Cloning and expression of a gene encoding a novel thermostable thiocyanate-degrading enzyme from a mesophilic alphaproteobacteria strain THI201

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Strain THI201, a member of the alphaproteobacteria, 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 hydrolyses thiocyanate to carbonyl sulfide and ammonia. Characterization of both native and recombinant SCNase revealed properties different from known SCNases regarding subunit structure and thermostability: SCNase of strain THI201 was composed of a single protein and thermostable. We cloned and sequenced the corresponding gene and determined 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. The specific activities of native and recombinant enzyme were 0.32 and 4–15 μmol min⁻¹ (mg protein)⁻¹, respectively. The maximum activity of SCNase was found in the temperature range 30–70 °C. The thiocyanate-hydrolysing activity in both enzymes was decreased by freeze–thawing, although 25–100 % of the activity of recombinant protein could be retrieved by treating the enzyme at 60 °C for 15 min. Furthermore, both native and recombinant enzymes retained the activity after pre-treatment of the protein solution at temperatures up to 70 °C.

INTRODUCTION

Thiocyanate (SCN") is used in various processes in the chemical industry, and is produced as a waste product in industrial processes such as coal gasification, photofinishing, and pesticide production. In the natural environment, thiocyanate is produced by the hydrolysis of glucosinolates in plants (Burrow et al., 2007) and is transformed by various indigenous micro-organisms such as Thiobacillus thioparus, a chemolithoautotrophic sulfur-oxidizing bacterium (Happold et al., 1958; Katayama & Kuraishi, 1978). In mammals, thiocyanate is produced through the detoxification of cyanide by thiosulfate cyanide sulfurtransferase, i.e. rhodanese (EC 2.8.1.1), and is degraded by the hydrogen peroxide/peroxidase system in body fluids such as saliva and milk (Vesey & Wilson, 1978; Wood, 1975). Although evidence of thiocyanate degradation and carbonyl sulfide (COS) emission in most environmental samples from aquatic and terrestrial...
origins indicated a wide distribution of thiocyanate-degrading microbes in nature (Yamasaki et al., 2002), research on the enzymic transformation of thiocyanate by bacteria has been limited (Katayama et al., 1992; Bezsdunova et al., 2007).

The first thiocyanate-transforming enzyme, thiocyanate hydrolase (EC 3.5.5.8, SCNase), was identified and isolated from Tb. thioparus THI115, which degrades thiocyanate up to a concentration of 60 mM under aerobic conditions (Katayama et al., 1992). The SCNase catalysed the hydration of thiocyanate to COS and ammonia (NH₃) (SCN⁻ + 2H₂O→COS + NH₃ + OH⁻), and is a tetrameric structure of α, β and γ subunits, (αβγ)₄. DNA sequences of the genes encoding these subunits reveal a close similarity to nitrite hydratase (NHas), which catalyses the hydration of various nitriles to the corresponding amides; the fused α and β subunits of the SCNase correspond to the β subunit of NHase, and the γ subunit of the SCNase corresponds to the α subunit of NHase (Katayama et al., 1998). SCNase of Tb. thioparus THI115 contains a non-corrin cobalt active centre and has two post-translationally modified cysteine ligands, i.e. cysteine sulfenic acid (or cysteine sulfenate) and cysteine sulfinate; the enzyme requires co-expression of an activator protein, P15K, for its functional expression (Kataoka et al., 2006; Katayama et al., 2006; Arakawa et al., 2007). The presence of a similar enzyme in the halophilic chemolithoautotroph Thiohalophilus (Th.) thiocyanoxidans has also been reported (Bezsudnova et al., 2007). Degradation of thiocyanate was found in a facultatively chemolithoautotrophic sulfur-oxidizing bacterium Paracoccus thiocyanatus strain THI011 (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.

We have isolated a novel facultatively chemolithoautotrophic thiocyanate-degrading bacterium, strain THI201, a member of the alphaproteobacteria, from an enrichment culture of lake water with thiocyanate (Yamasaki et al., 2002). This bacterium utilized thiocyanate and thiourea as the chemolithoautotrophic growth substrate, and produced sulfate as a metabolite. Part of the sulfur moiety of thiocyanate was recovered as COS in the headspace gas of THI201 culture, indicating that thiocyanate was metabolized through the COS pathway (Kelly et al., 1993). Similar to the P. thiocyanatus strain THI011, the ability of strain THI201 to degrade thiocyanate was decreased quickly, however not completely, by preparing crude cell extract. Therefore, we aimed to identify the enzyme responsible for thiocyanate degradation in this bacterium. This report describes the isolation of a novel SCNase protein from strain THI201, the cloning and expression of the gene in Escherichia coli, and some characterization of the SCNase. By treating the recombinant SCNase at higher temperatures than that required for the optimal growth of this bacterium, we revealed its unique property of heat tolerance.

