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Partial Purification and Characterization of an Extracellular Protease from *Xenorhabdus nematophilus*, a Symbiotic Bacterium Isolated from an Entomopathogenic Nematode, *Steinernema glaseri*

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Abstract Entomopathogenic nematodes are used for insect control. Herein, an extracellular protease was partially purified from a culture supernatant of *Xenorhabdus nematophilus*, a symbiotic bacterium of an entomopathogenic nematode, *Steinernema glaseri*, using precipitation with 80% v/v isopropyl alcohol followed by gel permeation chromatography with a packed Sephacryl S-300 HR media. The partially purified protease exhibited maximal activity at pH 7 in the presence of 1 mM $CaCl_2$. The protease was identified as a metallo-protease based on the inhibition of its activity by the metal chelating agent, EDTA.

Keywords: entomopathogenic nematodes, inhibitor, protease, symbiotic bacteria

INTRODUCTION

Entomopathogenic nematodes of the genera, *Steinernema* and *Heterorhabditis*, are of interests for the development of biological pesticides to replace chemical pesticides, the use of which has raised many environmental and biological concerns [1,2]. Entomopathogenic nematodes have the ability to infect a wide range of insect hosts, which are rapidly killed upon selective infection. They are, however, safe for non-target organisms, such as plants, animals and humans. The nematodes carry *Xenorhabdus* or *Photorhabdus* species in their guts, as symbiotic bacteria, which are released into the hemolymph of the insect hosts after infection [3]. The bacteria multiply rapidly killing the insect hosts within 24~48 h, and thus, provide suitable conditions for nematode reproduction. The bacteria exist in two forms; the primary (phase I) or secondary forms (phase II). The primary form is known to preferentially exist in the infective nematodes, while the secondary form develops in the *in vitro* culture medium of the primary form following a long incubation period [4,5], or when the bacteria exist in soil as free-living organisms outside the nematodes [6].

Several toxins produced from the symbiotic bacteria, *Photorhabdus luminescens*, were isolated, but have not yet been fully identified [7]. Protease is also considered a major toxic factor that contributes to the pathogenecity of the nematode/bacteria complex. Schmidt *et al.* [3] purified and characterized an extracellular protease from a culture of the primary form of *X. luminescens* grown in a gelatin medium. The protease was classified as an alkaline metallo-protease, based on its inhibition by metal chelating agents. On the contrary, Caldas *et al.* [8] reported that a major extracellular protease produced by *X. nematophilus* isolated from *Steinernema carpocapsae* is a serine protease because its activity is inhibited by serine protease inhibitors but not by EDTA. Ryu *et al.* [9] reported the production of an extracellular protease by *X. nematophilus*, which was isolated from an entomopathogenic nematode, *Steinernema glaseri*, by Park and Yu [10]. A high level of extracellular protease was produced from the symbiotic bacterium when tryptone and fructose were used as a nitrogen and a carbon source, respectively while no protease was produced when the bacterium was grown in the gelatine medium in which *X. luminescens* was reported to produce an extracellular protease [3]. In this work, an extracellular protease, produced from *X. nematophilus*, was partially purified using precipitation by organic solvents followed by gel permeation chromatography. The catalytic properties of the protease were investigated and compared with those of the proteases previously reported by others.

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MATERIALS AND METHODS

Organisms and Culture Conditions

An insect-pathogenic bacterium, *X. nematohpilus*, isolated from *Steinernema glaseri* by Park and Yu, was used for this study [10]. The stock culture of the bacterium was maintained at 25°C on NBTA agar medium, on which the primary and secondary forms could be distinguished by their green and dark red colors, respectively,

depending on the adsorption of bromothymol blue. The NBTA medium contained 20 g nutrient agar (Difco), 25 mg bromothymol blue (Sigma), and 40 mg triphenyl tetrazolium (Sigma) in 1 L distilled water. For a seed culture, the primary form of the bacterium, the green colored on the NBTA medium, was selectively transferred to a 250-mL flask, containing 50 mL yeast medium, and grown for 24 h at 25°C with shaking at 100 rpm. The yeast medium for the seed culture contained, per liter, 50 g yeast extract (Merck), 5 g NaCl, 0.5 g K_2HPO_4, 0.5 g $NH_4H_2PO_4$, and 0.2 g $MgSO_47H_2O$. A 2 mL of the seed culture was used as the inoculum for the protease production in a 250-mL flask containing 50 mL culture medium containing, per liter, 1.5 g tryptone, 3 g fructose, 5 g NaCl, 0.5 g K_2HPO_4, 0.5 g $NH_4H_2PO_4$, and 0.2 g $MgSO_4$·7H2O. The culture medium was incubated at 25°C for 40 h and shaken at 100 rpm.

