Characterization of a Novel Intracellular Endopeptidase of the α/β Hydrolase Family from Streptomyces coelicolor A3(2)

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Received 3 June 2002/Accepted 21 October 2002

In a proteasome-lacking mutant of Streptomyces coelicolor A3(2), an intracellular enzyme with chymotrypsin-like activity, absent from the wild type, was detected. Complementation that restored proteasome function did not suppress expression of the endopeptidase. Since the enzyme was not found in two other S. coelicolor proteasome mutants, its expression probably resulted from a secondary mutation arisen in the proteasome mutant. Purification of the endopeptidase revealed its identity to SCO7095, a putative hydrolase encoded by the S. coelicolor A3(2) genome with no known homologue. Based on the prediction of a Ser-Asp-His catalytic triad and an α/β hydrolase fold, SCO7095 was assigned to peptidase clan SC. N-terminally His-tagged SCO7095 was efficiently expressed in Escherichia coli cells and purified for further characterization. Although SCO7095 is distantly related to several proline iminopeptidases, including Thermoplasma acidophilum tricorn-interacting F1, no aminopeptidase activity was detected. On synthetic substrates, the monomeric enzyme exhibited not only chymotrypsin-like activity but also thrombin-like activity.

Proteases are key players in various processes occurring in eukaryotic and prokaryotic cells. For instance, proteolysis plays a global role in the bacterial cell cycle (13). Since the cellular environment is crowded with proteins, tight control of intracellular protein breakdown is vital. This may be the result of a strict substrate specificity of the intracellular proteases degrading only few target proteins. Another control mechanism is provided by the architecture of self-compartmentalizing proteases. These multisubunit assemblies dispose of internal active sites that are not accessible to folded proteins. However, substrates can be targeted to the proteolytic chamber following interaction with protease-associated ATP-dependent “unfoldases.” Well-characterized prokaryotic proteases operating according to this principle include ClpP (34), HslV (38, 50), and the 20S proteasome (4, 49). Evolution has given rise to various combinations of these self-compartmentalizing proteases in the proteolytic complements of individual prokaryotes, with HslV and 20S proteasomes apparently being mutually exclusive (8). Although ubiquitous in Archaea (27), in Bacteria the 20S proteasome is confined to the actinomycetes, a lineage of high-GC gram-positive bacteria (23, 30, 35, 42).

The actinomycete genus Streptomyces has proven a valuable resource for a wide variety of peptidases and proteases as well as potent inhibitors of such enzymes (19, 48). The complete genome sequence of Streptomyces coelicolor A3(2) has provided a comprehensive picture of the protease complement of this bacterium (5). As might be anticipated from the large size of the genome, encoding almost 8,000 proteins, this analysis has revealed a highly complex proteolytic network. In addition to 60 predicted secreted proteases and peptidases, the intracellular proteolytic system comprises the 20S proteasome (30), five ClpP-like proteins (47), several other ATP-dependent proteases (including two FtsH proteins and Lon protease [14]), two tricorn proteases (41), and more than 60 putative protein/peptide-hydrolyzing enzymes.

In this paper we report on the characterization of a novel enzyme that is part of the intracellular proteolytic machinery of S. coelicolor A3(2). This new member of the α/β hydrolase family, not annotated as a protein- or peptide-hydrolyzing enzyme but classified as a putative hydrolase (SCO7095), was identified in a S. coelicolor mutant lacking the 20S proteasome and characterized as a serine peptidase with unusual specificity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are described in Table 1. Escherichia coli strains were routinely cultured in Luria-Bertani medium at 37°C. Where appropriate, antibiotics were added to the medium as follows: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml. S. coelicolor strains were grown in CYG medium without NaCl (Casitone peptone, 10 g/liter; yeast extract, 5 g/liter; glucose, 5 g/liter) at 30°C. Antibiotics were added as follows: thiostrepton, 10 µg/ml (in liquid medium) or 50 µg/ml (in solid medium); kanamycin, 50 µg/ml. To generate spores, Streptomyces cells were sprayed on the CYG plates and incubated for 7 days at 30°C. The CYG medium was supplemented with 5 mM MgCl2 and 0.5% glycine to grow cells for the preparation of protoplasts.

