Purification and properties of an alkaline protease from alkalophilic Bacillus sp. KSM-K16

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Abstract Alkaline protease (EC 3.4.21.14) activity, suitable for use in detergents, was detected in the alkaline culture medium of Bacillus sp. KSM-K16, which was originally isolated from soil. The enzyme, designated M protease, was purified to homogeneity from the culture broth by column chromatographies. The N-terminal amino acid sequence was Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Arg-Val-Gln-Ala-Pro-Ala-Ala-His-Asn-Arg-Gly-Leu-Thr-Gly. The molecular mass of the protease was 28 kDa, and its isoelectric point was close to pH 10.6. Maximum activity toward casein was observed at 55 °C and at pH 12.3 in 50 mM phosphate/NaOH buffer. The activity was inhibited by phenylmethylsulfonyl fluoride and chymotrypsin. The enzyme was very stable in long-term incubation with liquid detergents at 40 °C. The enzyme cleaved the oxidized insulin B chain initially at Leu15-Tyr16 and efficiently at ten more sites. Among various oligopeptidyl p-nitro-anilides (pNA) tested, N-succinyl-Ala-Ala-Pro-Phe-pNA was efficiently hydrolyzed by M protease. M protease was precipitated in (NH4)2SO4-saturated acetate buffer (pH 5.0) as plank-like crystals.

Introduction

Bacillus spp. produce many different extracellular hydrolytic enzymes, which include proteases (Markland and Smith 1971; Priest 1977). The industrial use of proteases has been recognized mainly in terms of their applications for use in laundry detergents (Maase and van Tilburg 1983; Ainsworth 1994). Alkaline proteases are added to detergents to facilitate the release of proteinaceous materials in stains such as those due to grime, blood and milk. To withstand the alkaline conditions in detergents, many alkaline serine proteases have been isolated from strains of Bacillus (Ottesen and Svendsen 1971; Horikoshi 1971; Nijenhuis 1977; Shimogaki et al. 1991). Recently, we isolated an alkalophilic Bacillus sp. KSM-K16 that produces an alkaline protease with properties that fulfill the essential requirements for enzymes to be used in both powder and liquid detergents. Since 1991, a hyperproducing mutant of the strain has been used for the large-scale industrial production of the protease, as well as an alkaline cellulase (Yoshimatsu et al. 1990; Ito et al. 1991), for use in commercial, compact detergents.

In this report, we describe a detailed study of an alkaline protease from the alkalophilic Bacillus sp. KSM-K16. This study was undertaken not only to enhance our immediate understanding of this enzyme, but also to provide a foundation for future investigations of the tertiary structure and mechanism of action of the enzyme (Hakamada et al. 1994). For these purposes, we purified, crystallized and characterized the alkaline protease, designated M protease.

Materials and methods

Isolation and identification of the organism

Bacillus sp. KSM-K16 was isolated from a soil sample in our laboratory. It was a producer of alkaline protease on an alkaline agar plate composed of (w/v) 1.0% glucose, 0.2% yeast extract (Difco), 1.0% hair keratin (Kanto Chemical), 1.0% carboxymethyl-cellulose, 0.1% KH2PO4, 0.02% MgSO4·7H2O, 1.0% Na2CO3 (separately autoclaved), and 1.5% agar (pH 10.5). Microbiological characteristics of the isolate were examined by the methods of Gordon et al. (1973) and Claus and Berkeley (1986).

Culture conditions

The organism was propagated at 30 °C for 2 days, with shaking, in 50-ml aliquots of an alkaline medium in 500-ml flasks. The medium...
contained (w/v) 2.0% glucose, 1.0% meat extract (Wako Pure Chemical), 1.0% soybean meal (Ajinomoto), 0.1% K₂HPO₄, and 1.0% Na₂CO₃ (pH 10). After removal of cells by centrifugation (8000 g, 20 min) at 4 °C, the supernatant (1 l) was concentrated by ultrafiltration on a membrane (YM-5, 5000-M cut-off, Amicon). The concentrate was then dialyzed against 5 mM Tris/HCl buffer (pH 8.0) that contained 2 mM CaCl₂. The retentate was used as the crude extract for purification of the enzyme.

Purification of the enzyme

The retentate was loaded on a column of DEAE-Bio-Gel A (2.5 x 16 cm) that had been equilibrated with 10 mM Tris/HCl buffer (pH 8.0) plus 2 mM CaCl₂. The column was washed with the same buffer and unadsorbed fractions that contained alkaline protease activity were combined and concentrated (to 20 ml) by ultrafiltration. The concentrate was then dialyzed against a column of CM-Bio-Gel A (2.5 x 16 cm) that had been equilibrated with 10 mM Tris/HCl buffer (pH 8.0) plus 2 mM CaCl₂. The column was washed with the equilibrating buffer and proteins were eluted first with a 450-ml linear gradient of 0–30 mM KCl and then with a 450-ml linear gradient of 30–100 mM KCl in the same buffer. Fractions of 6.5 ml were collected at 44 ml h⁻¹ cm⁻² from the start of the wash of the column. A peak of protease activity (tubes 77–101) was eluted between 35 mM and 75 mM KCl. The active fractions were concentrated, dialyzed against the equilibrating buffer, and concentrated to 7.7 ml by ultrafiltration. The concentrate was loaded again on a column of CM–Bio–Gel A (1.6 x 16 cm) that had been equilibrated with 10 mM borate/NaOH buffer (pH 9.5) plus 2 mM CaCl₂. The column was washed with the equilibrating buffer (100 ml) and proteins were eluted with a 300-ml linear gradient of 0–100 mM triethanolamine HCl (TEA-HCl) in the same buffer, at a flow rate of 24 ml h⁻¹ cm⁻². Fractions of 3 ml were collected from the start of the gradient. The active fractions (tubes 48–75), eluted between 50 mM and 80 mM TEA-HCl, were concentrated to a small volume by ultrafiltration. The concentrate was dialyzed against 10 mM Tris/HCl buffer (pH 8.0) plus 2 mM CaCl₂ and the retentate was concentrated by ultrafiltration. The resultant retentate was used exclusively for further experiments as the final preparation of purified enzyme. The purified enzyme was stored in 20% (v/v) glycerol at 20 °C when necessary.

