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Cloning, overexpression, purification, and characterization of *O*-acetylserine sulfhydrylase-B from *Escherichia coli*

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Abstract

O-Acetylserine sulfhydrylase-B (OASS-B, EC 2.5.1.47) is one of the two isozymes produced by *Escherichia coli* that catalyze the synthesis of L-cysteine from *O*-acetyl-L-serine and sulfide. The *cysM* gene encoding OASS-B was cloned and the enzyme was overexpressed in *E. coli* using pUC19 with a *lac*UV5 promoter. The enzyme was purified to homogeneity, as evidenced by SDS–PAGE. Approximately 300 mg of purified OASS-B was obtained from 1600 mL of culture broth with a purification yield of 60% or higher. The purified OASS-B was characterized and its properties compared with OASS-A. OASS-B did not form a complex with *E. coli* serine acetyltransferase (SAT, EC 2.3.1.30) and showed a wide range of substrate specificity in nonproteinaceous amino acid synthesis.

Keywords: Cysteine synthase; *O*-Acetylserine sulfhydrylase-B; Recombinant *Escherichia coli*; Nonproteinaceous amino acid

L-Cysteine is essential for the synthesis of protein, secondary metabolites, coenzymes, and related compounds. The biosynthesis of L-cysteine in microorganisms [1] and higher plants [2] is usually catalyzed in two steps. The first step involves the enzyme serine acetyltransferase (SAT; EC 2.3.1.30) [3], which catalyzes the formation of *O*-acetyl-L-serine (OAS)[1] from L-serine and acetyl CoA, while the second step involves *O*-acetylserine sulfhydrylase-A (OASS-A; EC 2.5.1.47; *O*-acetylserine (thiol)-lyase) under aerobic conditions and OASS-B (EC 2.5.1.47) under anaerobic conditions, both of which catalyze the formation of L-cysteine from OAS and sulfide. The existence of two OASS isozymes, A and B, has been detected in a variety of bacteria [3–5]. OASS-A, the gene for which is coded for by *cysK*, has been extensively studied with respect to properties, structure, and complex formation with SAT [3,6–13]. In particular, the molecular mechanism and role of complex formation between SAT and OASS-A have been extensively studied in *Escherichia coli* [14,15] and *Haemophilus influenza* [16].

Nakamura et al. [17] purified OASS-B from wild-type *Salmonella typhimurium* cells and showed it to be a homodimeric protein with one-tightly bound pyridoxal 5′-phosphate (PLP) per subunit, similar to OASS-A. Sirko et al. [18] identified, for the first time, the *E. coli cysM* gene encoding OASS-B, which revealed that the overall sequence identity of the A- and B-isozymes is approximately 40%. Tai et al. [19,20] studied the reaction mechanism for OASS-B from *S. typhimurium*. Quite recently, the three-dimensional structure of OASS-B has been determined [21], and shown to be different from that of OASS-A. However, OASS-B has not yet been sufficiently characterized compared to OASS-A. Furthermore, OASS-B has a broad range of substrate specificity, which would be useful in the synthesis of

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[1] *Abbreviations used:* DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani; NAS, *N*-acetyl-L-serine; OAS, *O*-acetyl-L-serine; OASS-B, *O*-acetylserine sulfhydrylase-B; β-PA, β-pyrazole-1-yl-L-alanine; PCMB, *p*-chloromercuribenzoate; PLP, pyridoxal 5′-phosphate; PMSF, phenylmethylsulfonyl fluoride; SAT, serine acetyltransferase; β-TA, β-triazole-1-yl-L-alanine.

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nonproteinaceous amino acids, that is, β-substituted L-amino acids from OAS and nucleophiles [22]. Maier utilized recombinant *E. coli* cells that expressed OASS-B in the synthesis of various nonproteinaceous amino acids, for use as building blocks in the synthesis of various pharmaceuticals [22]. We report here on the cloning, overexpression, and high-yield purification of OASS-B using recombinant *E. coli* cells and its characterization with respect to properties, substrate specificity, and complex formation with SAT.

Materials and methods

Materials

Acetyl CoA trilithium salt, L-serine, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chemical Industries (Osaka, Japan). OAS hydrochloride and PLP were obtained from Sigma Chemical (St. Louis, MO). The enzymes used in the DNA manipulations were products of Takara Shuzo (Kyoto, Japan). A GenElute Bacterial Genomic DNA kit was purchased from Sigma (St. Louis, MO). A PCR kit was purchased from Toyobo (Osaka). A GENECLEAN SPIN kit was purchased from Qbiogene (Irvine, CA). All other reagents were purchased from Wako Pure Chemical Industries.

