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Functional expression of thiocyanate hydrolase is promoted by its activator protein, P15K

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Abstract **Thiocyanate hydrolase (SCNase) is a cobalt-containing enzyme with a post-translationally modified cysteine ligand, γCys131-SO_2H. When the SCNase α, β and γ subunits were expressed in *Escherichia coli*, the subunits assembled to form a hetero-dodecamer, $(\alpha\beta\gamma)_4$, like native SCNase but exhibited no catalytic activity. Metal analysis indicated that SCNase was expressed as an apo-form irrespective of the presence of cobalt in the medium. On the contrary, SCNase co-expressed with P15K, encoded just downstream of SCNase genes, in cobalt-enriched medium under the optimized condition ($SCNase_{(+P15K)}$) possessed 0.86 Co atom/αβγ trimer and exhibited 78% of the activity of native SCNase. $SCNase_{(+P15K)}$ showed a UV–Vis absorption peak characteristic of the SCNase cobalt center. About 70% of $SCNase_{(+P15K)}$ had the γCys131-SO_2H modification. These results indicate that $SCNase_{(+P15K)}$ is the active holo-SCNase. P15K is likely to promote the functional expression of SCNase probably by assisting the incorporation of cobalt ion.**

Keywords: Thiocyanate hydrolase; Nitrile hydratase; Non-corrin cobalt; Activator protein; Post-translational modification; Cysteine-sulfinic acid; Cysteine-sulfenic acid; Apo-protein

1. Introduction

Thiocyanate is commonly produced from cellular thioglucosides in plant tissues [1] and exists as a major constituent of waste water from coke oven factories. The degradation of thiocyanate is an important subject in green chemistry. Thiocyanate-degradating organisms are believed to be distributed widely. Thiocyanate hydrolase (SCNase) [2], catalyzing the hydrolysis of thiocyanate to carbonyl sulfide and ammonia ($SCN^- + 2H_2O \rightarrow COS + NH_3 + OH^-$), was first purified from *Thiobacillus thioparus* THI 115. SCNase is composed of α, β and γ subunits. The SCNase γ subunit is highly homologous with the α subunit of nitrile hydratase (NHase), while the SCNase β and α subunits comprise the N- and C-terminal halves of the NHase β subunit, respectively [3]. NHase [4] contains a non-corrin cobalt or non-heme iron center, which is bound to a motif, V-C1-X-L-C2-S-C3 of α subunit. C2 and C3 are post-translationally modified to cysteine-sulfinic acid (Cys-SO_2H) and -sulfenic acid (Cys-SOH), respectively [5,6]. The motif is completely conserved in the SCNase γ subunit, V^{127}-C-T-L-C-S-C^{133} [3]. Very recently, we have shown that SCNase possesses one Co ion per αβγ hetero-trimer with a similar ligand field with Co-type NHases [7]. We have also discovered that γCys131, corresponding to C2 in the motif, was post-translationally modified to Cys-SO_2H based on mass spectrometric measurements [7]. Although the presence of the Cys-SOH modification has not been confirmed, these results strongly suggest that SCNase belongs to the same protein superfamily as NHases.

To characterize SCNase biochemically and structurally, we tried to express SCNase in *Escherichia coli*. When only the genes coding SCNase α, β and γ subunits were introduced into *E. coli*, no active SCNase was obtained even in the cobalt-enriched medium. It is known that both Co- and Fe-type NHases require the co-expression of their specific accessory proteins [8–13]. We named them NHase activators [10]. Fe-type NHase activator has a putative metal-binding motif, CXCC, and the replacement of one of the three cysteines with serine impaired functional expression of NHase in *E. coli* [14]. In contrast, Co-type NHase activators are small proteins with a molecular mass of 14–17 kDa and exhibit no sequence

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Abbreviations: SCNase, thiocyanate hydrolase; NHase, nitrile hydratase; Cys-SO_2H, cysteine sulfinic acid; Cys-SOH, cysteine sulfenic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC-MALS, size exclusion chromatography-multiangle laser light scattering; ESI-LC/MS, electrospray ionization-liquid chromatography/mass spectrometry; CM, carboxy methylated

