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Purification and Characterization of an Intracellular Protease from *Pseudomonas carboxydohydrogena*

Lee, Hye Soog[1] and Young Min Kim*
Department of Biology, College of Science, Yonsei University, Seoul 120-749, Korea

*Pseudomonas carboxydohydrogena*에서 분리 정제된 세포내 단백질 가수분해효소의 특성

이혜숙[1] · 김영민*
연세대학교 이과대학 생물학과

ABSTRACT: An intracellular protease from cells of *Pseudomonas carboxydohydrogena* grown on nutrient broth was purified to better than 95% homogeneity in five steps using azocaseine as a substrate. The molecular weight of the native enzyme was determined to be 125,000. Sodium dodecyl sulfate-gel electrophoresis revealed at least two non-identical subunits of molecular weight 70,000 and 56,000. The enzyme activity was completely inhibited by phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate. The enzyme was also inhibited by Mg^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and Fe^{2+}, but was stimulated by iodoacetamide. Maximal reaction rate of the enzyme was observed at pH 8.0 and 30℃. The isoelectric point of the enzyme was found to be 7.5. The enzyme was unable to hydrolyze carbon monoxide dehydrogenase.

KEY WORDS ☐ Intracellular protease, Carboxydobacteria, *Pseudomonas carboxydohydrogena*

INTRODUCTION

It is well known that several bacteria produce intracellular and/or extracellular proteases (Levy and Goldman, 1969; Morihara, 1970). Intracellular proteases are known to be involved in the complex cellular processes such as maturation of enzymes, inactivation of functional proteins, secretion of extracellular proteins, and degradation of abnormal or malfunctional proteins (Bond and Butler, 1987; Goldberg and St. John, 1976; Goldschmidt, 1970; Pine, 1972), but the physiological role and target of many proteases and the mechanism of regulation of proteolysis are not completely known (Bond and Butler, 1987; Holzer and Heinrich, 1980).

Carboxydobacteria are a group of aerobic bacteria which are able to grow on carbon monoxide (CO) as sole carbon and energy sources. Utilization of CO by carboxydobacteria requires CO dehydrogenase (CO-DH) which is inducible by CO in most carboxydobacteria, except in *Acinetobacter* sp. strain JC1 (unpublished result) and *Pseudomonas carboxydoflava* (Kim and Hegeman, 1983; Meyer, 1989), indicating that certain protein turnover mechanisms specific for this group of bacteria may present. In this regards, Lee and Kim (1989) and Kim and Kim (1990) studied intracellular serine-type proteases in *Pseudomonas carboxydovorans* and *Acinetobacter* sp. strain JC1, respectively.

In this study, an intracellular protease of *Pseudomonas carboxydohydrogena*, a carboxydobacterium,, was purified and examined in an effort to assist studies on the mechanism of regulation of protein turnover in this and other carboxydobacteria in the future.

MATERIALS AND METHODS

Bacterial strain and cultivation

P. carboxydohydrogena DSM 1083 was cultivated at 30℃ in nutrient broth or in mineral medium (Kim and Hegeman, 1981) supplemented with 0.2 % sodium succinate, sodium pyruvate, sodium acetate or a gas mixture of 30% CO and 70% air. Growth was measured at 580 nm. Cells were

*Corresponding author.
[1] Present address Molecular Biology Laboratory. Genetic Engineering Research Institute, Korea Institute of Science and Technology, Taejeon, Korea.

harvested during the late exponential growth phase and washed twice in 0.05 M Tris hydrochloride buffer (pH 7.5; standard buffer) and stored at 20℃.

Enzyme assay

Protease activity was assayed by a modified method of Jensen *et al.* (1980) using azocasein as a sustrate as described previously (Lee and Kim, 1989), except that azocasein and $CaCl_2$ solutions were prepared in the standard buffer. One unit of the protease activity was defined as the amount of enzyme needed to increase an absorbance of 0.1 at 370 nm in 1 min under the assay conditions.

CO-DH activity was assayed by measuring the CO-dependent decrease of absorbance of thionin dye at 595 nm as decribed by Kim and Hegeman (1981).

Protein determination

Proteins were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Proteins in crude extracts were estimated by the same method after the sample were boiled in 20% NaOH solution for 10 min (Meyer and Schlegel, 1978).

Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) of the native enzyme in gels containing 7.5% acrylamide and denaturing PAGE of the purified enzyme in gels containing 12.5% acrylamide in the presence of sodium dodecyl sulfate (SDS) were performed by the method of Laemmli (1970) with several modifications as described previously (Kim *et al.*, 1989). Proteins were stained with Coomassie brilliant blue R-250 (CBB) by a modified method (Kim and Hegeman, 1981) of Weber and Osborn (1969).

Protease purification

All purification steps were carried out at 4℃. A 53-g portion of washed cells grown on nutrient broth was suspended in 104 m*l* of standard buffer and disrupted by ultrasonic treatment (10 s/m*l*). The suspension was centrifuged at 10,000×g for 30 min. The supernatant fluid (crude extract) was treated with protamine sulfate to a final concentration of 0.054% (w/v). The fluid was left in ice for 10 min, and then centrifuged at 100,000×g for 90 min. The resulting supernatant (soluble fraction) was next made 80% saturated with respect to ammonium sulfate, left for 2 h, and then centrifuged at 15,000×g for 30 min. Pellets were dissolved in a small volume of standard buffer and dialyzed against three 3-*l* changes of the same buffer of 18 h. The dialysate was then applied to a DEAE-Sephacel column (3.1×8.4 cm) equilibrated with standard buffer. Elution was carried out with 500 m*l* of a linear 0 to 0.4 M NaCl gradient in standard buffer. Fractions were collected at a flow rate of 1.5 m*l*/cm^2 per *h*, and fractions with high specific activity were pooled and made 80% saturated with ammonium sulfate. After 2 h, the solution was centrifuged at 15,000×g for 30 min, and the precipitates were resuspended in a small volume of standard buffer. The suspension was dialyzed against three 2-*l* changes of the same buffer, and then applied to an Ultrogel AcA44 column (1.3×60 cm) equilibrated with standard buffer. Elution was done with standard buffer at a flow rate of 5.4 m*l*/cm^2 per h with 40 cm hydrostatic pressure. Fractions containing peak protease activity (purified protease) were pooled and stored at −20 ℃ under air.

RESULTS

Cultivation conditions and protease activity

It was found that *P. carboxydohydrogena* grown on nutrient broth contained intracellular protease, but no extracellular protease, which is active on azocasein. The protease was most active in cells growing at the late exponential phase. The activity was not detected in cells grown on succinate, pyruvate, acetate or CO.

Purification

The intracellular protease was purified 16-fold in five steps with a yield of 1.8% and a specific activity of 1.6 units per mg of protein (Table 1).

The purified enzyme was revelaled as a single band on nondenaturing polyacrylamide gel (Fig.

Table 1. *Purification of protease from P. carboxydohydrogena*

Purification step	Total protein[a] (mg)	Sp. act.[b]	Purification fold	Total activity[c]	Recovery (%)
Crude extract	1407.6	0.1	1.0	140.8	100
Soluble fraction	887.4	0.13	1.3	115.4	81.9
Ammonium sulfate (0-80%)	642.2	0.2	2.0	107.0	76.0
DEAE-Sephacel	16.8	1.1	11.0	18.0	12.8
Ultrogel AcA44	1.1	1.6	16.0	1.8	1.3

[a] Lowry *et al.* (1951).
[b] Units per mg of protein. One unit of the enzyme activity was defined as the amount of enzyme needed to increase an absorbance of 0.1 at 370 nm in 1 min.
[c] Units.

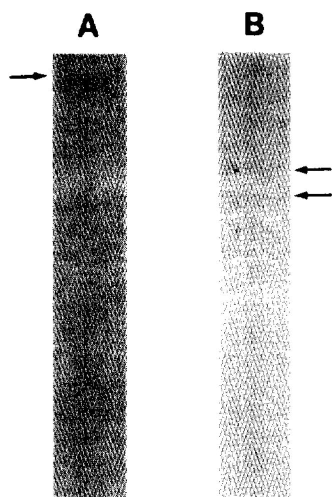

Fig. 1. *(A) Nondenaturing PAGE of the purified protease. The purified enzyme was subjected to PAGE on 7.5% acrylamide gel by the method of Laemmli (1970) without SDS and stained with CBB as described in the text. (B) Dissimilar subunits in the purified proetase. Denaturing PAGE (12.5% acrylamide, 0.1% SDS) of the purified enzyme was carried out according to Laemmli (1970) and stained with CBB. Arrows indicate the native enzyme (A, 1.0 μg) and subunits (B, 2.0 μg).*

1A). The enzyme was judged better than 95% homogeneous after analysis using densitometer of the gels stained with CBB.

