Purification and Some Properties of an Intracellular Protease from *Pseudomonas carboxydovorans*

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Pseudomonas carboxydovorans 의 세포내 단백질 가수분해 효소의 정제 및 특징

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ABSTRACT : A soluble intracellular protease from cells of *Pseudomonas carboxydovorans*, a carboxydobacterium, grown on nutrient broth was purified 68-fold in five steps to better than 95% homogeneity with a yield of 2.4% using azocasein as a substrate. The enzyme activity was not detected from cells grown on pyruvate, succinate, acetate, or CO as a sole source of carbon and energy. The molecular weight of the native enzyme was determined to be 53,000. Sodium dodecyl sulfate-gel electrophoresis revealed the purified enzyme a monomer. The enzyme was found to be a serine-type protease. The enzyme activity was inhibited completely by several divalent cations such as Cd^{2+}, Cu^{2+}, Hg^{2+}, and Fe^{2+}. The enzyme was also inhibited by EGTA, but was stimulated by iodoacetamide. The optimal pH and temperature for the enzyme reaction were found to be 8.0 and 50℃, respectively. The enzyme was inactive on CO dehydrogenase.

KEY WORDS ☐ **Carboxydobacteria - *Pseudomonas carboxydovorans* - Intracellular protease**

It has been reported that several microorganisms produce intracellular or extracellular proteases (Jensen *et al.*, 1980 ; Levy and Goldman, 1969 ; Morihara, 1974; Vitkovic and Sadoff, 1977). Intracellular proteases are known to be involved in the degradation of abnormal proteins, in the maturation of enzymes, in the secretion of proteins, and in the inactivation of functional proteins (Goldberg and St. John, 1976 ; Mella *et al.*, 1988 ; Pine, 1972 ; Voellmy and Goldberg, 1980).

Pseudomonas carboxydovorans is a carboxydobacterium which is able to grow aerobically with carbon monoxide (CO) as a sole source of carbon and energy (Kim and Hegeman, 1983 ; Meyer *et al.*, 1986 ; Meyer and Schlegel, 1983). The use of CO as a growth substrate by this bacterium is dependent on the presence of CO dehydrogenase (CO-DH) which is inducible by CO ; the enzyme is not present in heterotrophically grown cells (Kim and Hegeman, 1983 ; Meyer *et al.*, 1986 ; Meyer and Schlegel. 1983), indicating that there may be a specific turnover mechanism for CO-DH in this bacterium. Furthermore, it has been reported that the small plasmid (pYK100) of this bacterium contains gene(s) encoding protease which is responsible for the modification of the βsubunit of CO-DH (Kwon *et al.*, 1986 ; Kwon and Kim, 1985 ; Kim and Song, 1989).

In this study, we have examined a purified intracellular protease of *P. carboxydovorans* in some detail in an effort to assist studies on the regulation and specificity of protein turnover in this bacterium in the future.

MATERIALS AND METHODS

Bacterial strain and cultivation

Pseudomonas carboxydovorans DSM 1083 was a gift from Dr. O. Meyer, Universität Bayreuth,

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Bayreuth, FRG. The cell was cultivated at 30℃ in nutrient broth or in standard mineral medium (Kim and Hegeman, 1981) supplemented with 0.2% (w/v) sodium pyruvate, sodium succinate, sodium acetate, or a gas mixture of 30% CO-70% air. CO (99.5%, v/v) was purchased from Ulsan gas Co., Ulsan, Korea.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Proteins in crude cell extracts were determined by the same method after the extracts were boiled in 20% NaOH for 10 min (Meyer and Schlegel, 1978).

