Gene cloning and characterization of novel thermostable thiocyanate hydrolase of *Afipia* sp. strain THI201

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Thiocyanate is one of the major constituents of wastewater from factories of coal gasification. Removal of thiocyanate in biological treatment, however, is not stable and the untreated thiocyanate in effluents often contributes to an increase in COD. *Afipia* sp. strain THI201 is a novel thiocyanate (SCN⁻)-degrading bacterium isolated from lake water enriched with potassium thiocyanate (KSCN). This bacterium carries the enzyme thiocyanate hydrolase (SCNase) that hydrolyzes thiocyanate to carbonyl sulfide and ammonia. The ability of strain THI201 to degrade thiocyanate was decreased quickly, however not completely, by preparing crude cell extract. Due to this instability, the study of enzyme became difficult. Therefore, the aim of this research was to identify the enzyme responsible for thiocyanate degradation in this bacterium. This report describes the cloning and expression of the SCNase gene (*scn*) in *Escherichia coli*, and some characterization of the SCNase.

The *scn* was cloned into pUC118 vector by sub-genomic library preparation, and one positive clone designated as C14 was obtained by colony hybridization technique. Nucleotide sequencing analysis of the plasmid pUC118C14 isolated from clone C14 revealed that the SCNase protein was within an ORF of 1470 bp. The *scn* gene encodes a protein of 457 amino acids of molecular mass 50,267 Da. Presence of a twin-arginine (Tat) signal sequence of 32 amino acids was found upstream of SCNase. The deduced amino acid sequence of SCNase showed 83% identity to that of a putative uncharacterized protein of *Thiobacillus denitrificans* ATCC 25259, but no significant identity to those of three subunits of SCNase from *Thiobacillus thioparus* strain THI115.
ORF of SCNase was sub-cloned into expression vector pGEX-6P-1. An expression plasmid, pGEX-SCNase, which encoded a GST-fused SCNase (GST-SCNase) of strain THI201 with the recognition sequence for the protease factor Xa (IEGR), was constructed and expressed in *E. coli*. However, during purification, the fusion protein was found mostly in inclusion bodies, and could not be purified. To improve the recovery of the fusion protein in the soluble fraction, plasmids pG-KJE8, pKJE7, and pGRO7 were separately co-introduced with pGEX-SCNase into *E. coli* strain BL21(DE3). In these combinations, only pGRO7 with pGEX-SCNase produced the fusion protein abundantly in the soluble fraction and was used to purify the recombinant SCNase. GST-SCNase and the chaperone proteins GroEL and GroES were isolated by GS4B bead-based affinity column chromatography. Treatment with Mg-ATP and casein sodium eliminated almost all the chaperone proteins and digestion with factor Xa resulted in the separation of GST-SCNase into GST and SCNase. Thus, 25-55 µg of purified recombinant protein was obtained from 100 ml of *E. coli* culture. Enzyme activity was measured by detection of ammonia by Nessler’s reagent. The specific activities of recombinant enzyme were 4-15 µmol min⁻¹ mg⁻¹ of protein.

Enzymatic characterization was performed with the purest fraction of the recombinant SCNase. SCNase activity was observed in the pH range 5–8, and the highest activity of 5.4 µmol min⁻¹ mg⁻¹ of protein was found in the pH range 6.0–6.5. The enzyme showed activity at a broad range of temperatures from 10°C–100°C with the highest activity of 4 µmol min⁻¹ mg⁻¹ of protein always in the range 30°C–70°C. Analysis of the substrate concentration dependence of enzyme activity showed maximum activity at 20–40 mM thiocyanate. The reaction rate of ammonia formation was proportional to the amounts of SCNase up to 0.8 µg of protein per assay, and was linear with time up to 15 min. The experiment of thermostability showed that the enzyme activity of recombinant SCNase was retained after heat treatment at temperatures up to 70°C but decreased by heating at 80°C for 5 min. Similar thermostolerance was observed for native SCNase. The thiocyanate-hydrolyzing activity in both native and recombinant enzymes was decreased by freeze-thawing, though 25-100% of the activity of recombinant protein could be retrieved by treating the enzyme at 60°C for 15 min. Because recombinant SCNase showed the unexpected property of heat tolerance and heat reactivation after storage in a cold environment, the effect of heat treatment on crude cell extract of strain THI201 was also examined. Heating the crude cell extract at 60°C, 70°C, or 80°C for 5 min resulted in SCNase activity of 3.2, 3.5, or 4.5 µmol min⁻¹ mg⁻¹ of protein, respectively. However, intact cells of strain THI201 grew at temperatures of up to 35°C, but not at 40°C or higher. This result indicated that though the *Afipia* sp. strain THI201 is a mesophilic bacterium, it possess a thermostable enzyme.

The previously isolated SCNases from *Thiobacillus thioparus* THI115 and *Thiohalophilus thiocyanoxidans* contain protein of three subunits and was heat labile. Thus, characterization of THI201 SCNase revealed properties different from known SCNases regarding subunit structure and thermostability: SCNase of strain THI201 was composed of a single protein and thermostable. This is the first example of a thiocyanate-degrading enzyme consisting both thermostable and thermo-enhanced properties.
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CHAPTER 1

INTRODUCTION
Chapter 1

INTRODUCTION

1-1 Thiocyanate and thiocyanate degrading bacteria

Thiocyanate (SCN⁻) is an electronegative polyatomic ion widely distributed in nature. In the natural environment, glucosinolates (β-thioglucoside-N-hydroxysulfates) are found predominantly in plants of the order Brassicales which includes the common vegetables such as cauliflower, brussels sprouts, cabbage and broccoli (Fahey et al., 2001). The function of glucosinolates in plants are anti-herbivore defense, and upon plant tissue damage, isothiocyanate and thiocyanate are produced by the hydrolysis of glucosinolates (Burow et al., 2007). It is also present in mammalian body fluids such as saliva and blood, and degraded by the lactoperoxidase, myeloperoxidase, methemoglobin and oxyhemoglobin (Vesey & Wilson, 1978; Wood, 1975). In addition, thiocyanate acts as an anti-thyroid substance, and incorporation of iodine that is catalyzed by thyroid peroxidase is inhibited by thiocyanate (Coval and Taurog, 1967).

However, not only the plants and animals as common source, but being an anthropogenic pollutant thiocyanate is spreading from the industrial effluents rapidly. Thiocyanate is used in various processes in the chemical industry, and is produced as a waste product in factories such as coal gasification, photofinishing, and pesticide production. The concentration of SCN⁻ arising from industrial processes is normally in the range of 5-110 mg/l (Mudder & Whitlock, 1984). Earlier scientific studies indicate that in general, SCN⁻ is approximately 7 to 10 times less toxic than free cyanide species. Industry producing cyanide in its waste are steel manufacturing, metal mining, and electroplating units, where the cyanide reacts readily with sulphur to produce less toxic SCN⁻. Numerous technologies are currently employed to detoxify SCN⁻ bearing effluents; and the most widely being used is direct alkaline chlorination or addition of hypochlorite. However, this method produces large aggregates of chemical sludge, which does not have any further utilization and is environmentally hazardous to handle (Lanza and Bertazzoli, 2002). Bioremediation using metabolically active microorganisms is one such effective alternative for the detoxification of toxic chemical wastes. Use of metabolically passive (dead or inactive) microorganisms for
the removal and recovery of metal-cyanides and SCN have been reported (Gaddi and Patil, 2011; Patil, 2012; Patil and Paknikar, 1999; Thakur and Patil, 2009).

Thiocyanate is toxic to many microorganisms at relatively low concentrations (1–2 mM) and in natural environment it is degraded by various indigenous microorganisms including a number of chemolithoautotrophic and chemoorganotrophic bacteria. Among the obligate chemolithoautotrophs, *Thiobacillus* (*Tb.*) *thioparus* (Happold *et al.*, 1958; Katayama & Kuraishi, 1978), *Tb. denitrificans* (Wood, 1975), and *Thiohalophilus* (*Th.*) *thiocyanoxidans* (Bezsudnova *et al.*, 2007) use thiocyanate as an energy source for growth. Some chemoorganotrophic bacteria such as a *Pseudomonas stutzeri*-like bacterium and *Arthrobacter sp.* were reported to degrade thiocyanate though do not use it as an energy source (Betts *et al.*, 1979; Stafford & Calley, 1969).

Two alternative pathways for the initial cleavage of thiocyanate have been identified in heterotrophs (Kelly *et al.* 1993; Mason *et al.* 1994; Stratford *et al.* 1994; Mason 1995):

1. The carbonyl sulfide (COS) pathway, produced carbonyl sulfide and ammonia (NH₃) as the first intermediates, the latter subsequently being hydrolyzed to sulfide, which is oxidized to sulphate:

   \[
   \text{KSCN} + 2 \text{H}_2\text{O} \rightarrow \text{COS} + \text{NH}_3 + \text{KOH} \\
   \text{COS} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{S} + \text{CO}_2 \\
   \text{H}_2\text{S} + 2 \text{O}_2 \rightarrow \text{H}_2\text{SO}_4
   \]

2. The cyanate (CNO) pathway, producing cyanate and hydrogen sulfide as initial products of thiocyanate hydrolysis, with the cyanate being subsequently hydrolyzed to release ammonia:

   \[
   \text{KSCN} + \text{H}_2\text{O} \rightarrow \text{KOCN} + \text{H}_2\text{S} \\
   \text{KOCN} + 2 \text{H}_2\text{O} \rightarrow \text{NH}_3 + \text{CO}_2 + \text{KOH}
   \]

Both the “COS pathway” and “CNO pathway” were found in *Tb. thioparus* and the degradation of thiocyanate was caused by the enzyme thiocyanate hydrolase (EC 3.5.5.8, SCNase) and cyanase (EC 4.2.1.104), respectively.
The SCNase was found as inducible enzyme in *Tb. thioparus*, which metabolized thiocyanate to sulfate as an energy source; and the enzyme hydrolyzed 1 mol of thiocyanate to form 1 mol of COS and 1 mol of NH$_3$ (Katayama *et al.*, 1992). The use of cyanate as a nitrogen source was observed in *Escherichia coli* and in a *Pseudomonas* species, both of which cleave cyanate using cyanase enzymes (Anderson & Little, 1986; Kunz & Nagappan, 1989; Anderson *et al.*, 1990).

Thiocyanate degradation and COS emission was measured in environmental bacteria collected from different locations, such as, lake, river, and brackish water, activated sludge, and soil of Japan. Evidences of thiocyanate degradation and COS emission was found in almost all samples examined, indicating wide distribution of thiocyanate in various natural and man-made environments (Yamasaki *et al.*, 2002). However, research on the enzymatic transformation of thiocyanate by bacteria has been limited; one is *Tb. thioparus* THI115 utilizing SCNase and the other is *Th. thiocyanoxidans* utilizing both SCNase and cyanase for the degradation of thiocyanate (Katayama *et al.*, 1992; Bezsudnova *et al.*, 2007). Thiocyanate degrading bacterium *Thiohalobacter thiocyanaticus* was obtained from hypersaline chloride–sulfate lakes in Russia using aerobic enrichment culture at 1 M NaCl with thiocyanate as substrate. During growth on thiocyanate, cyanate was identified as an intermediate (Sorokin *et al.*, 2010).

### 1-2 Previously isolated thiocyanate hydrolase

The first thiocyanate-transforming enzyme, thiocyanate hydrolase, was identified and isolated from *Tb. thioparus* THI115 (Katayama *et al.*, 1992), which degrades thiocyanate up to a concentration of 60 mM under aerobic conditions (Katayama and Kuraishi, 1978). The SCNase catalyzes the hydration of thiocyanate to carbonyl sulfide and ammonia (SCN$^- + 2$H$_2$O $\rightarrow$ COS + NH$_3$ + OH$^-$), and is important as a key enzyme in the COS pathway. SCNase in THI115 is a tetrameric structure of $\alpha$, $\beta$, and $\gamma$ subunits, ($\alpha\beta\gamma$)$_4$. DNA sequences of the genes encoding these subunits reveal a close similarity to nitrile hydratase (NHase), which catalyzes the hydration of various nitriles to the corresponding amides (Nishiyama *et al.*, 1991): the fused $\alpha$ and $\beta$ subunits of the SCNase correspond to the $\beta$ subunit of NHase, and the $\gamma$ subunit of the SCNase corresponds to the $\alpha$ subunit of NHase (Katayama *et al.*, 1998). SCNase of *Tb. Thioparus* THI115 contains a noncorrin cobalt active center and has two
post-translationally modified cysteine ligands, namely cysteine sulfenic acid (or cysteine sulfinate) and cysteine sulfininate; the enzyme requires coexpression of an activator protein, P15K, for its functional expression (Arakawa et al., 2007; Kataoka et al., 2006; Katayama et al., 2006). The presence of a similar enzyme in the halophilic chemolithoautotroph, Th. thiocyanoxidans has also been reported. It is capable of growth with thiocyanate as an electron donor at salinity up to 4 M NaCl and hydrolyzes thiocyanate to sulfide and ammonia under anaerobic conditions (Bezsudnova et al., 2007). There are some differences between SCNase of Th. thiocyanoxidans and SCNase of THI115. The molecular masses of purified SCNases are 126 and 140 kDa, consisting three subunits with masses of 19, 23, 32 kDa, and 17, 19, 29 kDa from Th. thiocyanoxidans and strain THI115 respectively. The enzyme of Th. thiocyanoxidans was a Co, Fe containing protein, whereas that of THI115 contained five-coordinate cobalt center. Unlike SCNase of THI115, Th. thiocyanoxidans does not require activator protein for its functional expression (Bezsudnova et al., 2007; Katayama et al., 1992).

To know the diversity of SCNase in bacteria, SCN-degrading enzyme was obtained from bacterium that can use both inorganic and organic substances for the energy source. Paracoccus thiocyanata strain THI011 is a facultatively chemolithoautotrophic sulfur-oxidizing bacterium (Katayama et al., 1995), however identification of the responsible enzyme protein was unsuccessful because of loss of activity during preparation of the cell-free extract. Strain THI201 is another facultative chemolithotrophic bacterium isolated from the lake water of Sagami. Differing from strain THI011, SCN-degrading activity of this bacterium was detectable partially after the cell disruption. Therefore, I have started to identify and isolate responsive enzyme from this bacterium.

1-3 Aim of this study and composition of this dissertation

Thiocyanate is one of the major constituents of waste water from factories of coal gasification. Removal of thiocyanate in biological treatment, however, is not stable and the untreated thiocyanate in effluents often contributes to an increase in chemical oxygen demand (COD). Thiocyanate toxicity appears in many developing countries where the industrial effluents are directly discarded in natural water bodies. When the thiocyanate comes in contact with chlorine it is converted to highly toxic and volatile cyanogen chloride (13). Thus, drinking and
using of such water can lead to injury of eyes and respiratory organ, paralysis and even death (67). Although thiocyanate is approximately seven times less toxic than cyanide, increased thiocyanate concentration in the body resulting from chronic cyanide exposure can adversely affect the thyroid gland (13). Thiocyanate was found to be mildly neurotoxic for human at serum levels of 60 mg/L and became life threatening when the levels are 3 or 4 times higher than that (47). In contrast, the production of carbonyl sulfide during thiocyanate degradation contributes a role as sulfate aerosol in stratosphere and influences the climate (Crutzen, 1976). Therefore, study of thiocyanate and its related enzyme is quite necessary to contribute a significant role in elimination of toxic thiocyanate from environment. In this point, thiocyanate hydrolase enzyme of strain THI201 plays an important role by degrading the thiocyanate to COS.

