CHARACTERIZATION OF A PROTEIN INHIBITOR OF INTRACELLULAR PROTEASE FROM *BACILLUS SUBTILIS*

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1. Introduction

A few years ago an intracellular serylprotease present during sporulation was characterized in *Bacillus subtilis* [1]. The proteolytic activity determined by Azocoll hydrolysis was not detectable in growing cells. We now report results which support the existence of a specific inhibitor of this protease present in the cells both during growth and sporulation.

2. Experimental

2.1. *Materials*

Bovine serum albumine (fraction V) was purchased from Armour, Pronase E from Merck, Azocoll from Calbiochem, ribonuclease B (type III) from Sigma and deoxyribonuclease I from Calbiochem.

2.2. *Strain and growth conditions*

Mutant 512 derived from *B. subtilis* Marburg strain was used in this study. This mutant is devoid of extracellular metalloprotease [2]. The strain was grown in Difco nutrient broth [3] at 30°C in 1 litre Erlenmeyer flasks or in a 15 litre Biolafitte fermentor.

2.3. *Preparation of proteolytic enzymes*

The intracellular serylprotease was prepared as described previously [1]. Bacteria were harvested 3 h after the end of exponential growth. Routinely, the crude enzyme preparation obtained after ammonium sulfate precipitation and dialysis was used.

The extracellular serylprotease was prepared as described previously [4]. Since the 512 strain is devoid of metalloprotease the serylprotease was used after chromatography on DEAE-cellulose.

2.4. *Enzymatic assays*

The extracellular or intracellular serylprotease activity was assayed with Azocoll [5] as substrate: 30 mg of Azocoll in 6 ml Tris–HCl buffer 0.1 M, pH 7.3, containing 2 mM $CaCl_2$. Incubation was carried out at 30°C. One unit of activity was equivalent to the hydrolysis of 1 μg of Azocoll/min at 30°C.

The esterolytic activity was assayed with *Z*-tyrosine *p*-nitrophenylester as substrate [6].

2.5. *Assay of inhibitory activity*

Inhibitory activity was expressed according to Betz *et al.* [7] as the amount of enzyme activity inhibited per millilitre of inhibitor solution:

$$\text{Inhibitor activity} = \frac{x - y}{\text{ml inhibitor solution}}$$

where x = protease activity without inhibitor and y = protease activity with inhibitor. Values of y, between 30% and 60% inhibition were used for calculation. Routinely 30 units of intracellular serylprotease were used for the determination of inhibitory activity. The proteolytic enzyme was incubated with inhibitor solution for 5 min at 30°C and then buffer and Azocoll were added.

2.6. *Protein estimation*

Protein was estimated by the method of Lowry et al. [8] using bovine albumin as a standard.

3. Results

3.1. *Isolation and partial purification of the inhibitor from growing cells*

In order to eliminate contaminating extracellular compounds and enzymes the cells were washed twice, first with a buffer containing 20 mM Tris–HCl, pH 7.3, 2 mM $CaCl_2$ and 1 M KCl, then with the same buffer but free of KCl. Washings and other steps of purification were carried out at 4°C.

Washed cells (10 mg dry weight/ml) were suspended in 0.2 M Tris–HCl buffer, pH 7.3, containing 2 mM $CaCl_2$ (Tris–Ca). Crude extract was prepared by sonication in a Branson sonifier Model B-12 followed by centrifugation at 40 000 × *g* for 30 min.

The supernatant was heated at 95°C in a water bath for 5 min, then cooled in ice. The heavy precipitate was removed by centrifugation. The preparation of crude extract by sonication of the cells can be omitted. Cells suspended in Tris–Ca buffer (10 mg dry wt/ml) were heated in a boiling water bath for 20 min and immediately cooled in ice. The suspension was then centrifuged at 40 000 × *g* for 30 min. The supernatant was treated as described in the following paragraph.

The supernatant was brought to 15% saturation (w/v) with 100% trichloracetic acid and stirred for one hour at 4°C. The suspension was centrifuged at 40 000 × *g* for 30 min. The precipitate was dissolved in Tris–Ca buffer. The pH was adjusted to 7.3 with 1 N KOH.

The resulting solution was applied to a DEAE–Sephadex A 25 column equilibrated with Tris–Ca buffer. The column was eluted with the same buffer. Inhibitory activity was eluted in the first protein peak. As shown in table 1, inhibitor is purified about 20-fold from crude extract with an overall yield of about 68%.

3.2. *Some properties of the protease inhibitor*

Inhibitor solution, 0.2 ml, was incubated with 2 μg of pronase in Tris–Ca buffer or with 2 μg of deoxyribonuclease I in Tris–HCl 0.1 M, pH 7.3, containing 2 mM $MgCl_2$ or with 2 μg of ribonuclease B in Tris–HCl 0.1 M, pH 7.3. After 3 h incubation at 30°C each sample was heated at 95°C for 5 min in order to inactive the enzymes then cooled. The remaining inhibitory activity was assayed. Only the sample incubated with pronase lost virtually all the inhibitory activity (97%). The two others retained the same inhibitory activity as before incubation.

The molecular weight roughly estimated by Biogel P 60 filtration was between 15 000–20 000.

Both proteolytic and esterolytic activity of intracellular serylprotease are sensitive to the inhibition. The inhibitor has no effect on proteolytic and esterolytic activities of extracellular serylprotease even then 10-times higher concentrations of inhibitor are used.

3.3. *Inhibitor in cells during sporulation phase*

An inhibitory activity was detectable in the cells taken 3 h after the end of exponential growth. This activity was 20-times higher than in growing cells.

4. Discussion

Several protein protease inhibitors have been isolated from eukaryotic cells [9]. The inhibitor described here was isolated from a prokaryotic organism. Like the previously described inhibitors, the inhibitor isolated from *B. subtilis* is a protein compound, heat stable and trichloracetic resistant. Two different serylproteases are synthetized by *B. subtilis* after growth [1,2]. One, which is extracellular has a large specificity [10]. The other, which is intracellular has a more restricted specificity and is a chymotrypsin-

Table 1
Partial purification of *Bacillus subtilis* protease inhibitor

Step	Volume (ml)	Proteins (mg)	Specific activity (units/mg protein)
Crude extract	190	608	3.1
Boiled extract	190	200	11.5
TCA precipitation	20	72	44.9
DEAE Sephadex A 25	20	20	65.0

like enzyme [1]. The inhibitor isolated here is active on the intracellular serylprotease but not on the extracellular serylprotease.

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