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Purification and Characterization of a Puromycin-Hydrolyzing Enzyme from Blasticidin S-Producing *Streptomyces morookaensis*

Motohiro Nishimura,*,[1] Hiroaki Matsuo,† Akiko Nakamura,* and Masanori Sugiyama†

Department of Chemical and Biological Engineering, Ube National College of Technology Tokiwadai, Ube, Yamaguchi 755; and †Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima, Hiroshima 734

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Blasticidin S-producing *Streptomyces morookaensis* JCM4673 produces an enzyme which inactivates puromycin (PM) by hydrolyzing an amide linkage between its aminonucleoside and *O*-methyl-L-tyrosine moieties [Nishimura *et al.* (1995) *FEMS Microbiol. Lett.* 132, 95-100]. In this study, we purified to homogeneity the enzyme from the cell-free extracts of *S. morookaensis*. The molecular weight of PM-hydrolyzing enzyme, estimated by SDS-PAGE and gel filtration, was 68 and 66 kDa, respectively, suggesting that this protein is monomeric. The PM-hydrolyzing activity was strongly inhibited by Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, and *N*-bromosuccinimide, but was stimulated by DTT. The optimum pH and temperature for PM-hydrolyzing activity were 8.0 and 45°C, respectively. Several L-aminoacyl-β-naphthylamides were good substrates for the enzyme, suggesting that the PM-inactivating enzyme has an aminopeptidase activity. The N-terminal sequence of the first 14 amino acids (Val-Ser-Thr-Ala-Pro-Tyr-Gly-Ala-Trp-Gln-Ser-Pro-Ile-Asp) of the enzyme showed no significant homology with any published hydrolase sequences.

Key words: aminoacyl-β-naphthylamide, aminopeptidase, inactivating enzyme, puromycin, *Streptomyces morookaensis*.

The antibiotic-resistance mechanisms in actinomycetes have been studied with respect to enzymatic inactivation (*1-4*), resistance of target site (*5-7*), and membrane permeability (*8, 9*). The well known modes of antibiotic inactivation are acetylation (*1*), phosphorylation (*3*), adenylation (*10*), and hydrolysis by modifying enzymes. Recently, antibiotic inactivation by an antibiotic-binding protein has been found in bleomycin (BLM)-producing *Streptomyces verticillus* (*11*).

Puromycin (PM), a nucleoside antibiotic, is produced by *Streptomyces alboniger* (*12, 13*) and inhibits the growth of prokaryotic and eukaryotic cells. Its mode of action is ascribed to the inhibition of protein synthesis by substituting for aminoacyl t-RNA and serving as an acceptor for the nascent peptide chain of ribosome-bound peptidyl t-RNA (*14*). PM is also a competitive inhibitor of aminopeptidases in various mammalian tissues. The PM-producing microorganism has been reported to produce an *N*-acetyltransferase which inactivates its own antibiotic in the presence of acetyl coenzyme A (*15-17*). The acetylation site in the PM molecule was the 2″-amino group of *O*-methyl-L-tyrosine moiety (*16*).

Blasticidin S (BS) is also a nucleoside antibiotic and inhibits peptide-chain elongation in bacterial and mammalian cells (*18, 19*). Acetylation and deamination of the cytosine moiety in BS have been proposed as mechanisms of enzymatic inactivations of the drug in the resistant microorganisms, *Streptomyces morookaensis* (*20*) and *Aspergillus fumigatus* (*21*), respectively.

So far, little is known about the hydrolytic antibiotic-inactivation mechanism, except for a hydrolase which removes the dichloroacetyl group of chloramphenicol (*22*), an esterase which hydrolyzes a lactone ring of erythromycin (*23*), BLM hydrolases from several mammalian sources (*24-26*) which act on the carboxamide bond of the β-aminoalaninamide moiety on the BLM molecule and β-lactamases which hydrolyze the β-lactam antibiotics. Interestingly, we have found that BS-producing *S. morookaensis* JCM 4673 produces an enzyme catalyzing the hydrolysis of an amide linkage between the aminonucleoside and *O*-methyl-L-tyrosine moieties in PM (*27*) (Fig. 1). This PM-inactivating enzyme has not yet been purified and characterized.