*Afipia* sp. strain THI201 is a newly isolated strain and the characteristics of its enzyme thiocyanate hydrolase are still unknown. Although the enzyme could be isolated, it could not be used for further study due to its unstable activity. It is thought that if the gene of enzyme could be cloned and expressed, it is possible to produce it abundantly. In addition, the enzyme can be used for studying and enhancing enzyme stability. Furthermore, cloning will facilitate the determination of nucleotide sequence of enzyme as well as perform the identity (%) study with known thiocyanate hydrolase. Other than these, the purified enzyme can be used to prepare probe for detecting thiocyanate-degrading bacteria in natural environment.

With this point of view, the present study has been designed with the following objectives:

1. Gene cloning of SCNase enzyme in to *E. coli* and determination of ORF (open reading frame)
2. Sub-cloning in to expression vector and over-expression of fusion protein
3. Production and purification of fusion protein
4. Measurement of enzyme activity
5. Characterization of enzyme based on optimum temperature, pH, thermostability, dose dependence of enzyme, dose dependence of KSCN, and time course of ammonia formation.
The objectives are described in the following five chapters:

Chapter 2 gives a brief description of isolation procedures of SCNase enzyme from *Afipia* sp. strain THI201

Chapter 3 deals with cloning and sequencing of *scn* gene. A detail method of cloning of a target gene and the results of experiments are shown here.

Chapter 4 narrates the procedures and results of expression of *scn* in *E. coli*. It also describes the effective methods for abundant production of recombinant enzyme in soluble form.

Chapter 5 represents the major characteristics of SCNase. It also traces out the uncommon natures of SCNase of strain THI201.

Finally, The Conclusion section summarizes the major findings of current study. Composition and preparation of different media and solutions have been described in appendix section, at the end of this thesis.
Chapter 2

CHARACTERIZATION OF

AFIPIA SP. STRAIN THI201 AND

ISOLATION OF SCNASE ENZYME
Chapter 2

CHARACTERIZATION OF AFIPIA SP. STRAIN THI201 AND
ISOLATION OF SCNASE ENZYME

2-1 INTRODUCTION

Strain THI201 that I used in this research was isolated from surface water of lake Sagami and its 16S r RNA gene sequence was determined by T. Hayashi (74). Physiological characteristics especially on the thiocyanate degradation were examined by M. Nameki (75) and A. Sekine (76). Furthermore, enzyme for the degradation of thiocyanate was isolated by M. Saito (55). Based on these results, I have started to clone the thiocyanate degrading enzyme from strain THI201. Identity (%) studies showed that the isolated protein did not match with the previously known thiocyanate degrading enzyme, SCNase, from *Thiobacillus thioparus* THI115, therefore I tried the expression of the cloned *scn* gene in *E. coli* cells for detailed studies on the unique enzyme.

For the background of my research, a brief description of previous findings about strain THI201 was summarized in this chapter.

2-2 ISOLATION OF STRAIN THI201

The thiocyanate degrading bacteria is ubiquitous and its isolation technique depends on the source. For example, bacteria from activated sludge was homogenized and diluted by sterilized distilled water and plated outon SM agar medium (Katayama *et al*., 1982) containing 20 mM sodium thiosulfate. *Thiobacillus*-like colonies having precipitated sulfur were isolated, and the thiocyanate-degrading activities were tested on TC medium [0.5 g/l K$_2$HP0$_4$, 0.05 g/l MgSO$_4$.7H$_2$O, 0.01 g/l FeCl$_3$.6H$_2$O, 0.01 g/l CaCl$_2$.2H$_2$O, 1.0 g/l (NH$_4$)$_2$SO$_4$, 1.0 g/l potassium thiocyanate, 10 ml of trace metal solution (Katayama & Kuraishi, 1978), pH 7.0] (Katayama *et al*., 1992). Strain THI201 was isolated from lake water by using an enrichment culture supplemented with thiocyanate (Yamasaki *et al*., 2002).
2-2-1 Materials and methods

2-2-1-a Culture conditions:

Strain THI201 was isolated by streaking the enriched culture LS5 (Yamasaki et al., 2002) on TC5 medium [0.5 g of K2HPO4, 1 g of (NH4)2SO4, 0.05 g of MgSO4·7H2O, 0.01 g of FeCl3·6H2O, 0.01 g of CaCl2·2H2O, 10.0 ml of trace metal solution (Katayama & Kuraishi, 1978), 0.5 g of potassium thiocyanate (KSCN) per liter, pH 7.0].

2-2-1-b Assay of SCN degradation by whole cells of THI201:

Thiocyanate degradation and production of COS and sulfate was measured during growth with thiocyanate and malic acid. Growth of strain THI201 was measured at OD660 in a culturing flask (650 ml) containing 100 ml of MmTC12 medium. The amount of COS emission was estimated from head space (550 mL) of flask by gas chromatograph. Amounts of thiocyanate and sulfate were measured from the same flask by colorimetric (Katayama & Kuraishi, 1978) and turbidimetric (Sörbo, 1987) methods, respectively.

2-2-2 Results:

Surface water sampled from the Lake Sagami was incubated aerobically with 1 mM of KSCN until all thiocyanate supplemented to the medium was consumed. Re-inoculation in the fresh medium was repeated several times to enrich thiocyanate-degrading microorganisms. Strain THI201 was isolated by streaking the enriched culture LS5 (Yamasaki et al., 2002) on TC5 medium.

In a mixotrophic medium of MmTC12 containing thiocyanate of 12 mM and malic acid, strain THI201 used thiocyanate in four days, and the sulfur moiety of thiocyanate was recovered almost as equimolar amounts of sulfate. During the degradation of thiocyanate, carbonyl sulfide corresponding 6 to 10% of the degraded thiocyanate was transiently detected in the head space of a culture vessel, indicating transformation of the substrate by COS pathway (Kelly et al., 1993) (Fig. 1).
2-3 PHYSIOLOGICAL CHARACTERISTICS AND PHYLOGENETIC POSITION OF STRAIN THI201

Morphological and physiological characteristics of strain THI201 were examined to know the growth condition of this bacterium for the isolation of thiocyanate degrading enzyme. Phylogenetic position of strain THI201 was determined based on the sequencing of 16S rRNA gene.

2-3-1 Methods

Optimal temperature and pH for strain THI201 were examined in liquid medium of YmTC10. Phylogenetic position of the isolate was assessed based on the sequence data of the 16S rRNA gene.

2-3-2 Results

Afipia sp. strain THI201 was a gram-negative, motile, short rod bacterium with a polar or a sub-polar flagellum. It was a facultatively chemolithoautotroph: grew chemoorganotrophically with an organic substance such as organic acids and amino acids, and chemolithoautotrophically in a liquid medium of mineral-salts supplemented with thiocyanate. Strain THI201 was mesophilic that grew optimally around 30°C with ranges between 25 to 35°C, but unable to grow at 40°C. This bacterium grew optimally in pH 7.0 to 7.5 (initial medium pH) with ranges between pH 6.0 to 8.5. Phylogenetic affiliation based on the sequence data of almost full length of 16S rRNA showed that strain THI201 is a member of the Bradyrhizobium-Agromonas-Nitrobacter-Afipia (BANA) cluster in the Alphaproteobacteria. Phylogenetic analysis based on rpoB gene (Kanaya et al., paper in preparation) indicated that strain THI201 is a new species in the genus Afipia.
2-4 ISOLATION OF SCNASE

*Afipia* sp. strain THI201 utilizing thiocyanate as the chemolithoautotrophic growth substrate produced COS and NH₃ by thiocyanate degradation. Because the SCNase of THI201 is an intracellular enzyme, isolation of enzyme was performed by cell disruption with lysozyme and sonication. Utilizing the knowledge of previously isolated SCNase, the enzyme of THI201 was successfully isolated by Hydroxyapatite column chromatography; and the activity was measured by estimating COS in native enzyme.

2-4-1 Materials and methods

2-4-1a Isolation of SCNase:

Strain THI201 was grown in MmTC10 medium, which had the same composition as YmTC10 medium except that 8.3 mM L-malic acid was used instead of yeast extract. The bacterium was harvested by centrifugation and rinsed with buffer A [50 mM potassium phosphate, pH 7.5]. The cells were resuspended in 4 volumes of buffer A containing 25% (v/v) glycerol and disrupted by using an ultrasonic homogenizer (25 W for a total of 2 min, Model VP-5, TAITEC Inc.) on ice. After centrifugation of the disrupted cell suspension at 7,700g at 4°C for 30 min, the supernatant was harvested as the crude cell extract. All remaining manipulations were conducted at 4°C. The crude cell extract was diluted with buffer B [10 mM potassium phosphate, pH 7.5] containing 10% (v/v) glycerol and applied to a hydroxyapatite column (12 × 58 mm; gel volume, 3 ml; Bio-Gel HTP, Bio-Rad Inc.) that had been equilibrated with the same buffer B-glycerol solution. Proteins were eluted by applying a 24-ml linear gradient of increasing potassium phosphate concentrations from 10 mM to 300 mM. The active fractions were combined, dialyzed in buffer B and the hydroxyapatite column chromatography step was repeated. SDS-PAGE was performed as described by Laemmli (Laemmli, 1970) with a 12% polyacrylamide gel, and the proteins bands were stained with Coomassie Brilliant Blue R-250.
2-4-1b Enzyme assays:

Thiocyanate hydrolase activity can be estimated by measuring either of the reaction products, COS or NH₃. The enzyme activity during the isolation process was monitored by measuring the formation of COS with the use of a gas chromatograph (GC-14B, Shimadzu Corporation) equipped with a flame photometric detector and a glass column packed with Porapak QS (50–80 mesh; Nihon Waters K.K.) as described previously (Katayama et al., 1992). The assay mixture (100 μl) contained 10 mM KSCN, buffer A, and the enzyme solution. A 6-ml polypropylene tube with a headspace volume of 4.4 ml was used for the assay, after the tube was sealed with a butyl cap. The incubation was performed at 30°C for 20 min. Headspace gas (50 μl) was obtained by using a gas-tight micro syringe, and then injected into a gas chromatograph. Protein concentrations were estimated by the method of Lowry et al. (Lowry et al., 1951). The specific activity of SCNase was expressed as nmol or μmol of COS produced min⁻¹ mg⁻¹ protein.

2-4-2 Results and Discussion:

Thiocyanate degradation activity in cell-free extract was estimated by product ion of COS and found to be 11.7 nmol min⁻¹ mg⁻¹ protein. However, after the consumption of all thiocyanate, though cell growth continued using malic acid, the activity decreased to 0.29 nmol min⁻¹ mg⁻¹ protein. Therefore, cells used for isolation of the enzyme were harvested when almost all thiocyanate was consumed.

The thiocyanate-degrading activity of the crude cell extract of strain THI201 was considerably decreased by freeze-thawing. Addition of glycerol or polyethylene glycol partly protected the loss of the activity (data not shown). Therefore, the buffer used to disrupt the cells was supplemented with 25% (v/v) glycerol, and the frequency of freeze/thawing steps was minimized. Hydroxyapatite column chromatography of the crude cell extract was effective for the isolation of proteins harboring thiocyanate-degrading activity, and repeating the procedure resulted in partially purified proteins in SDS-PAGE (Saito, 2006. Master thesis). The specific activity of the isolated fraction was 0.32 μmol min⁻¹ mg⁻¹ of protein.

By using this isolated SCNase, cloning of the scn gene was performed.
Fig. 1. Thiocyanate degradation and production of COS and sulfate by *Afipia* sp. strain THI201 during growth with thiocyanate and malic acid. Growth of strain THI201 (▲) was measured at OD$_{660}$. Data were expressed as μmol/flask because the amount of COS (●) emission was estimated from head space of flask. Amounts of thiocyanate (□) and sulfate (●) were measured from the same flask. The error bars of sulfate production show the standard deviations based on duplicate experiments.
Chapter 3

CLONING AND SEQUENCING OF SCN GENE
Chapter 3

CLONING AND SEQUENCING OF SCN GENE

3-1 Introduction

Gene cloning refers to the procedure of isolating a defined DNA sequence and obtaining multiple copies of it in vivo. Now a day, this technique has become an important tool to study and reveal the genetic structure of any unknown gene. Many benefits to human have come about as a result of gene cloning and following research in molecular biology. These includes identification of human disease genes, production of important enzymes and hormones like insulin, production of transgenic crops and animals for better yield and so on (Russell et al., 2001, molecular cloning).

Gene is usually cloned in a well-known microorganism like E. coli which makes the further studies easier. Five basic steps are involved in gene cloning (Russell et al., 2001, molecular cloning):

1. Isolation of DNA containing the desired gene
2. Digestion of DNA and vector (i.e., plasmid vector) with the same restriction endonuclease
3. Ligation of DNA and vector with DNA ligase enzyme
4. Incorporation of recombinant DNA into a host cell
5. Screening of desired gene from the host cell

A desired gene can be cloned in a known organism in various ways. Among them three most widely used methods are: Genomic library construction, sub-genomic library preparation, and inverse PCR. I tried all three methods and became successful to clone the desired gene by sub-genomic library preparation.

Genomic library is used to screen and locate the specific genes in order to determine the DNA sequence and express it through expression vector. To construct a genomic library, chromosomal DNA of an organism is digested with restriction enzyme and all the fragments are inserted to host cell through vector. Therefore each host cell contains a different segment of DNA from the original organism. Taken together, these cells represent a DNA library (Russell et al., 2001, molecular cloning). In contrast, to prepare a sub-genomic library of a bacterium,
portions of genomic DNA are cloned instead of whole genome. As a result, screening of the target gene is easier than that of genomic library. At first, genomic DNA is completely digested with restriction endonuclease and from the whole DNA digests, portions of DNA fragments are selected by screening with antibody or probe. The following diagram represents an outline of cloning into a plasmid vector.

Fig. A: Cloning into a plasmid vector. Here, both the plasmid and foreign DNA were digested with restriction enzyme  \textit{HindIII}. Then the resulting sticky ends were ligated by enzyme DNA ligase and transformed into bacterial cell. Bacteria were grown in antibiotic containing media where only the clone containing antibiotic resistance gene can grow.
Kim Sang-Hoon and Patrick Oriel described cloning and expression of nitrile hydratase (NHase) and amidase gene from *Bacillus* sp. BR449 into *E. coli*. They performed southern hybridization to locate the fragments containing target gene, then constructed sub-genomic library with the isolated DNA fragments. The positive clones were selected by colony hybridization using labeled probe. The NHase gene sub-cloned in to expression vector and expressed in *E. coli* DH5α could produce significant levels of active enzyme (Sang & Patrick, 2000). Similarly, in this study, southern hybridization with DNA probe was carried out to select the target gene. The selected fragments were inserted into vector, then the vector was transferred to host cell like *E. coli*. Because only selected fragments were cloned, screening of the gene was possible with smaller numbers of clone compared to genomic library.

