Short Communication

A Novel Thermostable, S-PI
(Pepstatin Ac)-insensitive
Acid Proteinase from
Thermophilic *Bacillus novosp.*
Strain MN-32

Sawao MURAO, Keizoh OHKUNI,
Masanori NAGAO, Kohei ODA*
and Takashi SHIN

Department of Applied Microbial Technology,
Kumamoto Institute of Technology,
Ikeda 4-22-1, Kumamoto 860, Japan

*Department of Agricultural Chemistry,
College of Agriculture,
University of Osaka Prefecture,
Sakai, Osaka 591, Japan

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Several thermostable proteolytic enzymes have been purified from various microorganisms.¹⁻³⁾ These enzymes are classified as metal or serine type proteinases. There is no report on the occurrence of thermostable acid proteinases in microbes or animal tissues.

The authors have reported various acid (carboxyl) proteinases from yeast,⁴⁾ fungi imperfecti,⁵⁾ basidiomycetes,⁶⁾ and bacteria.^{7,8)} While screening for acid-proteinase-producing microorganisms, we found proteolytic activity in the culture filtrate of thermophilic bacteria MN-32 strain, isolated from acidic hot springs in Kumamoto Prefecture, Japan.

The strain was classified into the genus *Bacillus* on the basis of its morphological and physiological characteristics and tentatively named *Bacillus novosp.* strain MN-32. The growth temperature of MN-32 strain is between 50°C to 70°C (optimum temperature, 60°C), the growth pH range is pH 2.0~7.5 (optimum, pH 3.0~5.0).

The proteolytic activity was measured by the casein-Folin method at 70°C, pH 3.0 for 30 min, as reported previously.⁹⁾

For the production of acid proteinase, the

strain was aerobically cultured at 60°C in a medium comprising 1.5% polypeptone, 0.1% yeast extract, 0.3% NaCl, 0.5% KH₂PO₄, 0.05% MgSO₄·7H₂O, and 0.01% CaCl₂ at pH 3.5.

The procedures for purification of MN-32 acid proteinase were as follows. The culture filtrate (100 l) was fractionated with ammonium sulfate (80% saturation at pH 4). The precipitate, recovered by centrifugation (10,000 × g, 10 min), was dissolved in 0.05 M sodium acetate-HCl buffer (pH 4.7), and then dialyzed overnight against the same buffer. The dialyzed solution was put on a DEAE-Sephadex CL-6B column (7 × 27 cm), which was previously equilibrated with 0.05 M sodium acetate-HCl buffer (pH 4.7). The adsorbed enzyme was eluted by a linear gradient in the same buffer from 0 to 0.5 M NaCl, the active eluate was concentrated by a UF-10PS membrane (Tosoh Co.). The resulting enzyme solution was further purified by gel filtration on a Sephadex G-100 column (2.45 × 80 cm). The column was eluted with 0.05 M sodium acetate-HCl buffer (pH 5.5). Further purification was achieved by fractionation by high-performance liquid chromatography (HPLC), using a TSKgel DEAE-5PW column (20 × 100 mm). The final preparation was thoroughly dialyzed against distilled water and then lyophilized. The final enzyme yield was 25 mg, activity yield was 10% from the culture filtrate, and specific activity was increased 8300-fold.

The homogeneity of the enzyme preparation was proved by electrophoresis (see Fig. 1) and HPLC (TSKgel DEAE-5PW column).

The molecular weight of the enzyme was estimated to be 40,000 by gel filtration on a Sephadex G-100, and 41,000 by SDS-polyacrylamide gel electrophoresis. The pI value of the enzyme was pH 3.5 by isoelectric focusing.

The optimum pH for the enzyme activity was pH 3.0 (casein), the temperature optimum was at 70°C (pH 3.0). The enzyme is fairly acid stable and thermostable, retaining 100% of initial activity at pH 2~5 in 24 hr of incubation at 50°C, and 60% activity in 10 min of incubation at 80°C (pH 4).

FIG. 1. Polyacrylamide Disc Gel Electrophoresis of Purified MN-32 Acid Proteinase.

Electrophoresis was done under the following conditions. A: 4 mA for 2 hr, pH 2.3, 7% gel; B: 5 mA for 3 hr, pH 8.8, 7.5% gel; C: 5 mA for 3 hr, pH 8.8, 10% gel with SDS after reduction and denaturation.

After electrophoresis, the protein (40 μ g) was stained with Coomassie Brilliant Blue.

The effects of various proteinase inhibitors on MN-32 acid proteinase were observed. As shown in Table I, the enzyme was not inhibited by serine (PMSF, Chymostatin,¹⁰ Leupeptin,¹¹ or MAPI¹²), metallo (EDTA or *o*-phenanthroline), thiol (iodoacetic acid or Thiolstatin¹³) or carboxyl proteinase inhibitors (S-PI¹⁴ [Pepstatin Ac], DAN¹⁵ [diazoacetyl-DL-norleucine methylester] or EPNP¹⁶ [1,2-epoxy-3-(*p*-nitrophenoxy)propane]). Thus, the MN-32 acid proteinase is a member of the S-PI-insensitive carboxyl proteinase group, like the carboxyl proteinases isolated from *Scytalidium lignicolum*,^{17,18} various edible mushrooms,¹⁹ *Pseudomonas* sp.,⁷ and *Xanthomonas* sp.,⁸ that we have reported.

This is the first report of the natural occurrence of a thermostable acid proteinase. Furthermore, it is noteworthy that the acid proteinase of MN-32 strain is insensitive to various carboxyl proteinase inhibitors.

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TABLE I. EFFECTS OF VARIOUS PROTEINASE INHIBITORS ON MN-32 ACID PROTEINASE

The enzyme (1.2 μ g) was incubated at 50°C for 30 min with reagents at pH 3.0 (EDTA was incubated at pH 5.0), and the residual enzyme activity was assayed at the optimum pH.

Reagent	Concentration (mM)	Remaining activity (%)
None	—	100
PMSF	1	101
PCMB	10	98
Chymostatin	0.1	100
Leupeptin	0.1	100
MAPI	0.1	103
IAA	10	90
Thiolstatin	0.1	100
EDTA	10	105
<i>o</i> -Phenanthroline	10	107
S-PI	0.1	108
DAN	0.1	100
EPNP	0.1	100

PMSF, phenylmethanesulfonyl fluoride; PCMB, *p*-chloromercuribenzoate; IAA, iodoacetic acid; EDTA, ethylenediaminetetraacetic acid.

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