Molecular properties

The molecular weight of the native enzyme was determined to be 125,000 after Sephadex G-100 column (2.5×75 cm) chromatogrphy of the enzyme according to Andrews (1964) with several reference proteins of known molecular weight. Denaturing PAGE revealed the presence of two nonidentical subunits in the native enzyme (Fig. 1 B). The molecular weight of the subunits were estimated to be 70,000 and 56,000 by using SDS-PAGE with several molecular weight references.

Isoelectric point

The isoelectric point of the native enzyme was determined to be 7.5 by isoelectric focusing in PhastGel IEF 3-9 (Pharmacia) using a Phastsystem (Pharmacia) with reference proteins of known isoelectric points.

Effect of various chemicals

The effects of protease inhibitor, group specific reagent, divalent cation, and chelating agents on the purified protease were tested. The protease activity was inhibited completely by diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), but was stimulate by iodoacetamide (Table 2). The enzyme activity was very sensitive to Hg^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}. Mn^{2+} was found to be slightly inhibitory to the purified enzyme (Table 3). The enzyme was not affected by Ba^{2+}, Co^{2+}, and Mg^{2+}. Among those four metal-chelating agents tested, EDTA and EGTA showed some inhibitory effect, but KCN and NaN_3 did not (Table 3).

Table 2. *Effect of protein modification reagent and inhibitor on the protease activity*[a]

Addition	Conc. (mM)	Relative activity (%)[b]
None		100
DFP[c]	10	0
PMSF[d]	10	0
Iodoacetamide	10	136

[a] Protease activity was measured after 10 min of incubation of the enzyme at room temperature with the chemicals as described in text.
[b] Activity in the absence of chemicals was taken as 100%.
[c] Diisopropyl fluorophosphate.
[d] Phenylmethylsulfonyl fluoride.

Table 3. *Effect of divalent cations and metal chelating agents on the protease activity*[a]

Addition	Conc. (mM)	Relative activity (%)[b]
None		100
Cations[c]		
Ba^{2+}	10	104
Ca^{2+}	10	95
Cd^{2+}	10	9
Co^{2+}	10	109
Cu^{2+}	10	12
Fe^{2+}	10	38
Hg^{2+}	10	0
Mg^{2+}	10	114
Mn^{2+}	10	68
Zn^{2+}	10	0
Chelating agents		
NaN_3	10	103
KCN	10	109
EDTA	10	71
EGTA	10	45

[a] Protease activity was assayed after 10 min of incubation of the purified protease with metal ions or chelating agents at room temperature.
[b] Activity in the absence of additions was taken as 100%.
[c] Metal ions were added as chloride salt except for Fe^{2+} and Cu^{2+} which were added as sulfate salt.

Effect of pH and temperature

The purified enzyme showed high activity at pHs between 7.0 and 10.0. It was most active at

pH 8.0. When the enzyme was incubated for 1 h at 37℃, it was most stable at pHs 8.0 and 9.0; the enzyme retained almost over 80% of the initial activity at these pHs. The enzyme, however, lost its activity completely in 10 min at pH 5.0.

The optimal temperature for enzyme reaction was found to be 30℃. The enzyme exhibited over 50% of the original activity when it was stored for 1 h at 30℃. The enzyme was inactivated completely in 10 min at 50℃.

DISCUSSION

P. carboxydohydrogena is a carboxydobacterium which uses a CO-inducible CO-DH for the utilization of CO as the sole source of carbon and energy (Kim and Hegeman, 1981 and 1983). We found in this experiment that *P. carboxydohydrogena* grown on nutrient broth, not on minimal media, produces an azocasein-hydrolyzing protease, which is the same as those of *P. carboxydovorans* (Lee and Kim, 1989) and *Acinetobacter* sp. strain JC1 (Kim and Kim, 1990). It was also found that *P. carboxydohydrogena*, like the two other carboxydobacteria, does not produce extracellular protease active on azocasein under any cultivation conditions tested in the present study.

Meyer and Schlegel (1983) reported that *P. carboxydehydrogena* aggregate to form rosettes actively from the beginning of late exponential growth phase. The coincidence of growth phase for maximal protease activity and active rosette formaiton suggests that intracellular protease may be involved in the rosette formation in *P. carboxydohydrogena* during growth on nutrient broth.