3-2 Materials and methods

3-2-1 Bacterial strains and cultural conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The mixotrophic thiocyanate degrading bacterium *Afipia* sp. strain THI201 was grown in MmTC10 (modified malate thiocyanate) and YmTC10 (modified yeast extract thiocyanate) medium, where 10 indicates the presence of 10 mM KSCN in media. The compositions of media have been introduced in appendix section. Strain THI201 was grown in YmTC10 medium for 7-8 days by shake culture at 30°C to obtain the complete growth of cells and also to isolate the chromosomal DNA. Strain THI201 was stored in YmTC10 broth containing 50% glycerol and at -80°C.

*Escherichia coli* strain JM109 (Table 1) was used as host for the cloning of *sen* gene. *E. coli* was grown in Luria-Bertani (LB) medium supplemented with 100 μg mL⁻¹ ampicillin. Both the cells of strain THI201 and *E. coli* were grown aerobically with reciprocal shaking at 30°C.
3-2-2 Preparation of DNA probe:

DNA probe of 81 bp was prepared based on the N-terminal amino acid sequence. Degenerate oligonucleotide mixtures of F1 (5’-GAYATGWSIAARGGRCICARCAY-3’, where R is A or G; S is C or G; W is A or T; Y is C or T) and R1 (5’-GCISWIACIGTICCRTTRTAYTT-3’) were designed based on the N-terminal amino acid sequence of SCNase. Genomic DNA of *Afipia* sp. strain THI201 was isolated according to the method of Saito and Miura (Saito & Miura, 1963) and subjected to PCR with the oligonucleotide mixtures F1 and R1 and Hot Star Taq DNA polymerase (QIAGEN K.K., Tokyo, Japan) under the annealing temperature of 50°C according to the manufacture’s instruction. Nucleotide sequence of the resultant 92-bp DNA fragment was determined by using oligonucleotide mixtures F1 and R1 as sequencing primers and used for designing additional oligonucleotides F02 (5’-AGCAAGGAGGCGCAGCAC-3’) and R02 (5’-CACGGTGCCGTTGTACTT-3’). The 92-bp DNA fragment was used as a template for PCR with oligonucleotides F02 and R02. The resultant DNA fragment of 81 bp in size was labeled with AlkPhos Direct labeling and detection systems (GE Healthcare Japan, Tokyo, Japan) according to manufacturer’s instructions, and then used as a probe to clone SCNase gene.

3-2-3 Southern blotting with the probe:

Southern hybridization was performed to detect the fragment of *scn* in chromosomal DNA of THI201. Chromosomal DNA of strain THI201 was digested with *Pvu*II, *Stul*, *Hpa*I, *Eco*RI, *Eco*RV, *Pst*I, *Hind*III, *Xho*I, *Kpn*I, or *Sau*3AI, then separated on a 0.7% agarose gel and transferred to a nylon membrane (GE Healthcare) by capillary action. Hybridization was performed with the above-mentioned AlkPhos-labeled 81-bp probe at 55°C, as suggested by the manufacturer’s specifications. The membrane image was obtained with a luminescent imaging analyzer (LAS-3000, Fuji Film).
3-2-4 Construction of sub-genomic library

The aim of sub-genomic library was to clone only the selected fragments of genomic DNA carrying the target gene. Genomic southern hybridization of *Afipia* sp. strain THI201 showed that, *HindIII* digested DNA fragments of 5.5-6.0 kb contain the *sca* gene. Therefore, the genomic DNA of strain THI201 was completely digested with *HindIII* enzyme and a sub-genomic library of strain THI201 was prepared by using DNA fragments of length 5.0-6.5 kb and pUC118/*HindIII*/BAP vector (Takara Bio Inc.).

3-2-4-a Preparation of insert DNA

Hundred micro grams of genomic DNA of strain THI201 was completely digested with 2,000 units of *HindIII* (Nippon gene) in presence of 36 µL of 10× buffer B (Nippon gene) in the final volume of 360 µL. The reaction mixture was incubated at 37ºC for overnight and the enzyme was inactivated at 65ºC for 30 min. Then agarose gel (0.7%) electrophoresis of 20 µL (5.6 µg) of digested DNA was done. From agarose gel, DNA bands of 5.0-6.5 kb was extracted with Recochip kit and recovered by ethanol precipitation. The DNA pellet was dissolved in 50 µL of sterilized miliQ and the concentration was measured with nanodrop spectrophotometer.

3-2-4-b Checking the presence of target gene

The prepared insert DNA was checked and confirmed by two ways. One was by 0.7% agarose gel electrophoresis. Here isolated purified DNA (45 ng) was applied to agarose gel to check the presence of 6.0 kb DNA and also to check if the DNA is degraded or not.
The other method was doing PCR with the insert DNA to check the presence of 81 bp N-terminal sequence of \textit{scn}. PCR was done with primer F02/R02 (prepared from N-terminal sequence of \textit{scn}) and insert DNA as template. Positive control was done with 20 ng genomic DNA of strain THI201. Reaction mixture of PCR was prepared by following method:

- 10x PCR buffer 2 \mu L
- 2mM dNTP 2 \mu L
- Hot star Taq 0.1 \mu L
- Primer 4 \mu L (2 \mu M)
- Template DNA 10 ng
- Double distilled water to make the final volume 20\mu L

PCR conditions: Activation at 95\degree C, 15 min; denaturation at 94\degree C, 0.5 min; annealing at 50\degree C, 0.5 min; extension for 1 min at 72\degree C. Number of cycle was 35 and final extension was 10 min, 72\degree C.

PCR product was confirmed by electrophoresis of 10\mu L of the reaction mixture by 2% agarose gel and staining with ethidium bromide.

### 3-2-4-c Ligation

\textit{HindIII} digested insert DNA (95 ng) was ligated to dephosphorylated, \textit{HindIII} digested vector pUC118 (50 ng) by DNA ligase in molar ratio of vector: insert =1:1. Vector and insert DNA were taken together in an eppendorf tube and heated at 60\degree C for 3 min. The purpose of heat treatment was to prevent self ligation of DNA. The DNA mixture (6.83 \mu L) was then cooled rapidly in ice and DNA ligase of 6.83 \mu L was added. Total 13.66 \mu L reaction mixture containing 145 ng DNA was incubated at 16\degree C for 2 hours. It was used for transformation soon.
3-2-4-d Transformation of competent *E. coli* JM109 by recombinant DNA

Recombinant DNA (ligation product) of 13.66 µL was divided into 0.7, 5.0, and 7.96 µL and used for transformation. Competent cell of *E. coli* JM109 of 100 µL and ligated DNA of 0.7, 5.0, or 7.96 µL were mixed together lightly (by micropipette) in an eppendorf tube and incubated in ice bath for 30 min. The reaction mixture was then heated at 42°C for 45 sec and cooled in ice bath for 2 min. The SOC media was added to a final volume of 1 mL and the mixture was incubated 1 hour at 37°C in a shaker. Inoculation on LB agar supplemented with ampicillin (100 µg ml⁻¹), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 µg ml⁻¹) and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.035 mM) by spread plate technique was done with 200 µL bacterial suspension per plate. The plates were incubated overnight at 37°C. Transformants were selected by blue-white screening technique. White colonies were collected, and positive clone was selected by colony hybridization method. Two negative controls were done in parallel with transformation of recombinant plasmid. One negative control was done by transformation with 0.5 ng pUC118 plasmid to check the accuracy of transformation method. The other one was performed by plating the competent *E. coli* into LB/amp media to check the perfection of media preparation. As the *E. coli* cells do not comprise the ampicillin resistant gene, it will not grow in the LB media containing ampicillin.

3-2-5 Screening of positive clone from library

The resultant sub-genomic library was screened for *scn* by colony hybridization with AlkPhos-labeled 81-bp probe.

3-2-5-a Preparation of probe DNA:

The alkaline phosphatase method was followed for probe preparation. The method is simple and requires only 10ng/µL of PCR product to label with alkaline phosphatase.

PCR was performed using primers F02/R02 (designed from N-terminal amino acid of SCNase) and 6.0 kb DNA fragment containing *scn*, as template DNA. After PCR, agarose gel electrophoresis of PCR product was done and
81 bp DNA was collected from gel by using Ricochip Kit. DNA solution eluted from gel was recovered by centrifugation and concentrated with ethanol precipitation, and the DNA concentration was measured with nanodrop. Then the DNA was diluted to 10ng/μL, to use as probe DNA.

For labeling of probe, 50μL (10ng/μL) of probe DNA solution was denatured by heating 5 min in boiling water bath. After cooling on ice for 5 min, 50μL of reaction buffer was added. Then, 10μL of alkaline phosphatase and 50μL of cross linker solution were added and mixed. The solution was incubated at 37ºC for 30 min and stored with 50% glycerol for later use. This probe can be stored at -20ºC for up to 6 months.

3-2-5-b Colony hybridization

Colony hybridization is a technique for isolation of cloned DNA that contains a specific gene. By this method, a very large number of colonies of *Escherichia coli* carrying different hybrid plasmids can be rapidly screened to determine which hybrid plasmids contain a specified DNA sequence or genes. Here, from the library, 70 colonies were picked and checked for presence of *scn* gene by colony hybridization. The 81 bp PCR product was used as positive control and bacterium without any insert DNA (a blue colony from transformation plate) was used as negative control.

**Materials:**

1. Denaturation buffer: 1.5 M NaCl, 0.5 M NaOH

2. Neutralization buffer: 3 M NaCl, 0.5 M Tris. The pH was adjusted to 7.5 with concentrated HCl

3. 2X SSC: 300 mM NaCl, 30 mM Na-citrate. pH was adjusted to 7-8.

4. 10% SDS

5. 1 M MgCl₂

6. Hybridization buffer (working solution):
NaCl, 2.92 g/100 mL buffer; blocking reagent, 4.0 g/100 mL buffer (Supplied with GE health care kit)

7. Primary wash buffer (1 L)
   Urea 120 g
   SDS 1.0 g
   0.5M Na phosphate (pH 7.0) 100 ml
   NaCl 8.7 g
   1.0 M MgCl₂ 1.0 mL
   Blocking reagent 2.0 g
   pH was adjusted to 7.0 by NaOH Solution can be kept for 1 week in 2-8ºC.

8. Secondary wash buffer (20 × stock)
   Tris base 121 g/L
   NaCl 112 g/L
   pH was adjusted to 10 by HCl; can be kept for 4 months in refrigerator at 2-8ºC. Reheating should be avoided.

9. Blotting paper

10. Nylon membrane

11. LB/amp plate with number marking

12. Sterile toothpick

Methods:

1. Bacterial colonies were inoculated into the number marking two LB/amp plates with sterile toothpick and incubated overnight at 37°C.
2. A piece of nylon membrane was cut to the size of the petri dish to be probed
3. The blotting papers were soaked carefully into one of the 3 solutions (10% SDS, denaturation buffer and neutralization buffer) by avoiding over wetting the paper.
4. The piece of nylon membrane was pressed onto the plate to be probed and 3 spots were marked on both
the membrane and plate for orientation.

5. The blotting papers were put on a wrap and the piece of nylon membrane was placed as cells
side up over the 4 blotting papers. The blotting papers were soaked in their respective solutions in
the order, and for the incubation times, indicated below:

<table>
<thead>
<tr>
<th>ORDER</th>
<th>SOLUTION</th>
<th>INCUBATION TIME (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>10% SDS</td>
<td>3</td>
</tr>
<tr>
<td>Second</td>
<td>Denaturation buffer</td>
<td>5</td>
</tr>
<tr>
<td>Third</td>
<td>Neutralization buffer</td>
<td>3</td>
</tr>
<tr>
<td>Fourth</td>
<td>Neutralization buffer</td>
<td>3</td>
</tr>
</tbody>
</table>

6. The membrane was washed vigorously in 2XSSC

7. The membrane was placed, DNA side up, over a dry piece of blotting paper and air dried for 30 min

8. The membrane was wrapped with saran wrap and placed in to an optimized UV-crosslinking machine
(0.07 joules/cm²) for 3 min

9. The membrane was placed in to a plastic bag and hybridization was done in 3 steps. First, the membrane
was pre-hybridized with pre heated (at 55ºC) buffer for 20 min at 55ºC. The labeled probe was added and
overnight incubation at 55ºC was performed in a hybridization oven.

Then the membrane was transferred to preheated (at 55ºC) primary wash buffer for post hybridization stringency
washes. Two repeated washes with primary wash buffer were performed for 10 min at 55ºC, with gentle
agitation. Again two repeated washes with secondary wash buffer was done for 5 min at room temperature with
gentle agitation.

Finally signal generation and detection was performed by draining the excess secondary wash buffer and
pipetting detection reagent CDP-Star (GE healthcare) of 35μL/cm² on the membrane for 5 minutes reaction.
The membrane was wrapped in saran wrap and placed in the tray of LAS 3000 (luminescent image analyzer,
Fuji film) to see the image of southern hybridization.
10. The colonies showing the positive result were sub-cultured in to a fresh LB/Amp broth for further study.

**Fig. 1. Colony hybridization.** The microbial colonies are transferred (blotted) to a membrane. The cells are lysed in place to release the nucleic acids. The DNA (after denaturation) is fixed to the filter and hybridized with a labelled probe. Blocking reagent was added prior to the probe to prevent unspecific binding. Excess probe is washed away and the membrane is visualized by UV or autoradiography.

**3-2-5-c Confirmation of positive clone**

The colonies showing positive response in colony hybridization were used for plasmid extraction by Pure yield™ plasmid miniprep system (Promega). Plasmid DNA concentration was measured by nanodrop and used as template of PCR to check the presence of 81 bp DNA of *scn*.

Thus, bacterial clone designated as C14 was obtained and plasmid extraction was done. The recombinant plasmid which contained 6.0 kb DNA fragment of strain THI201 was designated pUC118C14 and used to check again the presence of 6.0 kb DNA by *HindIII* treatment. Plasmid DNA of 2.1 µg was treated with 10 U of *HindIII* enzyme. The reaction mixture was incubated 1 hour at 37°C and half of the reaction mixture was applied to 0.8% agarose gel.

**3-2-5-d Checking the position of *scn* in C14 clone:**
The position of \textit{scn} in plasmid pUC118C14 was checked to determine the existence of ORF of SCNase. Long Range PCR was done with pUC118C14 as template DNA and the following Primers:

- M13F and M13R, commercial primers designed from vector pUC118
- F02, R02, IP1, and IP2: Primers designed from N-terminal amino acid sequence of SCNase

The sequence of the primers are shown in Table 2. Qiagen Long range PCR kit and its protocol were used.

3-2-5-e Growth and KSCN degradation of C14 clone in mMTC media:

By colony hybridization, C14 was found as a positive clone containing \textit{scn}. This experiment was done to check the expression of \textit{scn} in this recombinant bacterium. The mMTC10 media was used to grow \textit{Afipia} sp. strain THI201. This media contains 10 mM KSCN which was degraded by THI201. Therefore, same media was used to determine the growth curve and KSCN degradation by C14 clone.

Bacterium C14 was inoculated into a Sakaguchi flask with 100 ml mMTC10 medium and incubated for 3 days at 30°C with reciprocal shaking. Control was done with bacterium of blue colony (without any insert DNA). Growth was determined by measuring OD at 660 nm and KCSN degradation was measured by colorimetric method (Katayama & Kuraishi, 1978). The experiment was performed in duplicate and the result was presented in average.