Measurement of Protease Activity

The protease activity was measured according to the method described by Schmidt *et al.* [3]. Typically, a 0.1 mL protease containing sample was added to 1.4 mL buffer solution (50 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0), containing 3 mg Hide Powder Azure (HPA) (Sigma) as a complex substrate for proteases. The mixture was incubated for 2 h at 30°C and shaken at 200 rpm in a constant-temperature shaker. After incubation, the mixture was centrifuged to remove the undigested HPA. The protease activity was represented by the increase in the supernatant optical density at 595 nm, as measured by a spectrophotometer (Hewlet-Packard 8402A).

RESULTS AND DISCUSSION

Partial Purification of Protease

Cells were separated from the fermentation broth by centrifugation at 0.5°C and 8,000 rpm for 30 min. After centrifugation, the supernatants containing the extracellular protease were collected and stored at 4°C . The extracellular protease produced from *X. nematohpilus* was partially purified by precipitation with organic solvents followed by gel permeation chromatography. To investigate the optimum conditions for the precipitation of the protease, several organic solvents, such as isopropyl alcohol, acetone, and ethanol, were used at various concentrations. To the cold supernatant, an organic solvent was slowly added at -10°C with gentle stirring. The supernatant-organic solvent mixtures were stored at 4°C for 1 h then centrifuged at 4°C and 7,000 rpm for 30 min to recover the precipitates. The residual organic solvents contained within the precipitates were further removed under vacuum. The final precipitates were subjected to measurement of the protease activity to determine the protease activity yield, which is based on the protease activity initially present in the fermentation supernatant. Fig. 1 shows the protease yields when 30 to 80% v/v isopropyl alcohol, acetone, or ethanol were used. Isopropyl alcohol was found to be the most efficient organic solvent for the precipitation of the protease, with a maximum yield of 56.7% when 80% v/v isopropyl alcohol was used. For further purification, the protease recovered by precipitation with 80% v/v isopropyl alcohol from the fermentation broth was applied to a gel permeation column (2.5 cm × 90 cm) packed with Sephacryl S-300 HR (Pharmacia). The column was initially eluted with 90 mL Tris-HCl buffer (10 mM, pH 8.1) without collection, then 6 mL of the eluting solution was collected in individual test tubes. Fig. 2 shows a typical chromatogram

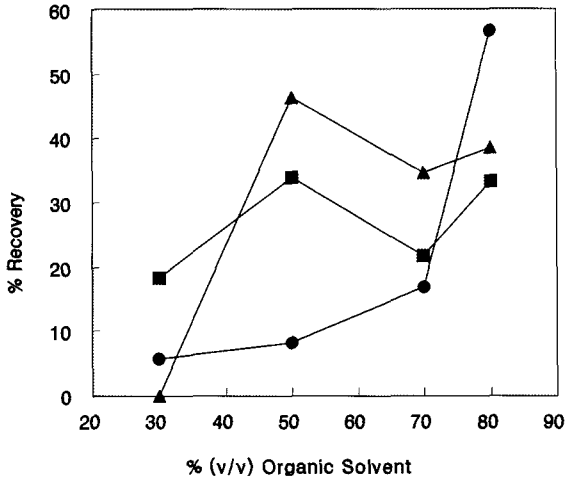

Fig. 1. Effects of the organic solvents, isopropyl alcohol (●), ethanol (■), and acetone (▲), on the protease activity from the culture supernatant of *X. nematophilus* recovered by precipitation.

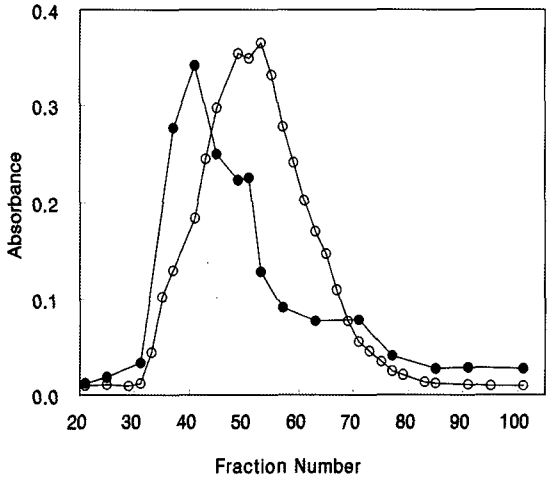

Fig. 2. The result of the column chromatography of the protease precipitates separated from the culture supernatant of *X. nematophilus*. A gel permeation column (2.5 cm × 90 cm) packed with Sephacryl S-300 HR was used. For each collected sample, protein content was represented by measuring the absorbance of the sample at 280 nm (○) and the protease activity (●) was determined as described in the text.