Enzyme assay

Protease activity was measured by a modified method of Jensen *et al.* (1980) using azocasein as a substrate. The reaction mixture contained 1.5 ml of azocasein solution (0.5% solution in distilled water adjusted to pH 7.5 and filtered through Whatman No.1 filter paper), 1.5 ml of 0.02 M Tris hydrochloride buffer (pH 7.5) containing 0.002 M $CaCl_2$ and 100 μl of enzyme solution. The reaction was stopped after 15 min of incubation at 37℃ by the addition of 3.0 ml of 10% trichloroacetic acid. After standing for 30 min at 21℃, the precipitate was removed by filtration and the absorbance at 370 nm of the filtrate was measured. One unit of the activity was defined as the amount of enzyme needed to increase an absorbance at 370 nm of 0.1 in 1min under the assay conditions.

CO-DH activity was assayed photometrically at 30℃ by measuring the CO-dependent reduction of thionin dye in 0.05 M Tris hydrochloride buffer (pH 7.5) at 595 nm using anaerobic cuvette as described previously (Kim and Hegeman, 1981).

Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) of the native enzyme was performed in gels containing 7.5% acrylamide by the method of Laemmli (1970), but without sodium dodecyl sulafte (SDS), as described previously (Kim and Hegeman, 1981). SDS-PAGE of the purified enzyme in 12.5% acrylamide gel was conducted by the procedure 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 modification (Kim and Hegeman, 1981) of the method of Weber and Osborn (1969).

Enzyme purification

All purification steps were carried out at 4℃. Cells grown on nutrient broth were harvested in early stationary growth phase, washed twice in 0.01M Tris hydrochloride buffer (pH 7.5), and stored at -20℃. A 54-g portion of thawed cells was suspended in 210 ml of 0.01 M Tris hydrochloride buffer (pH 7.5) and disrupted by sonic treatment (10 s/ml) in portions of 30 ml. The suspension was centrifuged at 10,000 x *g* for 30 min. The resulting supernatant (crude extract) was then treated with protamine sulfate to a final concentration of 0.054%, left in ice for 10 min, and then centrifuged at 100,000 x *g* for 90 min. The supernatant fluid (soluble fraction) was next made 35% saturated with respect to ammonium sulfate. After 2 h, this fraction was centrifuged at 15,000 x *g* for 30 min. The resulting supernatant was further treated with ammonium sulfate to achieve a final concentration of 55% of saturation. After 2 h, the solution was centrifuged again at 15,000 x *g* for 30 min, and the precipitate was suspended in a small volume of cold 0.01M Tris hydrochloride buffer (pH 7.5). The suspension was then dialyzed against three 2-liter changes of the same buffer for 5 h. The dialyste was then applied to a DEAE-Sephacel column (4.8 by 4.0 cm). The column was prewashed with at least 3 total bed volumes of 0.01M Tris hydrochloride buffer (pH 7.5) before application of the sample. Elution was carried out with 500 ml of a linear 0.1 to 0.4 M NaCl gradient in the same buffer. Fractions were collected at a flow rate of 0.7 ml/cm^2 per h, and the fractions with the highest specific activity were pooled and treated with ammonium sulfate to achieve a final concentration of 60%. After 2 h, the solution was centrifuged at 15,000 x *g* for 30 min, and the precipitate was resuspended in a small volume of 0.01M Tris hydrochloride buffer (pH 7.5). The suspension was dialyzed extensively against the same buffer, and then applied to a Sephadex G-75 column (2.5 by 60 cm). Elution was performed with 0.01M Tris hydrochloride buffer (pH 7.5) at a flow rate of 2.6 ml/cm^2 per h with 70 cm hydrostatic pressure. Fractions with the highest specific activity (purified protease preparation) were pooled and stored at -20℃ under air.

RESULTS

Protease activity

It was found that cells of *P. carboxydovorans* grown on minimal medium supplemented with succinate, pyruvate, acetate, or CO do not contain azocasein-hydrolyzing intracellular protease activity. The activity was present only in cells grown on nutrient broth. It was also found that *P. carboxydovorans* does not produce azocasein-dependent extracellualr protease under any growth conditions tested in this study (data not shown).