Here, we describe the purification and physico-chemical and enzymatic properties of the PM-hydrolyzing enzyme produced by *S. morookaensis* JCM 4673.

MATERIALS AND METHODS

Microorganisms and Culture—S. morookaensis JCM 4673 (=KCC S-0673) was obtained from the Japan Collection of Microorganisms (JCM) at the Institute of Physical and Chemical Research, Wako, Tokyo. The spores, grown on YEME medium [1% glucose, 0.5% polypeptone, 0.3% yeast extract, 0.3% malt extract, and 0.04% $MgCl_2 \cdot 6H_2O$, pH 7.0] containing 1.5% agar were suspended in 0.85% NaCl containing 20% glycerol and stored at −70°C until use. After the *S. morookaensis* spores were inoculated

[1] To whom correspondence should be addressed. Tel: +81-836-31-6111, Fax: +81-836-22-7801, E-mail: nisimura@ube-k.ac.jp

Abbreviations: BLM, bleomycin; BS, blasticidin S; IEF, isoelectric focusing; PIP, proline iminopeptidase; PM, puromycin.

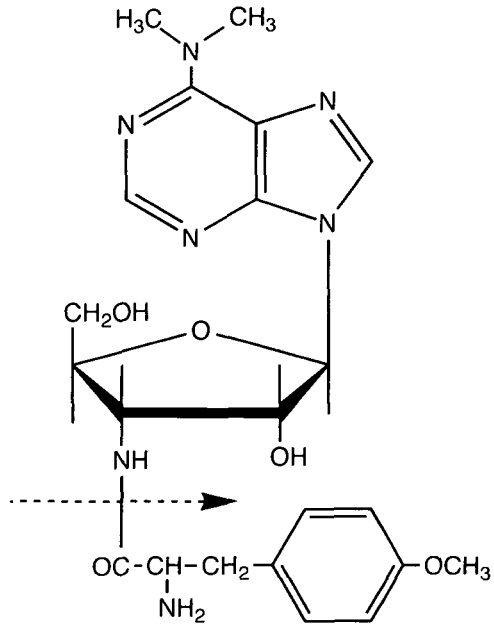

Fig. 1. **The chemical structure of puromycin.** ----► indicates the site which is hydrolyzed by puromycin-hydrolyzing enzyme from *S. morookaensis* JCM4673.

and cultured in 100 ml of YEME medium at 30°C for 48 h, a 2-ml portion of the culture was transferred to 100 ml of the same medium and incubated for an appropriate time at 30°C, with reciprocal shaking.

Bacillus cereus IFO 3001 and *Staphylococcus aureus* IFO12732, obtained from the Institute for Fermentation Osaka (IFO), Japan, were used to assay antibacterial activities as test organisms. These bacteria were grown on PMS medium [1% polypeptone, 0.5% meat extract, and 0.25% NaCl, pH 7.0] containing 1.5% agar at 37°C.

Assay for PM-Hydrolyzing Enzyme Activity—The PM-hydrolyzing enzyme activity was assayed by two methods (*27*): Method A involves determination of antibacterial activity of PM by bioassay. Enzyme solution (0.15 ml) was incubated at 37°C for 20 min together with 0.05 ml of Mixture I [80 mM Tris-HCl (pH 7.65), 10 mM Mg-acetate, 6 mM 2-mercaptoethanol, and 200 μg PM dihydrochloride (Sigma, USA)]. After heating at 70°C for 10 min, the reaction mixture was centrifuged at 4,700 $\times g$ for 5 min. A 100 μl portion of the supernatant fluid was subjected to the antibiotic bioassay using *B. cereus* IFO 3001 as a test organism. The PM-hydrolyzing activity was estimated from the residual antibacterial activity of the drug.