3-2-6 Nucleotide sequencing

Sequencing with pUC118C14 plasmid was performed by capillary sequencer (3130 genetic analyzer, Applied Biosystems). In order to sequence the upstream region and coding strand of plasmid clone, following 7 primers were sequentially used: M13R, M13RA, M13RA1, F02, F02A, F02B1, F02C. Sequencing of opposite strand was done with the following 8 primers: RF02D1a, RF02D, RF02B2, RF02A1, RF02B, RM13RA2, RM13RA3, RM13RA4 (Table 2). Thus, DNA sequence of the insert DNA was revealed by primer walking technique and each time sequencing was done by following 3 steps:

1. Sample preparation:

   Primer (2.5 µM) 1.3 µl (3.2 pmol/µl)
Big Dye terminator v3.1 (x2.5)  0.5 µl
Big Dye dilution buffer (x5)  1.75 µl
Template DNA  200 ng
Water to make the final volume 10 µl

2. Cycle sequencing reaction:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (ºC)</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
<td>10 sec</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>hold</td>
<td></td>
</tr>
</tbody>
</table>

3. Sample purification:

Reaction mixture of 10 µl was mixed with 3M sodium acetate 2.5 µl, 125 mM EDTA 2.5 µl, 95% ethanol 70 µl, and milli Q water 15 µl. The samples were kept at room temperature (RT) for 15 min and centrifuged at 14,000 rpm at RT for 20 min. The supernatant was discarded and the DNA was rinsed with 250 µl of 70% ethanol. The solution was centrifuged again at 14,000 rpm at RT for 5 min and dried for 10 min after discarding the supernatant. At this stage, the DNA can be stored at -20 ºC, up to 1 week. Finally, 20 µl of HiDi formamide was added and vigorous vortex was done to dissolve the DNA. The DNA solution was heated at 95ºC, 2 min and immediately put on ice. It was used for sequencing and sequence alignment was performed by the software “4 peaks.”
The percentage of identity was searched for the resultant sequence against the National Center for Biotechnology Information (NCBI) database by using the BLASTX program. Multiple-sequence alignments were performed with the ClustalW program accessed through the DNA Data Bank of Japan (DDBJ). The nucleotide sequence for the protein reported in this study has been submitted to DDBJ nucleotide sequence databases under accession no. AB674736.

3-3 Results and Discussion

3-3-1 Construction of sub-genomic library

Restriction digestion with enzymes except PstI produced single fragment of different sizes. This result indicated presence of a single copy gene of SCNase in the genomic DNA of Afipia sp. strain THI201. Among all the hybridized fragments, 6.0 kb fragment of HindIII digestion was found to be the most suitable size for transformation. A sub-genomic library of strain THI201 was prepared by using HindIII-digested DNA fragments of 5.0-6.5 kb in size and the HindIII-digested pUC118 vector (TAKARA BIO).

Southern hybridization showed the presence of scn in 6.0 kb fragment of HindIII digest, therefore, the 6.0 kb fragment was isolated from agarose gel (Fig. 2) and purified to use in ligation reaction with vector. In order to increase the ligation efficiency, concentrated (~ 100 ng), purified DNA is very important. Low concentrated DNA may cause self-ligation instead of DNA-Vector ligation. Consequently, the DNA isolation was done in large scale and the concentration was found to be 15 ng/µL.

The isolated and purified insert DNA was checked for presence of scn by PCR and agarose gel electrophoresis (Fig. 3a, 3b). The 81 bp DNA band of PCR confirmed the presence of scn and Fig.3b confirmed that the DNA isolated was about 6.0 kb in size and not degraded. Therefore the insert DNA was used in ligation reaction with vector. After ligation and transformation, total 335 white colonies and 352 blue colonies appeared in 15 LB/amp plates as follows:

<table>
<thead>
<tr>
<th>Ligated DNA</th>
<th>White colonies</th>
<th>Blue colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>335</td>
<td>352</td>
</tr>
</tbody>
</table>

29
<table>
<thead>
<tr>
<th>Volume</th>
<th>Concentration</th>
<th>Number</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7 μL</td>
<td>7.43 ng</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>5.0 μL</td>
<td>53 ng</td>
<td>129</td>
<td>202</td>
</tr>
<tr>
<td>7.96 μL</td>
<td>85 ng</td>
<td>189</td>
<td>131</td>
</tr>
</tbody>
</table>

The reason for large numbers of blue colonies might be because of self-ligation of vector. Though the dephosphorylated vector was used to prevent self-ligation but still small numbers of vectors without dephosphorylation might exist to cause the blue colonies.

Two negative controls were also checked to confirm the perfection of the procedures. Transformation with pUC118 plasmid produced blue colonies, it was TNTC (too numerous to count). This result indicated that the transformation protocol worked perfectly. Plating of *E. coli* on the LB/amp plate produced no colonies, indicating that the presence of ampicillin inhibited the growth of ampicillin sensitive *E. coli*.

### 3-3-2 Screening of positive clone

Screening was done by southern hybridization method and positive clones were selected by signal generation in LAS3000. Several colonies produced bright signals, but first four colonies were selected for further experiments. In one plate, the colony number 14, 33, and 20 showed bright signal gradually with time and in other plate colony 65 first showed the bright signal. The positive control also produced bright signal and the negative control didn’t show any signal (Fig. 4).

Positive control along with the C14, C20, C33, and C65 clones were checked for the presence of *scn* by PCR with F02/R02 primers and the extracted plasmids as template DNA. Fig. 5a shows that only pUC118C14 contains the 81 bp sequence of *scn* which was similar to the positive control. The other clones showed very light band alike the negative control. Therefore, pUC118C14 was checked again by HindIII digestion. Agarose gel showed 6.0 kb DNA insert and 3.2 kb vector pUC118. The pUC118/HindIII/BAP vector was also applied to agarose gel as a control and showed the 3.2 kb band (Fig. 5b).
Presence of \textit{scn} ORF was checked in pUC118C14 by PCR with R02/M13R (D1), F02/M13F (D2), IP1/M13R (S1), and IP2/M13F (S2) primer sets (Figs. 6a, 6b). D2 gave \~4.5 kb band but D1 did not produce any band, the reason of D1 was unknown. So again PCR was done with S1 and S2 primer set to determine the size of DNA insert. S1 showed \~1.5 kb band. S2 presented multiple bands instead of only 4.5 kb band, the reason was also unknown. Therefore, the 1.5 kb band (S1) + 4.5 kb band (D2) = 6.0 kb insert was predicted and the position of ORF was presented in Fig. 7.

3-3-3 Expression of \textit{scn} in \textit{E. coli} C14 clone

Expression of \textit{scn} was checked in C14 clone by growing the recombinant \textit{E. coli} into mMTC10 media containing 10 mM KSCN. The growth of C14 was very slow in mMTC10, it could not degrade KSCN in 3 days (result not presented). The control bacterium without \textit{scn} also did not grow or degrade KSCN. As the 10 mM KSCN is considered toxic for many bacteria, experiment with lower concentration (1 mM) of KSCN was also tried but it produced the similar result. Growth and KSCN degradation of C14 strain was also tried with LB media containing 1 mM KSCN (LB1). Though the recombinant \textit{E. coli} could grow very fast (OD$\text{600} = 0.74$) in LB1 compared to mMTC10 (OD$\text{600} = 0.03$) or mMTC1 (OD$\text{600} = 0.04$), it could not at all degrade the KSCN. Therefore, it could be concluded that the \textit{scn} was not expressed in C14 and sub-cloning in to expression vector was necessary to express the \textit{scn} into recombinant \textit{E. coli}.

3-3-4 Nucleotide sequence of the THI201 \textit{scn} gene

Nucleotide sequencing analysis of the plasmid pUC118C14 isolated from clone C14 revealed that the SCNase protein was within an ORF of 1470 bp. Two Shine-Dalgarno sequences AGGGGA and AGGCGGA were predicted, with two different putative start codons (ATG) within an ORF. The first of the two start codons encoded a signal sequence of 32 amino acids with a Tat motif (RRTLL) and the SCNase protein of 457 amino acids (Fig. 8a). Prediction using TatP gives exactly the N-terminally determined start at the alanine residue as the most probable cleavage site. The calculated molecular mass (50,267 Da) of the protein was consistent with that
estimated from SDS-PAGE analysis. The deduced amino acid sequence of the N-terminal region in the protein matched completely with the chemically-determined sequence (Fig. 8b).

The identity (%) searches of the protein sequence of SCNase against the NCBI protein database revealed identities of 83% with a putative uncharacterized protein of *Thiobacillus denitrificans* ATCC 25259, 36% with a hypothetical protein of *Hydrogenobacter thermophilus* TK-6, and 35% with a hypothetical protein ThithDRAFT_3226 of *Thioalkalivibrio thiocyanoxidans* ARh 4 (Fig. 9), but no significant identity with three subunits of SCNase from *Tb. thioparus* THI115. The complete nucleotide sequence of *scn* gene and the deduced amino acid were presented in Fig. 8a.
Table 1: Bacterial strains and plasmids used in scn cloning

<table>
<thead>
<tr>
<th>Strains or Plasmids</th>
<th>Relevant characteristic(s)*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>THI201</td>
<td>SCNase-producing bacteria</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td></td>
<td>Takara</td>
</tr>
<tr>
<td></td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17(rK– mK+), e14– (mcrA–), supE44, relA1, Δ (lac-proAB)/F[traD36, proAB+, lac I, lacZΔM15]</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC 118 HindIII / BAP</td>
<td>Amp, Cloning vector (HindIII digested, dephosphorylated)</td>
<td>Takara</td>
</tr>
<tr>
<td>pUC118C14</td>
<td>Amp, pUC118 plasmid carrying 6.0 kb DNA fragment of THI201</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Amp, ampicillin.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5′-3′)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>GAYATGWSIAARGARGCICARCY</td>
</tr>
<tr>
<td>R1</td>
<td>GCISWIACIGTIICRTRTRTAY TT</td>
</tr>
<tr>
<td>F02</td>
<td>AGCAAGGAAGGCGAGCAGC AC</td>
</tr>
<tr>
<td>R02</td>
<td>CACGGTGCCGTTGTACTT</td>
</tr>
<tr>
<td>M13R</td>
<td>AACAGCTATGACCATG</td>
</tr>
<tr>
<td>M13RA</td>
<td>AGCACAATATCCAGCGGC</td>
</tr>
<tr>
<td>M13RA1</td>
<td>TCAAGGCGCACAGTAAGGAC</td>
</tr>
<tr>
<td>F02A</td>
<td>CGGCGACTTCAAGATGTTC</td>
</tr>
<tr>
<td>F02B1</td>
<td>ACTTCCCTGCGCTCACAACAA</td>
</tr>
<tr>
<td>F02C</td>
<td>CCAGTACATCCACACGGTCTA</td>
</tr>
<tr>
<td>RF02D1a</td>
<td>GTTTCAAGCGGACGTTGATC</td>
</tr>
<tr>
<td>RF02D</td>
<td>TGCAAGTCCAACGCGATC</td>
</tr>
<tr>
<td>RF02B2</td>
<td>GCAGCGCGAGATACCTTTG</td>
</tr>
<tr>
<td>RF02A1</td>
<td>GAAATCGAACTTCTTGGTCG</td>
</tr>
<tr>
<td>RF02B</td>
<td>GCAGTGATGGCGGAATC</td>
</tr>
<tr>
<td>RM13RA2</td>
<td>TACGTCCTGTCTTCGGAATC</td>
</tr>
<tr>
<td>RM13RA3</td>
<td>TGCTTCACCGCATAGGAG</td>
</tr>
<tr>
<td>RM13RA4</td>
<td>AGAGGAACACTCACATGTC</td>
</tr>
<tr>
<td>IP1</td>
<td>GTGCTGCGCGCCCTCCTTTG</td>
</tr>
<tr>
<td>IP2</td>
<td>AAGTACAACGGACCGT</td>
</tr>
</tbody>
</table>

Table 2: Primers used in sequencing of 6.0 kb DNA insert from *Afipia* sp. strain THI201
Fig. 2. Complete digestion of THI201 DNA by *HindIII*. M1 is marker 2.5 kb ladder, M2 is marker λ DNA/*HindIII* digest, D is the genomic DNA digest of THI201. The position of 6.0 kb DNA fragment was indicated by black box.
Fig. 3a. Agarose gel to check the presence of *scn* gene in the isolated DNA fragment. The PCR was done with the insert DNA (~6.0 kb) as template and F02/R02 as primers. Agarose gel shows 81 bp PCR product (D). Here, M1, M2, N, and P are marker Molecular mass standard, marker φ/HaeIII digest, negative control and positive control, respectively.

Fig. 3b. Agarose gel to check the isolated insert DNA. Here D is the isolated purified DNA fragment (~ 6.0 kb) and M is the marker λ/HindIII digest.
Fig. 4. Colony hybridization with probe. N and P indicate negative and positive control, respectively. The numbers indicate clone numbers that showed bright signals and were checked for presence of the target gene. Only the C14 was found to be positive.
Fig. 5. Agarose gel electrophoresis to check the presence of *scn*. (a) Checking the *scn* in C14, C20, C33 and C65, (b) agarose gel showing *HindIII* digested pUC118C14. Four colonies showing bright signal (Fig. 4.) were checked for presence of 81 bp *scn* by plasmid extraction from these colonies and PCR with the plasmid DNA and F02/R02 primers. Here, M1, M2, P, and N are the marker φ/HaeIII digest (Takara), marker Molecular mass standard (Bio-Rad), positive and negative control, respectively. The recombinant plasmid (RP) pUC118C14 after *HindIII* digestion showed the insert DNA band (~6.0 kb) and plasmid band (3.2 kb). The control (C) is pUC118 plasmid DNA and M is the marker λ/HindIII digest.
Fig. 6. Checking the position of *scn* in recombinant plasmid pUC118C14 by PCR (a) with R02/M13R (D1) and F02/M13F (D2) primer set, (b) with IP1/M13R (S1) and IP2/M13F (S2) primer set. M1, M2, and M3 were the marker λ/HindIII digest, 2.5 kb ladder, and φ/HaeIII digest, respectively. The positive control (P) was done with plasmid pUC118C14 and M13F/M13R primers.
Fig. 7. Map of recombinant pUC118C14 plasmid containing 6.0 kb DNA fragment of *Afipia* sp. strain TH1201. The position of ORF of *scn* was predicted from Fig. 6a and 6b. Here, the primers M13F/M13R were from vector pUC118; F02/R02 and IP1/IP2 were from 81 bp N-terminal sequence of *scn*. The 1.47 kb ORF of *scn* is presented by red line.
Fig. 8a. Nucleotide sequence of *scn* gene of *Afipia* sp. strain THI201. The predicted two Shine-Dalgarno sequences, AGGGGA and AGGCGGA were underlined, the start codons (ATG) and the corresponding amino acids (M) of their related ORF were bolded. The N-terminal amino acid sequence of SCNase was shown by dash-line. The N-terminal amino acid (A) of SCNase and the corresponding codon (GCT) were in bold italics. The significant Tat motif (RRTLL) was indicated by white box and the sequence of Tat signal protein was underlined. An asterisk indicates the termination codon (TAG).
Fig. 8b. A portion of nucleotide and deduced amino acid sequence of the *scn* gene of *Afipia* sp. strain THI201. The positions and sequences of the degenerate oligonucleotides F1 (5′-GAYATGWSNAARGARGCNCARCAY-3′) and R1 (3′-TTYATRTRCCNTGNCANWSNC-5′) were shown. The nucleotide sequences corresponding to those of the oligonucleotides F02 and R02 were indicated as lowercase and lowercase italics, respectively.
Fig. 9. Alignment of SCNase of strain THI201 with uncharacterized proteins of *Thiobacillus* (Thb.) *denitrificans* ATCC 25259, *Thioalkalivibrio* (Ta.) *thiocyanoxidans*, and *Hydrogenobacter* (H.) *thermophilus*. The accession numbers of each amino acid sequence in the NCBI database were as follows: unnamed protein product of *Thb. denitrificans* ATCC 25259, YP_314672.1; hypothetical protein ThithDRAFT_3226 of *Ta. thiocyanoxidans* ARh 4, ZP_08931351.1; and conserved hypothetical protein of *H. thermophilus* TK-6, ADO45410.1. Identical and similar amino acid residues among the four sequences were marked with asterisks and dots, respectively.
Chapter 4

EXPRESSION OF SCN IN E. COLI
EXPRESSION OF SCN IN E. COLI

4-1 Introduction

Escherichia coli is one of the most widely used hosts for the production of heterologous proteins as its genetics are far better characterized than those of any other microorganisms. Recent progress in the fundamental understanding of transcription, translation, and protein folding in E. coli, together with serendipitous discoveries and the availability of improved genetic tools are making this bacterium more valuable than ever for the expression of complex eukaryotic proteins.