Table 1. *Purification of intracellular protease from P. carboxydovorans*

Purification step	Total protein[a] (mg)	Sp. act.[b]	Purification fold	Total activity[c]	Recovery (%)
Crude extract	5565	0.1	1.0	556.5	100
Soluble fraction	2876	0.15	1.5	431.4	77.5
Ammonium sulfate (35~55%)	719	0.3	3.0	215.7	38.8
DEAE-Sephacel	147	0.4	4.0	58.8	10.6
Sephadex G-75	2	6.8	68.0	13.6	2.4

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

Purification

A soluble intracellular protease was purified 68-fold in five steps, with a yield of 2.4% and a specific activity of about 6.8 units per mg of protein (Table 1).

Nondenaturing PAGE of the purified enzyme revealed only a single band, indicating the purity of the enzyme preparation (Fig 1). The enzyme was judged better than 95% homogeneous after densitometric analysis of the gel stained with CBB.

Molecular weight and structure

The molecular weight of the native enzyme was estimated to be 53,000 by using Sephadex G-100 column (2.5 by 55 cm) chromatography according to the method of Andrews (1964) with reference proteins of known molecular weight. SDS-PAGE gave corresponding molecular weight of 55,000, indicating that the purified enzyme is a monomer (Fig. 2).

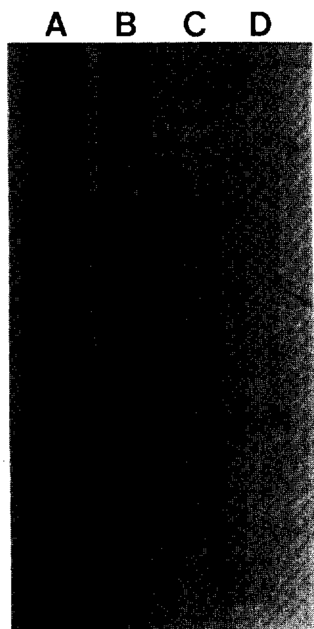

Fig. 1. *Protease after nondenaturing PAGE during purification. Gels containing 7.5% acrylamide were run according to Laemmli (1970) without SDS and stained with CBB. Crude extracts (90μg) (lane A), soluble fraction (90μg) (lane B), DEAE-Sephacel fraction (18μg) (lane C) and Sephadex G-75 fraction (1.0μg) (lane D).*

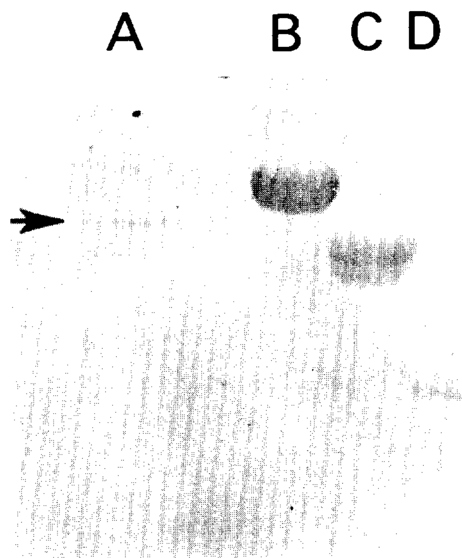

Fig. 2. *Mobility of protease during SDS-PAGE. Denaturing PAGE (12.5% acrylamide, 0.1% SDS) with several references of known molecular weights was carried out to determine the subunit composition of the purified protease as described in methods. Purified protease (0.5μg) (lane A), bovine serum albumin (68 kd, 9μg) (lane B), ovalbumin (45 kd, 6μg) (lane C), and chymotrypsinogen A (25.7 kd, 1.5μg) (lane D).*

Table 2. *Effect of protein modification reagents on the intracellular protease activity*[a]

Reagents	Concentration (mM)	Relative activity(%)
None		100
PMSF[b]	10	0
DFP[c]	10	0
Iodoacetamide	10	153

[a]Protease activity was measured after 10 min of incubation of enzyme at 37℃ with protein modification reagent as described in methods.
[b]Phenylmethylsulfonyl fluoride.
[c]Diisopropyl fluorophosphate.