Construction of an S. coelicolor proteasome mutant. A pUC18-based DNA replacement vector was constructed by replacing an internal fragment of the previously cloned preB operon (30) with a thiostrepton resistance gene. The internal 1,261-bp ScaI-MluI fragment of the preB operon was removed from the insert of pFA2594 by digestion with the corresponding restriction endonucleases (partial digestion with SacI required due to an internal site of pUC18), and...
the overhanging MluI end of the plasmid remnant was filled up with the Klenow fragment of DNA polymerase. The thiorrethion resistance gene, tsr, originating from pJ346 was inserted from pFAJ2608 by digestion with EcoRI and PstI. The purified DNA fragment (1,090 bp) was blunted and ligated to the remnant of pFAJ2594. This resulted in a plasmid, pFAJ2727, not replicating in Streptomyces cells and carrying a marker-resistant displaced operon promoter. Preparation and transformation of S. coelicolor protoplasts were carried out by the method of Oh and Chater (32). Thiorrethion-resistant colonies were checked by colony PCR using primers corresponding to the 5'-end of preB (5'-TGAGGCAACAACCTCGTAGACCAGC-3') and the 3'-end of preA (5'-CTACTCTCTGTGCGACCAGGACTC-3'). Putative double homologous recombinants showing only the PCR product of the size predicted for the marker-disrupted preB operon were selected (3 colonies of 100 screened). For these strains, the absence of the delivery vector pUC18 and the replacement of the Scl-MluI DNA fragment of the thiorrethion operon with the thiorrethion resistance gene were verified by Southern hybridization on total DNA digested with Stul and EcoRI, using the digoxigenin-labeled DNAs (Roche Applied Science) of the target fragments (pUC18, Scl-MluI fragment, or tsr gene) as probes. The absence of the 20S proteasome in the mutants was confirmed by Western blotting, using a cross-reactive polyclonal antibody raised against the Rhodococcus erythropolis 20S proteasome.

**Phenotypic characterization of the S. coelicolor proteasome mutant.** The ability of cells of S. coelicolor A3(2) and the proteasome-deficient mutants to grow under different kinds of environmental stress were analyzed. Growth was assessed quantitatively on solid media since the mycelial growth characteristics and clumping did not allow a reliable quantitative analysis based on culture turbidity measurements. A 20-μL volume of spore suspension was spread on the surface of solid CYG medium, and plates were incubated at 30°C for 5 days. The conditions used were essentially those described by Knipfer and Shradler (23). The CYG medium was supplemented with ethanol (7%, vol/vol), cadmium chloride (0.1 mM), paromycin (0.1 or 0.01 mg/ml), or sodium chloride (6 or 7%, wt/vol). To visualize the lack of proteasome activity and detect possible other changes induced in the mutants, zymograms using different synthetic substrates were prepared for wild-type and mutant cells. Cellular extracts were obtained by a mild extraction method. Cells were cultured for 48 h in the growth medium used to prepare protoplasts, collected by centrifugation, washed twice, and resuspended in 5 ml of 25 mM Tris- HCl (pH 7.5) supplemented with 1 mg of lysozyme per ml. The cell suspension was incubated on ice for 2 h, and 60 U of endonuclease (Sigma) was added to degrade DNA and RNA. Cell debris was removed by centrifugation (27,000 × g for 30 min) at 4°C. Cell extracts were analyzed by loading aliquots containing 30 μg of protein onto a native polyacrylamide gradient gel (4 to 20%) run as recommended by the supplier (Novex). After completion of the run, the gel was overloaded with 5 ml of 25 mM Tris-HCl (pH 7.5) supplemented with 0.1 mM of fluorogenic substrate (Bachem): Z-GGL-AMC (benzoyloxyacaronyl-Gly-Gly-Leu-7-amido-4-methylcoumarin), Suc-LLVY-AMC (succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin), or Suc-AAF-AMC (succinyl-Ala-Ala-Phc-7-amido-4-methylcoumarin). After incubation for 15 min at 37°C, the peptidease activity was visualized under UV illumination.

