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Extracellular proteases from *Bacillus licheniformis*: partial purification and characterization

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*Bacillus licheniformis*의 세포막 프로티아제 부분 정제 및 특성

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ABSTRACT: Extracellular proteases of *Bacillus licheniformis* were partially purified using ammonium sulfate fractionation and Sephadex G-75 gel filtration chromatography. The partial purification permited the separation of two different protease activities, type Ⅰ and type Ⅱ. Protease type Ⅰ is an enzyme with rather high protealytic activity toward casein and was highly susceptible to organofluoride and EDTA inhibitions.

It showed maximal proteolytic activity at pH 7.5 and was rapidly denatured at 71℃. Protease type Ⅱ is a protease with relatively lower proteolytic activity than the type Ⅰ. It was also inhibited by 10 mM of EDTA and 1 mM of PMSF by 30 min incubation. The enzyme showed maximal activity at pH 8.0 and was denatured relatively slowly at 71℃.

KEY WORDS □ *B. licheniformis* proteases.

The genus Bacilli secrete a variety of soluble extracellular enzymes and one of them is protease including exo-and endopeptidase. Several types of proteases has been observed and purified from various strains of *Bacillus* species. Proteases were isolated, for example, from *Bacillus licheniformis* (Hall *et al.*, 1966; Aiyappa *et al.*, 1977), *Bacillus subtilus* Marburg (Millet, 1970), Bacillus cereus (Levishon and Aronson, 1967), and alkalophilic *Bacillus* species (Tobe *et al.*, 1975). The major role of extracellular proteases in nature, in common with other extracellular depolymerizing enzymes, is nutritional, that is to hydrolyze large polypeptide substrates into smaller molecular entities which the bacterial cell can absorb. The proteases have many biotechnological applications, e.g. in dairy industry, protein hydrolysis, peptide synthesis, and as a loundary detergent additives.

Therefore, it has became one of the most widly used family of enzymes.

The extracellular proteases from *B. licheniformis* were divided into several types according to their catalytic nature. These are aminopeptidase, metal protease, serine protease, and serine-metal protease (priest, 1977). Here we report the partial purification and characterization of proteases from *B. licheniformis* in order to establish an experimantal procedure for isolation of extracellular proteases form cell culture.

MATERIALS AND METHODS

Growth conditions

Organism used was *B. licheniformis* (ATCC No 27811) and was maintained as spores on juiced potato agar slants. Culture medium used for enzyme production was LB media supplemented with final concentration of 1 mM $CaCl_2$. It was adjusted pH around 7.0 with NaOH. Cells were grown for 16 hours at 32℃.

The cells were precipitated with centrifugation at 8,000×g for 10 min, and supernatant fluid was collected.

Protease assay

Proteolytic activity in the culture supernatnat and in the fractions during partial purification was assayed by a slight modification of the method of Aiyappa *et al.* (1977) with casein as substrate. One ml of 1% casein in 50mM sodium phosphate buffer (pH 7.5) was incubated with 0.25ml of enzyme solution for 30min at 37℃. Reaction was stopped by addition of 2ml of trichloroacetic acid (TCA) solution. After standing for 10min at 4℃, the reaction

mixture was centrifuged at 13,000×g for 15min. The absorbance of the supernatant at 280 nm (A_{280}) was measured. Control mixtures were similarly prepared except that the TCA solution was added before incubation with the enzyme solution. One unit of protease activity was defined as an increase in A_{280} of 0.1 under the assay conditions.

In some cases, the protease activity was assayed in Eppendorf tube by mini-scale (one-fifth scale of the standard method). The substrate solution of casein was prepared based on the method of Reimerdes *et al.* (1976). Protein concentrations were estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Partial purification of proteases

Ammonium sulfate fractionation : All prodcedures were carried out at 4℃. The culture supernatant was brought to 20% saturation with powdered ammonium sulfate added gradually with stirring on an ice-bath and held for at least 1 hour.

Precipitates were collected by centrifugation at 15,000 x *g* for 10 min. The supernatant fluid was then brought to 85% saturation with ammonium sulfate on an ice-bath and was allowed to stand for 3 to 4 hours. The mixture was then centrifuged as before. The precipitates were dissolved in a minimum volume of 0.01 M Tris-HCl buffer (pH 7.2) containing 2 mM $CaCl_2$ and eluted with the same buffer at a flow rate of 3.5 ml/h and 2.8 ml fractions were collected. Protein content of the fractions was estimated by their A_{280} values.