Table 4. *Effect of metal chelating agents on the protease activity*[a]

Chelating agents	Concentration (mM)	Relative activity(%)
None		100
EDTA	10	71
EGTA	10	0
NaN_3	10	113
KCN	10	99

[a]Protease activity was measured after 10 min of incubation of the enzyme at room temperature with several chelating agents as described in methods.

Effect of protein modification reagent

Several protein modification reagents were tested for their effects on the purified protease activity. The protease was found to be stimulated by iodoacetamide, but was inhibited completely by both phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) (Table 2).

Effects of divalent cations and chelating agents

The effect of various divalent cations on the purified protease activity is shown in Table 3. It is clear that the protease is very sensitive to Cd^{2+}, Cu^{2+}, Hg^{2+}, and Fe^{2+}. The enzyme, however, was not affected by Co^{2+}, Ca^{2+}, Ba^{2+}, and Mg^{2+}. Mn^{2+} and Zn^{2+} were found to be slightly inhibitory to the purified enzyme. Among the four chelating agents tested, EGTA inhibited the enzyme completely (Table 4).EDTA showed some inhibitory effect,but NaN_3 and KCN did not.

pH dependence and stability

The protease was most active at pH 8.0 in 0.01M Tris hydrochloride buffer (Fig. 3). The enzyme was found to be more active in alkaline condition than in acidic one. When stored at 37℃ for 1h, the enzyme was most stable at pHs between 7.0 and 9.0 (Fig. 4).

Temperature dependence and stability

Maximum protease activity was found at 50℃

Table 3. *Effect of divalent cations on the intracellular protease activity*[a]

Cations	Concentration (mM)	Relative activity(%)
None		100
Co^{2+}	10	95
Ca^{2+}	10	102
Cd^{2+}	10	9
Cu^{2+}	10	5
Ba^{2+}	10	102
Mg^{2+}	10	104
Mn^{2+}	10	88
Hg^{2+}	10	0
Fe^{2+}	10	0
Zn^{2+}	10	77

[a]Protease activity was measured after 10 min of incubation of the enzyme at room temperature with several cations as described in methods.

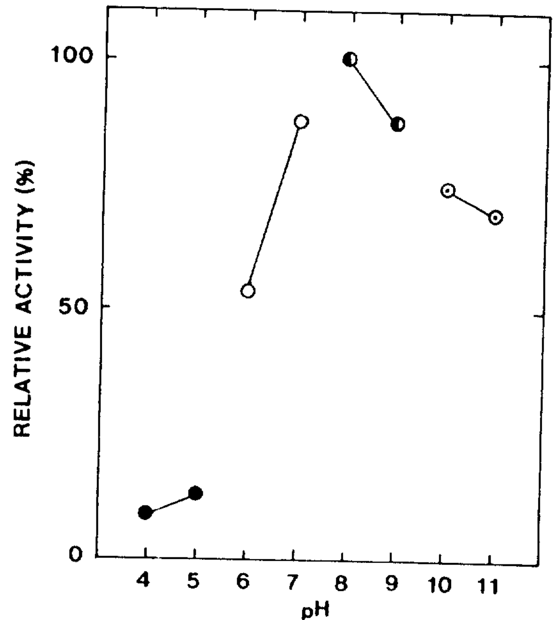

Fig. 3. *Effect of pH on the protease activity. The optimal pH for the enzyme activity was determined by the standard assay method using various pH buffers (0.01M) of acetate (pH 4 and 5), phosphate (pH 6 and 7), Tris hydrochloride (pH 8 and 9), and carbonate-bicarbonate (pH 10.3 and 11). Symbols : acetate buffer (-●-), phosphate buffer (-○-), Tris hydrochloride buffer (-◐-), and carbonate- bicarbonate buffer (-⊙-).*

