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# Extracellular Production and Characterization of Two *Streptomyces* L-Asparaginases

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**Abstract** L-Asparaginase (ASNase) has proved its use in medical and food industries. Sequence-based screening showed the thermophilic *Streptomyces* strain *Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270 (14270 ASNase) to positive against predicted ASNase primary sequences. The 14270 ASNase gene and four L-asparaginase genes from *Streptomyces coelicolor*, *Streptomyces avermitilis*, and *Streptomyces griseus* (SGR ASNase) were expressed in *Streptomyces lividans* using a hyperexpression vector: pTONA5a. Among those genes, only 14270 ASNase and SGR ASNase were successful for overexpression and detected in culture supernatants without an artificial signal peptide. Comparison of the two *Streptomyces* enzymes described above demonstrated that 14270 ASNase was superior to SGR ASNase in terms of optimum temperature, thermal stability, and pH stability.

**Keywords** Asparaginase · *Streptomyces* · Extracellular expression · Secretion · Asparagine

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## Introduction

L-Asparaginases (ASNases, EC 3.5.1.1) catalyze the conversion of L-Asn to L-Asp and ammonium. Since Broome first demonstrated that ASNase inhibits growth of various tumors that require L-asparagine for protein synthesis [1], various bacterial ASNases have been characterized [2–7]. It is also an important problem that potentially toxic acrylamide is derived largely from heat-induced reactions between L-asparagine and glucose in plant-derived foods [8]. In fact, ASNases are noted as a means to reduce L-asparagine concentration, a precursor of acrylamide [9]. As described above, ASNases have received much attention for use in medical and food industries.

Two related families of ASNase are designated type I and type II according to the terminology in *Escherichia coli*. Of them, ASN I is a low-affinity enzyme found in the cytoplasm, whereas ASNase II, which is used for cancer treatment, is a high-affinity periplasmic enzyme. Therefore, their purification from cell lysate is a complicated and expensive matter. For that reason, Khushoo et al. reported that a recombinant *E. coli* ASNase was secreted with pelB signal peptide [10, 11].

Recently, we developed a hyperexpression vector for streptomycetes: pTONA5a. Using pTONA5a and *Streptomyces lividans*, we achieved overexpression and extracellular production of two *Streptomyces* aminopeptidases, which possessed no signal peptide for secretion [12]. We plan overexpression and extracellular production of various useful *Streptomyces* enzymes in *S. lividans* using pTONA5a.

In this study, (1) we performed sequence-based screening of *Streptomyces* ASNase. (2) We cloned and expressed an ASNase gene from *Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270 (14270 ASNase) and those from three other *Streptomyces* species whose genomes had already been sequenced (one from *Streptomyces coelicolor* [http://www.sanger.ac.uk/Projects/S_coelicolor/], one from *Streptomyces avermitilis* [http://avermitilis.ls.kitasato-u.ac.jp/], and one from *Streptomyces griseus* [http://streptomyces.nih.go.jp/]). (3) We attempted extracellular production of five ASNases from *S. thermoluteus* subsp. *fuscus* NBRC 14270, *S. coelicolor*, *S. avermitilis*, and *S. griseus* (SGR ASNase). Two (14270 ASNase and SGR ASNase) were in vitro truncated forms: These enzymes and ASNase II from *E. coli* (EC ASNase) were compared for biochemical characterization.

## Materials and Methods

### Sequence-Based Screening

Genomic DNA of thermophilic *Streptomyces* NBRC-type culture strains (*S. thermoluteus* NBRC 14269, *S. thermoluteus* subsp. *fuscus* NBRC 14270, *Streptomyces thermocyaneoviolaceus* NBRC 14271, and *Streptomyces thermocyaneomaculatus* NBRC 14272), which had been cultured at 45 °C, were prepared using a method described in an earlier study [13]; they were used as templates for screening. Two degenerated primers (a sense and an anti-sense primers used were 5′-TGCWSSGGSAAGCACGC -3′ and 5′-CTCGAASCCGTCCTTSCC -3′) were designed from alignment of primary sequences of predicted ASNases from *S. avermitilis* (gene ID: SAV1316), *Saccharopolyspora erythraea* (UniProtKB/TrEMBL accession number: A4FK40), *Arthrobacter aurescens* (UniProtKB/TrEMBL accession number: A1RB61), and *Corynebacterium glutamicum* (UniProtKB/TrEMBL accession number: A4QHW1). Then, PCR was performed using

1 μM primers and LA Taq DNA polymerase with GC buffer I (TaKaRa Holdings Inc.). The PCR program was as follows: 1 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 20 s at 72 °C, and final extension of 5 min at 72 °C.