To complement the proteasome deficiency of mutant FAJ2038, protoplasts were transformed with pFAJ2762 and then subjected to regeneration. Kanamycin-resistant transformants were selected, and expression of the proteasome was verified by zymography using Suc-LLVY-AMC as a substrate.

**Partial purification of the enzyme hydrolyzing Z-GGL-AMC.** The enzyme hydrolyzing the synthetic substrate Z-GGL-AMC was partially purified by fast protein liquid chromatography. All purification steps were performed at 4°C. During the different purification steps, enzymatic activity was monitored by Z-GGL-AMC zymogram analysis. Crude cell extract was prepared from 12 g of S. coelicolor FAJ2038 cells as described above. A 20-ml volume of supernatant containing 200 mg of protein was loaded onto a Q-Sepharose column (2 by 10 cm; Amersham Biosciences) and eluted by 200 mM NaCl linear gradient. Active fractions, eluting at approximately 350 mM NaCl, were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7). The dialyzed sample was loaded onto a DEAE-Sephacel column (2 by 8 cm [Amersham Biosciences]) equilibrated with the same buffer, and bound proteins were eluted with a 0 to 600 mM NaCl linear gradient. Active fractions, eluting at approximately 350 mM NaCl, were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7). The dialyzed sample was loaded onto a hydroxyapatite column (1.4 by 6 cm [Bio-Rad]) equilibrated with the same buffer, and bound proteins were eluted with 0 to 300 mM potassium phosphate buffer (pH 7). The active fractions, eluting at approximately 180 mM phosphate, were pooled, dialyzed against 25 mM Tris-HCl (pH 7.0), and loaded on to a MonoQ column (0.5 by 5 cm [Amersham Biosciences]). Bound proteins were eluted with a gradient of 0 to 600 mM NaCl in the same buffer. The enzyme, emerging at approximately 220 mM NaCl, was concentrated by centrifugation in a Centrifior-10 apparatus (Amicon) and then loaded onto a Superose 6 HR column (1 by 30 cm [Amersham Biosciences]) equilibrated with 25 mM Tris-HCl (pH 7.0). The active fractions were
combined, and this sample was subjected to native gel electrophoresis to locate the band associated with Z-GGL-AMC-hydrolyzing activity. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the excised band confirmed that this activity corresponded to the major protein in the active Superoxide 6 fraction, indicating that proteolytic cleavage was required for the mature enzyme, the major band was subjected to Edman degradation, but the protein appeared to be N-terminally blocked. Therefore, the active band from native gel was subjected to in-situ digestion with trypsin. The resulting peptides were separated by high-performance liquid chromatography before being subjected to Edman degradation (42).

