the following two-step column procedure at 0–4°C using the potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. The cell-free extracts were fractionated with ammonium sulfate (30–60% saturation), followed by dialysis against 0.1 M buffer containing 80 μM PLP. The dialyzed enzyme solution was applied to a DEAE-Sephacel column (2.6 × 25 cm) and eluted with increasing concentrations of KCl (0 to 0.2 M). The enzyme solution was concentrated with an Amicon YM-10 membrane (Amicon Corp., Danvers, USA). The resulting enzyme solution was applied to a Sephacryl S-200 HR column (0.9 × 100 cm), and eluted with 0.01 M buffer containing 0.2 M KCl.

Enzyme assay. The standard assay mixture consists of 10 mM KCN, 80 μM PLP, 10 mM *O*-acetyl-L-serine, 0.1 M potassium phosphate buffer (pH 7.5) and an appropriate amount of enzyme solution. The reaction was carried at 30°C for 10 min and stopped by adding 20 μl of 2 M HCl. One unit of activity was defined as the amount of β-CNAla synthase required to produce 1 μmol of β-CNAla from amino acid per minute. The detection of the amount of β-CNAla was carried out by using high-performance liquid chromatography (HPLC) after modification of the reaction product with *o*-phthalaldehyde (OPA). OPA-amino acids were detected by using a fluorometer (Hitachi, Ltd., Tokyo, Japan). The excitation and emission wavelengths for the OPA-amino acids were 340 and 455 nm, respectively.

Preparation of apoenzyme. The apo form of the enzyme was obtained by treating holoenzyme with 1 mM hydroxylamine, followed by dialysis against 10 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM DTT. Reconstitution was carried out by dialyzing the apoenzyme against 10 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM DTT and 0.01 mM PLP.

Determination of PLP binding site. The purified enzyme was dialyzed against 5 mM potassium phosphate buffer (pH 7.5), and then against 5 mM $NaBH_4$. After the yellow color had disappeared, the enzyme solution was dialyzed against 5 mM potassium phosphate buffer (pH 7.5). The $NaBH_4$-reduced enzyme was denatured with 8 M urea in 20 mM Tris-HCl buffer (pH 9.0) at 37°C for 60 min, and subsequently digested with lysyl-endopeptidase at 37°C for 10 h. Pyridoxyl peptides were isolated and purified with Sephasil C8 (2.1 × 100 mm) and μRPC C2/C18 (2.1 × 100 mm) connected to Smart System (Pharmacia, Sweden). The sample was eluted with a liner gradient of acetonitrile (0–80%, v/v) in the presence of 0.1% trifluoroacetic acid. The amino acid sequences of pyridoxyl peptides were analyzed by automated Edman degradation.

Construction of the co-expression plasmid for β-CNAla synthase and nitrilase in E. coli. The 1.1 kb *Eco*RI-*Pst*I fragment containing the β-CNAla synthase gene from pCP40E was inserted into the blunt-ended *Eco*RI site of pNK30 containing the nitrilase gene to yield pCEAD10. *E. coli* JM109 harboring pCEAD10 was cultured in the presence of IPTG at 37°C.

Fig. 2. Phylogenetic analysis of β-CNAla synthase.

Phylogenetic analysis was carried out according to the method described in Section "Materials and Methods". *Pseudomonas ovalis* No. 111 and *Bacillus stearothermophilus* (Omura et al., 2003) shown in bold have been characterized by us. CAS indicates β-CNAla synthase. CS, CysK and CysM indicate cysteine synthase. CBS indicate the cystathionine-β-synthase. The abbreviation in parentheses shows subcellular localization of protein. C, cytoplasmic; M, mitochondrial; IM, integral membrane.

The 1.4-kb *Pvu*II fragment from pCP40E was inserted into the blunt-ended *Hind*III site of pNK30 to yield pCEAD20. *E. coli* JM109 harboring pCEAD20 was cultured in the presence of IPTG at 37°C.

Construction of the co-expression plasmid for β-CNAla synthase and H-NHase in R. rhodochrous ATCC12674. The 1.1 kb *Eco*RI-*Pst*I fragment containing the β-CNAla synthase gene from pCP40E was inserted into the blunt-ended *Spl*I site of pHJK19 containing the H-NHase gene form *R. rhodochrous* J1, to yield pCEAN192 containing both enzyme genes.