Method B involves determination of residual PM by HPLC after the enzyme reaction. This method was employed only in inhibitory and kinetic studies of the enzyme. The reaction mixture (20 μl) was subjected to HPLC using a Wakosil 5C18-200T column (4.6×250 mm, Wako Pure Chemical, Osaka) at a flow rate of 1.0 ml/min at 45°C. Elution was done using 6% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The amount of residual PM, monitored by measuring the absorption at 260 nm, was calculated from the relative peaks of known amounts of PM standards. In methods A and B, one unit of the enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of PM per min. The specific activity was expressed as unit per mg of protein, the latter being measured according to the method of Lowry *et al.* (*28*).

Elution of PM-hydrolyzing enzyme from columns was monitored in terms of the formation of PM hydrolysates, both its aminonucleoside and *O*-methyl-L-tyrosine. A portion of the reaction mixture was subjected to a silica gel 60 F_{254}-precoated TLC (Merck, USA) using *n*-butanol/methanol/2 N ammonia (6:1:1, by volume) as a solvent system (*27*) and the hydrolysates were detected by measuring the ultraviolet absorption at 254 nm.

Purification of PM-Hydrolyzing Enzyme—All operations were done at 6°C unless otherwise noted.

Step 1. Preparation of cell-free extract: Cells grown for 4 d and collected by centrifugation at 5,500 $\times g$ for 10 min were washed with buffer I [10 mM Tris-HCl (pH 7.65), 1 M KCl, 10 mM Mg-acetate, 6 mM 2-mercaptoethanol, and 5 mM Mg-titriplex (Merck)] and then twice with buffer II [10 mM Tris-HCl (pH 7.65), 30 mM NH_4Cl, 10 mM Mg-acetate, 6 mM 2-mercaptoethanol, and 5 mM Mg-titriplex]. The washed cells were ground with quartz sand and extracted with the same volume of buffer II. The cell extracts were centrifuged at 3,000 $\times g$ for 5 min to remove cell debris and quartz sand. The precipitate of centrifugation at 30,000 $\times g$ for 30 min was removed, and the resulting supernatant was used as S-30 fraction.

Step 2. Phenyl-Sepharose CL-4B chromatography: Solid ammonium sulfate was added to the S-30 fraction up to the concentration of 0.9 M. The sample was centrifuged at 30,000 $\times g$ for 20 min to remove the precipitate and applied to a column (2.6×18 cm) of Phenyl-Sepharose CL-4B (Pharmacia, Sweden) equilibrated with buffer A [20 mM Tris-HCl (pH 7.65), 10 mM $MgCl_2$, and 1 mM DTT] containing 0.9 M $(NH_4)_2SO_4$. The elution was done with a linear gradient of $(NH_4)_2SO_4$ from 0.9 to 0 M in buffer A. Enzyme fractions (12 ml) were mixed with polyethylene glycol 6000 and dialyzed against buffer A containing 10% (v/v) glycerol.

Step 3. DEAE-Sepharose CL-6B chromatography: The dialyzate was applied to a column (1.8×23 cm) of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with buffer A containing 10% (v/v) glycerol and washed thoroughly with the same buffer. The enzyme activity was eluted with a linear gradient of 0-1.0 M NaCl dissolved in the same buffer. Enzyme fractions (7 ml) were pooled and dialyzed against buffer A containing 1.4 M $(NH_4)_2SO_4$.

Step 4. Ether-Toyopearl 650 chromatography: The enzyme fraction from the above column chromatography was applied to an Ether-Toyopearl 650 (TOSOH, Tokyo) column (1.5×5.5 cm) equilibrated with buffer A containing 1.4 M $(NH_4)_2SO_4$. After the column had been washed thoroughly with the same buffer, elution was done with a linear gradient of $(NH_4)_2SO_4$ from 1.4 to 1.2 M in buffer A. Enzyme fractions (4 ml) were pooled, concentrated by ultrafiltration using a USY-5 (ADVANTEC, Tokyo) and dialyzed against buffer A containing 10% (v/v) glycerol.