Cloning of the S. coelicolor gene encoding the Z-GGL-AMC-hydrolyzing enzyme SCO7095. The amino acid sequences of five internal fragments resulting from trypsin digestion of the Z-GGL-AMC-hydrolyzing enzyme were determined: (a) DHVQDHPEVADYAATLTTR, (b) DYTVAHYAHFLHAVOHVL, (c) WAALHILHEFLK, (d) ALTVLEEAAGHLHLEOPAR, and (e) TPEDDSAPLSPGTHTTITVP. Since the relative positions of the internal amino acid sequences was not known, degenerate forward (F) and reverse (R) primers corresponding to the sequences (a) through (d) were designed: aF 5'-GAGTCAAGCCAACCCCGTGGGAGTGTGAAGTGGTGGTGGAGGAGGAGG-3', bF 5'-GACTACACGTTGCACTAACCAGCCTAGGGCCTAACCACTTCCCACTTCTGGGAGTCCCACTTGACG-3', cF 5'-TGGGCGGCGGCTTGGCACTAACCAGCCTAGGGCCTAACCACTTCCCACTTCTGGGAGTCCCACTTGACG-3', dF 5'-CGGAGGAGGAGGACGACGCTTGGGAAGGACGACGCTTGGGAAGGACGACGCTTGGGAAGGACGAC-3', and eF 5'-GGTGGCTGTAGGCGGGAGG-3'. From the primer combinations that produced DNA bands in PCR reactions using Phi polymerase, the PCR product (604 bp) produced by primer combination bF and dR was selected, phosphorylated with T4 polynucleotide kinase (New England Biolabs), cloned into the Smal site of pUC18, and verified by sequence analysis. A digoxigenin-labeled probe was synthesized by PCR on this DNA fragment and used in a Southern hybridization on total DNA of S. coelicolor digested with EcoRI, Smal, PstI, or BglII. Based on the size estimates of the hybridizing bands, it was decided to construct a minilibrary in pUC18 containing fragments of about 5 kb from PstI of the hybridizing bands, it was decided to construct a minilibrary in pUC18, and dR (5'-GCC-3') and d.R (5'-GCC-3') with the primers 5'-GCC-3'). In silico analyses of DNA and protein sequences was carried out using the program GenDoc (http://www.bsc.edu/biomed/genedoc/).

RESULTS AND DISCUSSION

Construction and phenotypic characterization of protease-lacking S. coelicolor mutants. Proteasome knockout mutants were isolated by exchanging an internal fragment of the prcBA operon, encoding the β and α subunits of the 20S proteasome, with a thiostrepton resistance marker through double homologous recombination. Three such mutants (FAJ2037, FAJ2038, and FAJ2053) with the predicted gene disruption were obtained. The absence of the 20S proteasome was confirmed by zymography (using Suc-LLVY-AMC as a substrate) and by Western analysis. Growth of the mutants on rich or minimal medium at 37 and 42°C was not impaired. In addition, the mutants could not be distinguished from the wild-type strain based on sensitivity to ethanol (organic solvent stress), cadmium ions (heavy metal stress), puromycin (generating misfolded puromycin-containing peptides), or high salt concentration (osmotic stress). The lack of an obvious growth- or stress-related phenotype was previously reported for a protease mutant of Mycobacterium smegmatis (23). In the archean Thermoplasma acidophilum, specific inhibition of protease activity impaired cell viability following heat shock but not under normal growth conditions (36). In E. coli, several proteases, including the self-compartmentalizing protease

bovine plasma at 1,700 NIH U/mg [Sigma]) at 0.096 mg/l was serially diluted twofold using 50 mM Tris-HCl (pH 8.3). Then 50 µl volumes of these dilutions were added to a 96-well plate. Subsequently, 50 µl of 0.4 mM S-2238 was added and the mixtures were incubated at 37°C. After a 30-min incubation, the absorbance at 405 nm was measured using an EL 808 Ultra microplate reader (BioTek Instruments Inc).

Carboxypeptidase activity was determined spectrophotometrically using the 3-(2-furyl)acryloyl-dipeptides FA-AR, FA-GL, and FA-RL as substrates (51). Purified recombinant enzyme (2 or 8 µg/ml) was incubated at 37°C with the respective substrates (100 µM final concentration) in 0.1 mM Tris-HCl (pH 7.5). The reaction was stopped after 30 or 60 min with an equal volume of 10% SDS. Activity was measured from the decrease in absorption at 340 nm. Esterase activity was assayed by the method of Jackman et al. (17). The peptide degradation assay used oxidized insulin B-chain as a substrate was described previously (40). The protease database MEROPS (5) was used as a resource for information about the activities of proteases on synthetic substrates.

The inhibitory effect of phenylmethylsulfonyl fluoride (PMSF) and i- or p-Pro-Arg-chloromethylketone (PPACK) on the activity of SCO7095 was analyzed by determining the residual activity after preincubation of the enzyme in the presence of inhibitor (40). Purified enzyme (20 ng) was incubated with different concentrations of PMSF or PPACK in 50 µl of Tris-HCl (pH 7.5) at 37°C for 15 min. The reaction was initiated by addition of 50 µl of 50 mM Tris-HCl containing 10 nmol of Z-PR-AMC, and the mixture was incubated at 37°C for 30 min. Reactions were terminated by addition of 100 µl of 10% SDS, and the amount of AMC released was measured.