Transformation of R. rhodochrous ATCC12674. A mid-exponential culture of *R. rhodochrous* ATCC12674 was harvested and washed three times with deionized cold water. Cells were then concentrated 20-fold in cold water and kept on ice. Ice-cold cells (100 μl) were mixed with 1 μg DNA in 1 μl of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) in a 1-mm-gapped electrocuvette (Bio-Rad), and subjected to a 2.0 kV electric pulse from a Gene pulsar (Bio-Rad) connected to a pulse controller (25 μF capacitor; external resistance, 400 Ω). Pulsed cells were diluted immediately with 1 ml of PYM medium and incubated for 2 h at 28°C. They were then spread on PYM medium containing 100 μg/ml kanamycin.

Preparation of cell-free extracts from R. rhodochrous ATCC12674. *R. rhodochrous* ATCC12674 transformants

Table 1. Purification of β-CNAla synthase from recombinant *E. coli*.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Fold	Yield (%)
Cell-free extract	2460	5160	2.10	1	100
Ammonium sulfate	1090	4930	3.01	1.43	66.7
DEAE-Sephacel	875	3020	3.45	1.64	58.5
Sephacryl S-200HR	320	1860	5.82	2.77	36.1

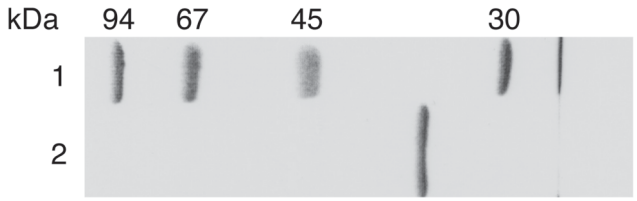

Fig. 3. SDS-PAGE of the purified β-CNAla synthase from recombinant *E. coli*.

Lane 1, molecular mass marker proteins. Lane 2, the purified β-CNAla synthase. The relative molecular mass of the enzyme was calculated from the mobilities of the marker proteins, phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

were grown at 28°C for 48 h in PYM medium containing 0.01 g/l $CoCl_2 \cdot 6H_2O$ and 0.75 g/l urea, harvested by centrifugation at 20,600 × *g* at 4°C. The cells were suspended in 0.1 M KPB (pH 7.5), disrupted by sonication for 30 min (19 kHz, Insonator model 201M; Kubota, Tokyo), and centrifuged at 20,600 × *g* for 10 min at 4°C. The resulting supernatant was used in the enzyme assay.

Results and Discussion

Cloning and nucleotide sequence analysis of the structural gene for β-CNAla synthase

We cloned the DNA fragment, which hybridized to the partial gene of β-CNAla synthase, into the *Eco*RV site of pBluescript KS^+, and the resultant plasmid was designated pCP30 (Fig. S1). Determination of the nucleotide sequence of the fragment in pCP30 revealed that the amino acid sequences determined for peptide fragments (shown in Section "Materials and Methods") of the purified β-CNAla synthase approximately matched those predicted from the obtained DNA sequences. The identified open reading frame is 975 nucleotides long and encodes 324 amino acids. The nucleotide sequence data appear in the DDBJ/GenBank database under accession numbers LC122269 for *casA*. The calculated molecular mass from the deduced amino acid sequence was 34,263, which is in close agreement with that of 34,000 separated by SDS-PAGE.

A search of the protein database using the program blastp revealed that the deduced amino acid sequence of the β-CNAla synthase exhibits a high similarity to that of cysteine synthase, or β-CNAla synthase, of plants (Fig. S2). Cysteine synthase and β-CNAla synthase belong to a group of pyridoxal-5′-phosphate (PLP) dependent enzymes together with cystathionine-β-synthase which catalyzes the cysteine biosynthesis in animals. Phylogenetic analysis suggested that the β-CNAla synthase of *P. ovalis* No. 111 exists in the distinct clade from bacterial cysteine synthases (Fig. 2). Interestingly, the β-CNAla synthase of *P. ovalis* No. 111 embedded to a clade different from that of *B. stearothermophilus*, which is the bifunctional cysteine synthase/β-CNAla synthase (Omura et al., 2003).

Cloning of the structural gene for β-CNAla synthase and its heterologous expression in E. coli

The 1.1 kb region of the β-CNAla synthase-coding gene was amplified by PCR and cloned into the pUC18 vector to obtain pCP40E. *E. coli* JM109 was transformed with pCP40E, and the recombinant β-CNAla synthase was heterologously expressed and purified as described in Section "Materials and Methods".

The purified recombinant enzyme showed only one band on SDS-PAGE (Fig. 3). The size of the protein was consistent with that of the purified enzyme from *P. ovalis* (Kumano et al., 2016). The specific activity of the recombinant β-CNAla synthase was 5.82 units/mg (Table 1).