Step 5. Fractogel EMD DEAE-650 chromatography: The dialyzate was applied to a column (1.5×10 cm) of Fractogel EMD DEAE-650 (Merck) equilibrated with buffer A containing 10% (v/v) glycerol, and the column was washed thoroughly with the same buffer containing 0.15 M NaCl. Elution was done with a linear gradient of 0.15-0.35 M NaCl in buffer A containing 10% (v/v) glycerol. Enzyme fractions (3 ml) were pooled and concentrated to a small

volume by ultrafiltration as described above.

Step 6. Toyopearl HW-55 chromatography: The concentrate was applied to a Toyopearl HW-55 (TOSOH) column (2.0×89 cm), equilibrated with buffer A containing 0.1 M NaCl, and eluted with the same buffer. The enzyme fractions (3 ml) were pooled and stored in 20% (v/v) glycerol at −20°C until use.

Electrophoresis—SDS-PAGE was performed on a RAPIDAS AE-6400 (ATTO, Tokyo) apparatus with 12% (w/v) acrylamide gel. Phosphorylase B (97.4 kDa), bovine serum albumin (BSA, 66.3 kDa), aldolase (42.4 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa) were used as size markers (Daiichi Pure Chemicals, Tokyo).

Isoelectric focusing (IEF) was performed on a Model 111 Mini IEF Cell (Bio-Rad, USA) with 5% (w/v) acrylamide and 2% (v/v) ampholytes, pH 3-10 (Bio-Lyte, Bio-Rad). Focusing was carried out under the condition of constant voltage in a stepped fashion as follows: the first step, 100 V for 15 min; the second step, 200 V for 15 min; the final step, 450 V for 60 min. The isoelectric point of the enzyme was determined by using pI markers (Bio-Rad), *i.e.*, phycocyanin (pI=4.45, 4.65, and 4.75), β-lactoglobulin B (5.1), bovine carbonic anhydrase (6.0), human carbonic anhydrase (6.5), equine myoglobin (7.0), human hemoglobin A (7.1), human hemoglobin C (7.5), lentil lectin (7.8, 8.0, 8.2), and cytochrome *c* (9.6). Conditions and sample preparations for SDS-PAGE and IEF analyses were done according to the manufacturer's protocols. After the electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250.

Measurement of Molecular Weight—The molecular weight of the enzyme was estimated by gel filtration on a Toyopearl HW-55 column. As marker proteins, BSA (68 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25.7 kDa), and lysozyme (14.4 kDa) were used. The molecular weight of the enzyme was also estimated by SDS-PAGE.

Effects of pH and Temperature on PM-Hydrolyzing Activity—To examine the effect of pH on PM-hydrolyzing activity, method A was used, except that 80 mM Tris-HCl buffer (pH 7.65) in the reaction mixture was replaced with 80 mM sodium acetate buffer (pH 3.5 to 5.5), 80 mM sodium phosphate buffer (pH 5.5 to 8.0), 80 mM Tris-HCl buffer (pH 7.5 to 9.5), or 80 mM sodium carbonate buffer (pH 9.0 to 11.0). To investigate the effect of temperature on the enzyme activity, the reaction was carried out between 15 and 60°C for 20 min.

Inhibition Studies—Effects of metal ions and chemical reagents on the enzyme activity were determined by pre-incubating the purified enzyme (1.3 μg protein) at 37°C for 10 min with 1 mM of each compound. In the study with actinonin, amastatin, bestatin, and leupeptin, as inhibitors of aminopeptidase or protease, the enzyme was pre-incubated at 28°C for 10 min with 0.5 mM inhibitor. In both experiments, the residual activities after pre-incubation were assayed according to Method B.