The plasminogen activator inhibitor type 1 (PAI-1) inhibition assay was carried out as described previously (11). PAI-1 was diluted with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 8 mM NaH2PO4, 1.5 mM KH2PO4, [pH 7.4]) to 0.7 µM. Both thrombin (purified from bovine plasma at 1,700 NIH U/mg [Sigma]) and SCO7095 were diluted to 13.4 µM with phosphate-buffered saline. Equal volumes of PAI-1 and either thrombin or SCO7095 were mixed and incubated at 37°C for 25 min. The reaction was terminated by adding SDS (final concentration, 1%) and heating for 30 min at 100°C. Reaction products were analyzed by SDS-polyacrylamide gel electrophoresis.

The Bio-Rad protein assay, based on the method of Bradford, was used for determination of protein concentrations with bovine serum albumin as a standard. The apparent molecular weight of purified SCO7095 was determined by gel filtration on a calibrated Superdex 200 HR column (Amersham Biosciences) equilibrated with 50 mM sodium phosphate buffer (pH 7) containing 0.15 M NaCl. The average molecular mass was determined by electropray ion trap mass spectrometry (Esquire-LC-MS; Bruker Daltonics) and calculated from the results of four experiments with an average of at least 2,000 scans per experiment.
ClpP, are involved in the response to conditions that cause damage to cellular proteins (52) or when foreign proteins are overexpressed (9). The ClpP-encoding operons \textit{clpP1 clpP2} and \textit{clpP3 clpP4} of \textit{Streptomyces lividans}, however, are not inducible by heat shock (47). Likewise, the expression of proteasome genes in \textit{R. erythropolis} is constitutive and not enhanced by heat shock (I. Nagy and R. De Mot, unpublished data). At present, no specific function has been attributed to these self-compartmentalizing ATP-dependent proteases in actinomycetes.

The observation that the 20S proteasome is dispensable under a variety of growth conditions indicates that this system is dedicated to some, as yet unidentified, specialized function(s). It has been suggested that the actinomycetes may have expanded their proteolytic machinery, after separation from other gram-positive bacteria, by lateral gene transfer of proteasome genes from a eukaryotic organism (7, 25). Proteasomes have been identified in diverse representatives of the order \textit{Actinomycetales} (8): \textit{Frankia} (suborder \textit{Frankineae}), \textit{Mycobacterium} and \textit{Rhodococcus} (suborder \textit{Corynebacterineae}), and \textit{Streptomyces} (suborder \textit{Streptosporagneae}). Through analysis of data generated by (ongoing) genomic sequencing efforts, we can add \textit{Thermobifida} (suborder \textit{Streptosporangineae}) to this list (draft genome analysis of \textit{Thermobifida fusca} by the DOE Joint Genome Institute: http://www.jgi.doe.gov/JGI_microbial.html/thermobifida/thermob_homepage.html). However, this survey also revealed two notable exceptions. No homologues of the proteasome genes are found in the genomes of two \textit{Corynebacterium} species, namely, \textit{C. diphtheriae} (complete genome sequence by the Sanger Institute: http://www.sanger.ac.uk/Projects/C_diphtheriae/) and \textit{C. glutamicum} (33), although they belong to a family (\textit{Corynebacteriaceae}) that is phylogenetically close to the proteasome-containing \textit{Mycobacteriaceae} and \textit{Nocardiaceae} families (39). This suggests that the proteasome genes have been lost from \textit{Corynebacterium}, which is in line with the observation that the actinomycete 20S proteasome fulfills no essential cellular function(s).

