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Isolation, Identification, and Fermentation of a *Bacillus* Species Producing a Detergent-Stable Endopeptidase

Keywords: *Bacillus pumilus; endopeptidase; fermentation; detergent-stable protease*

INTRODUCTION

Bacteria belonging to *Bacillus* are prevalent in nature. *Bacillus* species are by far the most important source of several commercial microbial enzymes. They can be cultivated under extreme temperature and pH conditions to give rise to products that are in turn stable in a wide range of harsh environments (Arbige et al., 1993). Because of the large divergence in physiological types found in the *Bacillus* species, and the facts that most members of the genus are nonpathogenic, relatively easy to manipulate by genetics, good secreters of proteins and metabolites, and easy to cultivate, *Bacillus* species are a preferred host for microbial fermentations.

While screening for a transglutaminase-producing microorganism from soil samples, we isolated a bacterium that produced a highly detergent-stable extracellular endopeptidase. In this paper we describe the identification of the organism and partial characterization of the extracellular endopeptidase, especially its stability in concentrated sodium dodecyl sulfate (SDS) solutions. Characterization of the morphological, physiological, and biochemical features of the organism and the fermentation conditions for the production of the extracellular endopeptidase, tentatively named "protease Q", are also described.

MATERIALS AND METHODS

Soil samples were collected from several locations in the United States. Polypeptone, yeast extract, and other media components were obtained from Difco Laboratories (Detroit, MI). Casein and other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents used in this study were of analytical grade.

Screening and Fermentation. The protease-selective plate contained 4.0% (w/v) LB agar, 1.5% skim milk, and other selection factors, if required. Soil samples suspended in autoclaved water were heat-shocked for 10 min at 75 °C to kill all vegetative cells. The heat-shocked soil samples were then spread on the agar plates. The protease-positive organism, selected from a single colony on the agar plate, was cultured at 37 °C in 250 mL conical flasks using a fermentation medium containing 1.8% (w/v) peptone, 0.2% (w/v) K_2HPO_4, 0.1% (w/v) $MgSO_4$, 0.3% (w/v) yeast extract, and 0.02% (v/v) antifoam reagent. The initial pH of the medium was 7.06. The flasks were shaken in an environmental shaker (Lab-Line Instruments Co., Melrose Park, IL) at 120 rpm. Growth rate was monitored by measuring the turbidity of the medium at 610 nm using uncultivated medium as the blank.

Partial Purification of Protease Q. All isolation and purification steps of the enzyme were carried out at 5 °C if not otherwise mentioned. The fermentation broth was centrifuged at 12100*g* for 15 min, and the clear supernatant (1000 mL) was mixed directly with DEAE-Sephadex A-50, which had been previously equilibrated with 20 mM Tris-HCl buffer (pH 8.3) containing 1.0 mM $CaCl_2$ (buffer A). The suspension was filtered and the filtrate freeze-dried. The freeze-dried sample was then fractionated on a Sephacryl 100 gel permeation column (2.5 × 100 cm) using buffer A as eluent. The fractions with alkaline protease activity were collected and further purified on a Blue-Sepharose column (1.2 × 25 cm). The column was eluted with buffer A containing 1.0 M NaCl until the absorbance of the eluent at 280 nm approached zero. The column was then eluted with a 1.0−0 M NaCl gradient in buffer A. A 173-fold purification was achieved after the Blue-Sepharose column chromatography.

Assay of Proteolytic Activity. Proteolytic activity on casein was determined according to the method described by Walter (1984) with slight modification. The reaction mixture was composed of 100 μL enzyme solution, 500 μL of 0.1 M Tris-HCl buffer (pH 8.3), and 300 μL of 1.5% casein solution. Proteolysis was carried out at 37 °C for 20 min and was stopped by the addition of 300 μL of 12% (w/v) trichloroacetic acid (TCA). The sample was then incubated in ice for 10 min, and the absorbance of TCA-soluble materials was determined at 280 nm after centrifugation at 14000*g* for 10 min. One casein unit (CU) was defined as the amount of enzyme that increased the absorbance at 280 nm of TCA-soluble materials by 0.001/min.

Stability in SDS Solutions. The stability of protease Q in SDS solutions was determined by monitoring its proteolytic

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activity in 0−10% (w/v) SDS solutions using β-casein as substrate. The enzyme was preincubated for 10 min at room temperature in 0.1 M Tris-HCl buffer (pH 8.3) containing 0−10% concentration of SDS prior to mixing with β-casein, which was also in 0.1 M Tris-HCl buffer (pH 8.3) containing the same amount of SDS. The mixture was incubated at 37 °C for 20 min, and the proteolytic reaction was stopped by heating the solution in boiling water for 5 min. The samples were then analyzed on a Tricine−SDS−PAGE slab gel.