Substrate Specificity—To examine whether the PM-hydrolyzing enzyme can catalyze the hydrolysis of aminopeptidase substrates, L-aminoacyl-β-naphthylamides, the method of Hopsu *et al.* was employed (*29*). The enzyme solution (0.5 ml) was mixed with various concentrations of each L-aminoacyl-β-naphthylamide dissolved in 0.1 M Tris-HCl (pH 7.65) and incubated at 37°C for 10 min. The reaction was stopped by adding Fast Garnet GBC (sulfate salt, Sigma) in 1 M acetate buffer (pH 4.2) containing 10% (v/v) Tween 20. The color intensity, produced by the coupling of Fast Garnet GBC with β-naphthylamine, was monitored by measuring the absorbance at 520 nm. The kinetic parameters of enzyme activity were determined from Lineweaver-Burk plots.

To examine whether the PM-hydrolyzing enzyme has proteolytic activity, Hammarsten casein, gelatin, BSA, hemoglobin, and ovalbumin were used as substrates. Aliquots (1 ml) of the purified enzyme solution (25 μg protein/ml) were mixed with 1 ml of 1% (w/v) substrates in 50 mM Tris-HCl buffer (pH 7.65) and incubated at 37°C for 30 min. The reaction was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 0.2 M. The amounts of TCA-soluble peptides, obtained by centrifugation at 30,000×*g* for 15 min, were determined by the method of Lowry *et al.* (*28*).

Several antibiotics having an amide bond(s) in the molecule, such as ampicillin, amoxicillin, chloramphenicol, viomycin and BS, were used to examine the substrate specificity of the PM-hydrolyzing enzyme. In this experiment, the purified enzyme (78 μg) was mixed with each antibiotic dissolved in Tris-HCl (pH 7.65) and incubated at 37°C for 20 min. *Staphylococcus aureus* IFO 12732 was used as a test organism for the determination of the antibacterial activities of ampicillin, amoxicillin and chloramphenicol, and the activities of viomycin and BS were assayed using *B. cereus* IFO 3001.

N-Terminal Amino Acid Sequencing—The N-terminal amino acid sequence of purified enzyme was analyzed using an automated protein sequencer (Model PSQ-1, Shimadzu, Kyoto). The N-terminal amino acid sequence was compared to other protein sequences in the SwissProt, PIR, and PRF databases.

RESULTS

Purification and Properties of PM-Hydrolyzing Enzyme—The PM-hydrolyzing enzyme was purified from the crude cell-free extracts of *S. morookaensis* JCM 4673. The results of enzyme purification are summarized in Table I. SDS-PAGE of the enzyme solution from the final step revealed a single protein band (Fig. 2). The enzyme was purified approximately 307-fold with a yield of 28%. In a series of purification steps, hydrophobic chromatography on Ether-Toyopearl 650 (Fig. 3) resulted in high purification of the enzyme (Fig. 2 and Table I). The protein was estimated to have a molecular weight of 68 or 66 kDa by SDS-PAGE (Fig. 2) or gel filtration on Toyopearl HW-55, respectively (data not shown), suggesting that this enzyme

TABLE I. **Purification of puromycin-hydrolyzing enzyme from *S. morookaensis*.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Activity yield (%)
Cell-free extracts	6,394	172	0.03	1.0	100
Phenyl-Sepharose CL-4B	528	146	0.28	9.3	85
DEAE-Sepharose CL-6B	169	125	0.74	24.7	73
Ether-Toyopearl 650	24.9	94.3	3.79	126	55
Fractogel EMD DEAE-650	14.4	80.1	5.56	185	47
Toyopearl HW-55	5.2	47.9	9.21	307	28

is monomeric. The isoelectric point of the enzyme was 6.4. The PM-hydrolyzing enzyme retained more than 80% of its activity when preincubated in 50 mM Tris-HCl (pH 7.65) for 15 min at below 45°C, but completely lost its activity at 55°C. The enzyme was stable at pHs between 5.5 and 9.0, but was unstable at pHs below 5.0 and over 10.0. The temperature and pH optima for the enzyme activity were 45°C and 8.0, respectively.