**Identification of the putative hydrolase SCO7095 from \textit{S. coelicolor} as a novel endopeptidase of the \(\alpha/\beta\) hydrolase family.** For one of the proteasome-minus mutants, FAJ2038, an additional band was detected in the zymogram using Z-GGL-AMC as substrate ([30]). When plasmid pFD666 carrying the proteasome operon (pFAJ2761) was introduced in FAJ2038, proteasome expression was restored but expression of the peptidase hydrolyzing Z-GGL-AMC was not repressed. This lack of phenotypic complementation and the fact that the peptidase was absent from the other mutants indicate that its appearance in mutant FAJ2038 was probably not due to the lack of proteasome but may have been caused by secondary mutation.

The enzyme with Z-GGL-AMC-hydrolyzing activity was partially purified from mutant FAJ2038 cell extracts by a combination of chromatographic steps (Fig. 1A). The major band in this sample displayed the Z-GGL-AMC hydrolytic activity. Several internal amino acid sequences were determined following tryptic digestion of enzyme further purified by native gradient gel electrophoresis. Based on these sequences, degenerate primers were designed that allowed amplification of the corresponding gene and, using this as a probe, isolation of a genomic clone containing the entire gene and flanking sequences. The DNA sequence was confirmed on release of the entire sequence of \textit{S. coelicolor} A3(2) cosmid SC3A4 (GenBank accession number AL354616). The enzyme could thus unequivocally be identified as SCO7095 (= SC3A4.21c), annotated as a putative hydrolase (5).

Database searches (including the National Center for Biotechnology Information unfinished microbial genomes; version 30 August 2002; http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi) revealed no homologue for SCO7095. The current picture of the distribution pattern of SCO7095 among actinomycetes, however, matches the one of tricorn protease, both presently being reported only for \textit{S. coelicolor} (41). Between residues 73 and 306, the protein contains the Pfam motif PF00561, characteristic for members of the \(\alpha/\beta\) hydrolase family (15, 31). Borderline homology (\(<30\%\) amino acid sequence identity) was detected with several (putative) members of this family. The best matches were with putative \(\alpha/\beta\) hydrolases revealed by genomic sequencing of \textit{Sinorhizobium meliloti} (SMc04033; 28% identity in a 276-residue overlap) and of \textit{Mycobacterium tuberculosis} (Rv0840c; 26% identity in a 295-residue overlap). Both deduced gene products are related to several bacterial proline iminopeptidases (about 35% identity). Proline iminopeptidase hydrolyses peptide substrates containing proline at the amino terminus, releasing free proline. In Fig. 2, the SCO7095 sequence is aligned with those of some functionally characterized \(\alpha/\beta\) hydrolase family members with peptidase activity. These enzymes, originating from low-GC gram-positive bacteria (\textit{Bacillus} and \textit{Lactobacillus}), gram-negative bacteria (\textit{Xanthomonas}), and \textit{Archaea} (\textit{Thermoplasma}), are also proline iminopeptidases (EC 3.4.11.5). Despite the low overall homology of these proteins (\(<30\%\) amino acid similarity), the regions containing the residues of the catalytic triad Ser-Asp-His aligned well (Fig. 2). Considerable sequence divergence was apparent in the central part of SCO7095 (residues 150 to 230).
In the aminopeptidase from *Xanthomonas campestris* pv. *citri* (28) and the tricorn-interacting aminopeptidase F1 from *Thermoplasma acidophilum* (12), the corresponding segment constitutes a separate “upper” domain, capping the “lower” α/β hydrodase domain. The respective active sites of this enzyme, containing the catalytic triad, are located at the interface between the two domains. The upper domain plays a major role in determining enzymatic activity and specificity of the hydrodase (12, 28). Mutational analysis has confirmed the requirement of a conserved serine residue, acting as the active-site nucleophile, in the aminopeptidases from *Bacillus coagulans* (21) and *T. acidophilum* (44). The latter enzyme displayed broad substrate specificity as an aminopeptidase (44). Based on the conserved catalytic triad Ser-Asp-His (Fig. 2) and predicted hydrolase fold in SCO7095, this *S. coelicolor* enzyme can be assigned to clan SC of serine peptidases (24).

