Biochimica et Biophysica Acta 1794 (2009) 468–475

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

# Putative "acylaminoacyl" peptidases from *Streptomyces griseus* and *S. coelicolor* display "aminopeptidase" activities with distinct substrate specificities and sensitivities to reducing reagent

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ARTICLE INFO

*Article history:*
Received 21 July 2008
Received in revised form 11 December 2008
Accepted 15 December 2008
Available online 25 December 2008

*Keywords:*
Prolyl oligopeptidase
Phenylalanylaminopeptidase
Serine peptidase
Actinomycete
Substrate recognition property

ABSTRACT

Aminopeptidases from *Streptomyces griseus* (SGRAP) and *S. coelicolor* (SCOAP) were cloned and characterized to clarify their biochemical characteristics. Although both enzymes had been annotated as putative oligopeptidases of family S9 enzymes, they showed "aminopeptidase" activities, not "oligopeptidase" activities. Although their deduced amino acid sequences showed high similarity (69% overall sequence homology), they showed distinct substrate specificities and sensitivities to the reducing reagent dithiothreitol (DTT). The reaction pH and addition of DTT dramatically affected the substrate preference of SGRAP. Furthermore, SCOAP selectively hydrolyzed phenyalanine *p*-nitroanilide (Phe-*p*NA) in the presence or absence of DTT. The chimera protein between SGRAP and SCOAP was constructed to identify the region responsible for the properties described above. Furthermore, $Cys^{409}$ of SCOAP was identified as a functional residue responsible for activation by reducing reagent DTT.



## 1. Introduction

Prolyl oligopeptidase (POP) family enzymes (clan SC, family S9), members of serine proteases, are widely distributed in nature. Enzymes of this family are further classifiable into four distinct subfamilies including POP (subfamily S9a), oligopeptidase B (OPB, subfamily S9a), dipeptidyl peptidase IV (DPPIV, subfamily S9b), acylaminoacyl peptidase (AAP, subfamily S9c), and glutamyl endopeptidase (GEP, subfamily S9d) based on their amino acid sequences and biochemical characteristics. The subfamilies described above are distinct on substrate recognition: POP hydrolyzes the proline-containing peptide at the carboxyl side of the proline residue, OPB hydrolyzes the peptides at those of lysine and arginine residues, and DPPIV hydrolyzes the peptide to release the N-terminal dipeptide units. In fact, GEP was recently reported as a new member of the S9 family, in which the synthetic substrate Z-Leu-Leu-Glu-naphthylamide was hydrolyzed [1]. The enzymes of S9 family differ considerably from classical serine proteases—trypsin and subtilisin—because they cleave only short peptides comprising fewer than 30 amino acid residues [2]. The 3D structure of the well-characterized POP from porcine muscle clarified the unique mechanism of preventing hydrolysis of large proteins [3]. The enzyme consists of a characteristic β-propeller domain and an α/β hydrolase catalytic domain. The former domain blocks the large protein from entry into the latter. Therefore, family S9 enzymes including the entire subfamily are unable to hydrolyze large proteins.

The reason for the wide distribution and physiological roles of the POP family enzymes has been clarified. Nevertheless, the enzymes have remained the focus of pharmacological interests by many researchers. In fact, POPs, a member of the subfamily S9a, hydrolyzes neuropeptides including substance P and α-melanocyte-stimulating hormone being responsible for the modulation of the memory process and Alzheimer's disease [4]. Another member of subfamily S9a of POP family enzymes, OPB, is reportedly involved in the pathogenesis of *Trypanosoma brucei* and *T. evansi*, which cause trypanosome infection [5,6]. Therefore, subfamily S9a enzymes are emerging as therapeutic targets for human diseases.