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similarity to Fe-type ones [11–13,15,16]. It has no obvious amino acid sequence homology with known proteins except NHase β subunit and lacks known metal-binding motifs. Recently, Cameron et al. [13] proposed that P14K, the Co-type NHase activator of *Bacillus pallidus* RAPc8 functions as a subunit-specific chaperone. They speculated that P14K stabilized the structure of NHase α subunit to allow the incorporation of Co ion prior to α-β subunit association. Here, we identified the SCNase activator, P15K, sharing only limited homology with Co-type NHase activators. Only when the SCNase α, β and γ subunits were expressed together with P15K in *E. coli* in cobalt-enriched culture medium, the obtained recombinant SCNase exhibited SCNase activity. The results strongly suggested that P15K promoted functional expression of SCNase by assisting the incorporation of a cobalt ion into SCNase proteins.

2. Materials and methods

2.1. Construction of expression plasmids

To construct the plasmid for the expression of SCNase in *E. coli*, the encoding sequence was amplified by PCR using a cosmid clone, SCN-2, containing *scnB*, *scnA* and *scnC* genes encoding the β, α and γ subunits of SCNase of *T. thioparus* THI 115 [3] as the template. The primers used were 5′-TTTCATATGTCATCGTCCATCAGAGAAG-3′ and 5′-TTTGAGCTCTCAATGATCGTGATGCAC-3′. The PCR products were excised with *Nde*I and *Sac*I and subcloned into pET32a (Novagen) to create the plasmid pGE32. To construct the plasmid for the expression of P15K in *E. coli*, the encoding sequence was amplified by PCR using pUC118/T2 as the template. The primers used were 5′-AAGCATATGCCTGAGAACAACGTGGAAG-3′ and 5′-CCGAAGCTTTCAGACGCTCTTCAGTTTC-3′. The PCR products were excised with *Nde*I and *Hin*dIII and subcloned into pET30a (Novagen) to create the plasmid, pSAE30. As for the arabinose-induced expression, the expression vectors for SCNase subunits and P15K were constructed as follows: The *scnB*, *scnA* and *scnC* genes were amplified by PCR using pGE32 as the template with the primers, 5′-TTCATATGTCATCGTCCATCAGAGAAGAGGTGC-3′ and 5′-TTTGGATCCTCAATGATCGTGATGCACCGGCCT-3′. The PCR products were excised with *Nde*I and *Bam*HI and subcloned into pET30a (Novagen) to create the plasmid, pSCNabgE30. The P15K gene was amplified by PCR using pSAE30 as the template with the same primers as pSAE30. The PCR products were excised with *Nde*I and *Hin*dIII and subcloned into pET23b (Novagen) to create the plasmid, pSAE23b.

2.2. Preparation of native and recombinant SCNases

Native SCNase was purified from *T. thioparus* THI 115 cells as described previously [2]. *E. coli* BL21(DE3) cells harboring pGE32 were grown in Luria–Bertani (LB) medium containing ampicillin (100 μg/mL) at 30 °C. In the case of *E. coli* BL21(DE3) cells harboring both pGE32 and pSAE30, ampicillin (100 μg/mL) and kanamycin (80 μg/mL) were added to the LB medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) (1.0 mM) was added when the optical density at 600 nm (OD_{600}) reached 0.5. In the case of cobalt-enriched medium, cobalt(II) chloride was added at a final concentration of 0.40 mM together with IPTG. The cells were further cultivated for 5 h and harvested by centrifugation ($5000 \times g$, 10 min, 4 °C). Recombinant SCNase was purified from the lysates of the cells by three steps chromatography using a Butyl-Toyopearl 650M column (2.5 × 13 cm, Tosoh, Japan), a hydroxyapatite column (1.5 × 8 cm, Bio-Rad, USA) and a SuperQ-Toyopearl 650M column (2.5 × 14 cm, Tosoh). In the case of the arabinose-induced expression, *E. coli* BL21-AI (Invitrogen, USA) cells harboring both pSCNabgE30 and pSAE23b were grown in LB medium containing ampicillin (100 μg/mL) and kanamycin (80 μg/mL) at 30 °C. 0.2% (w/v) arabinose was added when OD_{600} reached 0.2, and IPTG (1.0 mM) and $CoCl_2$ (0.1 mM) were added when OD_{600} reached 0.4. The cells were further cultivated for 5 h and harvested by centrifugation ($5000 \times g$, 10 min, 4 °C). Recombinant SCNase was purified from the lysates of the cells by four steps chromatography using Butyl-Toyopearl 650M (2.5 × 13 cm, Tosoh), Superdex 200pg Hiload 26/60 (2.6 × 60 cm, Amersham Biosciences, USA), SuperQ-Toyopearl 650M (2.5 × 14 cm, Tosoh) and Butyl-Toyopearl 650S (2.5 × 20 cm, Tosoh). The purified SCNases were detected as a single band by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3. Size exclusion chromatography-multiangle laser light scattering