Electrophoresis. Two electrophoresis techniques, namely Tricine−SDS−PAGE and gelatin−SDS−PAGE, were used. Tricine−SDS−PAGE was performed on a slab gel using a discontinuous Tricine (*N*-tris[hydroxymethyl]methylglycine) buffer system as described by Schagger and von Jagow (1987). The enzyme band was transblotted onto a PVDF membrane for N-terminal sequencing.

Gelatin−SDS−PAGE, as described by Heussen and Dowdle (1980) and Hare et al. (1983), was performed for detecting in-run proteolytic activity (i.e., proteolytic reaction in the SDS−PAGE gel during electrophoresis). The protein substrate (1% gelatin) was mixed directly with polyacrylamide and SDS solutions and copolymerized into a slab gel. The final SDS and gelatin concentrations in the slab gel were 0.1 and 0.05%, respectively. The protease solution was mixed at a ratio of 1:1 with Tricine−SDS−PAGE sample buffer containing 4% SDS and 2% (v/v) 2-mercaptoethanol. The mixture was incubated at room temperature for 30 min before loading onto the gelatin−SDS−PAGE slab gel. Electrophoresis was carried out at 4 °C with constant voltage of 80 V for 24 h. After electrophoresis, the gel was incubated in 0.1 M Tris-HCl (pH 8.9) buffer for 30 min, stained with Coomassie Blue-G for 1 h, and destained with SDS−PAGE destaining solution (10% glacial acetic acid and 15% methanol).

Determination of Morphological and Physiological Characteristics. The morphological and physiological characteristics were carried out according to the procedure described in *Bergey's Manual of Systematic Bacteriology* (1994). The isolated unknown was identified from genus to strain by matching experimental results with the identification key for strain identification as described by Priest (1993). The following tests were conducted: microscopic characteristics of the cell's morphology, including cell shape, size, endospores, and Gram-stain reaction; growth characteristics including colonial morphology, mobility, anaerobic growth, appearance in liquid culture, and pigmentation; physiological characteristics including growing temperature range, Voges−Proskauer test, and pH tolerance range; metabolic characteristics of the growing cells, such as oxidase production, catalase production, utilization of citrate, hydrolysis of casein, hydrolysis of starch, lysine decarboxylation, deamination of phenylalanine, indole production, and utilization of different carbohydrate sources (e.g., glucose and lactose).

Determination of Fatty Acid Profiles. The cellular fatty acid profile was used as an identification marker to confirm the tentative identification of the unknown bacterial strain based on its morphological and physiological characteristics. The cellular fatty acid profile of the organism was determined by Analytical Services Inc. (Williston, VT). The cellular fatty acid profile of the unknown strain was then compared with a database of known bacterial strains.

RESULTS AND DISCUSSION

Fermentation. A bacterial single colony that showed strong proteolytic activity in the casein agar plates was isolated and grown in a liquid medium at 37 °C. Figure 1 shows the specific growth curve of the isolated organism and the time-dependent increase of extracellular proteolytic activity. Proteolytic activity in the fermentation broth appeared when the bacteria entered the stationary growth phase (∼40 h), and the activity reached a maximum level after 100 h. Thereafter, the proteolytic activity remained constant during prolonged fermentation, indicating that the enzyme was relatively stable against autolysis at 37 °C. During the 10 day fermentation, the pH of the fermentation broth increased from 7.0 to a maximum value of 9.0 and then returned to its original level. The time at which the pH reached a maximum value during fermentation coincided with the time at which the enzyme concentration reached its highest level. Thus, the pH of the fermentation can be used to monitor protease production.

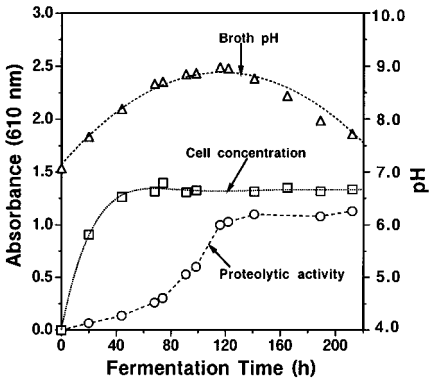

Figure 1. Typical fermentation kinetics of the unknown organism isolated from soil. The fermentation medium contained 1.8% (w/v) peptone, 0.2% (w/v) K_2HPO_4, 0.1% (w/v) $MgSO_4$, 0.3% (w/v) yeast extract, and 0.02% (v/v) antifoam agent.