Enzymes are proteins that catalyze chemical reactions and maintain the functional integrity of a cell. Microorganisms produce various enzymes to survive as well as adjust in a hostile environment. Purification of a microbial enzyme is a challenging task and can often results in an unstable or inactive product. Moreover, abundant production to characterize the enzyme is not always possible. In this instance, recombinant enzyme is produced to generate desired form of enzyme (abundant production, active form) by using specialized vector. Now a day, protein/peptide tag is fused to recombinant protein to facilitate its purification and also to improve the stability and solubility of enzyme. Selection of a suitable expression system depends on the desired scale of production, the resources available, and the intended use of the recombinant protein. In a protein expression system, three important factors should be considered (Recombinant protein purification handbook, GE Healthcare):

First one is a vector with an appropriate promoter and other regulatory sequences, along with the gene encoding the recombinant protein of interest. Vectors are available commercially for the expression of recombinant proteins either fused to a tag or untagged. Such expression vectors are designed with control regions to suit the specific host (for example, E. coli versus mammalian cells) and type of expression needed. The presence of resistance markers makes selection of the correct clones more straightforward.
Second one is to choose a suitable host. Many host systems are available including bacteria, yeast, plants, filamentous fungi, insect or mammalian cells grown in culture, and transgenic animals or plants. Each host system has its own advantages and disadvantages, and it is important to consider these before final selection of host. The choice of host affects not only the expression of the protein but also the way in which the product can be subsequently purified.

The third one is to select a tag. There are several affinity tags that can be used to simplify protein purification. The most common tag is the histidine tag, then the GST and MBP tags both of which are proteins.

In this study, pGEX-6P-1 vector (Fig. A), containing antibiotic resistance marker and GST (glutathione sepharose transferase) tag; and *E. coli* BL21(DE3) as a host were used. The purification was performed in a batch method by using affinity chromatography. There are several benefits of using GST tag, such as, purification procedure gives high yields, allows extremely high purity in a single purification step and may increase the solubility of the expressed protein. In addition, simple purification and very mild elution conditions minimize risk of damage to structure and function of the target protein.
**Escherichia coli** expression system, with its ability to grow rapidly and high expression level of recombinant proteins is the most ideal system for heterologous protein expression. However, the greatest disadvantage of this expression system is that the over-expression of plasmid-encoded genes triggers transcription of heat-shock genes and other stress responses; and as a result, aggregation of the encoded protein occurs as inclusion bodies (Jürgen *et al.*, 2000; Rinas, 1996). The recovery of biologically active products from the aggregated state is typically accomplished by unfolding with chaotropic agents, followed by dilution into optimized refolding buffers. However, many polypeptides (e.g. structurally complex oligomeric proteins and those containing multiple disulfide bonds) do not easily adopt an
active conformation following chemical denaturation. In such cases, maximizing the yields of recombinant proteins in a soluble and active form, in vivo, becomes an attractive alternative to in vitro refolding (Thomas & Baneyx, 1996). In this study, over-expression of the fusion protein encoded by the expression plasmid pGEX-SCNase was unsuccessful due to the formation of inclusion bodies. The problem was resolved by co-expression of selected chaperone-encoding genes along with the target gene. It is easier and can yield protein with improved folding and enhanced solubility (Georgiou & Valax, 1996; Wall & Plückthun, 1995). There are several chaperone plasmids (Fig. B) which are designed to enable efficient expression of multiple molecular chaperones known to work in cooperation in the folding process. These chaperone plasmids carry an origin of replication derived from pACYC and a chloramphenicol resistance gene (Cm). This arrangement allows their use with *E. coli* expression systems that utilize ColE1-type plasmids containing the ampicillin resistance gene as a marker, which are the most commonly used. The chaperone genes are situated downstream of either the araB or Pzt-l(tet) promoter. Thus, expression of target proteins and chaperones can be induced individually if the target gene is placed under the control of another promoter (e.g. lac).

The most extensively studied chaperones are the chaperonin GroEL and GroES from *E. coli* (Fenton and Horwich, 1997). Two most common strategies to maximize folded protein production in *E. coli* are the use of GroEL-GroES in combination with other folding accessory proteins like Dna K/Dna J (Gragerov et al., 1992); and the use of GroEL-GroES at low culture temperature (Park et al., 2005; Sun et al., 2005). In this study, recombinant protein purification was tried at low culture temperature, with pKJE7, pG-KJE8, or pGro7; and the successful result was found only with pGro7. Several studies have used chaperone-assisted protein purification: one similar study with nitrile hydratase (NHase) NI1 from *Comamonas testosterone* showed heterologous expression of NHase involving coexpression with the *E. coli* GroES and GroEL chaperones (Stevens et al., 2003); and another study with N-carbamoyl-D-amino acid amidohydrolase (D-carbamoylase) gene (*dcb*) from *Agrobacterium tumefaciens* AM 10 showed that coexpression of chaperones GroES and GroEL resulted in an active enzyme production that was 43-times that obtained using the wild-type strain (Sareen et al., 2001).
**Fig. B. Structure of chaperone plasmids** (Catalog, Takara-bio). The plasmids contain antibiotic resistant gene (Cm'or tetR); chaperones dnaK-dnaJ-grpE, groES-groEL, or tig;

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### 4-2 Materials and methods:

#### 4-2-1 Bacterial strains, culture conditions, and plasmids

*E. coli* strains (Table 1) Rosetta-gami B (Novagen) and BL21(DE3) (Takara Bio Inc.) were tried as host for the production of the recombinant SCNase. *E. coli* was grown in Luria-Bertani (LB) medium supplemented with 100 μg ml⁻¹ampicillin and/or 20 μg ml⁻¹ chloramphenicol when needed. Unless otherwise indicated, cells were grown aerobically with reciprocal shaking at 30°C.
Plasmid pGEX-6P-1 (Amersham) was used as expression vector. Plasmid pGEX-SCNase carrying SCNase ORF of THI201 was constructed in this study. Plasmids pG-KJE8, pKJE7, or pGRO7 (Takara Bio Inc.) were used as chaperone plasmids. Detail descriptions are shown in Table 1.

4-2-2 Construction of the expression plasmid

Expression plasmid was constructed with the ORF of SCNase. First, PCR was done with pUC118C14 as template and SCNaseF1/ SCNaseR1 as primer. The amplified DNA fragment was digested with EcoRI and XhoI and inserted between the EcoRI and XhoI sites of the expression vector pGEX-6P-1 (GE Healthcare) to construct the expression plasmid pGEX-SCNase. The detail procedures are described below:

4-2-2-a Preparation of insert DNA

Two oligonucleotides, SCNaseF1 (5′-CGGAATTCTAGAAGGTCGTGCTGTCGGCGATATGAGCAA-3′) and SCNaseR1 (5′-CGGCTCGAGCTACACCGAGGTACTACG-3′) were designed to amplify the DNA fragment encoding 457 amino acids of the SCNase. Nucleotide sequences corresponding to the recognition sites of EcoRI and factor Xa were introduced at the 5′ termini of oligonucleotide SCNaseF1, and sequence corresponding to the recognition site of XhoI was added at the 5′ termini of oligonucleotide SCNaseR1. Plasmid pUC118C14 (5 ng) was used as a template for PCR with SCNaseF1 and SCNaseR1 in a reaction mixture containing Phusion High-Fidelity DNA polymerase (New England Biolabs). Following 2 steps protocol was applied for amplification:

Denaturation 98°C, 30 sec

Denaturation 98°C, 5 sec

Extension 72°C, 30 sec

\[\text{35 cycles}\]

Final extension 72°C, 5 min
4°C, hold

The PCR product was checked by 1.5% agarose gel electrophoresis (Fig. 1). The DNA was purified from the gel by GFX kit (Takara) and the concentration was measured by nanodrop. Then the DNA (2.6 µg) was digested with 25 unit of each restriction enzyme EcoRI and XhoI and again purified with GFX kit. The concentration of the DNA was measured by agarose gel (Fig. 2) using Molecular mass standard (BIO-RAD) with densitogram (ATTO). The purified DNA was used as insert DNA to clone in to the expression vector.

4-2-2-b Preparation of vector DNA

Plasmid pGEX6P1 was used as vector of 1.37 kb insert after double digestion with restriction enzymes. Vector DNA of 2 µg was digested by enzymes Xhol (40U) and EcoRI (40U) in a reaction mixture of 100µL, and incubated at 37°C for 1 hour. The digested vector was then purified with GFX kit and the concentration was measured by nanodrop. The DNA band was checked by agarose gel (1%) electrophoresis (Fig. 3).

4-2-2-c Ligation

DNA ligase mix (mighty kit) was used to ligate vector and insert DNA in 1:3 molar ratio. Vector DNA of 4µL (21.6 ng) and insert DNA of 2.7µL (18 ng) were taken in an eppendorf tube and Ligase mix of 6.7µL was added. Total 13.4µL reaction mixture was incubated at 16°C for 3 hours. The ligated product was used for transformation.

4-2-2-d Transformation of competent cell Rosetta-gami B

Ligated product of 6.7µL was added to 20µL of bacterial cell & stirred gently. The tubes were placed on ice for 5 min, then incubated at 42°C for 30s and again placed on ice for 2 min. The SOC media of 80µL was added and the tube was incubated at 37°C shaker for 60 min. Finally, the cells were spread on LB/amp media. The plate was incubated at 37°C for 42 hours. Control was prepared by transformation of Rosetta-gami B by 0.5 ng of pGEX-6P-1 plasmid.
4-2-2-e Checking the presence of DNA insert in new clones:

The purpose of this experiment was to find out the bacteria which contain 1.37 kb DNA of SCNase. From the transformation plate, 24 colonies were randomly picked and inoculated in to LB/amp broth. After 22 hours incubation at 37°C shaker, plasmid was extracted by autoprep machine (PI-50 α, KURABO). Then the plasmid DNA was digested with 10 unit of XhoI and 10 unit of EcoRI enzyme. RNase A of 1 µl was also added to eliminate the RNA extracted along with plasmid DNA during plasmid extraction with autoprep machine. The reaction mixture was incubated at 37°C for 1 hr; then10 µL of reaction mixture was applied to 0.8% agarose gel to see the presence of vector DNA and DNA insert (Fig.4). From here, P1, P2, and P3 were selected to confirm the presence of scn by PCR with Phusion High-Fidelity DNA polymerase (Fig.5). PCR reaction was done by the 2 step protocol (conditions were described previously (4-1-2a); with the primer set SCNaseF1/SCNaseR1A (designed from the ORF of SCNase) and P1, P2 and P3 plasmid as the template DNA. Because the concentration of template DNA P1, P2, and P3 could not be measured by nanodrop due to presence of RNA, amount of template DNA was selected randomly. Template DNA of both 0.4 µl and 1/10 of the original concentration were used in PCR. Positive control was done with the same primer set and pUC118C14 plasmid DNA (5 ng) as template. Agarose gel of 1.5% was used to check the PCR product. Positive clone was selected by checking the presence of 1.37 kb DNA of SCNase, and the corresponding plasmid from the clone was designated as pGEX-SCNase. The nucleotide sequence of the inserted DNA fragment of pGEX-SCNase was confirmed by sequence analysis with pGEX5/pGEX3 (GE healthcare) primer set.

4-2-3 Purification of recombinant SCNase from Rosetta-gami B

Small scale purification of recombinant protein was done to check the fusion protein band in soluble fraction. The transformed bacteria T3 was grown to mid-exponential phase in 5 ml of LB containing ampicillin. IPTG was added to the culture at the final concentration of 0.1 mM to induce the production of glutathione S-transferase (GST)-fused SCNase. The cultures were incubated at 15°C for an additional 16 h. The cells were harvested by centrifugation (13,000 g, 4°C, 5min), washed twice with 500 µl of ice cold PBS (pH 7.3), and suspended in 300 µl of PBS (pH 7.3) containing 25% (v/v) glycerol. At this stage, the cell pellet can be stored at -20°C or used immediately. Then, PMSF,
DTT, and lysozyme at the final concentrations of 1 mM, 10 mM, and 1 mg ml⁻¹, respectively, were added, and cells were disrupted by ultra-sonication (Model VP-5, Taitec Inc., at 25 watts) for a total of 3 min on ice. The cell suspension was centrifuged at 10,000 g at 4°C for 15 min to obtain the supernatant (300 µl) as crude cell extract. The crude cell extract was incubated with 20 µl of 50% suspension of glutathione Sepharose 4B (GS4B) beads (GE Healthcare) on a rotating wheel at room temperature for 30 min. The GS4B beads were pre-equilibrated with PBS containing 25% (v/v) glycerol. The crude cell extract was washed with 100 µl of the same buffer after incubation with GS4B beads. The washed beads were then suspended in 10 µl of PBS containing 25% (v/v) glycerol and treated with 0.1 U µl⁻¹ factor Xa (Novagen) at room temperature for 2 h. After centrifugation at 2,100 rpm, 4°C, 5 min, the supernatant was collected as purified protein solution. Purification of proteins at each step was examined by 12% SDS-PAGE. The amount of protein was determined by the method of Lowry et al. (Lowry et al., 1951).

4-2-4 Chaperone assisted purification of recombinant SCNase from BL21(DE3)

Fusion protein was found as inclusion body rather than soluble protein during purification from Rosetta-gami B. Therefore, *E. coli* BL21(DE3) carrying chaperone plasmid was utilized for purification of recombinant SCNase. BL21(DE3), which is sensitive to chloramphenicol, was selected as host because both Rosetta-gami B strain and chaperone plasmids contain chloramphenicol resistant gene (Cm') and the screening of positive clone was not possible from Rosetta-gami B by using the selective media containing chloramphenicol.