Puromycin hydrolase (PMH) from *Streptomyces morookaensis* was recently identified and characterized as a putative member of POP family enzymes [7,8]. The deduced amino acid sequence resembled those of known POP family enzymes including POP from *Coxiella burnetii* and putative acylpeptide hydrolases (ACPH) of *S. avermetilis* MA-4680 and *S. coelicolor* A3 (2). Interestingly, PMH reportedly shows "aminopeptidase" activity, not "acylpeptide hydrolase" activity; that is, it releases the N-terminal amino acid from the *p*-nitroanilide derivatives of amino acids (a.a.-*p*NAs) or peptide substrates while not releasing the N-terminal acylaminoacyl amino acids (*N*-Ac-a.a.s) from peptide derivatives. Such a substrate preference was hitherto unknown among known POP family enzymes. Consequently, the question arose: are such new POP family enzymes possessing amino

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doi:10.1016/j.bbapap.2008.12.013

peptidase activity generally distributed in actinomycetes? The enzymes would be classified into a new subfamily of POP family enzymes if such were the case. Characterizations of such new subfamily enzymes will provide new insight into the biochemical study of and development of a selective inhibitor of POP family enzymes.

For this study, we cloned and characterized two PMH homologues of *S. griseus* NBRC13350 (SGRAP) and *S. coelicolor* A3 (2) (SCOAP). The enzymes showed "aminopeptidase activity," not "acylaminoacyl peptidase" activity, as expected. Although their amino acid sequences showed high similarity (69% overall sequence homology), they showed distinct substrate specificities and sensitivities to the reducing reagent DTT. We constructed a chimera protein between SGRAP and SCOAP, and C409A mutant of SCOAP to determine the functional region responsible for those properties.

## 2. Materials and methods

### 2.1. Materials, bacterial strain, and plasmids

For use in this study, Phe-*p*NA, Pro-*p*NA, Leu-*p*NA, and Lys-*p*NA were purchased from Sigma; Ala-*p*NA was obtained from the Peptide Institute, Inc. Other *p*NA derivatives of amino acids were purchased from Bachem AG. A Zero Blunt II TOPO PCR Cloning kit and casein, fluorescein conjugate (FITC-casein) were purchased from Invitrogen Corp. The plasmids pET28a (+), *E. coli* Rosetta 2 (DE3) pLysS, and *Escherichia coli* Rosetta 2 (DE3) were obtained from Novagen Inc.

### 2.2. Cloning of SGRAP and SCOAP gene

Genomic DNA was prepared from *S. griseus* NBRC13350 and *S. coelicolor* A3 (2) using methods described by Hopwood et al. [9]. The gene encoding SGRAP [ID of *S. griseus* genome project (http://streptomyces.nih.go.jp/griseus/): SGR1153] was amplified using PCR with a set of sense primers incorporating the NdeI site upstream of a start codon (5′-CATATGAACACCGTGCCAGCAGC-3′; the start codon is underlined) and anti-sense primer incorporating the HindIII site downstream of a stop codon (5′-AAGCTTCACGTGGTCAGCTCCAGGA-3′; the stop codon is underlined) and the genomic DNA of *S. griseus*. The gene encoding SCOAP (accession no. Q92BI6) was amplified similarly to the sense primer incorporating the NdeI site upstream of a start codon (5′-CATATGGGGGAGTCAGTGCGGAC-3′; the start codon is underlined) and anti-sense primer incorporating the HindIII site downstream of a stop codon (5′-AAGCTTCACCGGGTCAGCTCCAGGACCGGGACGCC-3′; the stop codon is underlined); also, the genomic DNA of *S. coelicolor* were used. In addition, a set of Prime Star GXL and Prime Star GXL buffers (Takara) was used for the PCR reaction. Routinely, PCR was carried out for 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, preceded by incubation for 1 min at 94 °C and followed by incubation for 5 min at 72 °C. The PCR products were cloned using a PCR cloning kit (Zero Blunt II TOPO; Invitrogen Corp.) and sequenced, with subsequent digestion using NdeI and HindIII, to obtain gene fragments encoding SGRAP and SCOAP. The obtained fragments were introduced into NdeI-HindIII gap of pET 28a (+) to generate the plasmids pET28-His$_6$-SGRAP and pET28-His$_6$-SCOAP. The expression vectors were designed for the N-terminal His$_6$-tag.

### 2.3. Construction of chimera protein SCO/SGR-AP

The plasmid pET28-His$_6$-SGRAP was digested using HindIII and SplI to obtain the 0.7 kbp fragment encoding the amino acid residues of 435–671 of SGRAP. The fragment was introduced into the HindIII-SplI gap of pET28-His$_6$-SCOAP to generate the plasmid pET28-His$_6$-SCO/SGR-AP followed by transformation into the *E. coli* Rosetta 2 (DE3) pLysS.