Table 1. Purification of Protease Q

sample	vol (mL)	protein (mg/mL)	activity units (mg)	yield (%)	purifn (-fold)
culture supernatant	1000	20.2	25.1	100.0	1.0
ion exchange	1120	2.42	178	95.2	7.1
Sephacryl 100	460	0.38	2275	78.4	90.6
Blue agarose	380	0.19	4336	61.7	173

Table 1 shows purification data for protease Q after each purification step. By passing the centrifuged fermentation broth through DEAE-Sephadex A-50 ion-exchange, Sephacryl-100 gel permeation, and Blue agarose columns, protease Q was purified ∼173-fold. Protease Q did not bind to DEAE-Sephadex A-50 at the pH of the fermentation broth (∼9.0), indicating that it was a basic protein. The gel permeation chromatography step was used immediately after the ion-exchange column because protease Q exhibited abnormal mobility in the gel permeation matrix (either Sephadex G-75 or Sephacryl 100) even when it was eluted with a buffer containing 0.5 M NaCl. The enzyme eluted on this column as a 10 kDa molecular mass protein, probably because of nonspecific interactions with the gel matrix. As a consequence, most of the contaminant proteins with a molecular mass >20 000 could be easily removed after this gel permeation step. Other contaminant proteins were removed via hydrophobic interaction chromatography by using the Blue agarose column.

Stability of Protease Q in SDS. Figure 2 shows proteolytic activity of protease Q in the gelatin−SDS−PAGE gel. The unstained region of the gel reflects hydrolysis of gelatin by protease Q as the enzyme migrated through the gel. It should be pointed out that prior to the gelatin−SDS−PAGE, the enzyme was preincubated for 30 min at room temperature in the SDS-PAGE sample buffer containing 2% SDS. Thus, hydrolysis of gelatin in the gelatin−SDS−PAGE clearly suggests that protease Q was not inactivated by 2% SDS.

The stability of protease Q in > 2% SDS solutions was investigated by using casein as substrate. Figure 3 shows the SDS−PAGE profile of proteolytic products of

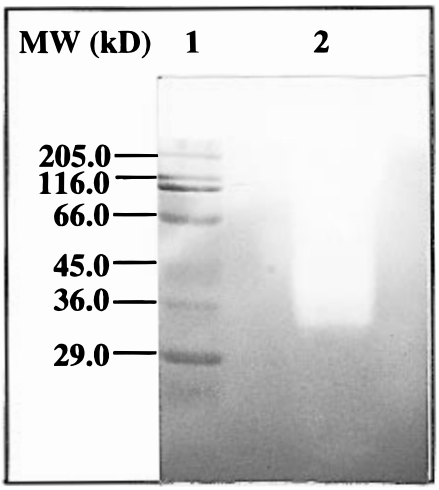

Figure 2. Gelatin−SDS−PAGE showing in-run proteolytic activity of protease Q in the presence of SDS: (lane 1) molecular mass markers; (lane 2) protease Q. The unstained area of the gel reflects hydrolysis of gelatin by protease Q as it moved through the gel.

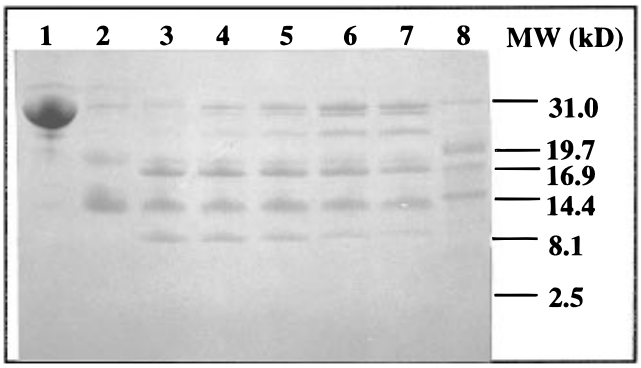

Figure 3. SDS−PAGE of β-casein treated with protease Q in the presence of 0−10% SDS: (lane 1) β-casein control; (lane 2) β-casein treated with protease Q without SDS; (lanes 3−7) β-casein treated with protease Q in the presence of 2, 4, 6, 8, and 10% SDS, respectively; (lane 8) molecular mass markers. The enzyme to substrate ratio was 5 casein units/mg of substrate. The enzyme was preincubated with SDS for 10 min at room temperature before it was added to the substrate, which was also in SDS solution.