## Cloning of 14270 ASNase Gene

The PCR product (360 nucleotides) was cloned into pGEM-T Easy (Promega Corp.) and sequenced. The genomic DNA of *S. thermoluteus* subsp. *fuscus* NBRC 14270 was digested with *Pvu*I and self-ligated. The ligation product was amplified using primer sets designed from the sequence obtained through PCR-based screening. The inverse PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen Corp.) and sequenced. The entire sequence of 14270 ASNase was assigned accession number AB469678 in the DDBJ database. Using sequence-based screening, we identified a 1,014-bp open reading frame from *S. thermoluteus* subsp. *fuscus* NBRC 14270, which encoded a predicted ASNase of 338 amino acids with 87.3% identities of the encoded SAV1316. The deduced 14270 ASNase shows 50.3%, 49.5%, and 50.2% identities, respectively, with those encoded SCO4085, SAV4025, and SGR3975 (SGR ASNase) in the primary structure.

## Construction of Expression Plasmids for *Streptomyces* ASNase

For construction of expression plasmids, 14270 ASNase was amplified using PCR with primer sets of a sense primer containing the *Nde*I site (CATATG; the start codon is underlined) and an anti-sense primer containing the *Hin*dIII site downstream of the stop codon. The resultant fragment was cloned; the correct cloning was confirmed by sequencing. The DNA fragment encoding the 14270 ASNase was digested using *Nde*I and *Hin*dIII, and ligated into the *Nde*I–*Hin*dIII gap of pTONA5a. Expression vectors for *S. coelicolor* (gene ID: SCO4085), *S. avermitilis* (gene ID: SAV1316 and SAV4025), and *S. griseus* (gene ID: SGR3975), which were annotated as putative ASNases, were constructed as similar to that of 14270 ASNase. We also constructed an expression vector of N-terminal truncated 14270 ASNase (14270 ▲N), from which 10 residues from N-terminal amino acid of 14270 ASNase were deleted (Fig. 1).

## Conjugation and Cultivation

The expression vectors were transformed in *E. coli* S17-1. The transformants were used to conjugate *S. lividans* 1326. The procedure resembles a method described in a previous report [12]. The resultant *S. lividans* transformants were inoculated and grown in 5 ml of culture medium containing 0.8% $K_2HPO_4$, 2.0% glucose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% polypeptone, and 0.5% yeast extract in a test tube at 30 °C for 5 days at 180 strokes per minute.

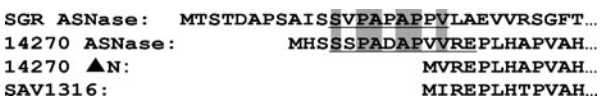


**Fig. 1** Comparison of N-terminal sequences of SGR ASNase, 14270 ASNase, SAV1316, and the deduced N-terminal sequence of 14270 ▲N. Shaded amino acid residues are conserved in N-terminal sequences of both SGR ASNase and 14270 ASNase

## Assay of ASNase Activity

Enzyme assay was performed at 37 °C using a spectrophotometer (Ultrospec 3300 pro; GE Healthcare Bio-Science) equipped with a thermal cell holder (10 mm path length). The ASNase activities were measured by determining the rate of ammonia formation using glutamate dehydrogenase [14]. The final assay volume of 800 μl contained 100 mM Tris–HCl buffer (pH 8.0), 5 mM L-Asn, 0.16 mM α-ketoglutaric acid, 0.25 mM NADH, four units of glutamate dehydrogenase, and a sample containing ASNase activity. One unit of ASNase activity is defined as the amount of enzyme that liberates 1 μmol of ammonia from L-Asn per minute at 37 °C. We used the EC ASNase for comparison with two *Streptomyces* ASNases. The enzyme was purchased from Sigma Co. Ltd.

## Purification of ASNases

Culture filtrate of 14270 ASNase or SGR ASNase was obtained using an ultrafiltration apparatus (0.45 μm pore size; Millipore Corp.). The filtrate was dialyzed against 25 mM Tris–HCl buffer (pH 8.0). After centrifugation, the sample was loaded onto a Vivapure-Q spin column (Millipore Corp.) that had been equilibrated using the same buffer used for dialysis. The bound protein was eluted with 0.25 M NaCl in 25 mM Tris–HCl buffer (pH 8.0). The eluate was loaded onto a gel filtration column (HiLoad 16/10 superdex 200 prep grade; GE Healthcare Bio-Science). The active fraction was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) and loaded onto a Mono Q HR5/5 column that had been pre-equilibrated with dialysis buffer. The ASNase was eluted using a linear gradient of NaCl from 0 to 0.25 M. The active fraction was dialyzed against 10 mM Tris-maleate buffer (pH 6.5). The resultant enzyme solution was used for characterization.