Effect of pH

The effect of pH on the protease activity was determined by adjusting the casein substrate solution to various pH values from 7.0 to 10.5. Since the casein substrate was precipitated at acidic pH, the effect of pH could not be examined at below pH 7.0. Buffers of various pH values were prepared with 0.05 M sodium phosphate buffer (pH8.8-10.5).

Effect of temperature

The effect of temperature was measured by assaying the enzyme solution with casein substrate for 30 min incubation at temperature ranging from 25℃ to 74℃ for an appropriate time intervals. After heating, the enzyme samples were withdrawn and chilled immediately in an ice-bath, and then the remaining activities were determened under the standard assay condition.

Effect of protease inhibitors

The sensitivity of the enzyme system toward protease inhibitors were tested using phenylmethansulfonyl floride(PMSF) and ethylenediaminetertraacetic acid (EDTA). PMSF inhibition was determined by mixing the enzyme solution with equal volumes of 2 mM PMSF dissolved in 30% (V/V) 2-propanol. After 30 min and 1 hour incubation at 37℃, aliquots of samples were withdrawn and assayed for remaining proteolytic activity. To test inhibition with EDTA 10 mM and 20 mM of EDTA solution at 37℃ for desired time. Effect of metal ions on the enzyme activity was also examined with the addition of Ca^{2+} and Mg^{2+}ions. Each of 1 mM, 5mM and 10mM of these ions concentrations were added to the reaction mixture prior to the addition of the casein substrate solution.

RESULTS AND DISCUSSION

Cell growth and protease activity

The relationship between cell growth and protease synthesis in nutrient broth was examined. Protease production (maximal protease activiry) was maximal when cell density at 570nm was aroud 1.2 which coresponds to the late exponential and the early stationary phases of cell growth (Table 1). This phenomenon observed for the *B licheniformis* is common in the production of most of the extracellular enzymes (Priest, 1977 ; Aunstrup, 1980). However, the protease synthesis in *B. subtilus Marburg* was occured only after the stationary phase when the cell was grown in nutrient broth (Millet, 1970).

Table 1. *Cell Growth and extracellular protease production.*

O. D. 570	protease activity (unit/ml)
0.44	8.84
1.00	18.0
1.20	23.0
1.30	18.4
1.35	6.52
1.46	4.08
1.75	1.92

Bl licheneformis was grown in LB broth supplemented with 1 mM $CaCl_2$. Growth was measured by turbidity determined, at 570nm at appropiate time intervals. Cells were removed by centrifugatin and the proteolytic activity present in the supernatant of the media was determined using the standard assay method.

Purification of proteases

For ammonium sulfate fractionation, Preliminary-test was performed to determine a proper concentration of ammonium sulfate. Various fractions obtained at between 20 to 90% of saturation were examined for maximum recovery. Most of the protease activity was found to be associated with the fraction between 20 to 85% of saturation, and this fraction provided pattern of ammonium sulfate was

Table 2. *Partial purifiction of proteases from B. licheniformis.*

Steps	Volume(ml)	Protein(mg)	Specific activity(unit/mg)
Culture media	800	6770.4	2.72
$(NH_4)_2SO_4$ fractionation (20 to 85%)	17.4	161.5	4.59
Sephadex G-75			
type I	9.5	8.5	5.41
type II	11.0	82.5	6.88

A unit of enzyme activity was defined as the amount of enzyme that caused an increase in absorbanc of 0.1 (280 nm) in 30 min at 37℃ under the standard assay condition.