The molecular weight of apo-SCNase was determined using size exclusion chromatography-multiangle laser light scattering (SEC-MALS) on Shodex PROTEIN KW-804 (0.80 × 30 cm, Showadenko, Japan) and KW-803 (0.80 × 30 cm, Showadenko) columns connected to a multiangle light-scattering detector (DAWN DSP, Wyatt Technology, USA) and a Shodex RI-71 differential refractive index detector (Showadenko). Potassium phosphate (50 mM, pH 7.3) containing 100 mM KCl was used as the mobile phase at a flow rate of 1.0 mL/min. 1.6×10^2 μg of apo-SCNase was injected into the column. The molecular weight and distribution of the recombinant protein were determined with the Program ASTRA [17] as described [18].

2.4. Size exclusion chromatography

The molecular weight and stoichiometry of the native and apo-SCNases and were examined by SEC on Superdex 200 10/300 GL (1.0 × 30 cm, Amersham Biosciences) equilibrated with 50 mM potassium phosphate, pH 7.8, containing 100 mM NaCl or with 50 mM potassium phosphate, pH 7.3, containing 100 mM KCl. The flow rate was 0.50 mL/min. The amounts of the native and apo-SCNases injected were 15 and 100 μg, respectively. The molecular weight and stoichiometry of $SCNase_{(+P15K)}$ was examined by SEC on the same column equilibrated with 50 mM potassium phosphate, pH 7.8, containing 100 mM NaCl. The flow rate was 0.50 mL/min. The amounts of $SCNase_{(+P15K)}$ injected was 80 μg.

2.5. Electrospray ionization-liquid chromatography/mass spectrometry

Electrospray ionization-liquid chromatography/mass spectrometry (ESI-LC/MS) was performed with a Finnigan LCQ ion trap mass spectrometer with an ESI probe, connected to a reversed-phase HPLC column, Mightysil C8 (Kanto-kagaku, Japan) using an Agilent model 1100 liquid chromatograph. The preparation of the peptide fragment containing the metal-binding motif and mass spectrometry were performed as described [19].

2.6. Enzyme assay

The SCNase activity was determined by the amount of ammonia produced using 40 mM potassium thiocyanate as the substrate [2]. The amount of ammonia was quantified by using Nessler reagent (Wako Pure Chemical Co., Japan). One unit of the activity is defined as the quantity of SCNase that produces 1 μmol of ammonia per minute. The specific activity of the purified native SCNase is about 32 U/mg-protein.

2.7. Other methods

Protein concentrations were determined based on Bradford's method [20] using bovine serum albumin as a standard or by measuring the absorbance at 280 nm (ε_{280} of the purified native SCNase = 2.3 mL mg^{-1} cm^{-1}). The amount of cobalt ions bound to the recombinant SCNase was determined with an inductively coupled plasma mass spectrophotometer (ICP-MS) (Agilent 7500a, Agilent Technology).

3. Results and discussion

3.1. SCNase was expressed as an apo-protein in E. coli

All SCNase subunits were highly expressed up to 5% of the total soluble proteins when *E. coli* cells harboring pGE32 were grown at 30 °C. However, the crude extract exhibited only trace SCNase activity (3.0×10^{-3} U/mg). The activity was only slightly enhanced (3.6×10^{-2} U/mg) when cobalt(II) chloride was added to the culture medium at a final concentration of 0.40 mM together with IPTG. The recombinant SCNase was purified from *E. coli* harboring pGE32 cultivated in the cobalt-enriched medium. The subunit stoichiometry of the