## Determination of N-terminal Amino Acid Sequences

The sample was electroblotted onto a polyvinylidene difluoride membrane after 12.5% SDS-PAGE under denaturing conditions. Then, it was loaded onto a protein sequencer (Model 491; Applied Biosystems) to identify its N-terminal amino acid sequence. The N-terminal sequences of secreted 14270 ASNase and SGR ASNase were, respectively, SSPADAPVVRE and SVPAPAPPV.

## Gel Filtration Analysis

Gel filtration was performed using a column (Superdex 200 10/300; GE Healthcare Bio-Science) that had been pre-equilibrated with 80 mM Tris–HCl (pH 7.0) containing 0.2 M NaCl. Molecular-weight marker proteins (glutamate dehydrogenase [290 kDa], lactate dehydrogenase [142 kDa], enolase [67 kDa], myokinase [32 kDa], and cytochrome C [12.4 kDa]) were purchased from Oriental Yeast Co. Ltd.

## Stability of ASNases

The thermal stability was investigated as follows: the purified enzyme solution (0.1 mg/ml in 10 mM Tris–HCl buffer (pH 8.0)) was incubated at different temperatures for 30 min. Subsequently, the samples were assayed for residual activity using the coupled enzyme assay method described above.

The residual activity was assessed relative to that when stored on ice. The $T_m$ value was determined from a plot of relative inactivation (%) versus temperature (°C). The $T_m$ value is the temperature at which 50% of the activity when stored on ice is lost after heat treatment.

The pH stability was investigated as follows: The purified enzyme solution (0.1 mg/ml in 10 mM Tris–HCl buffer (pH 8.0)) was incubated at 37 °C for 24 h. Subsequently, the samples were assayed for residual activity using the coupled enzyme assay method described above. The residual activity was assessed relative to that when stored at 4 °C and pH 8.0 for 24 h.

## Results and Discussion

### Extracellular Production of ASNases

In the six culture supernatants of transformants—14270 ASNase, 14270 ▲N, SAV1316, SCO4085, SAV4025, and SGR ASNase—the ASNase activities were detected only in the cases of 14270 ASNase and SGR ASNase. For 14270ASNase, the activity per culture volume of supernatant (6.24±0.29 U/ml) was 4.5-fold higher than that of cell lysate (1.39± 0.23 U/ml). In contrast, no activity exists in either the culture supernatant or the cell lysate in the case of 14270 ▲N (data not shown). Other ASNases (SAV1316, SCO4085, and SAV4025) were not detected; their activities in either the culture supernatants or the cell lysates, and their proteins were not also detected by SDS-PAGE. 14270 ▲N and SAV1316 were not obtained as the active form, although 14270ASNase showed a high similarity (87.3%) in the primary structure to that of SAV1316. Based on the results, the N-terminal region of ASNase was important for expression by using pTONA5a and *S. lividans* 1326. Many significant questions remain unanswered with regard to the relation between the N-terminal region and the secretion of the recombinants. Further research is planned to clarify these points. For SGR ASNase, the activity per culture volume of supernatant (17.51± 1.27 U/ml) was 3.2-fold higher than that of cell lysate (5.51±1.31 U/ml).

### Purification of 14270 ASNase and SGR ASNase

Purification of 14270 ASNase and SGR ASNase from culture filtrates is presented in Table 1. The SDS-PAGE pattern of the purified enzymes is depicted in Fig. 2. From N-terminal amino acid sequence analysis, both purified enzymes, were found to have had several amino acid residues truncated from the start Met. It is noteworthy that these enzymes had been digested between continuous Ser residues (Fig. 1). Using pTONA5a and *S. lividans*, we achieved overexpression and extracellular production of 14270 ASNase and SGR ASNase, although these enzymes possessed no signal peptide for secretion. Similarly, *Streptomyces* proline aminopeptidase and aminopeptidase P, which lacked signal peptides, were secreted using the same vector and host [12]. However, we have no understanding of their secretion mechanism. The SxPAxAPxV are conserved in the N-terminal amino acid residues of 14270 ASNase and SGR ASNase (Fig. 1). On the other hand, SAV1316 shows high homology to 14270 ASNase. Moreover, SCO4085 (77.2% identities) and SAV4025 (77.7% identities) resemble SGR ASNase in their primary structures. Nevertheless, these three enzymes cannot be produced in extracellular fractions. Regarding results of this study, some expression, folding, and secretion of proteins might depend on their N-terminal sequence using pTONA5a and *S. lividans*. However, much more research is necessary to clarify this phenomenon.