Hence, BL21(DE3) was transformed with pGEX-SCNase or pGEX-6P-1 plasmid (negative control) together with one of the chaperone plasmids pG-KJE8, pKJE7, or pGRO7 (Takara Bio Inc.) by following methods:

4-2-4-a Transformation of BL21(DE3) with chaperone plasmid

First, competent cells of BL21(DE3) were prepared by treatment with 100 mM CaCl₂. Cells were grown up to OD 0.4 and cooled on ice for 10 min. Cells were harvested by centrifugation and treated with 100 mM chilled CaCl₂. The
mixture was put on ice for 30 min, and then centrifuged. The supernatant was discarded and 50 µl of 100 mM CaCl$_2$ was added and mixed gently. The cell preparation was kept on ice and used for transformation within short time.

Transformation reaction was performed with 50 µl of competent BL21(DE3) and plasmid DNA (10 ng) of pG-KJE8, pKJE7, or pGRO7. The bacterium and the plasmid DNA was mixed gently and put on ice for 30 min. The mixture was incubated at 42°C for 2 min and cooled on ice for 2 min. LB medium of 500 µl was added and incubated at 37°C for 1 hour. The cells were spread on LB agar plate containing chloramphenicol (LB/Chl). Colony count was performed on next day after incubation of the plate at 37°C. Two negative controls were done; one was inoculation of the bacterium without any plasmid (control 1) into LB/chl, and the other was inoculation of bacterium without plasmid into LB without chl (control 2).

Plasmid extraction from the transformants was done by Promega kit and plasmid DNA band was checked by 0.8% agarose gel electrophoresis. Three transformants containing pG-KJE8, pKJE7, or pGRO7 plasmid were selected and designated as BLKJE8, BLKJE7 and BLGro7. Glycerol stock of each transformant was also prepared and stored at -80°C for future use.

4-2-4-b Transformation of BLKJE8, BLKJE7 and BLGro7 with pGEX-SCNase

Competent cells of BLKJE8, BLKJE7 and BLGro7 were prepared and transformed with pGEX-SCNase plasmid by following the procedure described in 3-1-4-a section. Again, plasmids were extracted by Promega kit and 0.8% agarose gel electrophoresis was performed to confirm the presence of both chaperone plasmids and pGEX-SCNase plasmid. Three transformants carrying three different chaperones together with pGEX-SCNase were selected and designated as BLKJE8-SCNase, BLKJE7-SCNase and BLGro7-SCNase. Two negative controls were prepared with BL21(DE3) containing pGEX-SCNase (C1) and BL21(DE3) containing pGEX6P1 (C2) to confirm that without chaperone, the fusion protein was found as inclusion body (C1) and the purification procedure was satisfactory (C2).
4-2-4-c Recombinant protein purification

Purification of GST-fused SCNase was tried with BLKJE8-SCNase, BLKJE7-SCNase and BLGro7-SCNase in small scale preparation. Only BLGro7-SCNase was found to produce soluble fusion protein and used for large scale protein purification.

The transformed bacterium BLGro7-SCNase was grown to mid-log phase in 100 ml of LB medium containing ampicillin and chloramphenicol. IPTG and arabinose were added to the culture at the final concentrations of 0.1 mM and 1.7 mM, respectively, to induce the production of glutathione S-transferase (GST)-fused SCNase and chaperone proteins, respectively. The culture was incubated at 15°C for an additional 16 h. The cells were harvested by centrifugation, washed with PBS (pH 7.3), and suspended in 10 ml of PBS (pH 7.3) containing 25% (v/v) glycerol. After the addition of PMSF, DTT, and lysozyme at the final concentrations of 1 mM, 10 mM, and 1 mg ml⁻¹, respectively, cells were disrupted by ultra-sonication for a total of 3 min on ice, and the suspension was centrifuged at 10,000 g at 4°C for 15 min to obtain the supernatant (11.1 ml) as crude cell extract. The crude cell extract was incubated with 1 ml of 50% suspension of glutathione Sepharose 4B (GS4B) beads on a rotating wheel at room temperature for 30 min. The GS4B beads were pre-equilibrated with PBS containing 25% (v/v) glycerol, and washed with the same buffer after incubation with crude cell extract. To remove chaperone proteins, the beads were incubated in PBS containing 10 mM ATP magnesium salt (Mg-ATP; Sigma-Aldrich) and 5 mg ml⁻¹ casein-sodium (Tokyo Chemical Industry Co.) for 30 min, and then washed twice with the same PBS buffer. The washed beads were then suspended in 500 µl of PBS containing 25% (v/v) glycerol and treated with 0.1 U µl⁻¹ factor Xa at room temperature for 2 h. After centrifugation, the supernatant was treated with Xarrest Agarose (Novagen) according to the manufacturer’s instructions to remove factor Xa. Purification of proteins at each step was examined by 12% SDS-PAGE. The amount of protein was determined by the method of Lowry et al. (Lowry et al., 1951).
4-2-5 Measurement of recombinant enzyme activity

The enzyme assay of recombinant SCNase was performed by measuring the product, NH$_3$. The standard reaction mixture for the assay of recombinant SCNase contained 40 mM potassium thiocyanate, 100 mM potassium phosphate buffer, pH 6.0, and purified enzyme (0.9 - 1.65 µg as protein) in final volume of 50 µl. The mixture was then incubated at 30°C for 10 min. An aliquot of the mixture (40 µl) was mixed with Nessler’s reagent (60 µl) and DW (3 ml) in a test tube. The tube was kept at room temperature for 15 min. The amount of ammonia formed by SCNase was quantified by measuring the absorbance at 420 nm. The specific activity of SCNase was calculated as µmol of NH$_3$ produced min$^{-1}$ mg$^{-1}$ protein.

4-3 Results and discussion:

4-3-1 Construction of the expression plasmid

The $scn$ was amplified by PCR with SCNaseF1/SCNaseR1 primer set and pUC118C14 plasmid as template. The PCR product was purified from agarose gel and the concentration was found to be 66.2 ng/µl. The purified DNA was digested with EcoRI and XhoI restriction enzymes, then again purified with GFX kit. The concentration of the DNA was measured to be 6.7 ng/µL and used as insert DNA to clone in to the expression vector. Plasmid pGEX6P1 was used as vector of 1.37 kb DNA insert and digested with the same enzymes. The double-digested vector was checked by agarose gel and 4.98 kb DNA band was found. Consequently, it was purified with GFX kit and the conc. was found to be 5.4 ng/µL. After ligation of insert and vector DNA, $E. coli$ Rosetta-gami B was transformed with the ligated DNA. Total 184 colonies appeared on the LB/amp plate. Control of Rosetta-gami B containing pGEX-6P-1 produced colonies TNTC (too numerous to count) which indicated that transformation procedure was perfect. From the transformed bacteria, 24 colonies were randomly selected for plasmid extraction. The extracted plasmids were checked for presence of insert DNA after digestion with EcoRI and XhoI. Fig. 4 shows the results obtained from 13
recombinant plasmids. Though vector DNA band of 4.98 kb was present in all 24 isolates, insert DNA was found in 19 isolates. In all isolates, two plasmid bands appeared in agarose gel, the top one was the digested one, and the lower one might be undigested plasmid. In insert DNA, also two bands appeared, the concentrated one was the target DNA, and the other one was unknown. From here, P1, P2 & P3 were selected to confirm the presence of *scn* by PCR with SCNaseF1/SCNaseR1 primer set. (Fig.5). P1 and P3 showed 1.37 kb band as similar as the positive control, but P2 showed a lighter band in P2a. Negative control without any template DNA showed no band. Sequence analysis was done with pGEX5/pGEX3 primer set and P1, P2, or P3 as template DNA. The *scn* was found in P1 and P3. Thus, P1 or P3 was constructed as an expression plasmid named pGEX-SCNase, which encoded a GST-fused SCNase (GST-SCNase) of *Afipia* sp. strain THI201 with the recognition sequence for the protease factor Xa (IEGR), and expressed in *E. coli* Rosetta-gami B.

4-3-2 Purification of recombinant SCNase from Rosetta-gami B

Small scale purification of GST-SCNase from Rosetta-gami B (Fig. 6) showed that IPTG induced the fusion protein production (lane 2). After sonication, presence of fusion protein was checked in both crude extract and pellet; but, the protein was mostly found as inclusion body in pellet (lane 3) instead of soluble protein in crude extract (lane 4). After incubation of crude extract with GS4B beads, beads were washed with buffer to remove the bead-unassociated proteins, but no protein bands were found in lane 5, the reason was unknown. The bead-associated proteins were checked in lane 6, GST-SCNase band was absent. Factor Xa treatment was done to separate GST and SCNase, but as the fusion protein was absent, SCNase band was not found in lane 7. Therefore, from this experiment it could be concluded that the recombinant GST-SCNase was found mostly in inclusion bodies and, as a result, could not be purified. Attempts were taken to optimize the conditions for the production of soluble protein by changing the IPTG concentration, induction time, and temperature. SDS-PAGE with the crude extracts prepared from *E. coli* cells induced with IPTG of 0.01, 0.05, 0.1, 0.5, 1.0, and 5.0 mM was performed to determine the optimum IPTG concentration for soluble GST-SCNase (Fig. 7). Though the fusion protein could be produced with any of the concentration of IPTG, none of them tested could improve the recovery of the fusion protein in the soluble fraction.
Similar results were found when variable induction time (8, 12, 16, and 24 hours) and incubation temperature (10, 15, 20, and 25°C) were tried with IPTG. SDS-PAGE analysis showed the presence of the fusion protein band in both the soluble and insoluble fractions, but mostly in the latter, and a faint band of fusion protein appeared after the protein was concentrated with GS4B beads (result not shown).

4-3-3 Protein purification from two controls

SDS-PAGE analysis was performed with the purified proteins from BL21(DE3) containing pGEX-SCNase (C1) and BL21(DE3) containing pGEX6P1 (C2) as two controls (Fig. 8). Comparison between lane 1 and lane 3 clearly showed that larger amount of fusion protein was found as insoluble protein. The GST-SCNase band was absent in both bead-unassociated and bead-associated proteins (lane 5 and 7). These results again confirmed that the GST-SCNase was found as inclusion bodies and could not be purified from crude extract by GS4B beads. Therefore, recovery of fusion protein was tried by co-expression of chaperone proteins together with GST-SCNase.

On the other hand, proteins purified from C2 showed that GST was present in crude extract (lane 2), though also found as insoluble protein (lane 4); it was absent in bead-unassociated proteins (lane 6); could be purified abundantly by GS4B beads and found as bead-associated protein (lane 8). These results indicated that the purification procedures for GST-fused protein by GS4B beads were working well except some fade bands those appeared along with the GST band in lane 8.

4-3-4 Chaperone assisted purification of recombinant SCNase from BL21(DE3)

To improve the recovery of the fusion protein in the soluble fraction, plasmids pG-KJE8, pKJE7, or pGRO7 were separately co-introduced with pGEX-SCNase into E. coli strain BL21(DE3).
Transformation of BL21(DE3) with pG-KJE8, pKJE7, or pGRO7 plasmid produced colonies of TNTC. In control 1, no colonies were found, which indicated the presence of antibiotic on the media that inhibited the growth of ampicillin sensitive *E. coli*. In control 2, colonies of TNTC appeared, and it indicated that the competent cells of *E. coli* were healthy. Two isolates from each transformation done with pG-KJE8, pKJE7, or pGRO7 plasmid were randomly selected and checked for presence of chaperone plasmids by agarose gel (result not presented). Three transformants named BLKJE8, BLKJE7 and BLGro7 were selected for transformation with plasmid pGEX-SCNase. The number of colonies found was TNTC, 185, and TNTC from BLKJE8, BLKJE7 and BLGro7 respectively. Two transformants were randomly selected from each plate and checked for presence of both chaperone plasmids and pGEX-SCNase, after plasmid extraction and agarose gel electrophoresis. Fig. 9 shows that in BLKJE7-SCNase and BLGro7-SCNase, the chaperone plasmid and the plasmid pGEX-SCNase (pGEX6P1 of 4.98 kb + SCNase of 1.47 kb = 6.45 kb) appeared in both transformant 1 and 2. In BLKJE8-SCNase, both chaperone and pGEX-SCNaseplasmid bands appeared in same position and it made the result unclear. Though the chaperone plasmids pKJE7, pG-KJE8, and pGRO7 are 7.2, 11.1, and 5.4 kb in size, respectively, but they appeared as smaller size in the agarose gel due to their closed, circular form.

First, the GST-fused SCNase was tried to be purified from recombinant bacteria BLKJE7-SCNase (Fig. 10). Though sonicate and crude extract (lane 1 and 2) exhibited the band similar to that of fusion protein, it was actually the chaperone dnaK (69 kDa). The identity of the protein band as dnaK instead of fusion protein was confirmed when it was also found in GS4B bead-unassociated proteins (lane 4), as the fusion protein binds to GS4B beads and does not appear as bead-unassociated proteins. The fusion protein might be present as insoluble protein in pellet (lane 3). Both the GS4B bead-unassociated proteins (lane 5) and bead-associated proteins (lane 6) were treated with factor Xa, so that if the GST-SCNase was present in either place, it would be separated by factor Xa, and SCNase band would appear. Here, no SCNase band appeared; and it indicated that the fusion protein was found as inclusion bodies even in presence of chaperone. Thus, the pKJE7 was unable to turn the insoluble protein to soluble form. In this figure, the factor Xa bands found in lane 6 and 7 were remarkable; because, factor Xa usually shows 2 bands of 34 kDa and 29 kDa instead of several bands presented here. This might be due to use of old and contaminated factor Xa. Hence,
recombinant protein purification was done from BLKJE8-SCNase and BLGro7-SCNase by using newly purchased factor Xa.