**METHODS**

*Isolation and phylogenetic affiliation of strain THI201.* Surface water sampled from Lake Sagami was incubated aerobically with 1 mM potassium thiocyanate (KSCN) until all thiocyanate supplemented to the medium was consumed. Reinoculation in the fresh medium was repeated several times to enrich thiocyanate-degrading micro-organisms. Strain THI201 was isolated by streaking the enriched culture LS5 (Yamasaki et al., 2002) on TC5 medium [0.5 g K₂HPO₄, 1 g (NH₄)₂SO₄, 0.05 g MgSO₄.7H₂O, 0.01 g FeCl₃.6H₂O, 0.01 g CaCl₂.2H₂O, 10.0 ml trace metal solution (Katayama & Kuraishi, 1978), 0.5 g KSCN per litre, pH 7.0].

Phylogenetic position of the isolate was assessed based on the sequence data of the 16S rRNA gene. Optimal temperature and pH for strain THI201 were examined in liquid medium YmTC10 [0.5 g K₂HPO₄, 0.1 g (NH₄)₂SO₄, 0.05 g MgSO₄.7H₂O, 0.01 g FeCl₃.6H₂O, 0.01 g CaCl₂.2H₂O, 10.0 ml trace metal solution (Katayama & Kuraishi, 1978), 1 g KSCN and 1 g yeast extract per litre, pH 7.0].

**Bacterial strains and culture conditions.** Strain THI201 was grown in YmTC10 medium. E. coli strains Rosetta-gami B (Novagen), JM109 (Takara) and BL21(DE3) (Takara) were used as hosts for the cloning of the SCNase gene (scn) and the production of the recombinant SCNase. E. coli was grown in LB medium supplemented with 100 µg ampicillin ml⁻¹ and/or 20 µg chloramphenicol ml⁻¹ when needed. Unless otherwise indicated, cells were grown aerobically with reciprocal shaking at 30 °C.

**Isolation of enzyme.** 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 four 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) on ice. After centrifugation of the disrupted cell suspension at 7700 g 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) that had been equilibrated with the same buffer. Proteins were eluted by applying a 24 ml linear gradient of increasing potassium phosphate concentrations from 10 to 300 mM. The active fractions were combined, dialysed in buffer B and the hydroxyapatite column chromatography step was repeated. SDS-PAGE was performed as described by Laemmli (1970) with a 12 % polyacrylamide gel and the proteins bands were stained with Coomassie brilliant blue R-250.

**Enzyme assays.** SCNase 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) equipped with a flame photometric detector and a glass column packed with Porapak QS (50–80 mesh; Nilon Waters) 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. (1951). The specific activity of SCNase was expressed in units of nmol of μmol COS min⁻¹ (mg protein)⁻¹.

The thermostability of SCNase in the crude cell extract was assessed by measuring the production of NH₃. Crude cell extract (3.42 g as protein in 10 µl) of strain THI201 was heat treated for 5 min at 60, 70 or 80 °C. Then, reaction mixture (40 µl) containing KSCN and
potassium phosphate buffer, pH 6.0, was added to give final concentrations of 40 and 100 mM, respectively. The mixture was then incubated at 30 °C for 10 min. An aliquot of the mixture was reacted with Nessler reagent and the amount of NH₃ formed by SCNase activity was quantified by measuring the absorbance at 420 nm. The specific activity of SCNase was calculated in units of µmol NH₃ min⁻¹ (mg protein)⁻¹.

### N-terminal amino acid sequencing

The partially purified enzyme preparation was separated by SDS-PAGE and electroblotted onto a PVDF membrane. The enzyme band was located by Coomassie brilliant blue R-250 staining and the protein was subjected to microsequencing in a sequencer (model 477A; Applied Biosystems).