Absorption spectra and activities of holo- and apoenzyme

The enzyme exhibited absorption maxima at 275 nm (ε 118,000) and 420 nm (ε 28,000). The occurrence of the absorption peak at 420 nm suggests that the formyl group of the bound PLP forms an azomethine linkage with an amino group of proteins, as shown in other PLP enzymes. The holoenzyme was completely converted to the apoenzyme by treatment with hydroxylamine and dialysis. The apoenzyme had no peak at 420 nm and no activity when PLP was not added to the reaction mixture. The apoenzyme was reconstituted by dialysis against a buffer containing PLP. About 83.9% of the initial activity could be restored at a given concentration of PLP (0.01 mM)

Determination of PLP content

The enzyme was dialyzed against the buffer, and the amount of PLP was determined, assuming that the molar absorption coefficient of the phenylhydrazone of PLP is 24,500 M^{-1} cm^{-1} at 410 nm. An average PLP content of 29 nmol/mg enzyme was obtained, indicating that 2 mol PLP are bound to 1 mol enzyme protein in the holoenzyme. Since the enzyme appears to be homodimer consisting of two identical subunits (Kumano et al., 2016), each subunit would contain PLP at a stoichiometric ratio of unity.

Determination of a PLP binding site

Reduction of the enzyme with $NaBH_4$ by the dialysis

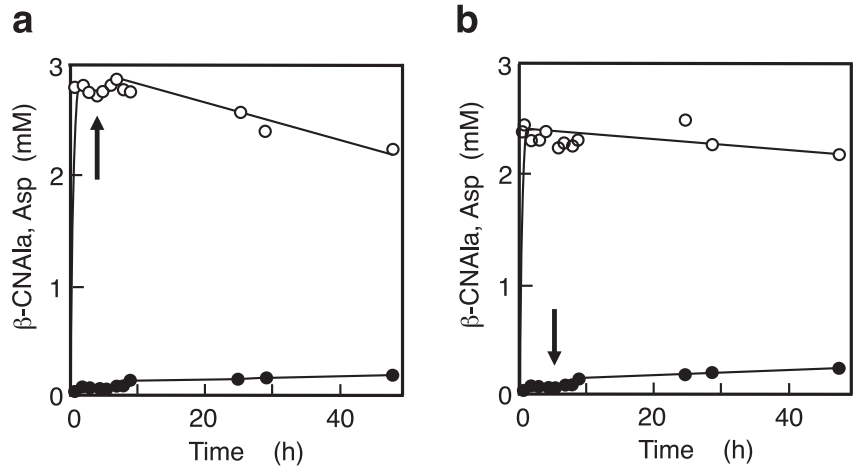

Fig. 4. Synthesis of aspartic acid by *E. coli* recombinant enzymes.

(a) The reaction was carried out with the supernatant prepared from *E. coli* containing pCEAD20. After 5 h, the cell-free extract was added (indicated by the arrow). (b) The reaction was carried out with the supernatant prepared from pCP40E. After 5 h, the supernatant prepared from *E. coli* containing pNK30 was added (indicated by the arrow). Symbols: (○) *β*-CNAla; (●) Asp.

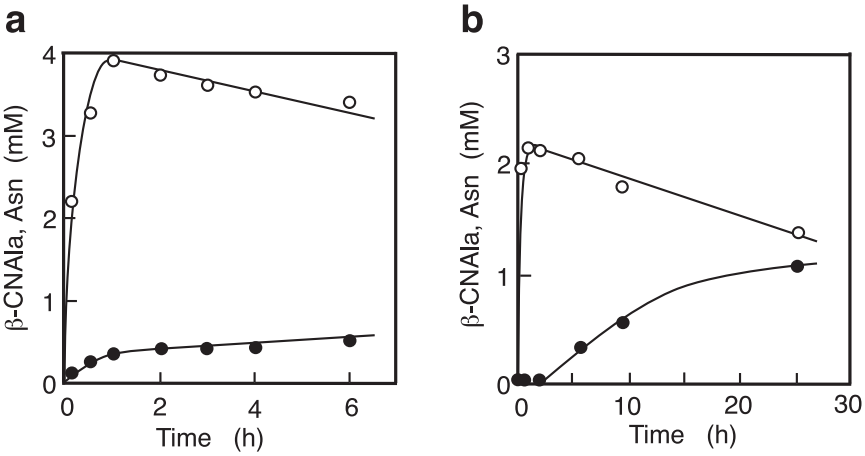

Fig. 5. Synthesis of asparagine by *R. rhodochrous* recombinant enzymes.