However, protein purification from BLKJE8-SCNase could not produce soluble GST-SCNase (result not shown). Only BLGro7-SCNase produced the fusion protein abundantly in the soluble fraction and was used to purify the recombinant SCNase (Fig. 11). The crude extract prepared from BLGro7-SCNase contained GST-SCNase, and the chaperone proteins GroEL, and GroES (lane 1). GST-SCNase, GroEL and GroES were isolated by GS4B bead-based affinity column chromatography (lane 2). Treatment with Mg-ATP and casein sodium eliminated almost all the chaperone proteins (lane 3). Digestion with factor Xa resulted in the separation of GST-SCNase into GST and SCNase (lane 4). The final SCNase preparation was obtained by treating the GS4B bead-unbound fraction with Xarrest agarose to remove factor Xa. Thus, 25-55 µg of purified recombinant protein was obtained from 100 ml of *E. coli* culture and only a trace amount of GroEL remained in the final preparations observed by SDS-PAGE (lane 5). The effect of the remaining chaperone protein on SCNase activity was checked by comparing two purified recombinant protein samples containing SCNase and GroEL in ratios of 1:1 (Fig. 12) and 6:1 (Fig. 11). Both the samples showed similar enzyme activity as well as similar properties such as optimum temperature, optimum pH, and thermostability. Therefore, it was assumed that the coexistence of a trace amount of GroEL had little or no influence on the function of SCNase *in vitro*. Though there are several other methods that could be used to remove the chaperone protein GroEL, the fastest method was selected to avoid loss of enzyme activity. In parallel, protein purification from negative control was done with BL21(DE3) cultures containing plasmids pGEX-6P-1 and pGRO7 (Fig. 13). GST and chaperone proteins expressed after induction with IPTG and arabinose, respectively (lane 2). Though GST was found in both soluble and insoluble fractions (lane 4 and 5), and all the GST could not bind with GS4B beads and some portion also found as GS4B-beads unassociated proteins (lane 6), abundant production of GST was successful after incubation with GS4B beads (lane 8). The GroES (10 kda) band appeared as different size in gel image. The fusion protein band was absent in protein preparations from this vector-only control culture and it confirmed that the only possible source of thiocyanate degradation was the gene encoded on the recombinant plasmid.
4-3-5 Measurement of enzyme activity:

Standard curve of NH₄Cl (Fig.14) was prepared with 0-0.15 µmole of NH₃ and used to calculate the NH₃ produced by SCNase. The NH₃ production was measured in the whole cell extracts of THI201 and in the recombinant enzyme prepared from *E. coli* harboring pGEX-SCNase and pGRO7. The enzyme activities of the native enzyme in the whole cell extract and the recombinant purified enzyme were 2.1 µmol min⁻¹ mg⁻¹ of total protein and 4-15 µmol min⁻¹ mg⁻¹ of protein, respectively.
Table 1. Bacterial strains and plasmids used in production of recombinant SCNase

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristic(s)*</th>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>Rosetta gami B……….F⁻, *ompT, hsdS_B (r_B⁻ m_B⁻), gal, dcm, lacY1, ahpC gor522::Tn10, trxB, pRARE (Cmᵀ, Kanᵀ, Tetᵀ)</td>
<td></td>
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<tr>
<td>BL21(DE3)……………..F⁻, *ompT, hsdS_B (r_B⁻ m_B⁻), gal, dcm</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pUC118C14</td>
<td>Ampᵀ, pUC118 carrying 6.0 kb DNA fragment of THI201</td>
</tr>
<tr>
<td>pGEX-6P-1</td>
<td>Ampᵀ, expression vector</td>
</tr>
<tr>
<td>pGEX-SCNase</td>
<td>Ampᵀ, GEX-6P-1 carrying <em>scn</em> ORF of THI201</td>
</tr>
<tr>
<td>pG-KJE8</td>
<td>Cmᵀ</td>
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<tr>
<td></td>
<td>Chaperone: dnaK-dnaJ-grpE, groES-groEL</td>
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<td></td>
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<td></td>
<td>Inducer: L-arabinose, tetracycline</td>
</tr>
<tr>
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<td>Cmᵀ</td>
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<td>Inducer: L-arabinose</td>
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*Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; Tet, tetracycline.
Fig. 1. Agarose gel electrophoresis to check and purify 1.37 kb DNA insert.

Insert DNA was prepared by PCR with SCNaseF1/SCNaseR1A primer set and pUC118C14 as template DNA. Here, M is the marker φ/HaeIII digest. The amplified DNA was cut and purified from gel by GFX kit.
Fig. 2. Concentration measurement of purified DNA. The concentration of double-digested, purified, insert DNA, was measured by comparing the DNA band intensity with marker DNA, using the densitogram. M1 is the marker φ/HaeIII digest and M2 is the marker Molecular mass standard. M2 was used to calculate the concentration of 1.37 kb digested DNA (DD). Undigested DNA (UD) was also run as control.
Fig. 3. Checking the vector DNA (D) after digestion with Xhol + EcoRI. Here M is the marker 2.5 kb ladder. A single DNA band confirmed that the vector DNA was not contaminated with DNase.
Fig. 4. Agarose gel to check the presence of 1.37 kb DNA insert. Plasmid DNA extracted from 24 transformants (P1-P24) were digested by enzymes XhoI and EcoRI. The double-digested DNA was applied to agarose gel to select the transformed bacteria containing vector DNA (4.98 kb) and DNA insert (1.37 kb). Plasmids from 13 isolates were shown. M1 is marker 2.5 kb marker and M2 is marker φ/HaeIII digest.
Fig. 5. Agarose gel to confirm the presence of 1.37 kb DNA of SCNase in recombinant plasmids P1, P2 and P3. Plasmids P1, P2, and P3 isolated from T1, T2 & T3 transformants respectively, were used as template DNA to confirm the presence of *scn* by PCR with the primer set SCNaseF1/SCNaseR1 (designed from the ORF of SCNase). Here M, P, and N represent the marker φ/HaeIII digest, positive and negative control respectively. “a” after each numbering means the PCR was done with template DNA diluted 1/10 of original concentration.
Fig. 6. Purification steps of recombinant SCNase from E. coli Rosetta-gami B harboring pGEX-SCNase. Lane 1, protein production before IPTG addition; lane 2, protein production by IPTG induction; lane 3, insoluble proteins found in pellet; lane 4, crude extract; lane 5, buffer wash; lane 6, GS4B bead-associated proteins, lane 7, GS4B bead-unassociated proteins after the beads were treated with factor Xa; lane 8, Molecular mass marker (kDa). Fusion protein of GST-SCNase (FP) of 76 kDa was found in lane 2, 3, and 4; but SCNase band was absent after final purification step (lane 7).
Crude cell extract was prepared with *E. coli* Rosetta-gami B containing IPTG of 0.01 (C1), 0.05 (C2), 0.1 (C3), 0.5 (C4), 1.0 (C5), and 5.0 (C6) mM to check the differences in soluble fusion protein. Fusion protein band (FP) of almost similar intensity was found in all crude extract. M is a molecular mass marker (kDa) and IP is insoluble proteins found in cell pellet of Rosetta-gami B, where fusion protein induction was done with 5.0 mM IPTG.
Fig. 8. SDS-PAGE showing purified proteins from BL21(DE3) containing pGEX-SCNase (C1) and BL21(DE3) containing pGEX6P1 (C2). Lane 1, crude extract from C1; lane 2, crude extract from C2; lane 3, insoluble proteins from C1; lane 4, insoluble proteins from C2; lane 5, GS4B bead-unassociated proteins from C1; lane 6, GS4B bead-unassociated proteins from C2; lane 7, GS4B bead-associated proteins from C1; lane 8, GS4B bead-associated proteins from C2; lane 9, molecular mass marker (kDa). Fusion protein (FP) of ~76 kDa was mostly found as insoluble form in C1 (lane 3) instead of as soluble protein; and as a result, no FP band was seen in lane 7. In C2, GST band (~26 kDa) was present in lane 8.
Fig. 9. Agarose gel electrophoresis to check the presence of chaperone plasmid and the plasmid pGEX-SCNase (6.45 kb), extracted from transformant 1 and 2. C1, C2, and C3 were the chaperone plasmids pKJE7, pG-KJE8, and pGRO7, respectively, used as control. M was the marker λ DNA/HindIII digest. In BLKJE7-SCNase and BLGro7-SCNase, both plasmids were present. But, in BLKJE8-SCNase, chaperone plasmid band appeared in the same position of pGEX-SCNase, and the presence of two plasmids could not be identified.
Fig. 10. Recombinant SCNase purification steps from *E. coli* BLKJE7-SCNase.

Lane 1, sonicate; lane 2, soluble proteins in crude extract; lane 3, insoluble proteins in pellet; lane 4, GS4B bead-unassociated proteins; lane 5, GS4B bead-unassociated proteins treated with factor Xa; lane 6, GS4B bead-associated proteins treated with factor Xa; lane 7, GS4B bead-unassociated proteins treated with factor Xa, lane 8, molecular mass marker (kDa). Fusion protein (FP) was present in insoluble fraction (lane 3) and could not be purified by GS4B beads. As a result, no SCNase band was found after factor Xa treatment (lane 6).
Fig. 11. SDS-PAGE analysis of recombinant SCNase of *Afipia* sp. strain THI201 at each step of purification. Lane 1, Crude cell extract prepared from *E. coli* harboring pGEX-SCNase and pGRO7; lane 2, proteins associated with glutathione sepharose 4B (GS4B) beads after the incubation of the crude cell extract with the beads; lane 3, GS4B bead-associated proteins after the beads were treated with Mg-ATP and casein sodium; lane 4, GS4B bead-associated proteins and unassociated proteins after the beads were treated with factor Xa; lane 5, final purified proteins after removal of factor Xa from the GS4B bead-unassociated proteins with the use of Xarrest Agarose. Positions of molecular mass markers (kDa) were shown on the left. Arrows on the right indicate the positions of GST-SCNase (A), GroEL (B), SCNase (C), factor Xa (D), GST (E), and GroES (F), respectively.
Fig. 12. SDS-PAGE analysis of recombinant SCNase from BLGro7 containing pGEX-SCNase, at each step of purification. Lane 1, Crude cell extract; lane 2, proteins associated with GS4B beads after the incubation of the crude cell extract with the beads; lane 3, GS4B bead-associated proteins and unassociated proteins after the beads were treated with factor Xa; lane 4, final purified proteins after removal of factor Xa from the GS4B bead-unassociated proteins with the use of Xarrest Agarose.; lane 5, molecular mass marker (kDa). Arrows on the right indicate the positions of GST-SCNase (A), GroEL (B), SCNase (C), factor Xa (D), GST (E), respectively. Positions of molecular mass markers (kDa) were also shown on the right.
Fig. 13. SDS-PAGE analysis of proteins purified from BL21(DE3) cultures containing plasmids pGEX-6P-1 and pGRO7. Lane 1, Proteins before induction with IPTG and arabinose; lane 2, proteins after induction with IPTG and arabinose; lane 3, sonicate after ultra-sonication; lane 4, crude extract; lane 5, insoluble proteins in pellet; lane 6, GS4B- beads unassociated proteins; lane 7, proteins washed in PBS buffer containing 25% (v/v) glycerol; lane 8, GS4B-beads associated proteins after the incubation of the crude cell extract with the beads. Positions of molecular mass markers (kDa) were shown on the left. Arrows on the right shows the positions of chaperones GroEL (60 kDa), and GroES (10 kDa), and GST (26 kDa) protein. GST protein was purified abundantly by GS4B beads in presence of chaperone proteins.
Fig 14. Standard curve of ammonia. Ammonia of different concentrations was reacted with 60 µl of Nessler’s reagent in a final volume of 3.1 ml. The absorption at 420 nm of the mixture was measured after incubation at room temperature for 15 min.
Chapter 5

CHARACTERIZATION OF RECOMBINANT SCNASE ENZYME OF

AFIPIA SP. STRAIN THI201
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CHARACTERIZATION OF RECOMBINANT SCNASE ENZYME OF *AFIPIA* SP. STRAIN THI201

5-1 Introduction

SCNase was initially purified from *Afipia* sp. strain THI201. However, the amount purified from the cells of THI201 was insufficient (0.1 µg) as well as the activity was quite instable. The specific activity was also low (0.32 µmol min\(^{-1}\) mg\(^{-1}\) of protein) compared to recombinant SCNase (4-15 µmol min\(^{-1}\) mg\(^{-1}\)). As a result, further study with the purified fraction was not possible. Cloning and expression of *scn* in *E. coli* produced the enzyme abundantly though unstability was still the same. Purification and characterization of enzyme again became challenging. Different recombinant enzyme preparations purified immediately before each experiment had to be used to achieve the maximum activity, however, these exhibited differences in degree of purification on SDS-PAGE.

According to the result of DNA sequence of SCNase, it is a novel enzyme; the sequence did not match with previously isolated SCNases, NHases or any other known enzymes. Therefore it was necessary to characterize this enzyme to reveal its enzymatic properties and compare it with previously isolated SCNases. Furthermore, optimum conditions will help to handle and utilize the enzyme more efficiently, with highest activity. Thus the known properties will ease the determination of unknown characterization; such as, for consisting heat reactivation property, the purified enzyme can be stored at low temperature and activated just before use. The purest fraction of the recombinant SCNase from each preparation was used to characterize it on the basis of optimal temperature, optimal pH, substrate concentration dependence, dose dependence, time course of ammonia formation, and thermostability. The uncommon properties of cold inactivation and heat reactivation were also observed during characterization.
5-2 Materials and Methods

5-2-1 Preparation of recombinant SCNase:
Recombinant SCNase was prepared just before the each experiment to obtain maximum activity of enzyme. The crude cell extract of *E. coli* carrying pGEX-SCNase and pGro7 was prepared by ultra-sonication. The crude cell extract was incubated with 1 ml of 50% suspension of GS4B beads and washed with PBS buffer containing 25% glycerol. Chaperone proteins were removed by treatment of crude extract with 10 mM ATP magnesium salt and 5 mg ml⁻¹ casein-sodium, and then washed twice with the same PBS buffer. The washed beads were then suspended in 500 µl of PBS containing 25% (v/v) glycerol and treated with 0.1 U µl⁻¹ factor Xa at room temperature for 2 h. After centrifugation, the supernatant (500 µl) was treated with 50% Xarrest Agarose in PBS (300 µl) according to the manufacturer’s instructions (Novagen) to remove factor Xa. Thus final purified enzyme of 650 µl was obtained and 20 µl of the enzyme was used in each assay.

5-2-2 Preparation of assay mixture:
The standard reaction mixture for the assay of recombinant SCNase contained 40 mM potassium thiocyanate, 100 mM potassium phosphate buffer, pH 6.0, and purified enzyme (0.9 - 1.65 µg as protein) in final volume of 50 µl. The mixture was then incubated at 30ºC for 10 min. An aliquot of the mixture (40 µl) was mixed with Nessler’s reagent (60 µl) and DW (3 ml) in a test tube. The tube was kept at room temperature for 15 min. The amount of ammonia formed by SCNase was quantified by measuring the absorbance at 420 nm. The specific activity of SCNase was calculated as µmol of NH₃ produced min⁻¹ mg⁻¹ protein. The negative control without SCNase was checked in each experiment to ensure no degradation of thiocyanate.
5-2-3 Determination of optimal temperature

The reaction mixture (50 μl) containing 100 mM phosphate buffer, pH 6.0, 40 mM KSCN, and purified enzyme of 900 ng was incubated in 1.5 ml eppendorf tube for 10 min by varying the incubation temperature of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100°C. The production of ammonia was measured by Nessler’s reagent.

5-2-4 Determination of optimal pH

The pH optimum was determined by using purified enzyme of 1.65 μg, 40 mM KSCN and 100 mM potassium phosphate buffer of pH 4-9 to adjust the final pH of the assay mixture to 5-8. Because the purified recombinant enzyme was dissolved in PBS of pH 7.3, and 20 µl of enzyme was used in each assay, the final pH of reaction mixture had to be adjusted prior to each assay. Thus the assay mixtures of pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0 were prepared for measurement of enzyme activity.

5-2-5 Substrate dependence of thiocyanate on the enzyme activity

The substrate concentration dependence of enzyme activity was studied in an assay mixture of 50 µl containing 1.0 µg of purified enzyme, 100 mM phosphate buffer, pH 6.0 and KSCN varying the concentration over the range of 10-60 mM (10, 20, 30, 40, 50, and 60 mM).