recombinant SCNase was estimated to be α:β:γ = 1:1:1 by visual estimation of SDS–PAGE, which was in agreement with that of the native SCNase. However, the specific activity of the recombinant SCNase was 0.20 U/mg, only 0.6% of that of the native SCNase. The amount of cobalt ions in the recombinant SCNase was determined to be 0.050 atom/αβγ heterotrimer by ICP-MS, clearly indicating that the recombinant SCNase was an apo-protein. Apo-enzymes of Co-type NHase from *Bacillus* sp. BR449 [16] and *B. pallidus* RAPc8 [13] were activated by incubating crude NHase extracts at 50 °C in the presence of 5 μM Co^{2+}. However, apo-SCNase could not be activated by the incubation at a temperature range of 4.0–50 °C in the presence of 5 μM cobalt(II) chloride (data not shown).

We determined the molecular weight and stoichiometry of apo-SCNase by SEC-MALS (Fig. 1). Apo-SCNase eluted as a mono-dispersed peak, indicating that apo-SCNase was not an aggregate of the subunits but an appropriately assembled complex. The molecular mass was estimated at 2.4×10^2 kDa, demonstrating that apo-SCNase behaved as a hetero-dodecamer, $(\alpha\beta\gamma)_4$. The result contradicted the previous observation [2] that native SCNase was a hetero-hexamer, $(\alpha\beta\gamma)_2$, based on SEC. To clarify the contradiction, the native and apo-enzymes of SCNase were analyzed by SEC on Superdex 200 10/300 GL (1.0×30 cm, Amersham Biosciences) equilibrated with the same buffer as that used in the previous study [2]. Each form of SCNase was eluted at 12.5 mL. From the SEC analysis, the molecular weight of the native and apo-SCNases were estimated as about 140 kDa. However, the elution profiles of both the native and apo-enzymes of SCNase did not changed even when the SEC column was equilibrated with the buffer used in SEC-MALS. In general, the molecular weight determination by SEC is not decisive because the elution volume of a protein was influenced not only by its molecular weight but also by its molecular shape. In addition, the elution time might be delayed when a protein interacted with the chromatography resin. In contrast, in SEC-MALS, the molecular weight was determined only by the measurement of light scattering and refractive index of the eluted proteins [17]. Thus, we concluded that native as well as apo-forms of SCNase have the heterododecameric structure, $(\alpha\beta\gamma)_4$. Very recently, we elucidated the crystal structures of the native and apo-forms of SCNase and found that both forms of SCNase have stable $(\alpha\beta\gamma)_4$ structure (Arakawa et al., manuscript in preparation).

3.2. Expression of SCNase with the putative activator protein, P15K

The above results suggest that SCNase requires an activator protein for functional expression, like most Co- as well as Fe-type NHases. Only NHase of *Bucillus* sp. BR449 was known to be expressed functionally independently of P12K, corresponding to Co-type NHase activator [16]. Generally, the genes of Co-type NHase activators are 0.3–0.4 kb and located immediately downstream of the NHase genes [11–13,15,16]. Thus, P15K encoded in *orf1*, locating just downstream of SCNase genes, was considered a candidate for the SCNase activator, even though it exhibited only limited homology (9–19% identity) with Co-type NHase activators (Fig. 2). When SCNase was co-expressed with P15K in *E. coli* cells harboring both pGE32 and pSAE30 at 30 °C, the SCNase activity of the crude extract was more than 100 times enhanced by the addition of cobalt(II) chloride at a concentration of 0.40 mM. The purified recombinant SCNase contained 0.56 Co atom/αβγ hetero-trimer and exhibited a level of the activity of 8.0 U/mg, 25% of that of the native enzyme. Thus, P15K is likely to promote the functional expression of SCNase in *E. coli*. The relatively low catalytic activity may be due to low expression level of P15K, although the P15K band could not be identified because of the same mobility with the SCNase α subunit in SDS–PAGE analysis. To increase the expression of P15K, we constructed the expression vectors, pSCNabgE30 and pSAE23b in which the SCNase genes and the P15K gene were subcloned downstream of T7*lac* promoter and T7 promoter, respectively. Using the plasmids, SCNase and P15K were co-expressed in *E. coli*, BL21-AI, where the expression of T7 RNA polymerase was strictly controlled by the addition of arabinose. By