(a) The reaction was carried out with the supernatant prepared from *R. rhodochrous* ATCC12674 containing pCEAN192. (b) The reaction was carried out with the purified *β*-CNAla synthase from *P. ovalis* No. 111 and NHase from *R. rhodochrous* J1. Symbols: (○) *β*-CNAla; (●) Asn.

affected both the absorption spectrum and activity. The reduced enzyme was catalytically inactive, and the addition of PLP into the reduced enzyme did not reverse the inactivation. These findings suggest that the $NaBH_4$ reduces the aldimine linkage formed between the 4-formyl group of PLP and an amino group of the protein to yield the aldamine bond.

To identify the amino acid residue to which PLP binds in the enzyme, the $NaBH_4$-reduced enzyme was digested with lysyl-endopeptidase, and the peptide that showed significant absorbance at 315 nm was purified by HPLC. The partial amino acid sequence of the resultant peptide was subjected to amino acid sequence analysis. Together with the predicted amino acid sequence information derived from the nucleotide sequence of *β*-CNAla synthase, the determined sequence 33-IEGRNPGYSVXCRIGANM-IWDAESSGK-60 revealed that PLP binds lysine-43 (Fig. S2).

Synthesis of aspartic acid by β-CNAla synthase and nitrilase expressed in E. coli

The addition of the cell-free extracts from *E. coli* JM109 containing pCP40E to the reaction mixture containing KCN and *O*-acetyl-L-serine resulted in an efficient production of *β*-CNAla with a conversion yield of more than 97%. In order to produce aspartic acid, we attempted tandem reactions using *β*-CNAla synthase and nitrilase. Namely, after the above 5 h reaction, the cell-free extracts from *E. coli* JM109 containing pNK30, which harbors the aliphatic nitrilase gene from *R. rhodochrous* K22 (Kobayashi et al., 1990) were added to the reaction mixture. Aspartic acid was produced, but its yield was low (Fig. 4a).

Synthesis of aspartic acid by β-CNAla synthase and nitrilase co-expressed in E. coli

The *β*-CNAla synthase gene from pCP40E was inserted

into the pNK30 containing the nitrilase gene to yield pCEAD10. When *E. coli* JM109 harboring pCEAD10 was cultured, β-CNAla synthase was overexpressed in the cells, but nitrilase was neither in the supernatant of the cell-free extracts nor in the precipitates.

In order to express both enzymes, we constructed pCEAD20 containing a *lac* promoter upstream of each gene. When *E. coli* JM109 harboring pCEAD20 was cultured, β-CNAla synthase and nitrilase were co-expressed. When the resultant cells were added to the reaction mixture containing KCN and *O*-acetyl-L-serine, β-CNAla was produced, but the resultant β-CNAla was not almost converted into aspartic acid. For the reaction, therefore, cell-free extracts were used instead of the cells. β-CNAla was immediately produced from KCN and *O*-acetyl-L-serine, and the resultant β-CNAla was transformed into aspartic acid, but the conversion yield was low (Fig. 4b). This low conversion ratio can be ascribed to the low activity of nitrilase for β-CNAla.

Synthesis of asparagine by β-CNAla synthase and nitrile hydratase (NHase) expressed in Rhodococcus rhodochrous ATCC12674

We constructed the co-expression vector pCEAN192 for β-CNAla synthase and high molecular mass-NHase (H-NHase) (Komeda et al., 1996b) in *Rhodococcus rhodochrous* ATCC12674. When *R. rhodochrous* ATCC12674 harboring pCEAN192 was cultured, both β-CNAla synthase and NHase were expressed. When the cells were added to the reaction mixture containig KCN and *O*-acetyl-L-serine, β-CNAla was formed, but most of the resultant β-CNAla was not converted into asparagine. For the production of asparagine from KCN, therefore, cell-free extracts were used instead of the cells. β-CNAla was rapidly produced from KCN and OAS, and the resultant β-CNAla was transformed into asparagine, but at a low conversion yield (Fig. 5a).

We next used the purified β-CNAla synthase for the production of β-CNAla, and then added the purified *R. rhodochrous* J1 NHase to the reaction mixture. Although asparagine was produced at a superior yield in comparison with the above case, the amount of the produced asparagine was low (Fig. 5b). This low conversion ratio can be ascribed to the low activity of NHase for β-CNAla.

This is the first report on the development of recombinant microorganisms that directly synthesize amino acids from cyanide in a "one-pot reaction". Cyanide is a cheap starting compound for the synthesis of biologically active tracers used *in vivo* for positron emission tomography (PET), which has received increasing attention in biomedical research. Our strategy is useful for the synthesis of labeled amino acids (e.g., aspartic acid and asparagine) via β-CNAla, the cyano group carbon of which is labeled with a positron-emitting radionuclide, by using labeled cyanide (e.g., ^{11}C) as a substrate.