Enzyme assays

Caseinolytic activity was measured as follows. A suitably diluted solution of the enzyme (0.1 ml) was mixed with 1.0 ml 50 mM borate/NaOH buffer (pH 10) that contained 10 mg Hammerstein casein (Merck). After incubation at 40 °C for 10 min, 2.0 ml 123 mM sodium acetate/369 mM acetic acid was added to terminate the reaction. The mixture was passed through no. 2 filter paper (Whatman) to remove denatured proteins. Acid-soluble materials in the filtrate were quantified as L-tyrosine by the method of Lowry et al. (1951). One unit (U) of caseinolytic activity was defined as the amount of enzyme that produced acid-soluble peptides equivalent to one micromole L-tyrosine per minute.

Protease activity was also monitored with oligopeptidyl p-nitroanilides (pNA; Sigma) as substrates. The assay mixture contained 50 mM Tris/HCl buffer (pH 9.0), 2 mM CaCl₂, and 5 mM oligopeptidyl pNA dissolved in dimethylsulfoxide. The reaction was started by adding a suitably diluted solution of enzyme (0.05 ml) to 0.95 ml assay mixture. After incubation at 30 °C for 5–30 min, the reaction was stopped by addition of 2.0 ml 5% (w/v) citric acid and then the amount of p-nitroaniline liberated was determined at 420 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme that liberated one micromole of p-nitroaniline per minute under the conditions of the assay.

Protein was quantified by the method of Lowry et al. (1951), with bovine serum albumin (Sigma) as the protein standard.

Electrophoretic analyses

Polyacrylamide gel electrophoresis (PAGE) was performed on 7.5% (w/v) acrylamide slab gels (8 x 10 cm, 1 mm thickness) by the method of Taber and Sherman (1964), with 25 mM Tris/192 mM glycin buffer (pH 8.3) as the running buffer. Gels were stained for proteins with Coomassie brilliant blue R-250 (Sigma) and destained with 7.0% (v/v) acetic acid. Protein bands having protease activity were visualized by use of an overlay agar sheet that contained 1.0% (w/v) skim milk, 50 mM borate/NaOH buffer (pH 10.0), and 2.0% (w/v) agar. The slab gel was laid on top of the agar sheet and was left at 40 °C for 2–3 h. The bands of protein that had protease activity were detected as clear zones on the overlay agar sheet after flooding with 5.0% (w/v) trichloroacetic acid. Sodium dodecyl sulfate (SDS)-PAGE was done in the conventional manner on 12.5% (w/v) slab gels with 25 mM Tris/192 mM glycine buffer (pH 8.3) that contained 0.1% (w/v) SDS as the running buffer, as described by Laemmli (1970).

The isoelectric point (pI) of M protease was measured in a 110-ml column for electrophoresis (LKB). The enzyme and carrier amphotolyte [Bio-lyte (Bio-Rad), pH 3.0–10.0; Servalyte (SERVA), pH 9.0–11.0] were placed in the column in a sucrose density gradient and subjected to electrophoresis, in accordance with the directions of the manufacturer of the column. After equilibrium had been reached 2 days later, the material in the column was drained in 2-ml fractions, and the pH and enzymatic activity of each fraction were measured.

Amino acid analysis

Amino acid analysis was performed on an amino acid autoanalyzer (model L-8500; Hitachi) after M protease had been hydrolyzed at 110 °C for 24–72 h in 6.0 M HCl that contained 0.01% (v/v) phenol and 40% (w/v) thioglycolic acid. The levels of tryptophan and cysteine in the protein were measured by the methods of Edelhoef (1967) and Yoshida et al. (1977) respectively. The N-terminal amino acid sequence was determined by Edman sequencing on a pulsed liquid-phase protein sequencer (model 477A; Applied Biosystems) equipped with an on-line phenylthiohydantoin analyzer (model 120A).

Measurement of circular dichroism

Circular dichroism (CD) spectra of M protease dissolved in distilled water were recorded at room temperature in a cell with 5 mm path length under constant flushing with nitrogen in an automatic recording spectrophotometer (model J-720; JASCO). Data were expressed in terms of the mean residue ellipticity. The mean residue weight of the enzyme was calculated from its relative mobility during SDS-PAGE. The a-helix content of M protease was estimated with a computer program (SSE-338; JASCO) from the equation of Yang et al. (1986).

Hydrolysis of the oxidized insulin B chain

For the determination of the initial cleavage sites, the oxidized insulin B chain (1.16 µmol; Sigma) was dissolved in 1.0 ml 20 mM borate/NaOH buffer (pH 9.0) that contained 2 mM CaCl₂, and the hydrolytic reaction was carried out at 30 °C for 10 min after the addition of 1.2 µg M protease to the solution. For the determination of all the cleavage sites, 1.16 µmol substrate was hydrolyzed by 12 µg