Inhibition Studies—We tested the effects of metal ions on the PM-hydrolyzing enzyme activity. The enzyme activity was inhibited substantially by Zn^{2+}, Fe^{2+}, Cu^{2+}, and Hg^{2+}, and partially by Co^{2+} (Table II).

The enzyme activity was markedly inhibited by *N*-bromosuccinimide and *N*-ethylmaleimide at the concentration of 1 mM, and slightly inhibited by iodoacetic acid, *p*-chloromercuribenzoic acid, phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (Table III). EDTA and *o*-phenanthroline had no effect. DTT significantly stimulated the enzyme activity. Neither aminopeptidase- nor protease-specific inhibitors tested affected the PM-hydrolyzing enzyme activity.

Substrate Specificity—We investigated the substrate specificity for the PM-hydrolyzing enzyme using L-amino-acyl-β-naphthylamides. The enzyme had high affinity for hydrophobic amino acid derivatives (Table IV), but not for hydrophilic and ionic amino acid derivatives. Proline (Pro)-β-naphthylamide was the best substrate. The k_{cat}/K_m value ($=5\times10^{-3}$ $mM^{-1}\cdot s^{-1}$) for PM was about 20 to 25×10^3 times lower than these for Pro-, Phe-, Tyr-, Leu-, Ala-, and His-β-naphthylamides. Lys-, Arg-, Asn, and Glu-β-naph-

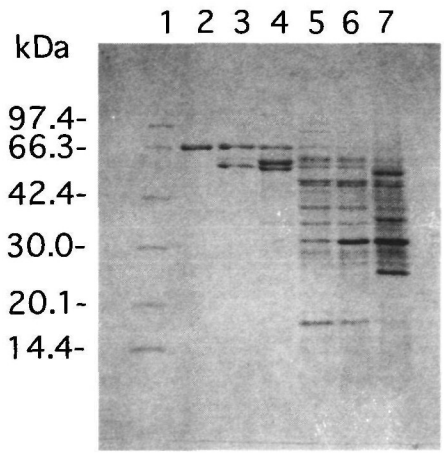

Fig. 2. **SDS-PAGE analysis of puromycin-hydrolyzing enzyme purified by a series of chromatographies.** The electrophoresis was carried out on a 12% polyacrylamide gel at pH 8.3 in Tris-glycine buffer containing 0.1% SDS. Lanes: 1, standard molecular mass markers (sizes indicated); 2, the enzyme fractions from the final step; 3, the active fractions from Fractogel EMD DEAE-650 column; 4, the active fractions from Ether-Toyopearl 650; 5, the active fractions from DEAE-Sepharose CL-6B; 6, the active fractions from Phenyl-Sepharose CL-4B; 7, the cell-free extracts from *S. morookaensis*.

TABLE II. **Effects of metal ions on puromycin-hydrolyzing enzyme activity.**

Metal ions (1 mM)	Residual activity (%)
None	100
$MnCl_2$	104
$CaCl_2$	103
$BaCl_2$	98
$MgCl_2$	97
$CoCl_2$	15
$HgCl_2$	6
$CuCl_2$	5
$FeCl_2$	3
$ZnCl_2$	0

TABLE III. **Effect of inhibitors on puromycin-hydrolyzing enzyme activity.**

Inhibitors	Concentration (mM)	Residual activity (%)
None	0	100
Ethylenediaminetetraacetic acid	1.0	100
	10.0	93
o-Phenanthroline	1.0	95
Dithiothreitol	1.0	138
Iodoacetic acid	1.0	86
p-Chloromercuribenzoic acid	1.0	66
Phenylmethylsulfonyl fluoride	1.0	57
Diisopropyl fluorophosphate	1.0	41
N-Ethylmaleimide	1.0	21
N-Bromosuccinimide	1.0	0