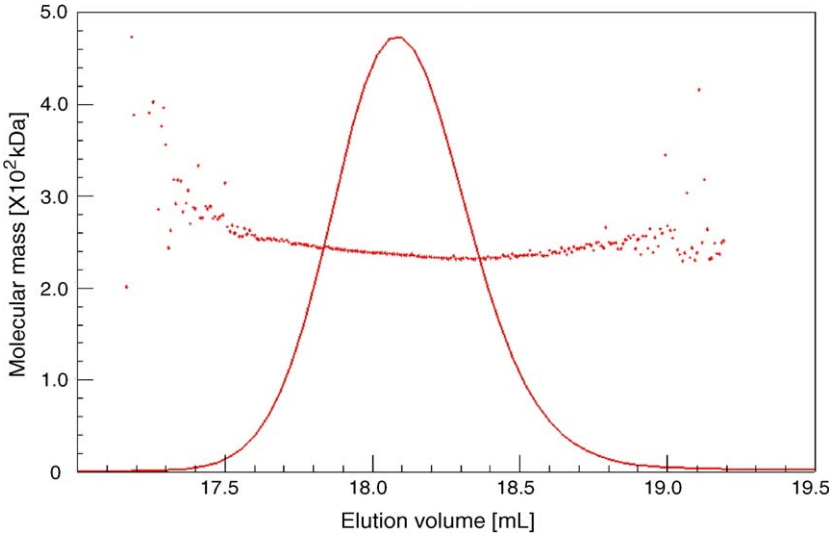

Fig. 1. Plot of the apparent molecular weight of apo-SCNase versus elution volume in SEC-MALS. The symbols represent the apparent molecular mass estimated from a light scattering measurement against a slice with a 16.7 μL volume. The curve indicates the elution profile of apo-SCNase from SEC monitored with the differential refractive index detector. The vertical scale of the curve is arbitrarily. Other experimental conditions were shown in Section 2.

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P15k      1 MPENNVEGRGGWQGTDTTPIPVLEGVRAHGRAWEELAPQYGVTNPDPPWKIDLETTCDML
SCNβ      1 MSSSIREEVHRHLGTVALMQPALH-QQTHAPAPTEITHTLFRAYTRVPHDVGGEADVPIE
putida    1 -----------MALCLTSLGSPRRLPWWS------ACTRPVSFSGKTGPRPSPPKSTLPR
BR449     1 -----------MKSCENQPNESLLANMS-------EEVAPPRKNGELEFQEPWERRSFG-
RAPc8     1 -----------MKSCENQPNESLLANMS-------EEVAPPRKNGELEFQEPWERRSFG-
nhhG      1 -------------MSEDTLTDRLPATG---------TAAPPRDNGELVFTEPWEATAFG-
nhlE      1 -----------MPRLNEQPHPGLEANLGDLVQNLPFNERIPRRSGEVAFDQAWEIRAFS-

P15k     61 AADSCVKSYDEIEPGSCVLPALE--------RRAEEDDLSETIYADVPFPERQLLALAHS
SCNβ     60 YHEKEEEIWELNTFATCECLAWRGVWTAEERRRKQNCDVGQTVYLGMPYYGRWLLTAARI
putida   44 SAGESVNDTYYRQWVSALEKLVA--------SLGLVTGGDVNSRAQEWKQAHLNTPHGHP
BR449    42 MTLALYEEKLYSSWEDFRSRLIE--------EIKGWETAKQKENSDWNYYEHWLAALERL
RAPc8    42 MTLALYEEKLYSSWEDFRSRLIE--------EIKGWETAKQKDNSDWNYYEHWLAALERL
nhhG     38 VAIALSDQKSY-EWEFFRQRLIH--------SIA-------EANGCEAYYESWTKALEAS
nhlE     49 IATALHGQGRF-EWDEFQSRLIE--------SIKQWEAEH-ATTEQWSYYERWMLALEEL