### 2.4. Construction of the SCOAP C409A mutant

The pCR-Blunt II-TOPO:*SCOAP* plasmid was amplified with inverse PCR using a set of sense primer (5′-CACCACCCCGGC (T→G, Cys→Ala)CCCGCGCCCCCGAC-3′) corresponding to 1213-1239 of *SCOAP*) and its complementary strand. The resultant PCR reaction mixture was diluted with $H_2O$, treated with DpnI (Toyobo Co. Ltd.) followed by cloning and sequencing with subsequent digestion using NdeI and HindIII to obtain gene fragments encoding C409A mutant of SCOAP. The fragment was introduced into the NdeI-HindIII gap of pET28a (+) to generate the plasmid pET28-His$_6$-SCOAP C409A followed by transformation into the *E. coli* Rosetta 2 (DE3).

### 2.4. Expression and purification of recombinant SGRAP, SCOAP, SCO/SGR-AP, and C409A variants of SCOAP

An overnight express system 1 (Novagen Inc.) was used for the overexpression of each protein. Several colonies of the Rosetta 2 (DE3) pLysS or Rosetta 2 (DE3) harboring each expression plasmid were inoculated into 50 ml of overnight express instant TB medium prepared according to the manufacturer's instructions. Cultivation and induction were carried out for 16 h at 30 °C with rotary shaking at 180 rpm; the cells were then collected by centrifugation and disrupted using sonication (Elestin NP035SP; Nepa Gene Co., Ltd.) at 1 min intervals for total sonication time of 30 min at maximum output. The obtained cell-free extracts were applied to purification with TALON metal affinity resin (Clontech) according to the manufacturer's instructions. The purified proteins were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under a reducing condition with Coomassie Blue staining.

### 2.5. Enzyme assays

The standard assays were performed as follows: 100 μl of the enzyme solutions were added to 900 μl of 1 mM of Phe-*p*NA or 2 mM of a.a.-*p*NAs in 50 mM Tris–HCl (pH 8.0) containing 1 mM DTT and incubated at 37 °C for 1–5 min. The increase in absorbance at 405 nm attributable to release of *p*-nitroaniline was monitored continuously using a spectrometer (U2800; Hitachi Ltd.). The initial rates of the hydrolytic activity were determined from the linear portion of the optical density profile ($\varepsilon_{405\ nm}$ of *p*NA = 10600 $M^{-1}$).

The effect of pH on enzyme activity was examined in 50 mM sodium acetate (pH 4.4–6.4), Tris-maleic acid (pH 5.7–7.7), Tris–HCl (pH 7.1–9.0), and Gly-NaOH (pH 9–10.1). In all cases, 1 mM DTT was added. Thermal stability testing was carried out as follows: the enzyme solutions were incubated at various temperatures (30–70 °C) for 30 min without DTT; then the residual activities were measured under the standard assay condition in which 1.0 mM Phe-*p*NA was used as a substrate. For determination of kinetic parameters ($K_{cat}$ and $K_m$) against Phe-*p*NA, 0.10–0.6 mM of substrates and 50 mM Tris-maleic acid (pH7.0) containing 1 mM DTT were used. For Ala-*p*NA, 1.5–4.7 mM of substrate in 50 mM Tris–HCl (pH 8.2) containing 1 mM DTT was used.

### 2.6. Enzyme inhibition study

100 μl of reaction mixture containing 0.17 nmol of SGRAP or SCOAP, and 500 nmol of *N*-ethylmaleimide (NEM), iodoacetic acid (IAA), or iodoacetamide (IAD) were incubated at 15 °C for 15 min. As a control reaction, the enzymes were treated similarly with $H_2O$. 5 μl of the solution (50 ng of protein) were added to 95 μl of 1 mM of Phe-*p*NA in 50 mM Tris–HCl (pH8.0) containing 5 mM of DTT (500 μmol) and incubated at 37 °C for 1–5 min to evaluate the residual enzymatic activities. In these procedures, small amounts of contaminated NEM, IAA or IAD (25 nmol each) were demonstrated to give no effect for the