**Table 1** Purification from culture filtrates

| Fraction | ASNase activity (U/ml) | Protein concentration (mg/ml) | Specific activity (U/mg) | Total volume (ml) | Total unit (U) | Yield (%) | Purity (-fold) |
|---|---|---|---|---|---|---|---|
| 14270 ASNase | | | | | | | |
| Culture filtrate dialysis | 4.9 | 0.88 | 5.6 | 15 | 74.1 | 100.0 | 1.0 |
| Vivapure-Q eluate | 4.4 | 0.46 | 9.6 | 10 | 44.1 | 59.5 | 1.7 |
| Gel filtration eluate | 6.6 | 0.17 | 38.9 | 5 | 33.0 | 44.5 | 6.9 |
| Mono Q eluate | 6.8 | 0.10 | 68.3 | 2 | 13.7 | 18.5 | 12.2 |
| SGR ASNase | | | | | | | |
| Culture filtrate dialysis | 7.3 | 1.61 | 4.6 | 15 | 110.1 | 100.0 | 1.0 |
| Vivapure-Q eluate | 4.2 | 0.50 | 8.5 | 10 | 77.0 | 69.9 | 1.8 |
| Gel filtration eluate | 5.1 | 0.15 | 34.1 | 5 | 46.5 | 42.2 | 7.4 |
| Mono Q eluate | 5.9 | 0.12 | 48.9 | 2 | 11.7 | 10.6 | 10.6 |

The enzyme activity was determined using 5 mM Asn and 100 mM Tris–HCl buffer (pH 8.0) at 37 °C

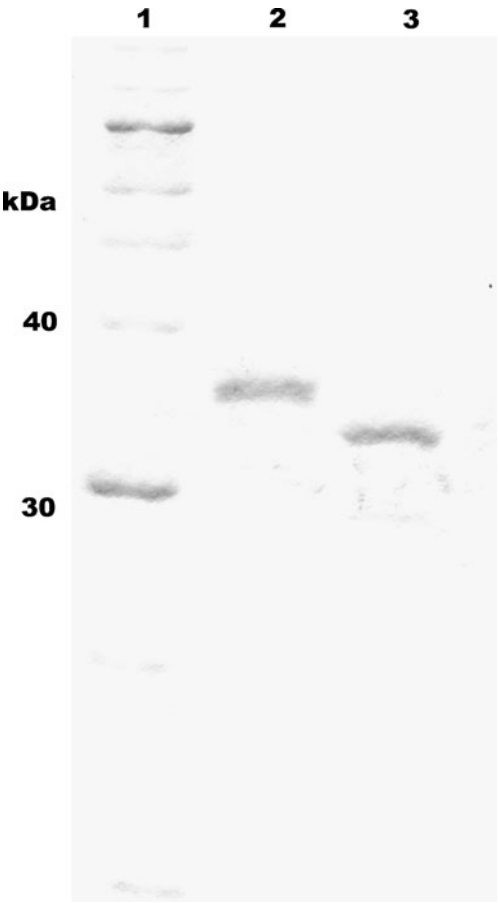


**Fig. 2** SDS-PAGE results of purified 14270 ASNase and SGR ASNase. Sample proteins (1.5 μg each) were loaded on 12.5% acrylamide gel. *Lane 1* shows molecular-weight protein markers. *Lanes 2* and *3*, respectively, portray purified 14270 ASNase and SGR ASNase

**Table 2** Biochemical properties of 14270 ASNase, SGR ASNase, and ECO ASNase

| Property | 14270 ASNase | SGR ASNase | EC ASNase |
|---|---|---|---|
| Molecular mass of protomer (kDa) | 35 | 33 | 38 |
| Composition | Dimer | Dimer | Tetramer |
| Optimum pH[a] | 8.0–9.0 | 8.0–9.0 | 8.0–9.0 |
| Optimum temperature (°C)[a] | 63.6 | 48.8 | 68.2 |
| $T_m$ (°C)[a] | 54.0 | 42.4 | 54.9 |
| Specific activity for L-Asn (U/mg) | 68.09 | 48.89 | 41.41 |
| $K_m$ for L-Asn (mM) | 1.83±0.22 | 2.33±0.47 | 0.34±0.06 |
| $V_{max}$ for L-Asn (μmol $min^{-1}$ $mg^{-1}$) | 92.73±4.30 | 71.38±7.10 | 44.59±2.27 |
| Specific activity for L-Gln (U/mg) | 0.65 | 0.09 | 4.06 |