P. carboxydovorans was reported to produce an intracellular protease of molecular weight 53,000 (Lee and Kim, 1989), while *Acinetobacter* sp. strain JC1 produce two different kinds of proteases of molecular weights 55,000 and 44,000 (Kim and Kim, 1990). Those proteases were found to consist of single polypeptides. The enzyme from *P. carboxydohydrogena*, on the other hand, is larger than those found in the two carboxydobacteria and consists of two nonidentical polypeptides, indicating that proteases in carboxydobacteria are specific for each bacteria even though they share the uncommon physiological property, the aerobic CO oxidation.

The purified protease, like those from *P. carboxydovorans* (Lee and Kim, 1989) and *Acinetobacter* sp. strain JC1 (Kim and Kim, 1990), seems to be a kind of serine-type protease since it was inhibited completely by the well-known serine protease inhibitors, PMSF and DFP (Barret, 1977; Bond and Butler, 1987; Kurotsu *et al.*, 1982; Morihara, 1974).

Activation of the purified enzyme by the sulfhydryl group blocking reagent, iodoacetamide, suggests that free sulfhydryl residues are unlikely to participate in the enzymatic catalysis, which was also suggested for the CO-DH (Kim *et al.*, 1989) and a protease (Kim and Kim, 1990) from *Acinetobacter* sp. strain JC1 and a protease found in *P. carboxydovorans* (Lee and Kim, 1989). It may be possible to assume that iodoacetamide increase the enzyme activity through binding to the free sulfhydryl groups in the protease, preventing the nearby sulfhydryl groups from interacting each other. In the absence of iodoacetamide, the free sulfhydryl groups close to each other may interact to effect changes in the enzyme structure, which results in the decrease of the enzyme activity.

It is well known that several bacterial proteases are sensitive to inhibition by divalent cations and chelating agents (Jensen *et al.*, 1980; Kim and Kim, 1990; Kreger and Gray, 1978; Lee and Kim, 1989; Wretlind and Wadstrom, 1977). The purified protease was also inhibited by several divalent cations such as Hg^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and Fe^{2+}, which is similar to those from *P. carboxydovorans* (Lee and Kim, 1989) and *Acinetobacter* sp. strain JC1 (Kim and Kim, 1990). Partial inhibition of the enzyme activity by 10 mM EDTA and EGTA suggests that metal ions are not required for the enzyme activity. It seems that metal ions such as Mg^{2+}, Co^{2+}, Ba^{2+}, and Ca^{2+} are involved in the stabilization of the purified enzyme.

The isoelectric point together with the optimal pH for the stability and reaction of the purified enzyme indicates that the enzyme is an alkaline protease. The enzyme, however, may be a weakly alkaline serine protease like those from other carboxydobacteria (Kim and Kim, 1990; Lee and Kim, 1989) since several serine-type alkaline proteases are most active at pHs between 10 and 12 (Morihara, 1974; Sastry *et al.*, 1983; Vitkovic and Sadoff, 1977).

The purified protease was found to be inactive on CO-DH (data not shown). This together with the observation that the proteases purified from cells of *P. carboxydovorans* and *Acinetobacter* sp. strain JC1 grown on nutrient broth did not hydrolyze CO-DH (Kim and Kim, 1990; Lee and Kim, 1989) implies that the intracellular proteases produced in carboxydobacteria growing at the late exponential phase on nutrient broth are not involved in the turnover of CO-DH.

적 요

Nutrient broth에서 성장한 *Pseudomonas carboxydohydrogena*로부터 azocasein을 기질로 사용하고 다섯단계의 순화과정을 거쳐 95% 이상의 순도를 가진 세포내 단백질 가수분해 효소를 분리, 정제하였다. 정제된 효소의 분자량은 125,000

이었고, 70,000과 56,000의 분자량을 가지는 두개의 서로 다른 소단위로 구성되어 있었다. 이 효소는 phenylmethylsulfonyl fluoride와 diisopropyl fluorophosphate에 의해 활성이 완전히 억제되었다. 이 효소는 또한 Hg^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}에 의해서도 활성이 억제되었으나, iodoacetamide에 의해서는 활성이 증가 되었다. 정제된 효소의 pI는 7.5로 추정되었고, pH 8.0과 30℃에서 가장 높은 활성을 나타내었다. 정제된 효소는 일산화탄소 산화효소를 가수분해하지 못하였다.

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