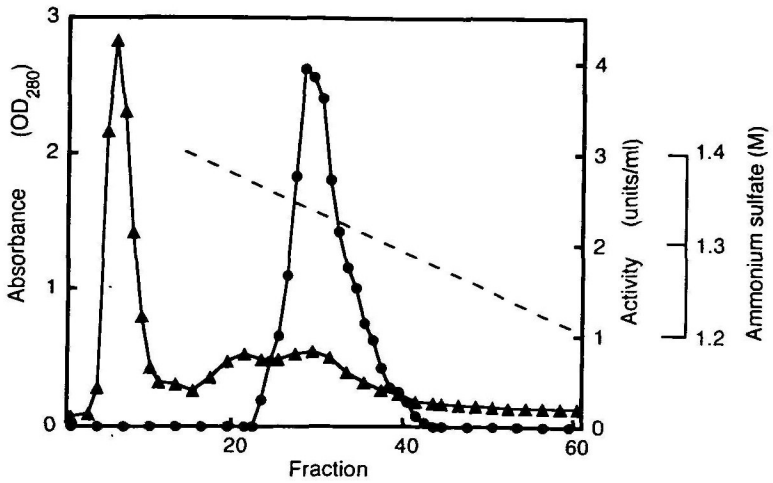

Fig. 3. **Elution pattern of PM-hydrolyzing enzyme by Ether-Toyopearl 650 column chromatography.** Elution of the enzyme activity was monitored by the formation of PM hydrolysates on a silica gel TLC. ●, PM-hydrolyzing enzyme activity assayed by Method A; ▲, absorbance at 280 nm; ·····, $(NH_4)_2SO_4$ concentration.

TABLE IV. **Substrate specificity of puromycin-hydrolyzing enzyme towards L-aminoacyl-β-naphthylamides.**

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Puromycin	864	0.004	0.005
His-β-naphthylamide	419	0.046	0.110
Ser-β-naphthylamide	929	0.302	0.325
Ala-β-naphthylamide	378	8.18	21.6
Tyr-β-naphthylamide	62.8	1.78	28.4
Leu-β-naphthylamide	108	3.83	35.5
Phe-β-naphthylamide	26.7	1.65	61.9
Pro-β-naphthylamide	11.5	1.44	125

thylamides did not serve as substrates for the enzyme (data not shown).

To examine whether the enzyme exhibits proteolytic activity, Hammarsten casein, gelatin, BSA, hemoglobin, and ovalbumin were used as substrates. However, none of these proteins was hydrolyzed by the enzyme, even with a 25 times greater amount of the enzyme than under the standard conditions (data not shown).

BS, ampicillin, amoxicillin, chloramphenicol, and viomycin, which have an amide bond(s) in their molecules, were not substrates for the enzyme (data not shown).

N-Terminal Amino Acid Sequence of the PM-Hydrolyzing Enzyme—The enzyme from the final purification step was analyzed for the amino acid sequence using an automated protein sequencer. Its N-terminal sequence was Val-Ser-Thr-Ala-Pro-Tyr-Gly-Ala-Trp-Gln-Ser-Pro-Ile-Asp. A homology search for the sequence showed a high degree of similarity (57.1% amino acid identity) to BZLF 1 protein, a viral transcriptional activator from Epstein-Barr virus (*30*). But, there were no hydrolases exhibiting significant homology to the N-terminal fragment of the PM-hydrolyzing enzyme.