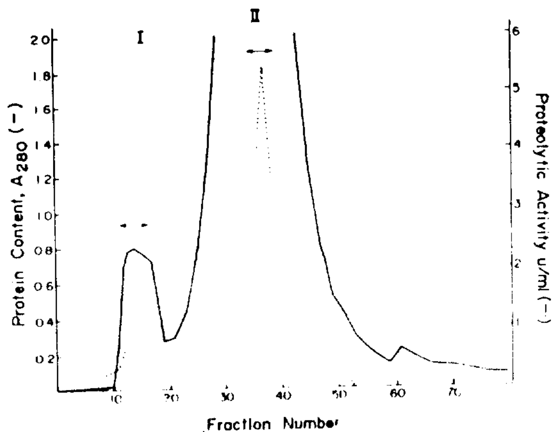

Fig. 1. *Gel permeation chromatography of proteases on Sephadex G-75 column.*

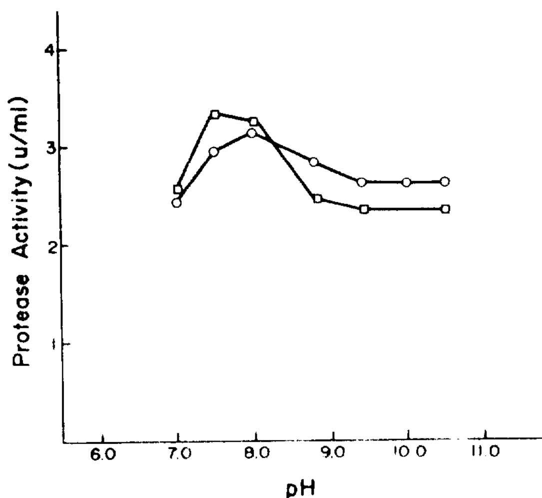

Fig. 2. *Effect of pH on the proteolytic activities of proteases., type I & type II.*

similar to the previously reported one (Aiyappa *et al.*, 1977). In Sephadex G-75 gel filtration chromatography, two protease activity peaks were separated and these fractions were collected separately (Fig. 1). The specific activities of protease type I and II were increased only marginally during the purification procedure, since the recovered total activities were reduced significantly.

Attempts to purify the enzymes further by use of DEAE-Sephadex and CM-cellulose ion exchange chromatogrphy have been tried. A summary of the partial purification of proteases from *B. licheniformis* is given in Table 2.

General properties

The effect of pH on the hydrolyzing activity of the enzymes was determened at 37℃ as a function of pH (Fig. 2). Maximum proteolysis occured at pH 7.5 for protease type I and at pH 8.0 for protease type II. At alkaline pH, the type II enzyme retained almost 85% of the maximum activity. This characteristics allowed to consider the type II enzyme as an alkaline protease (Lin et al., 1969). The effect of temprature on proteolytic activity of the enzymes were determined at various temperatures (Fig. 3). For protease type I, maximum hydrolysis was occured between 60℃ and 65℃, and the enzyme was rapidly inactivated above this temperature. The protease type II showed a maximum activity at 60℃. Fig. 4 shows heat inactivation pattern of the enzymes at 71℃ in 50mM sodium phosphate buffer (pH 7.5). The protease type I was rapidly inactivated at this temperature. It had lost all activity within 10 min. However, protease type II was moderately resistant to thermal denaturation; 50% of the ori-

Table 3. *Effects of various chemicals on the protease activities.*

Chemicals	Conc. (mM)	Relative activity	
		Protease I	Protease II
None		100	100
PMSF	2		
30min incubation		12	81
60min incubation		0	62
EDTA	5	45	128
	10	0	57
$CaCl_2$	5	100	65
$MgCl_2$	5	93	96

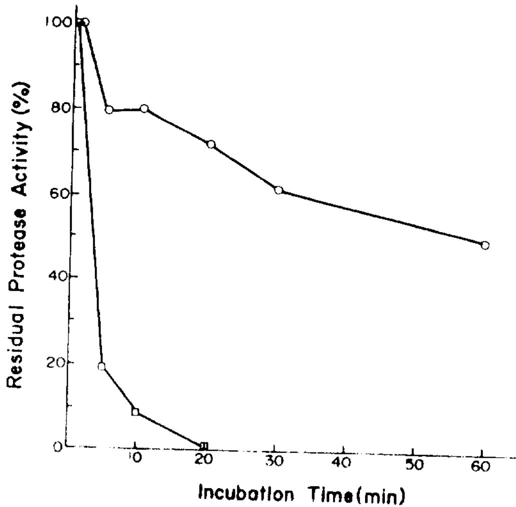

Fig. 3. *Effect of temperature on the proteolytic activities of proteases., protease type* I *& protease type* II.