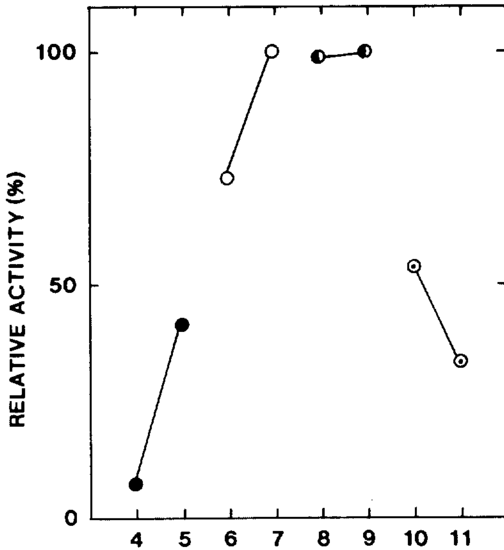

Fig. 4. *pH stability of the purified protease. A 100-μl enzyme solution which was adjusted to a given pH was incubated at 37℃ for 1 h. At the end of incubation, the residual enzyme activity was determined by the standard assay method. Symbols : 0.01M acetate buffer (●), phosphate buffer (○), Tris hydrochloride buffer(◐), and carbonate-bicarbonate buffer (⊙).*

(Fig. 5). This temperature, however, was not used for routine enzyme assay since almost 30% of the

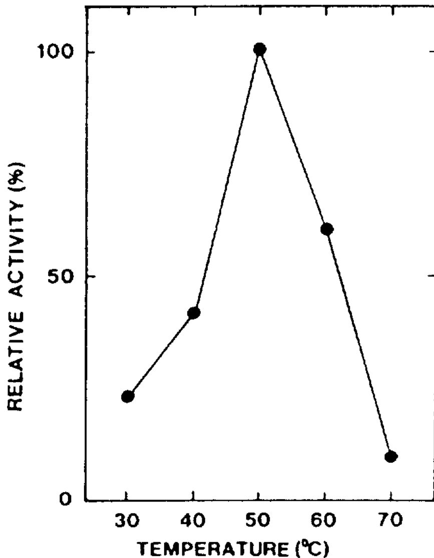

Fig. 5. *Effect of temperature on the protease activity. Temperature dependence of the enzyme activity was tested at various temperatures using the standard enzyme assay as described in methods.*

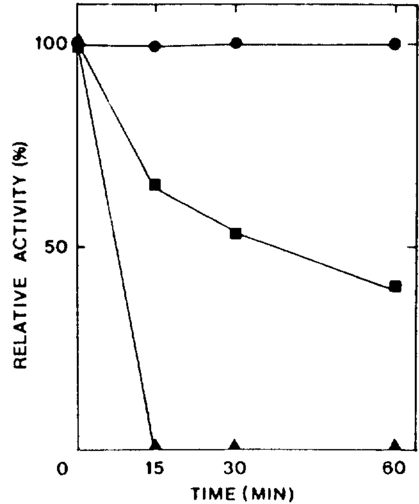

Fig. 6. *Thermal stability of the purified protease. Stability of the proteolytic activity of the purified enzyme at various temperatures was tested using the standard enzyme assay as described in methods. Symbols : 37℃ (●), 50℃ (■), and 60℃(▲).*

initial activity was inactivated in 15 min at this temperature (Fig. 6). The enzyme was very stable at 37℃ and this temperature was used for the enzyme assay, although the hydrolysis of azocasein at 37℃ proceeded three times slower than that at 50℃.

DISCUSSION

Carboxydobacteria are a group of aerobic bacteria which are able to grow on CO as sole energy and carbon sources (Kim and Hegeman, 1983 ; Meyer *et al.*, 1986 ; Meyer and Schlegel, 1983). *P. carboxydovorans* is one of several carboxydobacteria which have been studied extensively on the mechanism of CO oxidation at the level of physiology and biochemistry (Bray *et al.*, 1983 ; Kim and Kim 1984 ; Kim and Lee, 1986 ; Krüger and Meyer, 1986 ; Meyer, 1982 ; Meyer and Rajagopalan, 1984a and b : Meyer and Schlegel, 1979 and 1980, Rohde *et al.*, 1986 ; Meyer and Schlegel, 1979 and 1983) and that there may be a specific protease for the modification of a subunit of CO-DH in this bacterium (Kwon *et al.*, 1986 ; Kwon and Kim, 1985), suggesting the presence of specific protein turnover mechanism in *P. carboxydovorans*.

