Purification and Characterization of Two Extracellular Proteases from *Oligotropha carboxydovorans* DSM 1227

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Two extracellular proteases, EP I and EP II, from cells of *Oligotropha carboxydovorans* (formerly *Pseudomonas carboxydovorans*) DSM 1227 grown in nutrient broth were purified to greater than 95% homogeneity in five steps using azocasein as a substrate. The final specific activities of EPs I and II were 214.9 and 667.4 units per mg of protein. The molecular weights of native EPs I and II were determined to be 23,000. Sodium dodecyl sulfate-gel electrophoresis revealed the two enzymes to be monomers. The enzymes were found to be serine-type proteases. The activity of EP I was stimulated by Ca^{2+}, Mg^{2+}, and Ba^{2+}, but that of EP II was not. The enzymes were completely inhibited by Fe^{2+}, Hg^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+}. EDTA and EGTA exhibited a strong inhibitory effect on EP I. The optimal pH for the two enzymes was pH 9.0. The optimal temperatures for EP I and II were 60 and 50°C, respectively. The enzymes were stable under alkaline conditions. The thermal stability of EP I was higher than that of EP II. Cell-free extracts did not inhibit the purified enzymes. The enzymes were active on casein, azocasein, azocoll, and carbon monoxide dehydrogenase, but weakly active with bovine serum albumin.

Key words: Carboxydobacteria, extracellular protease, *Oligotropha carboxydovorans*

Microbial proteases have been studied since the beginning of enzymology and widely used in an industrial scale for protein degradation, peptide biosynthesis, and detergent production. Several microorganisms are known to produce intracellular and/or extracellular proteases (23, 30). Intracellular proteases are involved in complex cellular processes such as maturation of enzymes, degradation of abnormal or malfunctional proteins, secretion of extracellular proteins, and inactivation of functional proteins (3, 7, 8, 26, 31, 32). Extracelluar proteases, on the other hand, play an important role in the hydrolysis of other proteins or polypeptides present in the environment to enable the cells to absorb and utilize hydrolysis products as substrates for growth and also in the transformation of nonpathogenic materials to pathogenic molecules (13, 37).

It has been reported that the biosynthesis and secretion of extracellular proteases depends on nutritional condition (4, 12). *Serratia marcescens* is known to produce extracellular protease during starvation, but does not synthesize the protein when it is growing in media containing free amino acids, gelatin, or peptone (4). It is also noted that the ability to produce specific proteases can be used as a key in the classification of taxonomically close organisms (9).

Carboxydobacteria are a group of bacteria which are able to grow aerobically on carbon monoxide (CO) as a sole source of carbon and energy (17, 27). Several carboxydobacteria such as *Pseudomonas carboxydohydrogena* (21), *Oligotropha carboxydovorans* (formerly *Pseudomonas carboxydovorans* [28]) (2, 22), and *Acinetobacter* sp. strain JC1 (14) are known to produce one or two intracellular proteases during growth in nutrient broth or CO. Interestingly, no extracellular proteases have been reported for cells of *P. carboxydohydrogena* (21) and *Acinetobacter* sp. strain JC1 (14) grown in nutrient broth.

In this study, we examined cells of *O. carboxydovorans* to determine whether the inability to produce extracellular proteases is a common property of carboxydobacteria. As a result, we found and characterized two kinds of extracellular proteases from *O. carboxydovorans* grown in nutrient broth.

Materials and Methods

Bacterial strain and cultivation

O. carboxydovorans DSM 1227 was grown at 30°C

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in nutrient broth or mineral medium (16) supplemented with a gas mixture of 30% CO and 70% air. Growth was measured at 560 nm.

Enzyme assay

Protease activity was assayed by a modified method of Jensen *et al.* (13) using azocasein as a substrate. The reaction mixture contained 1 ml of a-zocasein solution (0.5% solution in distilled water adjusted to pH 7.5 and filtered through Whatman No. 1 filter paper), 1 ml of 50 mM Tris-hydrochloride buffer (pH 7.5; standard buffer) containing 2 mM $CaCl_2$ and 100 µl of enzyme solution. The reaction was stopped after 15 min of incubation at 40°C by the addition of 2 ml of 10% trichloroacetic acid. After standing for 30 min at 21°C, precipitates were removed using filter paper and the absorbance at 370 nm of the filtrate was determined. One unit of activity was defined as the amount of enzyme required to increase an absorbance at 370 nm of 0.1 in 15 min under the assay conditions.

