KOR. JOUR. MICROBIOL August, 1992 p. 299~304 Vol. 30, No. 4

Purification and Characterization of an Intracellular Protease from *Pseudomonas carboxydovorans* DSM 1227 Grown on Carbon Monoxide

Bae, Ki Ho and Young Min Kim*

Department of Biology, College of Science, Yonsei University, Seoul 120-749, Korea

An intracellular protease from cells of *Pseudomonas carboxydovorans* DSM 1227 grown on carbon monoxide was purified 57-fold in six steps to homogeneity with a yield of 4.3% using azocoll as a substrate. The molecular weight of the enzyme was determined to be 150,000. Sodium dodecyl sulfate-gel electrophoresis revealed the purified enzyme to be a dimer with two identical subunits of molecular weight 72,000. The enzyme was stimulated by Mg^{2+}, but was inhibited completely by Cd^{2+}, Fe^{2+}, Hg^{2+}, and Zn^{2+}. The enzyme activity was also inhibited by EDTA, EGTA, phenylmethylsulfonyl fluoride, and phenyl glyoxal, but was increased by 1-ethyl-3(dimethyl aminopropyl) carbodiimide, iodoacetamide and dithiothreitol. The optimal pH and temperature for the enzyme reaction were found to be 7~8 and 50°C, respectively. Casein and bovine serum albumin were hydrolyzed by the enzyme, but carbon monoxide dehydrogenase was not.

KEY WORDS □ **Carboxydobacteria, Carbon monoxide, Intracellular protease, *Pseudomonas carboxydovorans***

It has been known that several microorganisms produce intracellular and/or extracellular proteases (5, 8, 19, 26). Intracellular proteases are not only responsible for the inactivation of functional proteins and the degradation of abnormal proteins but also involved in the maturation of enzymes, the formation and germination of spores, and the secretion of proteins (4, 15, 21, 28, 29, 31), but little is known about the role and target of many proteases *in vivo* and the mechanism of regulation of proteolytic processes (2, 6, 27).

Pseudomonas carboxydovorans, a carboxydobacterium, is able to grow aerobically with carbon monoxide (CO) as a sole source of carbon and energy (13, 22). Utilization of CO by this bacterium is dependent on the presence of CO dehydrogenase (CO-DH) which is inducible by CO (23, 24). It has recently been reported that the bacterium grown on nutrient broth produces an intracellular and two extracellular proteases (9, 18). One of the two extracellular proteases was found to hydrolyze and inactivate the CO-DH.

We have examined in this study an intracellular protease which was purified from cells of *P. carboxydovorans* grown on CO to assist comparative studies on the mechanism of protein turnover in this bacterium grown under different nutritional conditions in the future.

*Corresponding author.

MATERIALS AND METHOD

Bacterial strain and growth condition

P. carboxydovorans DSM 1227 was grown at 30°C in mineral medium (12) supplemented with a gas a gas mixture of 30% CO and 70% air.

Enzyme assay

Protease activity was measured by a modified method of Kurotsu *et al.* (15) using azocoll as a substrate. The reaction mixture contained 5 mg of azocoll, 0.9 ml of 0.05 M Tris-hydrochloride buffer (pH 8.0, standard buffer), and 100 μl of enzyme solution. After incubation for 2 h at 37°C, the mixture was rapidly cooled to 0°C and centrifuged. The absorbance of supernatant fluid was then measured at 520 nm. One unit of the protease activity was defined as the ability to hydrolyze 1 μg of azocoll per min at 37°C. Complete hydrolysis of 5 mg of azocoll in the reaction mixture was taken to give an absorbance at 520 nm of 2.40. CO-DH activity was assayed by the method of Kraut *et al.* (14).

Protein determination

Protein was determined by the method of Lowry *et al.* (20) with bovine serum albumin as a standard.

Electrophoresis

Denaturing PAGE of the purified enzyme was carried out in gels containing 12.5% acrylamide and 0.1% sodium dodecyl sulfate (SDS) by the method of Laemmli (16) with several modifications (11). Proteins were stained with Coomassie brilliant blue R-250 (CBB) by a modification (12) of the method of Weber and Osborn (30).