### Preparation of the DNA probe.

Degenerate oligonucleotide mixtures of F₁ (5′-GAYATGWSNAARGCNARCA-CAY-3′) and R₁ (5′-GCNSWNAACNGTNCRCRTTAYT3′) were designed based on the N-terminal amino acid sequence of the SCNase of strain THI201 (Fig. 1). The annealing sites of oligonucleotide mixes were chosen to minimize the degeneracy of the oligonucleotides. Genomic DNA of strain THI201 was isolated according to the method of Saito & Miura (1963) and subjected to PCR with F₁/R₁ and Hot Star Taq DNA polymerase (Qiagen), under the annealing temperature of 50 °C according to the manufacturer’s instructions. The nucleotide sequence of the resultant 92 bp DNA fragment was determined using a bacterial alkaline phosphatase. The resultant subgenomic DNA fragment was labelled with the AlkPhos Direct fragment was used as a template for PCR with F₀₂ and R₀₂ (5′-AGCAAAATATCCACGGCGGC-3′). M₁₃RA (5′-TCAAGGCGCAGTAGTAAGGAGC-3′), F₀₂, R₀₂ (5′-CGCCGATCTTCAAGATGTTTCC-3′), F₀₂B (5′-ACCTTCTGCGCTGACACAAACC-3′) and F₀₂C (5′-CCAGTGACTACACCGGTTCA-3′); for the opposite strand: R₀₂D1a (5′-GGTTGACCGCAGCTGATGTC-3′), R₀₂D₂ (5′- TGCAAGTCCACCGGCAATC-3′), R₀₂B2 (5′-GCAAGCGGAGATGCATTGGTGA-3′), R₀₂A₁ (5′-GAAATGGAATGTTTCTGTGGACG-3′), R₀₂B₁ (5′-GCCGTGTAGTGCGGGAATC-3′), M₁₃RA₂ (5′-TACGGTCTGTTCGCCGGACATC-3′), M₁₃RA₃ (5′-TGCTTTCACCCGATAGGAG-3′) and M₁₃RA₄ (5′-AGAGAACACACTGACGTC-3′). The position 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 CLUSTAL W program accessed through the DNA Data Bank of Japan.

### Construction of the expression plasmid.

Two oligonucleotides, SCNaseF₁ (5′-CGGATTCATCAGGATGTTGCTCCGGCGGATA-TGACAAC3′) and SCNaseR₁ (5′-CGGCCCTGGACACTACCGGCG- GTACTACG-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 Xhol was added at the 3′ termini of oligonucleotide SCNaseR₁. Plasmid pUC118C14 was used as a template for PCR with SCNaseF₁ and SCNaseR₁ in a reaction mixture containing Phusion High-Fidelity DNA polymerase (New England Biolabs). The amplified DNA fragment was digested with EcoRI and Xhol, and inserted between the EcoRI and Xhol sites of the expression vector pGEX-6P-1 (GE Healthcare) to construct the expression plasmid pGEX-SCNase. The nucleotide sequence of the

### Southern hybridization.

Chromosomal DNA of strain THI201 was digested with PstI, Stul, HpaI, EcoRI, EcoRV, PstI, HindIII, XhoI, KpnI or Sau3A1, and 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-labelled 81 bp probe at 55 °C, as suggested by the manufacturer’s specifications. The membrane image was obtained with a luminous imaging analyser (LAS-3000; Fuji Film).

**Fig. 1.** A portion of the nucleotide and deduced amino acid sequence of the scn gene of strain THI201. The two predicted Shine-Dalgamo sequences, AGGGGA and AGGCAGA are underlined; the start codons (ATG) and corresponding amino acids (M) of their related ORF are in bold. The N-terminal amino acid sequence of SCNase is shown by a white box and the sequence of the Tat signal peptide is underlined. The positions and sequences of the degenerate oligonucleotides F₁ (5′-GAYATGWSNAARGCNARCA-CAY-3′) and R₁ (3′-TTYATRTRCTGNTGCANWSNCG-5′) are shown. The nucleotide sequences corresponding to those of the oligonucleotides F₀₂ and R₀₂ are indicated in lower case and lower case italics, respectively.

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**RAW TEXT END**
inserted DNA fragment of pGEX-SCNase was confirmed by sequence analysis.