**Biochemical characterization of SCO7095.** To facilitate the further characterization of purified SCO7095, the recombinant...
His-tagged protein was overexpressed in *E. coli* and purified by Ni-NTA affinity chromatography (Fig. 1B). The mass of the protein was determined by electrospray ion trap mass spectrometry to be 36,733.55 Da, corresponding to the His-tagged protein lacking the N-terminal methionine (calculated molecular mass of 36,733.60 Da). This was confirmed by N-terminal sequencing, yielding the sequence of the histidine tag with methionine cleaved off (GSSHHHHHH). On SDS-PAGE, the protein migrated as a single band with $M_r = 35,300$. The size estimated by gel filtration yielded a $M_r$ of 34,500. This indicates that the enzyme was purified as a monomer, as reported for the *Thermoplasma* F1 enzyme (44) and the *Xanthomonas* aminopeptidase (28).

The activity of SCO7095 on several peptidyl-AMC substrates was analyzed (Table 2). Although the enzyme is distinctly related to several proline iminopeptidases, no such activity (with H-P-AMC) or other aminopeptidase activity (with H-X-AMC, where X is a glycine, leucine, valine, arginine, glutamate, phenylalanine, or tyrosine) was detected for SCO7095. Lack of hydrolysis of several N-protected substrates with a P1 proline (Z-GP-AMC, Suc-GP-AMC, H-P-AMC, Z-GP-AMC, Suc-GP-AMC, and Suc-GPLGP-AMC) indicated that SCO7095 has no prolyl-endopeptidase activity. The divergent central part (Fig. 2), which probably folds into a separate “cap” domain (12, 28), most probably plays a major role in determining the catalytic activity, which is quite different from that of the distinctly related proline iminopeptidases.

On Z-GGL-AMC, the enzyme had the highest activity at pH 7.9, retaining 90% of this activity at pH 8.5 and pH 7.2. Only 10% of this activity remained at pH 5.5 (data not shown).

### Table 2. Hydrolysis of synthetic substrates by purified recombinant SCO7095

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sp act (nmol/min/mg of protein)</th>
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<tbody>
<tr>
<td>H-R-AMC</td>
<td>ND*</td>
</tr>
<tr>
<td>H-AR-AMC</td>
<td>0.3</td>
</tr>
<tr>
<td>H-PR-AMC</td>
<td>1.1</td>
</tr>
<tr>
<td>Z-R-AMC</td>
<td>1.6</td>
</tr>
<tr>
<td>Z-PR-AMC</td>
<td>24.0</td>
</tr>
<tr>
<td>Boc-(Obzl)P-AMC</td>
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</tr>
<tr>
<td>Z-R-ARR-AMC</td>
<td>0.4</td>
</tr>
<tr>
<td>Z-ARR-AMC</td>
<td>ND</td>
</tr>
<tr>
<td>Z-GGL-AMC</td>
<td>ND</td>
</tr>
<tr>
<td>Z-RR-AMC</td>
<td>0.4</td>
</tr>
<tr>
<td>Z-GGR-AMC</td>
<td>ND</td>
</tr>
<tr>
<td>Z-GGR-AMC</td>
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* a Boc, t-butyloxycarbonyl; Bz, benzoyl; Suc, succinyl; Obzl, benzyl ester; Z, benzyloxycarbonyl. For each of the substrates, the concentration was 100 μM. The values represent the mean of three independent determinations. ND, no activity was detected. This was also the case for the substrates: H-E-AMC, H-G-AMC, H-L-AMC, H-V-AMC, H-P-AMC, Z-GP-AMC, Suc-GP-AMC, and Suc-GPLGP-AMC.