β-casein treated with protease Q. In this case, the enzyme was preincubated for 10 min in 0.1 M Tris-HCl buffer (pH 8.3) containing 0−10% SDS before the addition of the substrate, which was also in the same buffer containing the same concentration of SDS. As shown in Figure 3, protease Q was able to hydrolyze β-casein even in the presence of 10% SDS. The extent of hydrolysis of the intact β-casein decreased only slightly as the SDS concentration was increased. However, when a higher enzyme to substrate ratio and longer reaction times were employed, the intact β-casein was completely hydrolyzed even in 10% SDS (data not shown), which demonstrated the unusual stability of protease Q in SDS.

The N-terminal sequence of protease Q was found to be A-Q-T-V-P-Y-G-I-P-Q-I-K-A-P-A-V-H-A-Q-G-Y-K-G-A-N-V-K-V-A-V. Protein data bank (SwissProt) search results showed no match; however, the sequence showed a high homology with subtilisin Carlsberg from *Bacillus licheniformis*. Thus, it appears that protease Q is a new member of endopeptidases.

Identification of the Unknown Organism. The identity of the unknown organism that produced the

Table 2. Differential Characteristics of Endospore-Forming Bacteria and Similar Genera[a]

characteristic	unknown	*Amphibacillus*	*Bacillus*	*Clostridium*	*Desulfotomaculum*	*Sporohalobacter*	*Sporolactobacillus*	*Sporosarcina*	*Sulfidobacillus*	*Syntrophospora*
rod-shaped in young cultures	+	+	+	+	+	+	+	−	+	+
rods of filaments curved	−	−	−	D	D	−	−	NA	−	ND
cocci in tetrads or packets	−	ND	−	−	−	−	−	+	−	−
endospores produced	+	+	+	+	+	+	+	+	+	+
motile	+	+	+	+	+	+	+	+	−	+
Gram-positive at least in young cultures	+	+	+	+	d	+	+	+	+	+
strict aerobes	+	−	D	−	−	−	−	+	+	−
facultative anaerobes or microaerophiles	−	+	D	−	−	−	+	−	−	−
strict anaerobes	−	−	−	+	+	+	−	−	−	+
carbohydrate fermentation producing lactate	ND	−	D	−	−	−	+	−	ND	−
sulfate actively reduced to sulfide	−	ND	−	−	+	ND	−	−	−	−
catalase	+	−	+	−	−	−	−	+	ND	ND
oxidase	+	−	D	−	ND	−	ND	+	ND	ND
marked acidity from glucose	+	+	+	D	−	+	+	−	ND	ND
nitrate reduced to nitrite	+	−	D	D	ND	ND	−	D	ND	−

[a] Symbols: +, ≥90% of strains are positive; −, ≤90% of strains are negative; d, 11−89% of strains are positive; D, substantial proportion of species differ; NA, not applicable; ND, not determined.

Table 3. Comparison of Characteristics of the Unknown Strain with Those of *B. pumilus*[a]

feature	unknown strain	B. pumilus
Gram-positive	+	+
motile	−	−
cell diameter > 1.0 μm	−	−
spore-forming	+	+
sporangium swollen	−	−
anaerobic growth	−	−
growth in 7% NaCl	+	+
growth in lysozyme	ND	D
catalase	+	+
growth		
10 °C	+	+
50 °C	+	+
55 °C	−	−
Voges−Proskauer test	−	−
pH in V−P broth	5.1 ± 0.2	4.8−5.5
acid from glucose	+	+
gas from glucose	−	−
lactose fermentation	−	−
hydrolysis of casein	+	+
hydrolysis of starch	−	−
utilization of citrate	+	+
lysine decarboxylation	+	ND
indole formation	−	−
nitrate reduction	−	−
degradation of tyrosine	−	−
allantoin or urate required	ND	−

[a] Symbols: +, ≥ 90% positive; −, ≥ 90% negative; d, 11−89% positive; D, substantial proportion of species differ; ND, not determined.