**Purification of recombinant SCNase.** *E. coli* strain Rosetta-gami B was transformed with pGEX-SCNase or pGEX-6P-1 plasmid, and *E. coli* strain BL21(DE3) was transformed with pGEX-SCNase or pGEX-6P-1 plasmid together with one of the chaperone plasmids pG-KJE8, pKJE7 or pGRO7 (Takara Bio). The transformed bacteria were grown to mid-exponential phase in 100 ml of LB medium containing ampicillin and chloramphenicol. IPTG and/or arabinose were added to the culture at the final concentrations of 0.1 and/or 1.7 mM, respectively, to induce the production of glutathione S-transferase (GST)-fused SCNase and/or chaperone proteins, respectively. The cultures were 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 final concentrations of 1 mM, 10 mM and 1 mg ml\(^{-1}\), respectively, cells were disrupted by ultrasonication 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 (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 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) 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 factor Xa μl\(^{-1}\) (Novagen) 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 Lowery et al. (1951).

**Characterization of recombinant SCNase.** The enzyme assay of recombinant SCNase was performed by measuring NH\(_3\). The standard assay conditions were as described above for the crude cell extract of THI201 and the reaction mixture contained the purified recombinant enzymes (0.9–1.65 μg protein) in a final volume of 50 μl.

Each preparation of the purified recombinant enzyme was used to determine the optimal conditions of activity. The pH optimum was determined by using potassium phosphate buffer of pH 4–9 to adjust the final pH of the assay mixture to 5–8. The optimum temperature was measured by varying the incubation temperature over the range 10–100 °C.

The substrate concentration dependence of enzyme activity was studied in an assay mixture of 50 μl containing about 1.0 μg of purified enzyme, 100 mM phosphate buffer of pH 6.0 and KSCN varying the concentration over the range of 10–60 mM. Dose dependence of enzyme was tested by changing the amount of enzyme in the range of 0.2–1.4 μg. Time course of product formation was examined by using 1.5 μg of enzyme in a final volume of 50 μl and NH\(_3\) was measured at different time intervals. Thermostability was checked by heating the purified enzymes at 30–100 °C for 5 min before enzyme assay. The change in activity after heating was determined 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.

**RESULTS**

**Taxonomic characteristics of strain THI201**

Strain THI201 was a Gram-negative, motile, short rod bacterium with a polar or a subpolar flagellum. It was a facultative chemolithoautotroph: it grew chemo-organotrophically 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 and 35 °C, but unable to grow at 40 °C. This bacterium grew optimally at pH 7.0–7.5 (initial medium pH) with a range between pH 6.0 and 8.5. Phylogenetic affiliation based on the sequence data of almost full-length 16S rRNA indicated that strain THI201 is a member of the *Bradyrhizobium–Agromonas–Nitrobacter–Afipia* (BANA) cluster in the alphaproteobacteria (Saito et al., 1998). Polyphasic identification of strain THI201 in the cluster is in progress.

**Degradation of thiocyanate by strain THI201**

In a mixotrophic medium of MmTC12 containing thiocyanate 12 mM and malic acid, strain THI201 used thiocyanate in 4 days, and the sulfur moiety of thiocyanate was recovered almost as equimolar amounts of sulfate. During the degradation of thiocyanate, COS corresponding to 6–10 % of the degraded thiocyanate was transiently detected in the headspace of a culture vessel, indicating transformation of the substrate by COS pathway (Kelly et al., 1993) (Fig. 2). Thiocyanate degradation activity in cell-free extract was estimated by production of COS and found to be 11.7 nmol min\(^{-1}\) (mg protein\(^{-1}\)). However, after the consumption of all thiocyanate, although cell growth continued using malic acid, the activity decreased to 0.29 nmol min\(^{-1}\) (mg protein\(^{-1}\)). Therefore, cells used for isolation of the enzyme were harvested when almost all thiocyanate was consumed.