Protease purification

All purification steps were carried out at 4°C. Cells grown on CO as the sole source of carbon and energy were harvested during the late exponential growth phase and washed twice in standard buffer. A 30-g portion of washed cells was suspended in 100 m*l* of standard buffer, disrupted by ultrasonic treatment (10 s/m*l*), and then centrifuged at 18,000×*g* for 30 min. The supernatant fluid (crude extract) was treated with protamine sulfate to a final concentration of 0.054 %, left in ice for 10 min, and then centrifuged at 100,000×g for 90 min. Ammonium sulfate was added to the resulting supernatant (soluble fraction) to achieve a final concentration of 20% of saturation. After 2 h, the solution was centrifuged at 27,000×*g* for 30 min, and the resulting supernatant was further made 60% saturated with respect to ammonium sulfate. After 2 h, the solution was centrifuged again at 27,000×*g* for 30 min, and the pellets were dissolved in a small volume of the standard buffer and dialyzed against three 2-liter changes of standard buffer for 18 h. The dialysate was then applied to a Sephacryl S-200 column (1.6×85 cm) equilibrated with standard buffer. Elution was carried out with standard buffer at a flow rate of 7.36 m*l*/cm^2 per h. Fractions with high protease activity were pooled and applied to a DEAE-Sephacel column (2.8×85 cm) equilibrated with standard buffer. Elution was performed with 500 m*l* of a linear gradient of NaCl (0 to 0.3 M in 500 m*l* standard buffer) at a flow rate of 1.95 m*l*/cm^2 per h. Fractions with high protease activity were pooled and applied to a Sepharose 6B column (1.6×92 cm) equilibrated with standard buffer. Elution was performed with standard buffer at a flow rate of 7.36 m*l*/cm^2 per h. Fractions containing protease activity were pooled and stored at −50°C.

RESULTS

Protease activity

It was found that *P. carboxydovorans* grown on CO produced an intracellular protease which is active on azocoll. The protease was most active in cells growing at the late exponential phase.

Purification of proteases

The intracellular protease was purified 57-fold in six steps with a yield of 4.3% and a specific activity of 45.4 units per mg of protein (Table 1). The purified enzyme migrated as a single band on denaturing polyacrylamide gel (Fig. 1).

Molecular weight and structure

The molecular weight of protease was determined to be 150,000 after Sepharose 6B column (1.6×92 cm) chromatography by the method of Andrews (1) with several reference proteins of known molecular weights. The molecular weight of the subunits was estimated to be 72,000 by using SDS-PAGE with several molecular weight references.

Effect of divalent cations

The effects of divalent cations on the activity of the purified protease is shown in Table 2. The enzyme activity was inhibited completely by 10 mM Cd^{2+}, Fe^{2+}, Hg^{2+}, and Zn^{2+}. Ba^{2+} and Cu^{2+} were also found to be inhibitory to the enzyme. The enzyme, however, was activated by Mg^{2+}. Ca^{2+} and Mn^{2+} showed no effect on the enzyme activity.

Effect of chelating agents

Several metal chelating agents were tested for their effect on the purified protease activity. EGTA at 10 mM inhibited the enzyme completely (Table 2). EDTA, KCN, and NaN_3 showed some inhibitory effect on the enzyme activity.

Effect of protein modification reagents

Among the eight protein modification reagents

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

Purification step	Total protein[a] (mg)	Total act.[b]	Sp. act.[c]	Recovery (%)	Purification (fold)
Crude extract	4459.4	3560.0	0.8	100.0	1.0
Soluble fraction	1941.6	1770.7	0.9	49.7	1.1
Dialysate	863.7	1318.4	1.5	37.0	1.9
Sephacryl S-200	622.8	1229.4	2.0	34.5	2.5
DEAE-Sephacel	87.0	281.4	3.2	7.9	4.1
Sepharose 6B	3.4	154.2	45.4	4.3	56.8

[a] Determined by Lowry (20) method. [b] Units. [c] Units per milligram of protein. One unit of the enzyme activity was defined as the ability to hydrolyze 1 μg of azocoll per min at 37°C.

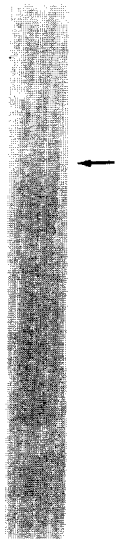

Fig. 1. *Purified protease after denaturing PAGE. Gels containing 12.5% acrylamide were run in the presence of 0.1% SDS according to Laemmli (16) and stained with CBB.*

Table 2. *Effect of divalent cations and chelating agents on the protease activity*[a]

Chemicals	Conc. (mM)	Relative act. (%)[b]
None	–	100
Cations[c]		
Ba^{2+}	10	12
Ca^{2+}	10	98
Fe^{2+}	10	0
Mg^{2+}	10	125
Cu^{2+}	10	19
Hg^{2+}	10	0
Cd^{2+}	10	0
Mn^{2+}	10	98
Zn^{2+}	10	0
Chelating agents		
KCN	1	76
	10	75
NaN_3	1	95
	10	70
EDTA	1	76
	10	35
EGTA	1	35
	10	0

[a] Protease activity was measured after 5 min of incubation of the enzyme at room temperature with cations and chelating agents. [b] Activity in the absence of chemicals was taken as 100%. [c] Ions were added as chloride salt except for Fe^{2+} and Cu^{2+} which were added as sulfate salt.