We found in this study that *P. carboxydovorans* does not produce extracellular protease which is active on azocasein. The bacterium was also found not to produce azocoll-dependent extracellular and

intracellular proteases (data not shown). The presence of protease activity only in nutrient broth-grown cells indicates that the protease is not the one involved in the modification of CO-DH since the CO-DH-modifying enzyme was found to present in cells grown either on minimal medium or on nutrient broth (Kwon *et al.*, 1986; Kwon and Kim, 1985).

It has been reported that the molecular weights of intracellular proteases fall between 20,000 and 800,000 (Bond and Butler, 1987). The molecular weight of purified protease from *P. carboxydovorans* was around 53,000. The enzyme was found to consist of a polypeptide.

It is well known that PMSF and DFP specifically inhibit serine-type proteases (Bond and Butler, 1987; Bromke and Hammel, 1978; Koide *et al.*, 1986; Kurotsu *et al.*, 1982). The purified protease was found to be a kind of serine-type protease since the enzyme was inhibited completely by both DFP and PMSF.

The protease was inhibited by several cations such as Cd^{2+}, Cu^{2+}, Hg^{2+}, and Fe^{2+}. It was also inhibited completely b, EGTA, but partially by EDTA. Inhibition of pseudomonal protease activity by cations and chelating agents also has been observed by several other workers (Jensen *et al.*, 1980; Kessler *et al.*, 1977; Kreger and Gray, 1978; Morihara *et al.*, 1965; Wretlind and Wadstrom, 1977). Complete inhibition of the purified enzyme by EGTA implies that the enzyve requires Ca^{2+} for normal reaction. Addition of Ca^{2+} to the enzyme assay mixture, however, did not stimulate the enzyme activity probably because the Ca^{2+} (1mM) present in the assay mixture was sufficient for the proper action of the purified enzyme.

It was found that the purified protease is an alkaline enzyme since it was more active and stable in alkaline pH than in acidic one; the enzyme activity was maximal at pH 8.0 and was most stable at pHs between 7 and 9. Considering that the activities of several serine-type alkaline proteases are optimal at pHs between 10 and 12 (Sastry *et al.*, 1983; Vitkovic and Sadoff, 1977), the purified protease may be a weakly alkaline enzyme. The enzyme was also found to be not stable to heat treatment.

Together with the result that the purified enzyme does not react on CO-DH (data not shown) which disappears upon transfer of cells growing with CO to the heterotrophic growth condition, the present results indicate that there are at least three kinds of proteases in *P. carboxydovorans*. The results also indicate that the protease studied in this work is a weakly alkaline serine-type protease which requires Ca^{2+} for normal activity. Identification of physiological target of the purified enzyme is undergoing to learn about the protein turnover mechanism in *P. carboxydovorans*.

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적 요

Azocasein을 기질로 사용하여 nutrient broth에서 성장한 *Pseudomonas carboxydovorans*로 부터 다섯 단계의 순화 과정을 거쳐 68배 순화된 세포내 가용성 단백질 가수 분해 효소를 얻었다. Pyruvate나 succinate, acetate, 또는 일산화탄소를 이용하여 성장한 세균들은 이 효소의 활성을 나타내지 않았다. 순화된 효소의 크기는 약 53,000이었고, 한개의 polypeptide로 구성되어 있었다. 이 효소는 serine계통의 단백질 가수분해 효소로 Cd^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}등의 2가 양이온과 EGTA에 의해 활성이 완전히 억제되었고, iodoacetamide에 의해 활성이 증가 되었다. 이 효소는 pH 8.0과 50℃에서 최대 활성을 나타내었으며, 일산화탄소 산화효소를 가수분해 시키지 못하였다.

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