detergent-stable protease Q was systematically studied. The isolated organism was a Gram-positive, endospore-forming, rod-shaped strict aerobe. The bacterial cell contained a single spore and sporulated spontaneously. Sporulation of the strain was not repressed by exposure to air. The spores were ellipsoidal and located centrally to subcentrally and did not distend the sporangium. On the LB agar plate, the bacterial colonies had a diameter of 2.5−4.4 mm and were bright-colored with a smooth to wavy edge. The size of germinating cells was ~0.6−0.8 μm. Some of the morphological and physiological properties of the isolated bacterial strain and other Gram-positive endospore-forming genus listed in *Bergey's Manual of Systematic Bacteriology* (1994) are summarized in Table 2. The isolated organism was a strict aerobe with both catalase and oxidase activity, which differentiated it from strict anaerobes, such as *Clostridium*, *Desulfotomaculum*, *Sporohalobacter*, and *Syntrophospora*. The rod shape of the organism also excluded it from *Sporosarcina*, which has a spherical or oval-shaped cell arranged mainly as diplococci and tetrads (*Bergey's Manual*, 1994). The isolated organism grew well in the fermentation broth at alkaline pH. Therefore, it was not a member of the genus *Sulfidobacillus*, which is nonmotile and grows optimally at a pH range of 1.9−2.4. Unlike *Sporolactobacillus*, which are facultative anaerobes without catalase (but show scanty growth in air), the unknown organism was a strict aerobe containing catalase. It was also not a *Amphibacillus*, a catalase-negative and oxidase-negative genus. All results summarized in Table 2 indicate that the unknown organism belonged to the genus *Bacillus*.

Species Identification. Additional physiological tests demonstrated that the isolated unknown organism could grow in 7% NaCl and produced acid from glucose fermentation (with no gas production). It was negative in the Voges−Proskauer test, indicating that it could not utilize starch and citrate, and in the nitrate reduction test. It grew at 10 and 50 °C but could not grow at 55 °C. The physiological characteristics of the isolated organism are summarized in Table 3. Comparison of its characteristics with those of *Bacillus pumilus* tentatively suggested that it was a strain of *B. pumilus*.

Table 4. Fatty Acid Constituents of the Unknown Bacterial Strain

name	%	name	%	name	%
13:0 iso	0.32	16:1ω7c alcohol	0.54	iso 17:1ω10c	2.64
14:0 iso	0.87	16:0 iso	2.18	sum in feature 5[a]	0.55
14:0	1.39	16:1ω11c	2.68	17:0 iso	11.09
15:0 iso	48.42	16:0	5.03	17:0 anteiso	5.05
15:0 anteiso	18.92				
15:0	0.31				

[a] 17:1 iso I/antei B 17: anteiso B/i.

Taxonomically, cellular fatty acid profiles have provided the greatest information on strain identification of microorganisms (Suzuki et al., 1993). Most fatty acids are found in the cytoplasmic membrane as constituents of polar and glycolipids (Kates, 1964) where they form an integral part of the lipid bilayer (Ratledge and Wilkinson, 1988). Extensive studies have shown that diversity in fatty acid types and their highly regulated production make them useful taxonomic markers. Gram-positive organisms contain three main groups of cellular fatty acids, namely straight-chain, branched-chain, and complex fatty acid types (O'Leary and Wilkinson, 1988). Those with branched-chain fatty acid types possess large proportions of iso- and anteiso-branched fatty acids but rarely unsaturated fatty acids (particularly anteiso-C15:0) as the predominant fatty acid (Kaneda, 1991). *Bacillus* species typically contain anteiso-C15:0 as their predominant fatty acid component (Kaneda, 1977). The cellular fatty acid profile of the unknown bacterial strain is summarized in Table 4. The cytoplasmic membrane of the isolated strain predominantly contained C15:0 iso (48.42%) and C15:0 anteiso (18.92%) fatty acids. Results of a computerized data bank search indicated that the isolated unknown matched at 0.767 with *B. pumilus* GC group B, at 0.237 with *Staphylococcus sciuri*, at 0.128 with *Staphylococcus intermedius* subgroup A, at 0.201 with *B. licheniformis*, and at 0.006 with *Bacillus brevis*. The results of cellular fatty acid profile and the results of conventional morphophological and physiological studies discussed above indicate that the isolated unknown bacterium is a strain of *Bacillus pumilus*.

Because of its remarkable stability in concentrated SDS, protease Q may have potential uses in several commercial applications, including detergent formulations, cleaning of ultrafiltration and microfiltration equipments in the food and pharmaceutical industries, and molecular biology techniques. Further studies on the biochemical and structural characteristics of protease Q produced by the isolated *B. pumilus* strain are under investigation.

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Xiao-Qing Han and Srinivasan Damodaran*

Department of Food Science, University of Wisconsin—Madison, 1605 Linden Drive, Madison, Wisconsin 53706

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* Author to whom correspondence should be addressed [telephone (608) 263-2012; fax (608) 262-6872; e-mail sdamodar@facstaff.wisc.edu].