Table 3. *Effect of protein modification reagents on the protease activity*[a]

Chemicals	Conc. (mM)	Relative act. (%)[b]
None	–	100
DTNB[c]	1	107
	5	123
PCMPS[d]	1	116
	5	102
PLP[e]	1	115
	10	120
phenyl glyoxal	1	84
	10	13
PMSF[f]	1	30
	10	26
EDC[g]	1	90
	10	188
iodoacetamide	1	96
	10	147
dithiothreitol	1	102
	10	138

[a] Protease activity was measured after 5 min of incubation of the enzyme at room temperature with several protein modification reagents. [b] Activity in the absence of chemicals was taken as 100%. [c] 5,5′-dithiobis(2-nitrobenzoic acid). [d] *p*-Chloromercuriphenylsulfonic acid. [e] pyridoxal 5-phosphate. [f] phenylmethylsulfonyl fluoride. [g] 1-ethyl-3(dimethyl aminopropyl) carbodiimide.

tested, phenyl glyoxal and phenylmethylsulfonyl fluoride (PMSF) showed strong inhibitory effect on the purified enzyme (Table 3). 1-ethyl-3 (dimethyl aminopropyl) carbodiimide (EDC), iodoacetamide and dithiothreitol at 10 mM, however, stimulated the enzyme activity.

Effect of pH and temperature

The purified protease was most active at pHs between 7.0 and 8.0. The optimal temperature for the enzyme reaction was found to be 50°C. The enzyme retained over 50% of the original activity when it was incubated for 30 min at 50°C, but lost its activity completely in 30 min at 70°C.

Substrate specificity

The purified enzyme was active on, in addition to azocoll, casein and bovine serum albumin (data not shown). The enzyme was most active on azocoll. The enzyme, however, could not hydrolyze CO-DH at all. Azocasein was found to be very resistant to the enzyme.

DISCUSSION

It has been reported that several carboxydobacteria grown on nutrient broth produce intracellular proteases which are active on

azocasein or azocoll; the proteases were not detected from cells grown on succinate, pyruvate, acetate, or CO as the sole source of carbon and energy (10, 17, 18). We, however, identified in this experiment an intracellular protease in cells of *P. carboxydovorans* grown on CO. It may be possible that the enzyme was not found in the previous study since the cultivation condition or the substrate for the enzyme assay was not suitably chosen as suggested in other reports (3, 5, 7).

The size (150,000) of the purified enzyme was found to be larger than those of *P. carboxydovorans* (53,000) (18), *Pseudomonas carboxydohydrogena* (125,000) (17), and *Acinetobacter* sp. strain JC1 (55, 000 and 44,000) (10) grown on nutrient broth. The enzymes purified from cells of *Acinetobacter* sp. strain JC1 and *P. carboxydovorans* grown on nutrient broth were found to consist of single polypeptides. The enzyme of *P. carboxydohydrogena* was found to consist of two nonidentical subunits of molecular weights 70,000 and 56,000. The enzyme from *P. carboxydovorans* grown on CO, on the other hand, was found to have two identical subunits of molecular weight 72,000.

It is well known that there are four types of proteases such as serine-type, cysteine-type, metal, and acidic proteases in microorganisms (25). The purified enzyme, like other proteases studied in carboxydobacteria (10, 17, 18), seems to be a serine-type protease since it was inhibited strongly by PMSF (2, 15, 27). PMSF has also been known to inhibit several cysteine-type proteases (2). The purified enzyme, however, was not considered as a kind of cysteine-type protease since the well-known cysteine protease inhibitors such as 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), *p*-chloromercuriphenylsulfonic acid (PCMPS), and iodoacetamide did not inhibit the enzyme activity. Iodoacetamide was found stimulatory rather than inhibitory to the purified enzyme as to other carboxydobacterial proteases (10, 17, 18). It may be deduced from the inhibition of the protease activity by phenyl glyoxal that arginine may present in or around the active site of the enzyme. Activation of the enzyme by EDC and dithiothreitol suggests that carboxyl and sulfhydryl groups may affect the enzyme activity by modification of the enzyme through ionic interaction and disulfide formation, respectively. Almost two-fold increase of the enzyme activity after treatment with EDC suggests an idea that it may possible to engineer the protease to make a better one through modification of proline, aspartate, or glutamic acid present in the purified enzyme.