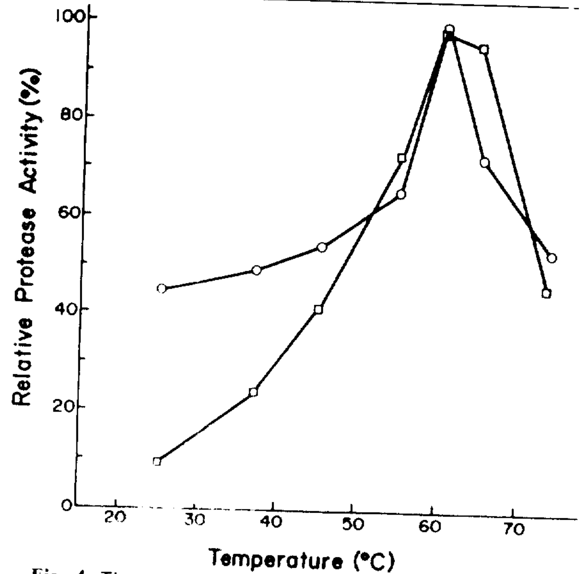

Fig. 4. *Thermal stabilities of protease type* I *and type* II. *The protease were incubated for 30 min at 71℃., type* I *& type* II.

ginal activity remained after incubation for 1h at 71℃.

Table 3 shows the effect of some chemicals on the proteolytic activity of protease type I and II. The proteolytic activity of protease type I was almost completely inhibited after incubation of the enzyme with 2 mM PMSF. While the activity of protease type II was decreased slightly by PMSF after 30 min incubation, but the activity was further reduced to 60% after extended 1 hour incubation. The activity of type I enzyme was almost half-reduced by 5 mM EDTA and was fully inhibited by 10 mM EDTA. The inhibitory effect of EDTA on type I enzyme was not simple as type II. At 5 mM concentration of EDTA, the protease activity was increased by about 30%, but at 10 mM EDTA, concentration, the activity was less than 60% of the reference sample. Effects of metals such as $Ca^{2+}$and Mg^{2+}on the proteases activities were also examined. For protease type I, the activity was not affected by 5 mM Ca^{2+} but Mg^{2+} ions inhibit the enzyme slightly. In the case of protease type II, both Ca^{2+} and Mg^{2+}ions at various concentrations resulted in significant decrease of proteolytic activity.

Main purpose of this study was to establish an experimental procedure for the isolation of cloned extracellular protease such as subtilisin (Rappaport *et al.*, 1965; Delange and Smith, 1968; Brown and Schleich, 1975). Subtilisin Carlsberg, the alkaline serine protease is one of the several extracellular proteases secreted from *B. licheniformis* (McPhalen *et al.*, 1985). The protease type I and type II studied here were somewhat diffetent from subtilisin and other proteases in several points of aspect (Hall *et al.*, 1966; Brown *et al.*, 1977; Karasaki and Ohno, 1979; Aunstrup, 1980). Since proteolytic activity of protease type I was inhibited by both PMSF and EDTA, this enzyme seems to have some properties

similar to those of known serine-metal proteases (Matta and Staley, 1974; Priest, 1977). However, the enzymatic activity was not stabilized by metal ions such as Ca^{2+} and was very sensitive to heat treatment. The Ca^{2+} has been considered to stabilize metal protease activity (Voordouw *et al.*, 1976). Although protease type Ⅱ was relatively stable in heat treatment, it did not show consistent response to protease inhibitors. Therefore, the present results could not clearly point out to which class of the protease type Ⅱ belongs (Priest, 1977).

적 요

*Bacillus licheniformis*에서 분비된 단백질 추출물에서 ammonium sulfate fractionation과 Sephadex G-75겔 여과 크로마토그라피를 사용하여 부분 정제된 2개의 서로 다른 활성을 갖는 단백질 분해 효소를 얻었다. 단백질 분해 효소 type I은 카제인에 대해 비교적 높은 활성을 보이며, organofluoride와 EDTA 에 의한 억제에 매우 민감했다. 이 효소의 최적 pH는 7.5였으며 71℃에서 빠르게 변성되었다. 단백질 분해효소 typeII는 type Ⅰ에 비해 낮은 카제인 분해 활성을 보이며, 1mM PMSF와 10mM EDTA용액과 함께 37℃에서 30분간 반응시키면 역시 활성이 억제되었다. 한편 이 효소는 pH 8.0에서 최대 활성을 나타내고, 71℃ 에서 상대적으로 늦게 불활성화 되었다.

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