P15k    113 MLKRGLFSEEELA------RQMEIVGQKLKSV------------------------
SCNβ    120 LVDKQFVTLTELHNKIVEMRERVASGQGLGEYLPPKAK------------------
putida   96 ILLAHALCPPAID------PKHKHEPQRSPIKVVAAMA------------------
BR449    94 VVETGMLN-------------------------------------------------
RAPc8    94 VVETGMLNKRDVD------TRTNEFLTGKRDEVFY----------------------
nhhG     82 VVDSGLISEDEIR------ERMESMAIID---------------------------
nhlE     99 LHDKGFVAGEELA------HRTEQVLATPAGAHHQHAVRDPIAVHAIGTRTTDSDG
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Fig. 2. Alignment of the amino acid sequences of P15K, the SCNase β subunit and Co-type NHase activators. Conserved and homologous amino acid residues between P15K and Co-type NHase activators are indicated by black and gray back grounds, respectively. Conserved amino acid residues between P15K and the SCNase β subunit were indicated by solid boxes. Gaps (hyphens) were introduced to maximize the homology. Co-type NHase activators are as follows: BR449, P12K from *Bacillus* sp. BR449 (16); RAPc8, P14K from *B. pallidus* RAPc8 (13); putida, P14K from *Pseudomonas putida* 5B (12); nhhG from *Rhodococcus rhodochorous* J1 H-NHase (11); nhlE from *Rhodococcus rhodochorous* J1 L-NHase (15).

optimizing the culture conditions, the activity of the crude SCNase extract 4.2 times increased. The purified recombinant SCNase possessed 0.86 Co atom/αβγ hetero-trimer and its catalytic activity was 25 U/mg, 78% of that of the native enzyme. We designated this active recombinant NHase as SCNase$_{(+P15K)}$.

3.3. Characterization of apo-SCNase and SCNase$_{(+P15K)}$

SCNase$_{(+P15K)}$ was eluted at 12.5 min in SEC on Superdex 200 10/300 GL (1.0 × 30 cm, Amersham Biosciences) equilibrated with 50 mM potassium phosphate, pH 7.8, containing 100 mM NaCl showing that SCNase$_{(+P15K)}$ is also $(\alpha\beta\gamma)_4$. UV–Vis absorption spectrum of apo-SCNase showed only one peak at 280 nm, while that of SCNase$_{(+P15K)}$ exhibited an extra shoulder peak above 300 nm (Fig. 3), which was very similar to that of native SCNase [7]. The magnitude of the shoulder at 330 nm was about 84% of that in native enzyme, which was in good agreement with the Co content. The characteristic shoulder is commonly observed in Co-type NHases [21] and has been assigned as a ligand-to-metal charge transfer band [22,23]. We investigated the post-translational modifications of the cysteine ligand residues in apo-SCNase and SCNase$_{(+P15K)}$. Each recombinant SCNase was treated with TPCK-trypsin after reduction and S-carboxymethylation and then analyzed by ESI-LC/MS. In apo-SCNase, the mass spectrum of the metal-binding peptide, H^{124}VVVCTLCSCYPR-PIL-GQSPEWYR^{147} (HR24), showed a mass peak of *m/z* 1491.7 (Fig. 4A). This corresponded to the calculated *m/z* value of the $[M + 2H]^{2+}$ ion of HR24 with three carboxymethyl (CM)-cysteines, indicating that no cysteine ligand was modified to Cys-SO_2H in apo-SCNase. In contrast, the magnitude of the *m/z* 1491 peak decreased dramatically and the intense peak at *m/z* 1478.4 as well as the peaks at *m/z* 1465.7 and 1485.2 were observed in the mass spectrum of HR24 of SCNase$_{(+P15K)}$ (Fig. 4B). Each peak was assigned by MS/MS analyses (see the legend of Fig. 4). The relative abundance of the *m/z* 1478 peak was estimated to be 74%, indicating that about 70% of SCNase$_{(+P15K)}$ had the Cys-SO_2H modification at γCys131 like native SCNase. The presence of the *m/z* 1466 and 1486 peaks indicated that some of SCNase$_{(+P15K)}$ had the different oxidation states in γCys131 or γCys133. However, we should note that these mass peaks were also observed in the mass spectrum of HR24 from native SCNase [7]. Thus, we concluded that SCNase$_{(+P15K)}$ was the active holo-form of SCNase. In other words, the functional heterologous expression of SCNase is promoted by P15K.