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Supplementary Materials

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

References

Gouet, P., Robert, X., and Courcelle, E. (2003) ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. *Nucleic Acids Res*., **31**, 3320–3323.

Hashimoto, Y., Hosaka, H., Oinuma, K., Goda, M., Higashibata, H. et al. (2005) Nitrile pathway involving acyl-CoA synthetase: Overall metabolic gene organization and purification and characterization of the enzyme. *J. Biol. Chem*., **280**, 8660–8667.

Herai, S., Hashimoto, Y., Higashibata, H., Maseda, H., Ikeda, H. et al. (2004) Hyper-inducible expression system for streptomycetes. *Proc. Natl. Acad. Sci. USA*, **101**, 14031–14035.

Higgins, D. G., Thompson, J. D., and Gibson, T. J. (1996) Using CLUSTAL for multiple sequence alignments. *Methods Enzymol*., **266**, 383–402.

Knowles, C. J. (1976) Microorganisms and cyanide. *Bacteriol. Rev*., **40**, 652–680.

Kobayashi, M., Yanaka, N., Nagasawa, T., and Yamada, H. (1990) Purification and characterization of a novel nitrilase of *Rhodococcus rhodochrous* K22 that acts on aliphatic nitriles. *J. Bacteriol*., **172**, 4807–4815.

Kobayashi, M. and Shimizu, S. (1998) Metalloenzyme nitrile hydratase: Structure, regulation, and application to biotechnology. *Nature Biotechnol*., **16**, 733–736.

Kobayashi, M., Izui, H., Nagasawa, T., and Yamada, H. (1993) Nitrilase in biosynthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile: Cloning of the Alcaligenes gene and site-directed mutagenesis. *Proc. Natl. Acad. Sci. USA*, **90**, 247–251.

Komeda, H., Hori, Y., Kobayashi, M., and Shimizu, S. (1996a) Transcriptional regulation of the *Rhodococcus rhodochrous* J1 nitA gene encoding a nitrilase. *Proc. Natl. Acad. Sci. USA*, **93**, 10572–10577.

Komeda, H., Kobayashi, M., and Shimizu, S. (1996b) Characterization of the gene cluster of a high-molecular-mass nitrile hydratase (H-NHase) induced by its reaction product in *Rhodococcus rhodochrous* J1. *Proc. Natl. Acad. Sci. USA*, **93**, 4267–4272.

Konishi, K., Ohta, T., Oinuma, K., Hashimoto, Y., Kitagawa, T. et al. (2006) Discovery of a reaction intermediate of aliphatic aldoxime dehydratase involving heme as an active center. *Proc. Natl. Acad. Sci. USA*, **103**, 564–568.

Kumano, T., Suzuki, T., Shimizu, S., and Kobayashi, M. (2016) Nitrile-synthesizing enzyme: Screening, purification and characterization. *J. Gen. Appl. Microbiol*., **62**, this issue, 167–173.

Nagasawa, T., Mathew, C. D., Mauger, J., and Yamada, H. (1988) Nitrile hydratase-catalyzed production of nicotinamide from 3-cyanopyridine in *Rhodococcus rhodochrous* J1. *Appl. Environ. Microbiol*., **54**, 1766–1769.

Nomura, J., Hashimoto, H., Ohta, T., Hashimoto, Y., Wada, K. et al. (2013) Crystal structure of aldoxime dehydratase and its catalytic mechanism involved in carbon-nitrogen triple bond synthesis. *Proc. Natl. Acad. Sci. USA*, **110**, 2810–2815.

Omura, H., Kuroda, M., Kobayashi, M., Shimizu, S., Yoshida, T. et al. (2003) Purification, characterization and gene cloning of thermostable *O*-acetyl-L-serine sulfhydrylase forming β-cyano-L-alanine. *J. Biosci. Bioeng*., **95**, 470–475.

Yamada, H. and Kobayashi, M. (1996) Nitrile hydratase and its application to industrial production of acrylamide. *Biosci. Biotechnol. Biochem*., **60**, 1391–1400.

Yip, W. K. and Yang, S. F. (1988) Cyanide metabolism in relation to ethylene production in plant tissues. *Plant Physiol*., **88**, 473–476.

Zhou, Z., Hashimoto, Y., Shiraki, K., and Kobayashi, M. (2008) Discovery of posttranslational maturation by self-subunit swapping. *Proc. Natl. Acad. Sci. USA*, **105**, 14849–14854.