Bacteria and plasmids

General cloning procedures, sequencing, and PCR were carried out as described elsewhere [23]. *E. coli* JM109 and plasmid pUC19 were obtained from the Takara Shuzo. *E. coli* NK3 (Δ*trpE5 leu-6 thi hsdR hsdM*$^+$ *cysK cysM*), a cysteine auxotroph lacking both OASS-A and OASS-B activities, was kindly donated by Dr. N.M. Kredich, Duke University Medical Center (Durham, NC).

CysM was amplified by PCR using genomic DNA from *E. coli* JM109 as a template with a pair of primers, CYSMS (5′-TTATGAGTACATTAGAACAAACAATAGGC-3′) and CYSMAS (5′-TTAAATCCCCGCCCCCTGGCTAA AATG-3′), which were designed from BEX (Tokyo, Japan) on the basis of the nucleotide sequence of *cysM* (GenBank Accession No. M32101) using ATG in place of GTG as an initiation codon. The PCR products were phosphorylated and cloned into a pUCP vector, prepared from pUC19 by adding a *lac*UV5 promoter and a ribosome-binding site from plasmid pOHE100 [24]. The sequence of the cloned *cysM* under the control of the *lac*UV5 promoter was confirmed by DNA sequencing (ABI PRISM 310 Genetic Analyzer, Milpitas, CA) and designated pUCcysM. *E. coli* NK3 was transformed with the resulting plasmid for the expression of OASS-B.

Expression and purification of OASS-B

Recombinant *E. coli* NK3 cells containing pUCcysM were grown in 1600 mL of Luria–Bertani (LB) medium containing 0.002% thiamine and 100 μg/mL ampicillin with shaking at 150 strokes/min and at 37 °C. The incubation was allowed to proceed until the optical density of the culture broth at 600 nm reached 0.6, at which time, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The culture was allowed to continue for a further 12 h and the cells were isolated by centrifugation at 3000*g* for 15 min at 4 °C followed by washing with 10 mM potassium phosphate buffer, pH 7.5. The washed cells were then resuspended in 80 mL of buffer A (50 mM potassium phosphate buffer, pH 7.5, containing 5 mM of 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM PLP) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated. The suspension was then centrifuged at 18,000*g* for 30 min at 4 °C to give the supernatant as a crude extract. The crude extract was subjected to the subsequent purification steps at 4 °C. Streptomycin sulfate, at a level of 2%, was added to the crude extract and after standing for 1 h with gentle stirring, the suspension was centrifuged at 18,000*g* for 20 min to precipitate the nucleic acids. Ammonium sulfate was then added to the supernatant to 70% saturation followed by standing at 4 °C for 12 h to allow proteins to precipitate. The precipitate was then recovered by centrifugation at 18,000*g* for 20 min and the pellet dissolved in 10 mL of buffer A followed by dialysis against a large amount of the same buffer. The dialyzate was applied to a DEAE Sephadex A-50 column (1.6 × 16 cm) that had been equilibrated with buffer A and the column was eluted by a linear increase in NaCl concentration in buffer A from 0 to 0.5 M at a flow rate of 0.22 mL/min. Fractions showing OASS activity were collected and dialyzed against buffer A containing 1.7 M ammonium sulfate adjusted to pH 7.5 with 5 M NaOH (abbreviated as buffer B). The dialyzate was applied to a phenyl-Sepharose CL-4B column (1.6 × 16 cm) that had been equilibrated with buffer B, and was eluted first by linearly decreasing the ammonium sulfate concentration in buffer A from 1.7 to 0 M and then by isocratic elution with buffer A at a flow rate of 0.22 mL/min. The collected active fractions were dialyzed against buffer B and then placed on an octyl-Sepharose CL-4B column (1.6 × 16 cm) that had been equilibrated with buffer B. The elution procedures were the same as those used in the Phenyl-Sepharose CL-4B chromatography as described above. Fractions showing OASS activity were pooled, dialyzed against buffer A, and finally stored at −80 °C until used. Protein concentrations were determined by the method of Lowry et al. [25]. The eluted protein was subjected to SDS–PAGE (12.5% gel) and the gel stained with Coomassie brilliant blue R-250 (Sigma–Aldrich, St. Louis, MO).

N-Terminal amino acid sequences were determined using a protein sequencer (Applied Biosystems, Model 491, Foster, CA).

Preparation of the SAT and OASS-A

SAT and OASS-A were purified by the same method as described previously using recombinant *E. coli* cells