It has been reported that several divalent cations and metal chelating agents affect protease activity in carboxydobacteria (10, 17, 18). The complete inhibition of the purified enzyme by Cd^{2+}, Fe^{2+}, Hg^{2+}, and Zn^{2+} and the strong inhibition of the enzyme by Ba^{2+} and Cu^{2+} are similar to those observed in other carboxydobacterial proteases. Complete inhibition of the purified enzyme by 10 mM EGTA implies that Ca^{2+} is required for the enzyme activity. This result is the same as that of *P. carboxydovorans* enzyme prepared from cells grown on nutrient broth (18). The purified enzyme, however, is different from the enzyme of nutrient broth-grown cells in that the former is also sensitive to KCN and NaN_3.

The optimal pH for enzyme reaction indicates that the purified enzyme is an weakly alkaline protease like other carboxydobacterial proteases (10, 17, 18). The optimal temperature was found to be the same as those of *Acinetobacter* sp. strain JC1 (10) and *P. carboxydovorans* (18) enzymes prepared from nutrient broth-grown cells.

The purified protease, like other carboxydobacterial proteases, was found to be inactive on CO-DH, implying that the proteases studied to date are not involved in the degradation of CO-DH. From the result that the purified protease was almost inactive on azocasein, it may be possible to explain the reason why the previous study using azocasein as a substrate for enzyme reaction could not detect protease activity from CO-grown cells of *P. carboxydovorans* (18).

The present study, together with the previous results obtained from *P. carboxydohydrogena* (17), *Acinetobacter* sp. strain JC1 (10), and *P. carboxydovorans* (18) grown on nutrient broth, indicates that intracellular proteases in carboxydobacteria are specific not only for each bacterium but also for nutritional conditions.

ACKNOWLEDGEMENTS

This work was supported by a research grant (88-0913-01) from the Korea Science and Engineering Foundation to YMK.

REFERENCES

1. **Andrews, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochem. J.* **91**, 222-223.
2. **Bond, J.S. and P.E. Butler.** 1987. Intracellular proteases. *Annu. Rev. Biochem.* **56**, 333-364.
3. **Bromke, B.J. and J.M. Hammel.** 1979. Regulation of extracellular protease formation by *Serratia marcescens*. *Can. J. Microbiol.* **25**, 47-52.
4. **Burnett, T.J., G.W. Shankweiler, and J.H. Hageman.** 1986. Activation of intracellular serine proteinase in *Bacillus subtilis* cells during sporulration. *J. Bacteriol.* **165**, 139-145.
5. **Gibb, G.D. and W.R. Strohl.** 1987. Physiological regulation of protease activity in *Streptomyces peucetius*. *Can. J. Microbiol.* **34**, 187-190.
6. **Goldberg, A.L. and L. Waxman.** 1985. The role

of ATP hydrolysis in the breakdown of proteins and peptides by protease La from *Escherichia coli*. *J. Biol. Chem.* **260**, 12029-12034.