**Isolation of SCNase**

The thiocyanate-degrading activity of the crude cell extract of strain THI201 was considerably decreased by freeze–thawing. Addition of glycerol or PEG 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 harbouring thiocyanate-degrading activity and repeating the procedure resulted in partially purified proteins in SDS-PAGE (data not presented). The specific activity of the isolated fraction was 0.32 μmol min\(^{-1}\) (mg protein\(^{-1}\)). Microsequencing of the enzyme protein that was cut off from a filter after blotting revealed the N-terminal amino acid sequence and 37 amino acid residues were unambiguously determined (Fig. 1).
Cloning and nucleotide sequence of the THI201

Genomic Southern hybridization of strain THI201 was performed by using the 81 bp DNA fragment (positions 217–297, Fig. 2) corresponding to the N-terminal amino acids of the isolated enzyme as a probe. Digestion of genomic DNA with each restriction enzyme except PstI resulted in the appearance of a single band, suggesting that the scn of strain THI201 was a single-copy gene. HindIII-digested DNA fragments of 5.0–6.5 kb were selected for the preparation of a subgenomic library. By colony hybridization screening, one positive clone designated C14 was obtained among 335 colonies. Nucleotide-sequencing analysis of the plasmid pUC118C14 isolated from clone C14 revealed that the SCNase protein was within an ORF of 1470 bp. We predicted two Shine–Dalgarno sequences, AGGGGA and AGGCGGA, 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. 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. 1). The identity (%) searches of the protein sequence of SCNase against the NCBI protein database revealed identities of 83 % with a putative uncharacterized protein of Thiomonas denitrificans ATCC 25259, 36 % with a hypothetical protein of Hydrogenobacter thermophilus TK-6 and 35 % with a hypothetical protein ThithDRAFT_3226 of Thioalkalivibrio thiocyanoxidans ARh4 (Fig. S1, available in Microbiology Online), 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 are presented in Fig. S2.

Production and purification of recombinant SCNase

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, the produced 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. Although
the fusion protein could be produced in adequate amounts, none of the conditions tested could improve the recovery of the fusion protein in the soluble fraction. 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. To improve the recovery of the fusion protein in the soluble fraction, plasmids pG-KJE8, pKJE7 and pGRO7 encoding chaperone proteins DnaK/DnaJ/GrpE and GroES/GroEL, DnaK/DnaJ/GrpE, and GroES/GroEL, respectively, 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 (Fig. 3). In parallel, we also ran the negative control of BL21(DE3) cultures containing plasmids pGEX-6P-1 and pGRO7. The fusion protein band was absent in protein preparations from the vector-only control culture and it confirmed that the only possible source of thiocyanate degradation was the gene encoded on the recombinant plasmid. GST–SCNase and the chaperone proteins GroEL and GroES were isolated by GS4B bead-based affinity column chromatography (Fig. 3, lane 2). Treatment with Mg-ATP and casein sodium eliminated bead-based affinity column chromatography (Fig. 3, lane 2).

Characterization of the SCNase enzyme

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. Purification and characterization of enzyme became challenging due to instability of enzyme. We used different recombinant enzyme preparations purified immediately before each experiment, which exhibited differences in degree of purification on SDS-PAGE. Thus, the purified enzyme showed a fourfold difference in specific SCNase activity that is reflected in the overall yield of ammonia in the comparable experiments shown in Fig. 4(a, c). The specific activity of the prepared enzyme ranged between 4 and 15 μmol min⁻¹ (mg protein)⁻¹. Later, we found that the activity of the SCNase purified at room temperature was 1.4 times that of the enzyme purified at 4 °C.

Enzymic characterization was performed with the purest fraction of the recombinant SCNase. 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 protein)⁻¹ was found in the pH range 6.0–6.5. The enzyme showed activity at a broad range of temperatures from 10 to 100 °C, with the highest activity of 4 μmol min⁻¹ (mg protein)⁻¹ always in the range 30–70 °C. The negative control without SCNase was checked to ensure no degradation of thiocyanate.

Analysis of the substrate concentration dependence of enzyme activity showed maximum activity at 20–40 mM thiocyanate (Fig. 4a). A distinct inhibition of enzyme activity by the substrate was observed at thiocyanate concentrations of 50 mM or greater. The reaction rate of ammonia formation was proportional to the amounts of SCNase up to 0.8 μg of protein (Fig. 4b) and was linear with time up to 15 min (Fig. 4c). The experiment to determine thermostability showed that the enzyme activity of recombinant SCNase was retained after heat treatment at temperatures up to 70 °C, but was decreased by heating at 80 °C for 5 min (Fig. 4d). Enzyme activity was absent in the no-enzyme control at both 70 and 80 °C. Similar thermostolerance was observed for native SCNase. 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. As

![Fig. 3. SDS-PAGE analysis of recombinant SCNase of strain THI201 at each step of purification. Lane 1, crude cell extract prepared from E. coli harbouring pGEX-SCNase and pGRO7; lane 2, proteins associated with 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) are 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.](http://mic.sgmjournals.org)
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, 70 or 80 °C for 5 min resulted in SCNase activity of 3.2, 3.5 or 4.5 nmol min⁻¹ (mg protein)⁻¹, respectively, whereas the control without heat treatment showed activity of 2.1 nmol min⁻¹ (mg protein)⁻¹. However, intact cells of strain THI201 grew at temperatures of up to 35 °C, but not at 40 °C or higher.