DISCUSSION

We have found an enzyme, which catalyzes the hydrolysis of an amide linkage between the aminonucleoside and *O*-methyl-L-tyrosine moieties on the PM molecule, in the cytoplasm of BS-producing *S. morookaensis* JCM 4673 (*27*). Several kinds of hydrolytic antibiotic-inactivating enzymes have been reported, but enzymes involved in amide bond hydrolysis are little known, except for BLM hydrolases and β-lactamases. The BLM-hydrolases, produced by animal cells, are believed to play an important role in protecting cells from the BLM-induced toxicity. These enzymes are aminopeptidases. The PM-hydrolyzing enzyme was unstable in buffers without DTT and sensitive to thiol-blocking reagents such as *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, and iodoacetic acid as well as divalent heavy metal ions, Zn^{2+}, Cu^{2+}, and Hg^{2+} (Tables II and III). These results suggest that its active center may contain a sulfhydryl group(s). The PM-hydrolyzing activity was completely inhibited by *N*-bromosuccinimide, and only slightly by phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate, suggesting that tryptophan and/or serine residues might also be present in the catalytic site of the enzyme. Judging from our observation that EDTA and *o*-phenanthroline did not affect the PM-hydrolyzing activity, metal ions are probably not essential for the expression of the enzyme activity.

Proline (Pro)-iminopeptidases (PIP; EC 3.4.11.5) which selectively hydrolyze substrates containing Pro at the N-terminus to release Pro, have been found in several microorganisms (*31-35*). However, PIP from *Thermoplasma acidophilum* can also hydrolyze substrates harboring neutral and hydrophobic amino acid residues (*31*). The substrate specificity of PM-hydrolyzing enzyme towards L-aminoacyl-β-naphthylamides revealed that the enzyme exhibited the highest affinity for Pro-β-naphthylamide ($k_{cat}/K_m = 125\ mM^{-1} \cdot s^{-1}$), although the enzyme could hydrolyze substrates harboring hydrophobic amino acids at the N-terminus, such as Phe-β-naphthylamide ($k_{cat}/K_m =$ 61.9) and Leu-β-naphthylamide ($k_{cat}/K_m = 35.5$). Most PIPs were previously reported to be a family of thiol enzymes (*32-35*), except for PIP purified from *Lactobacillus delbrueckii,* which is a serine proteinase (*36*). Moreover, in contrast with the fact that many aminopeptidases are metalloenzymes, PIPs are hardly affected by metal-chelating agents (*32-34, 37*). Thus, the PM-hydrolyzing enzyme is very similar to a PIP derived from *T. acidophilum,* exhibiting broad substrate specificity, rather than BLM hydrolases which have no ability to hydrolyze PIP substrates such as Pro-β-naphthylamide (*25*), Pro-*p*-nitroanilide (*24*), or Pro-methyl coumarylamide (*26*).

In a comparison of the catalytic efficiency (k_{cat}/K_m) for the hydrolysis of PM and L-aminoacyl-β-naphthylamides, the PM-hydrolyzing enzyme preferentially hydrolyzed β-naphthylamide derivatives to PM. Consequently, this enzyme might recognize the *O*-methyl-L-tyrosine moiety in the PM molecule as a substrate. This enzyme had no endopeptidase activity towards proteins (data not shown).

Several PIP genes have been cloned and sequenced (*31, 36-40*). A homology search among the primary sequences derived from eubacterial PIP genes showed significant similarity in their N-terminal half (*31*). However, the N-terminal amino acid sequence of PM-hydrolyzing enzyme showed no significant homology with reported sequences of PIPs. To evaluate the similarity of amino acid sequences among this enzyme and PIPs, further information on its sequence is required.

In conclusion, physico-chemical and enzymatic characterization of the PM-hydrolyzing enzyme revealed that the enzyme has some aminopeptidase-like properties closely related to those of PIPs. In order to perform further characterization of the enzyme, we are cloning a gene encoding the PM-hydrolyzing enzyme from the *S. morookaensis* chromosomal DNA, using an oligonucleotide probe designed from its N-terminal sequence.

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