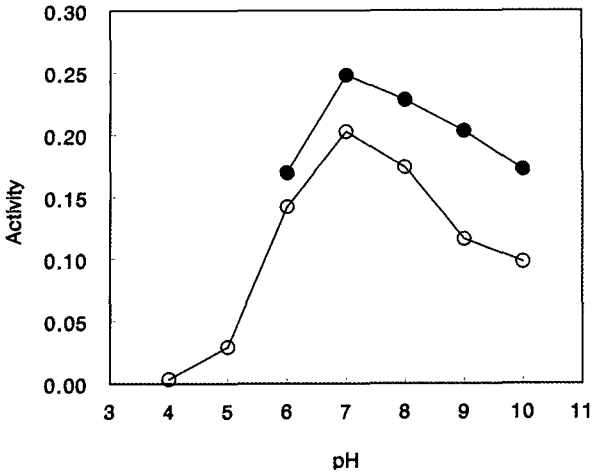

Fig. 3. Effects of pH on the activity of the partially purified protease from the culture supernatant of *X. nematophilus* in the presence (●) and absence (○) of $CaCl_2$ (1 mM). The buffers (100 mM) used were sodium acetate (pH 5, 6), bis-tris (pH 6, 7), Tris-HCl (pH 8), sodium borate (pH 9), and glycine (pH 10).

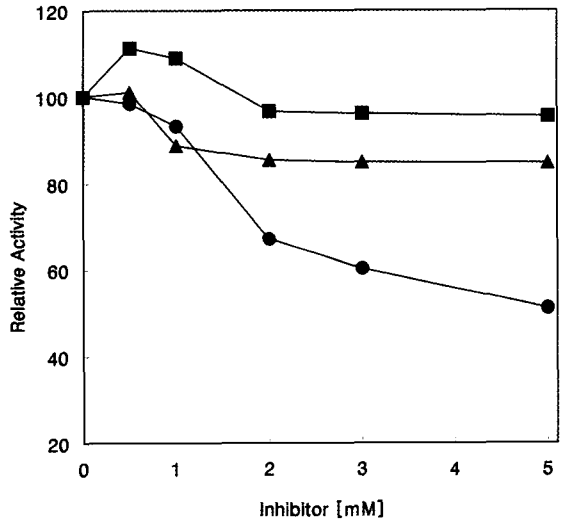

Fig. 4. Effects of different inhibitors on the activity of the partially purified protease from the culture supernatant of *X. nematophilus*. The inhibitors used were PMSF (■), PCMB (▲), and EDTA (●).

from the gel permeation column. Fractions with the highest protease activity (fraction numbers from 39 to 43 in Fig. 2) were collected for characterization.

Characterization of Protease

The effects of pH on the activity of the partially purified protease were investigated by varying the pH from 4 to 10 in the presence or absence of 1 mM $CaCl_2$. As shown in Fig. 3, the partially purified protease exhibited the maximal activity at pH 7, both with and without $CaCl_2$ in the assay solution. The activity of the protease decreased both in acidic and alkaline solutions, showing a typical bell-shaped curve for the pH-activity profile. The addition of 1 mM $CaCl_2$ to the assay solution increased the maximum protease activity by 25% in a pH 7 solution. The active site of the protease was characterized by studying the effects on the enzyme activity of three different inhibitors, phenylmethylsulfonyl fluoride (PMSF), *p*-chloromercuribenzoate (PCMB), and EDTA, which have specific inhibitory effect on serine, cystine, and metalloproteases, respectively. Among the three inhibitors tested, EDTA shows the greatest inhibitory effect on the activity of the protease, indicating that the partially purified protease from the culture supernatant of *X. nematophilus* belongs to the class of metallo-proteases. Other inhibitors, PMSF and PCMB, showed little inhibitory effects on the activity of the purified protease. This result is in agreement with the results previously reported by Schmidt *et al.* [3], where an extracellular protease purified from a symbiotic bacterium, *X. luminescens*, was characterized. Caldas *et al.* [8], however, reported that the extracellular protease produced by *X. nematophilus* isolated from another entomopathogenic nematode, *Steinernema carpocapsae*, was a serine protease. Such results that *X. nematophilus* isolated from two species of entomopathogenic nematodes, *Steinernema glaseri* and *Steinernema carpocapsae*, produce different classes of extracellular proteases raise interesting questions on the production of proteases by the symbiotic bacteria. Therefore, complete purification and characterization of the protease including the investigation on the contribution of the purified protease to the pathogenecity of the entomopathogenic nematodes are necessary and will be pursued in the future.

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