5-2-6 Dose dependence of the enzyme

Dose dependence of the enzyme was quantified by changing the amount of enzyme in the range of 0.2-1.4 μg as protein in a 50 µl of reaction mixture containing 40 mM of KSCN and 100 mM phosphate buffer, pH 6.0.
5-2-7 Time course of the reaction

Time course of the reaction was examined by measuring the ammonia, in a reaction mixture containing 1.5 µg of enzyme, 40 mM KSCN and 100 mM phosphate buffer, pH 6.0, in a final volume of 50 µl. NH₃ was measured by Nessler’s reagent at time intervals of 5 min, up to 25 min.

5-2-8 Determination of thermostability of enzyme

Thermostability was checked by heating 1.17 µg of purified enzymes at 30, 40, 50, 60, 70, 80, 90, and 100°C for 5 min before the assay. Change in the activity after heating was estimated in comparison with the original activity.

Heat activation of enzyme stored at −20°C for 1 week was assessed by heating the enzyme at 60°C for 15 min and examining the residual activity. Thermo tolerance was also checked in crude cell extract of *Afipia* sp. strain THI201 by heating at 60°C, 70°C, or 80°C for 5 min prior to enzyme assay.

5-3 Results

The recombinant SCNase showed the activity at a broad range of temperatures from 10°C–100°C with the highest activity of 5 µmol min⁻¹ mg⁻¹ of protein at 30°C (Fig. 1a). High activity was also found when heated at 60 and 70°C, it was 88 and 92% of original activity respectively.

SCNase activity, as measured by NH₃ production, was observed in the pH range 5–8, and the highest activity of 5.4 µmol min⁻¹ mg⁻¹ of protein was found in the pH range 6.0–6.5 (Fig. 1b).

Analysis of the substrate concentration dependence of enzyme activity showed maximum activity of 0.18-0.22 µmol/µg at 20–40 mM thiocyanate (Fig. 2a). A distinct inhibition of the enzyme activity by the thiocyanate was observed at 50 mM or greater.
The reaction rate of SCNase was estimated based on the formation of ammonia, was proportional to the amounts of SCNase up to 0.8 µg of protein (Fig. 2b), this result indicated that the 40 mM substrate became saturated with 0.8 µg of purified enzyme.

The reaction rate of ammonia formation was linear with time up to 15 min (Fig. 2c). Though the highest ammonia production was 0.055 µmol/µg after 15 min, the rate became constant soon after that. The highest enzyme reaction persists during 10 min and therefore incubation time of 10 min was followed during enzyme assay.

The experiment of thermostability showed that the enzyme activity of recombinant SCNase was retained after heat treatment at temperatures up to 70°C but decreased by heating at 80°C for 5 min (Fig. 2d). Enzyme activity was absent in no-enzyme control at both 70 and 80°C. Similar thermostolerance was also observed for native SCNase. Heating the crude cell extract at 60°C, 70°C, or 80°C for 5 min resulted in SCNase activity of 3.2, 3.5, or 4.5 µmol min⁻¹ mg⁻¹ of protein, respectively, whereas the control without heat treatment showed activity of 2.1 µmol min⁻¹ mg⁻¹.

Recombinant SCNase showed unexpected property of heat tolerance and heat reactivation after storage in a cold environment. Incubating recombinant SCNase on ice for 3 h reduced the enzyme activity to 2% of the original activity, and subsequent heating at 60°C for 15 min returned the activity to the original level. Furthermore, the enzyme activity decreased to 0% when the enzyme was stored at −20°C for 1 week, and 72% of the original activity was recovered by the heat treatment of 60°C for 15 min. Purification of the recombinant protein was initially performed at 4°C; but after 1 h incubation, the enzyme activity decreased to 63% of the original, and later it was found that the activity of the SCNase purified at room temperature was 1.4 times that of the enzyme purified at 4°C.
5-4 Discussion

Thiocyanate hydrolase of THI201 showed wide temperature range, 50% activity was found even at 100°C. Though in Fig.1a highest activity was found at 30°C, experiment with different enzyme preparation exhibited highest activity at 65 and 70°C, and it was concluded that optimal temperature was in the range 30-70°C.

Considering the favorable temperature of 30°C for the growth of strain THI201, enzyme assays for SCNase were carried out at 30°C. On the other hand, when the activity was measured at 70°C, surprisingly it was about 1.3 times that at 30°C. This result indicates that the mesophilic bacterium *Afipia* sp. strain THI201 contains a thermostable SCNase enzyme. Incubation of the enzyme protein at high temperature might initiate the conformational changes in the SCNase that accelerated the enzyme activity. Further studies such as x-ray crystallography of the purified protein may resolve the reason behind the heat tolerance. Similar studies of thermostable enzymes of mesophilic bacteria have been conducted for trithionate hydrolase of *Acidithiobacillus acidophilus* and sulfur oxygenase reductase of *Halothiobacillus neapolitanus* (Meulenberg et al., 1992; Veith et al., 2012).

Although the SCNase lost activity upon freezing, it could be partially reactivated by heating at 60°C for 15 min, however the percentage of recovery varied on the enzyme preparation and duration of freezing. This result suggested that the SCNase may have a special property of cold inactivation and heat reactivation. The characteristic of cold inactivation was also found in other enzymes such as *E. coli* tryptophanase (Erez et al., 1998), trithionate hydrolase of *Thiobacillus acidophilus* (reclassified as *Acidiphilium acidophilum*) (Meulenberg et al., 1992), and ribulose-1,5-biphosphate carboxylase-oxygenase from tobacco leaf (Chollet & Anderson, 1977). In the latter, the cold inactivation was the result of partial dissociation of the hydrophobic catalytic subunits of the enzyme (Chollet & Anderson, 1977).
The km and kcat of SCNase could not be determined. Because SCNase enzyme was very unstable and the experiment had to be performed within 1 hour of enzyme preparation, "substrate concentration dependence of enzyme activity" was determined only with some selected concentrations of KSCN. As a result, the data set was not good enough to draw a lineweaver-burk plot. Ensuring the stability of enzyme will enable us to measure the enzyme activity from more assay mixtures containing various concentrations of KSCN. Thus, if results are possible to obtain by using KSCN of concentration 2, 4, 8, 10, and 12 mM (Fig. 2a), it will help to determine the enzyme kinetics.
Fig. 1. Optimum conditions for recombinant SCNase of *Afipia* sp. strain THI201. (a) Optimum temperature. The temperature optimum for SCNase reaction was determined by incubating the 50 µl reaction mixtures containing 900 ng enzyme, 100 mM phosphate buffer, pH 6.0, and KSCN of 40 mM, at temperature varying 10-100°C for 10 min and measuring the formation of NH₃. (b) Optimum pH. Production of ammonia was measured from 50 µl reaction mixtures containing 1.65 µg of enzyme, 100 mM phosphate buffer of various pH, and 40 mM KSCN, at 30°C, for 10 min. The error bars express the standard deviations based on duplicate experiments.
Fig. 2. Characteristics of recombinant thiocyanate hydrolase of *Afipia* sp. strain TH1201. (a) Substrate concentration dependence of enzyme activity. KSCN of different concentrations were added in separate reaction mixtures of 50 µl containing 1 µg of enzyme and 100 mM phosphate buffer, pH 6.0. The mixtures were incubated at 30°C for 10 min and used to measure NH₃. (b) Dose dependence of enzyme. Several assay mixtures with various amounts of purified enzymes, 40 mM KSCN and 100 mM phosphate buffer, pH 6.0, were incubated at 30°C for 10 min and NH₃ was estimated. (c) Time course of ammonia formation. Production of ammonia was measured at various time intervals at 30°C, from 50 µl reaction mixtures containing 1.5 µg of enzyme and 100 mM phosphate buffer, pH 6.0. (d) Thermostability of enzyme. The purified recombinant enzyme was heat treated for 5 min at the temperatures specified and the residual activity was obtained by comparing the activity before and after the treatment; the original activity was considered to be 100%. The error bars show the standard deviations based on duplicate experiments.
CONCLUSIONS
CONCLUSIONS

This study narrates the cloning of a gene encoding a novel thiocyanate hydrolase enzyme from a mesophilic bacterium, *Afipia* sp. strain THI201, and the expression of the recombinant gene product in *E.coli*. Because SCNases have only been examined in obligately chemolithoautotrophic bacteria, i.e., *Tb. thioparus* THI115 and *Th. thiocyanoxidans* (Katayama et al., 1992; Bezsdunova et al., 2007), the aim was to characterize the enzyme from strain THI201 that can grow under mixotrophic growth conditions. The SCNase activity of native and recombinant enzyme, and also in the whole cell extracts of THI201 was examined by estimating NH$_3$.

By southern hybridization, *Hind*III-digested DNA fragments of 5.0 to 6.5 kb were selected for the preparation of a sub-genomic library. Screening by colony hybridization resulted one positive clone C14 and nucleotide sequencing analysis of the plasmid pUC118C14 isolated from C14 determined a protein of 457 amino acids of molecular mass 50,267 Da. The identity (%) studies of the NCBI database with the resultant amino acid sequence displayed highest (83%) identity with an uncharacterized protein of 488 amino acids derived from the sulfur-oxidizing Betaproteobacteria *Tb. denitrificans* ATCC 25259. The sequence homology analysis found no significant homology to other thermostable enzymes or previously purified SCNases or NHases. These results indicated the novelty of SCNase isolated from THI201.

By sequence analysis, presence of a twin-arginine (Tat) signal sequence with a Tat motif (RRTLL) was found upstream of SCNase. The Tat is a system specific to the transport of fully folded proteins and the motif is involved in sec-independent protein targeting in *E. coli* (Nicolas et al., 2001). Other relevant study with Tat motif showed that either replacing one or both of the arginine residues with lysine, or replacing one of the hydrophobic residues with alanine, led to a block in translocation (Kwan et al., 2010). In *Pseudomonas aeruginosa*, secretion of two extracellular
Expression of \( \text{scn} \) was done in \( E. \text{coli} \) by constructing pGEX-SCNase plasmid and purification of SCNase was carried out by GS4B-bead based affinity chromatography. The recombinant SCNase was found as inclusion bodies and could not be purified. However, protein aggregation is a common issue during the production of heterologous proteins in \( E. \text{coli} \) (Gatti-Lafranconi et al., 2011) and in this instance, selection of the IPTG concentration is an important task (Jana et al., 2005). Accordingly, production of soluble fusion protein was tried with various concentration of IPTG but it was unsuccessful. Another study showed that, the optimal IPTG concentration varied depending on strain, scale (shake scale or bench scale) and culture conditions, and should be optimized for each production system and recombinant protein (Hernández et al., 2013). Therefore, optimization of conditions was also tried by changing induction time and temperature. Unfortunately, neither of the conditions could change the solubility of protein. It was presumed that, as production of recombinant protein was tried with shake scale and batch culture, other cultural conditions like bench scale or continuous culture might produce fruitful result.

A widely used technique to aid production of biologically active heterologous proteins is to overexpress simultaneously one or more chaperone proteins, which constitute a diverse family, many of which belong to the group of heat shock proteins and are up-regulated when the cell is under stress (Thomas et al., 1997; Baneyx, 1999; young et al., 2004). In this report, chaperone assisted expression of \( \text{scn} \) and consequently, abundant production of active SCNase were described. Three chaperone systems were tried for co-expression with SCNase: GroEL/GroES (plasmid pGro7), DnaK/DnaJ/GrpE (plasmid pKJE7), and GroEL/GroES together with DnaK/DnaJ/GrpE (plasmid pG-KJE8). Only GroEL/GroES with GST-fused SCNase successfully produce soluble SCNase. The mechanism was: poorly folded proteins are targeted to the GroE complex, composed of a barrel with a hydrophobic core of GroEL and a lid of GroES; conformational changes in GroEL, driven by ATP, force proteins within the barrel into more compact,
properly folded conformations (Thomas et al., 1997). Several studies explained the efficiency of GroEL/ES in purification of recombinant protein. One relevant study showed that, when the GroEL/GroES system was co-expressed with the target gene, 6-fold greater enzyme yield resulted than in the absence of co-expressed chaperones (Nicholas et al., 2013). Another similar study revealed that the co-overexpression of the bacterial chaperone system GroEL/ES along with \( \zeta \)-crystallin could significantly enhance the yield of soluble protein (Goenka & Rao, 2001). Thus it can be concluded that, the GroEL/ES system used in this study was worthwhile in producing adequate amount of soluble SCNase.

Characterization of recombinant SCNase determined various optimal conditions for maximum enzyme activity, beside the uncommon property of heat tolerance. The optimum temperature was found within the range of 30-70°C and thermostability study showed that the SCNase of THI201 can withstand even 100°C (50% of the original activity was found). A temperature optimum of 70°C is a common property of (extreme) thermophiles (Brock, 1978; Gupta, 1991), but has not often been described for proteins from a mesophile like strain THI201. As a result, reports of protein with similar characteristic are very limited. A related study was found with enzyme sulfur oxygenase reductase from the mesophilic bacterium *Halothiobacillus neapolitanus* that explained the reason of thermostability as a result of a rigid protein core together with the stabilizing effect of the 24-subunit hollow sphere (Veith et al., 2012). Hence, reason behind the thermo tolerance of SCNase can also be revealed by detecting its secondary and quaternary structure.

Comparison of the SCNase of *Afipia* sp. strain THI201 with the previously isolated SCNases revealed some remarkable differences. The results of SDS-PAGE (not shown) analysis of the partially purified native enzyme showed a single band of SCNase (50.3 kDa), whereas both the SCNases of *Th. thioparus* THI115 (140 kDa) and *Th. thiocyanoxidans* (126 kDa) contain three subunits (Katayama et al., 1992; Bezsudnova et al., 2007). Furthermore, characterization of the recombinant enzyme of strain THI201 showed that it had a pH optimum of 6.0–6.5, whereas the other two SCNase enzymes have an optimum pH range of 7.0–7.5 (Katayama et al., 1992; Bezsudnova et al.,
The SCNases from *Tb. thioparus* THI115 and *Th. thiocyanoxidans* are reported to be heat labile (Katayama et al., 1992; Bezsudnova et al., 2007), but the purified recombinant SCNase of THI201 was a thermostable enzyme exhibiting heat tolerance up to 70°C. Thus these results confirmed that the SCNase of THI201 is a novel enzyme.

The instability of SCNase made the study difficult and each experiment was carried out soon after purification of each preparation. The reason behind the instability has not been determined yet, may be it needed assistance of activator or cofactor. In plant polyphenol oxidases, the provision of CuCl₂ in the growth medium was necessary to produce an active enzyme (Mareike et al., 2013). Another study with recombinant N-acetylgalactosamine-6-sulfate sulfatase showed that, enzyme activity in the culture media was only detected when signal protein (SP) was presented and the culture was carried out under semi-continuous mode. When native SP was presented, higher enzyme activity levels were observed in both soluble and inclusion bodies fractions, and its removal had a significant impact on enzyme activation (Hernández et al., 2013). In this study, *scn* was expressed without SP, and the production of SCNase was done only by batch culture. However, it was assumed that, expression of SP and changing of culture conditions might enhance the enzyme stability. Now, the stabilization of this enzyme is mandatory for further characterization, such as determination of kinetic parameters, substrate specificity, activators, and inhibitors. This report represents valuable information regarding nucleotide sequence and characterization, and becomes an important milestone on the way to a detailed understanding of novel SCNase.


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