[a] The activities were determined using 5 mM Asn as a substrate

## Biochemical Characterization of 14270 ASNase, SGR ASNase, and EC ASNase

Basic properties of the enzymes such as optimum temperature, optimum pH, composition, $T_m$ value, and specific activities toward L-Asn, L-Gln, and kinetic parameters for L-Asn are presented in Table 2. Figure 3 shows pH stabilities of the enzymes. In fact, 14270 ASNase is superior to SGR ASNase in terms of the properties listed above, especially optimum temperature and stabilities against heat and pH (Table 2). However, 14270 ASNase is inferior to EC ASNase in pH stability. Furthermore, comparison of the kinetic parameters toward L-Asn shows that 14270 ASNase is better than SGR ASNase. In fact, 14270 ASNase was superior to EC ASNase in specific activity and the $V_{max}$ value toward L-Asn. However, 14270 ASNase shows a 5.4-fold higher Km value toward L-Asn than that of EC ASNase (Table 2). Although known bacterial ASNases consist of homotetramers (dimers of dimers), present *Streptomyces* ASNases are homodimers, as revealed by gel filtration analysis (Table 2).

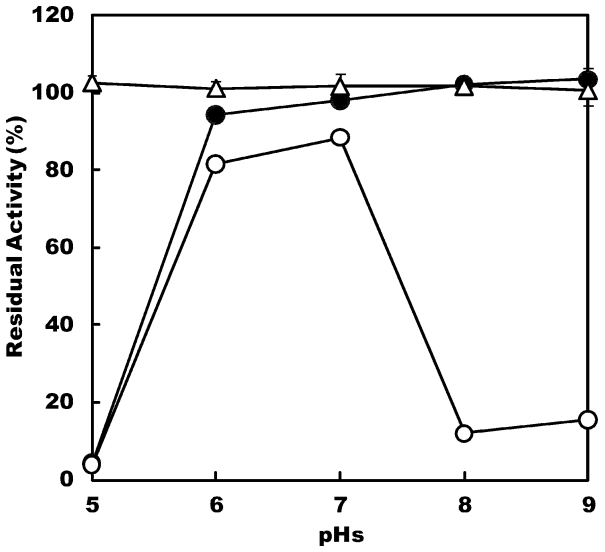


**Fig. 3** The pH stability of purified 14270 ASNase, SGR ASNase, and EC ASNase. *Closed* and *open circles*, respectively, represent 14270 ASNase and SGR ASNase. The *open triangle* represents EC ASNase

Both EC ASNase and *Erwinia carotovora* ASNase (EW ASNase) have been used in leukemia treatment. Thus, both enzymes were important from the medical and industrial points of view; however, they have some shortcomings. In short, their L-glutaminase side activity limits their use and causes harmful effects such as L-Gln depletion [15]. Actually, EW ASNase shows a low glutaminase activity (about 1.5% of the ASNase activity); EC ASNase has glutaminase activity that is approximately 10% of the ASNase activity (Table 2) [16, 17]. Both 14270 ASNase and SGR ASNase exhibit much lower (about 100-fold) glutaminase activities than their ASNase activities (Table 2). Furthermore, a limiting factor of ASNase treatment is the development of hypersensitivity ranging from mild allergic reactions to anaphylactic shock [18]. Because 14270 ASNase shows very low similarity to the EC ASNase (12.3% identities, UniProtKB/TrEMBL; accession number, B1IT50) and the EW ASNase (31.8% identities, UniProtKB/TrEMBL; accession number, Q6Q4F4) in the primary sequence, 14270 ASNase is expected to be immunologically distinct from EC ASNase and EW ASNase. Actually, 14270 ASNase is better than SGR ASNase in terms of thermal stability, pH stability, and specific activity toward L-Asn (Table 2). However, as a result of kinetic study, 14270 ASNase showed low affinity toward L-Asn in comparison of EC ASNase (Table 2). Based on these results, 14270 ASNase is not suitable for an antileukemic agent, unfortunately. Further work is needed to improve its affinity toward L-Asn by protein engineering.

Recently, the reduction of dietary content and toxicity of acrylamide has become an important problem that must be solved [8]. The use of ASNase is one means to prevent acrylamide formation in heated foods [9]. Because 14270 ASNase has highly hydrolytic activity and specific activity toward L-Asn (Table 2), it is expected to be useful as a tool for selective removal of L-Asn, which is an acrylamide precursor. Additionally, it is convenient for industrial production because of success in extracellular production of 14270 ASNase. The validity of additional effects of ASNases in processing of heated foods remains to be tested.

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