**DISCUSSION**

Here, we report the cloning of the gene encoding a novel SCNase enzyme from a mesophilic bacterium, strain THI201, and the expression of the recombinant gene product in E. coli. As SCNases have only been examined in obligately chemolithoautotrophic bacteria, i.e. *Tb. thioparus* THI115 and *Th. thiocyanoxidans* (Katayama et al., 1992; Bezsudnova et al., 2007), we aimed to isolate the enzyme from strain THI201 that can grow under mixotrophic growth conditions. We examined the SCNase activity of native and recombinant enzyme by estimating COS and NH₃, respectively. The NH₃ production was also measured in the whole-cell extracts of THI201. The enzyme activities of the native enzyme in the whole-cell extract and the recombinant purified enzyme were 2.1 and 4–15 nmol min⁻¹ (mg protein)⁻¹, respectively.

Comparison of the SCNase of 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, whereas both the SCNases of *Tb. thioparus* THI115 and *Th. thiocyanoxidans* 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., 2007). 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 (Fig. 4d). Considering the favourable temperature of 30 °C for the growth of strain THI201, enzyme assays for SCNase were also carried out at

![Fig. 4. Characteristics of recombinant SCNase. (a) Substrate concentration dependence of enzyme activity. KSCN of different concentrations was 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%. Error bars show the SD based on duplicate experiments.](image-url)
30 °C. However, surprisingly, when the activity was measured at 70 °C, it was ~1.3 times that at 30 °C. This result indicates that the mesophilic 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).

The identity (%) studies of the NCBI database with the amino acid sequence of the strain THI201 SCNase 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. In addition, the characteristic cobalt-binding motif, V-C1-X-L-C2-S-C3, present in previously characterized SCNases and NHases (Nagashima et al., 1998; Miyanaga et al., 2001; Katayama et al., 2006), was absent in the SCNase here. In contrast, addition of 1 mM Co²⁺ in the crude extract of THI201 decreased the original enzyme activity by 26%. Thus, it may need the addition of other co-factors to enhance the stability of enzyme. 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 depending 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-bisphosphate 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).

In recombinant bacteria, the overexpression of plasmid-encoded genes triggers transcription of heat shock genes and other stress responses; as a result, aggregation of the encoded protein occurs as inclusion bodies (Rinas, 1996; Jürgen et al., 2000). This problem can be solved by co-expression of selected chaperone-encoding genes along with the target gene; therefore, we tried with plasmid pGRO7, which encodes the genes for chaperonin GroEL and its regulator GroES. Several studies have used chaperone-assisted protein purification: one similar study with NHase NI1 from *Comamonas testosterone* showed heterologous expression of NHase involving co-expression with the *E. coli* GroES and GroEL chaperones (Stevens et al., 2003); and another study with the N-carbamoyl-d-amino acid amidohydrolase (d-carbamoylase) gene (*dbc*) from *Agrobacterium tumefaciens* AM 10 showed that co-expression of chaperones GroES and GroEL resulted in an active enzyme production that was 43 times that obtained using the WT strain (Sareen et al., 2001).

The SDS-PAGE analysis of the purest protein fraction showed that a trace amount of GroEL protein remained with the recombinant SCNase. 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 and 6:1 (result not shown). 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 co-existence of a trace amount of GroEL had little or no influence on the function of SCNase *in vitro*. Although there are several other methods that we could have used to remove the chaperone protein GroEL, we chose the fastest method to avoid loss of enzyme activity. Nevertheless, stabilization of the novel SCNase is now mandatory for further characterization, such as determination of kinetic parameters, substrate specificity, activators and inhibitors. The high-level expression system described here and the heat-tolerant property of this novel SCNase can be used to help reveal the structure and function of the enzyme, and determine its role in biogeochemical cycles.

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**REFERENCES**


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