Chymotrypsin-like activity, inferred from cleavage of Z-GGL-AMC (100% relative activity), was confirmed by the ability to hydrolyze H-AAF-AMC (56%), Suc-LY-AMC (43%), and Suc-LLVY-AMC (7%), albeit at a reduced rate. Changing the hydrophobic P1 residue into a positively charged amino acid (Z-GGL $\rightarrow$ Z-GGR) or introducing a negatively charged N-terminal blocking group (H-AAF $\rightarrow$ Suc-AAF) abolished activity. Hydrolysis of the chymotrypsin-like tripeptide substrates Z-GGL-AMC and H-AAF-AMC has also been reported for the distantly related nonspecific aminopeptidase F1 from *T. acidophilum* (43, 44). This enzyme also showed little or no activity on Z-GGR-AMC and Suc-AAF-AMC.

The highest endopeptidase activity was found with Z-PR-AMC (226%). Several other N-protected substrates with a P1 arginine (Z-R-AMC, Z-RR-AMC, and Boc-LRR) were hydrolyzed at a low rate (15% or less compared to Z-GGL-AMC). Conversely, no activity was measured with Z-ARR-AMC, Z-GGR-AMC, and Boc-VGR-AMC. The presence of a free amino terminus in the Pro-Arg dipeptide strongly affected the activity of the enzyme (>20-fold reduction). Apparently, the presence of an amino-terminally substituted proline at position P2 promotes the endopeptidase activity of SCO7095. The importance of the nature of the P3 residue is apparent from the relatively high activities with Boc-VPR-AMC (79%), and Z-GPR-AMC (30%) and the lack of detectable activity with Boc-D(Obzl)PR-AMC (Table 2).

The postarginine cleavage of the X-P-R-Y motif is reminiscent of the activity of thrombin on polypeptides in the coagulation cascade (46). According to the MEROPS database, post-VPR cleavage is known for thrombin (acting on coagulation factor XIII) and for activated coagulation factor C from the horseshoe crab but is not documented for a prokaryotic enzyme. Unlike SCO7095, these coagulation-active serine endopeptidases have a His-Asp-Ser triad and belong to a different clan (24). Toward S-2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline hydrochloride), a thrombin-specific chromogenic substrate (26), SCO7095 revealed only low activity (0.01% of that of thrombin). The enzyme was unable to degrade oxidized insulin B-chain that is a substrate for tricorn protease from *S. coelicolor* (41).

The activity of SCO7095 on Z-PR-AMC was inhibited by PMSF (Fig. 3A), consistent with our prediction that SCO7095 is most probably a serine hydrolase. Such inhibition was also observed with Z-GGL-AMC as a substrate (data not shown). Inhibition by PMSF was also reported for aminopeptidase F1 from *Thermoplasma* (12). Z-PR-AMC hydrolysis was also inhibited in a dose-dependent fashion by the irreversible thrombin inhibitor PPACK (6), but the concentration required to attain a comparable level of inactivation was about 10-fold higher than for PMSF (Fig. 3B). A similar inhibition curve was obtained with Boc-VPR-AMC as a substrate (data not shown). The *Streptomyces* enzyme was not capable of forming a complex with PAI-1. As a member of the serpin (serine protease inhibitor) family, PAI-1 inhibits certain serine proteases, such as thrombin, by forming a covalent 1:1 complex, resulting from the formation of an ester bond between the P1 residue (i.e., Arg346 in PAI-1) and the active-site serine of the protease (10).

Using benzoyl-tyrosine ethyl ester as a substrate, esterase activity was demonstrated for SCO7095 (data not shown). Es-
ter hydrolysis by an endopeptidase is not an unusual feature, since the catalytic triad in α/β hydrolases, comprising a nucleophile, an acidic residue, and a conserved histidine, enables different types of reactions, including carboxyl ester hydrolysis and peptide hydrolysis (16, 45). No carboxypeptidase activity was detectable with three different N-blocked furylacryloyl dipeptides that have been used as substrates for carboxypeptidases B, U, N, or Y.

Although SCO7095 displays thrombin-like activity on some synthetic substrates, the poor inhibition by PPACK and the observations that the reactive site loop and the tachykinins. Probable identity with the lysosomal proteases tachykinins. Probable identity with the lysosomal proteases

REFERENCES


INTRACELLULAR ENDOPEPTIDASE FROM *STREPTOMYCES*