7. **Jensen, S.E., I.T. Fecycz, and J.N. Campbell.** 1980. Nutritional factors controlling exocellular protease production by *Pseudomonas aeruginosa*. *J. Bacteriol.* **144**, 844-847.
8. **Jensen, S.E., L. Phillippe, J.T. Tseng, G.W. Stremke, and J.N.** Campbell. 1979. Purification and characterization of exocellular proteases produced by clinical isolate and a laboratory strain of *Pseudomonas aeruginosa*. *Can. J. Bacteriol.* **26**, 77-86.
9. **Kang, B.S.** 1990. Purification and characterization of two extracellular proteases from *Pseudomonas carboxydovorans*. Master thesis, Yonsei University.
10. **Kim, E.B. and Y.M. Kim.** 1990. Purification and some properties of two intracellular proteases from *Acinetobacter sp. strain JC1 DSM 3803*. *Kor. Biochem. J.* **23**, 8-314.
11. **Kim, K.S., Y.T. Ro, and Y.M. Kim.** 1989. Purification and some properties of carbon monoxide dehydrogenase from *Acinetobacter* sp. strain JC1 DSM 3803. J. Bacteriol. **171**, 958-964.
12. **Kim, Y.M. and G.D. Hegeman.** 1981. Purification and some properties of carbon monoxide dehydrogenase from *Pseudomonas carboxydohydrogena*. *J. Bacteriol.* **148**, 904-911.
13. **Kim, Y.M. and G.D. Hegeman.** 1983. Oxidation of carbon monoxide by bacteria. *Intl. Rev. Cytol.* **81**, 1-32.
14. **Kraut, M., I. Hugendieck, S. Herwig, and O. Meyer.** 1989. Homology and distribution of CO dehydrogenase structural genes in carboxydotrophic bacteria. *Arch. Microbiol.* **152**, 335-341.
15. **Kurotsu, T., M.A. Marahiel, K. Müller, and H. Kleinkauf.** 1982. Characterization of an intracellular serine protease from sporulating cells of *Bacillus brevis*. *J. Bacteriol.* **151**, 1466-1472.
16. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**, 680-685.
17. **Lee, H.S. and Y.M. Kim.** 1991. Purification and characterization of an intracellular protease from *Pseudomonas carboxydohydrogena*. *Kor. J. Microbiol.* **29**, 167-171.
18. **Lee, J.H. and Y.M. Kim.** 1989. Purification and some properties of an intracellular protease from *Pseudomonas carboxydovorans*. *Kor. J. Microbiol.* **27**, 237-244.
19. **Levy, C.C. and P. Goldman.** 1969. Bacterial peptidases. *J. Biol. Chem.* **244**, 4467-4472.
20. **Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall.** 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
21. **Maeda, H. and A. Molla.** 1989. Pathogenic potentials of bacterial proteases. *Clin. Chim. Acta.* **185**, 357-368.
22. **Meyer, O.** 1989. *In* Autotrophic bacteria (Schlegel, H.G. and B. Bowien, eds.), pp. 331-350. Science Tech Publishers, Springer-Verlag, Berlin.
23. **Meyer, O. and H.G. Schlegel.** 1978. Reisolation of the carbon monoxide utilizing hydrogen bacterium *Pseudomonas carboxydovorans* (Kistner) comb. nov. *Arch. Microbiol.* **118**, 35-43.
24. **Meyer, O. and H.G. Schlegel.** 1980. Carbon monoxide: methylene blue oxido-reductase from *Pseudomonas carboxydovorans*. *J. Bacteriol.* **141**, 74-80.
25. **North, M.J.** 1982. Comparative biochemistry of the proteinases of eucaryotic *microorganisms*. *Microbiol. Rev.* **46**, 308-340.
26. **Pine, M.J.** 1972. Turnover of intracellular proteins. *Annu. Rev. Microbiol.* **26**, 103-126.
27. **Ruppen, M.E., G.L.V. Alstine, and L. Band.** 1988. Control of intracellular serine protease expression in *Bacillus subtilis*. *J. Bacteriol.* **170**, 136-140.
28. **Swamy, K.H.S. and A.L. Goldberg.** 1981. *E. coli* contains eight soluble proteolytic activities, one being ATP dependent. *Nature*. **292**, 652-654.
29. **Switzer, R.L.** 1977. The inactivation of microbial enzymes *in vivo*. *Annu. Rev. Microbiol.* **31**, 135-157.
30. **Weber, K. and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate polyacylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406-4412.
31. **Wretlind, B. and T. Wadström.** 1977. Purification and properties of a protease with elastase activity from *Pseudomonas aeruginosa*. *J. Gen. Microbiol.* **103**, 319-327.

(Received July 7, 1992)
(Accepted August 3, 1992)

초 록: 일산화탄소를 이용하여 성장한 *Pseudomonas carboxydovorans* DSM 1227에서 분리 정제된 세포내 단백질 가수분해효소의 특징

배기호 · 김영민 (연세대학교 이과대학 생물학과)

일산화탄소를 유일한 에너지 및 탄소원으로 이용하여 성장한 *Pseudomonas carboxydovorans* DSM 1227로부터 azocoll을 기질로 사용하여 약 57배 정제된 세포내 단백질 가수분해효소를 얻었다. 정제된 효소의 분자량은 150,000이었고, 72,000의 분자량을 지닌 두 개의 동일한 소단위로 구성되어 있었다. Mg^{2+}이온은 이 효소의 활성을 증가시켰으나 Cd^{2+}, Fe^{2+}, Hg^{2+}, Zn^{2+} 등은 이 효소의 활성을 완전히 억제하였다. 이 효소는 EDTA, EGTA, phenylmethylsulfonyl fluoride, phenyl glyoxal 등에 의해 활성이 억제되었고, iodoacetamide와 dithiothreitol, 1-ethyl-3(dimethyl aminopropyl) carbodiimide에 의해서는 활성이 증가되었다. 정제된 효소는 pH 7~8과 50°C에서 가장 높은 활성을 나타내었다. 이 효소는 casein과 bovine serum albumin은 가수분해하였으나 일산화탄소 탈수소효소는 가수분해하지 않았다.