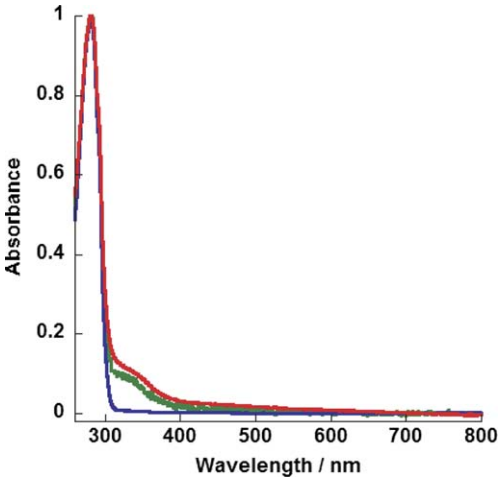

Fig. 3. Absorption spectra of native SCNase, apo-SCNase and SCNase$_{(+P15K)}$. Blue, red and green lines represent the absorption spectra of native SCNase, apo-SCNase and SCNase$_{(+P15K)}$, respectively. The concentration of each sample was 0.37 mg/mL in 50 mM potassium phosphate, pH 7.5. All spectra were recorded at room temperature with a Cary50 spectrophotometer (Varian, USA).

The present study indicates that P15K is responsible for the incorporation of Co ion into the SCNase protein. Considering the weak amino acid sequence homology between P15K and the SCNase β subunit (Fig. 2), it is possible that P15K has a chaperone-like activity and interacted with the SCNase γ sub-

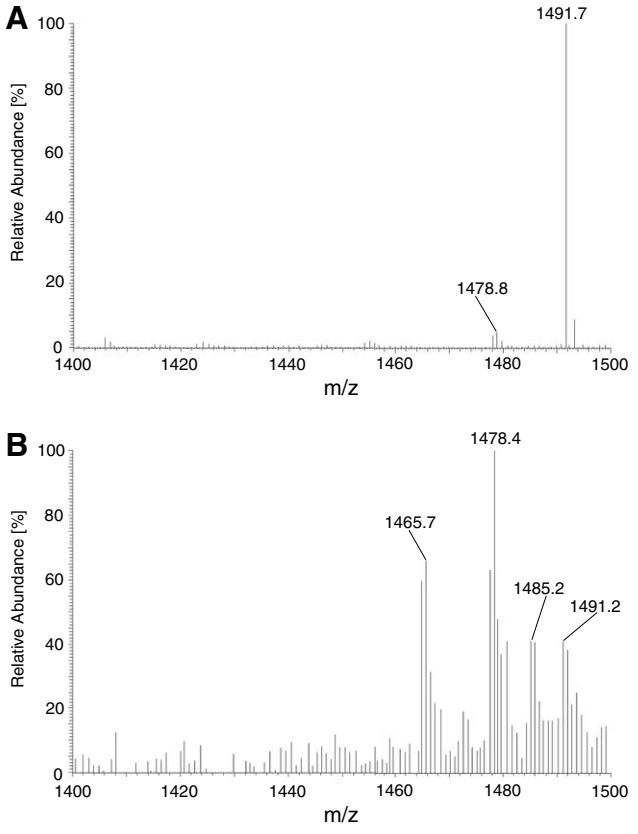

Fig. 4. ESI-LC/MS spectra of the metal-binding peptide, HR24, of apo-SCNase (A) and $SCNase_{(+P15K)}$ (B). The mass peaks with *m/z* values of 1491, 1486, 1478 and 1466 correspond to the $[M + 2H]^{2+}$ ion of HR24 with three CM-cysteines (the calculated *m/z* value = 1491.7), that with γCys131-SO_3H and two CM-cysteines (the calculated *m/z* value = 1486.9), that with γ Cys131-SO_2H and two CM-cysteines (the calculated *m/z* value = 1478.9), and that with CM-γCys128, γCys131-SO_2H and γCys133-SO_2H (the calculated *m/z* value = 1466.1), respectively. The detailed condition of ESI-LC/MS was described previously [20].

unit to assist the incorporation of Co ion before the assembly with α and β subunits as postulated in *B. pallidus* RAPc8 NHase [13]. Alternatively, we could not exclude the possibility that P15K directly mediate the insertion of Co ion into the SCNase protein. In the recombinant SCNase expressed in *E. coli* harboring pGE32 and pSAE30, the catalytic activity and relative abundance of the Cys-SO_2H modification were low, compared with its Co-content. The modification of cysteine residues might occur after the incorporation of Co ion. The biochemical characterization of P15K is in progress in our laboratory.

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