CO dehydrogenase (CO-DH) activity was determined photometrically at 30°C by measuring the CO-dependent reduction of thionin dye (ε_{595}=4.2×10^4 M^{-1} cm^{-1}) in standard buffer at 595 nm as described previously (16).

Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed in gels containing 7.5% acrylamide by the method of Laemmli (20), but without sodium dodecyl sulfate (SDS), as described previously (16). Denaturing PAGE was conducted using 12.5% gels following the procedure of Laemmli (20) with several modifications as described by Kim *et al.* (15). Proteins were stained with Coomassie brilliant blue R-250 (CBB) by a modified method (16) of Weber and Osborn (38). Activity staining of CO-DH was carried out in standard buffer, using a nondenaturing gel strip in the presence of 0.05% phenazine methosulfate, 0.25% nitroblue tetrazolium, and CO (16).

Protein determination

Protein was determined by the method of Lowry *et al.* (24) using bovine serum albumin as a standard. Proteins in crude cell extracts were measured by the same method after samples were boiled for 10 min in 20% NaOH solution (29).

Enzyme purification

Purification was carried out at 4°C. Cultures growing at the late exponential growth phase in nutrient broth were centrifuged at 15,000×*g* for 10 min. The proteins in cell-free supernatant fluid (crude protease) were then concentrated using Amicon YM10 ultrafil-

tration membrane (concentrated crude protease). The concentrated crude protease preparation was next 40% saturated with ammonium sulfate, left for 2 h, and centrifuged at 15,000×*g* for 30 min. The resulting supernatant fluid was further treated with ammonium sulfate to achieve a final concentration of 60% saturation. After 2 h, the solution was centrifuged at 15,000×*g* for 30 min, and the precipitate was suspended in a small volume of cold standard buffer. The suspension was then dialyzed against three 2-liter changes of standard buffer for 8 h. The dialysate was applied to a Sephadex G-75 column (2.3×80 cm) prequilibrated with standard buffer. Elution was carried out with standard buffer at a flow rate of 2 ml/cm^2 per h. Fractions with high protease activity were pooled, concentrated with YM10 membrane filters, and dialyzed against three 2-liter changes of 10 mM sodium phosphate buffer (pH 7.5). The dialysate was then applied to a CM-cellulose column (4×11 cm) prewashed with at least 3 total bed volumes of 10 mM sodium phosphate buffer (pH 7.5). Elution was performed with 400 ml of a linear 10 to 100 mM sodium phosphate buffer (pH 7.5) gradient at a rate of 2.4 ml/cm^2 per h. Two groups of fractions with high protease activity were pooled separately and concentrated with Amicon YM10 membranes (purified protease). The concentrates were then dialyzed against three 2-liter changes of standard buffer for 6 h and stored at −20°C under air.

Crude cell-free extracts preparation

Cells grown in nutrient broth or CO were harvested during the late exponential growth phase and washed twice in standard buffer. The washed cells were suspended in standard buffer, disrupted by ultrasonic treatment (10 s/ml), and centrifuged at 15,000×*g* for 30 min. The resulting supernatant fluids were used as cell-free extracts.

Results

Presence of extracellular protease activity

It was found that *O. carboxydovorans* grown in nutrient broth exhibits azocasein-hydrolyzing extracellular protease activity. The activity was high in culture supernatant of cells growing at the late exponential growth phase. It was not detected from cells grown in minimal medium supplemented with CO.

Purification

Two extracellular proteases, EP I and EP II, were purified 86- and 267-fold in five steps with 4.6 and 4.2% activity yields and specific activities of 214.9 and 667.4 units per mg of protein, respectively (Table 1).

Table 1. Purification of extracellular proteases from *O. carboxydovorans* grown in nutrient broth

Purification step	Total protein[a] (mg)	Sp. act.[b]	Purification fold	Total activity[c]	Recovery (%)
Crude protease	2,198	2.5	1.0	5,440	100
Concentrated crude protease	228	13.4	5.4	3,059	56.2
Dialysate	14.6	66.5	26.6	928	17.1
Sephadex G-75	7.4	103.4	41.4	763	14.0
CM-cellulose					
EP I	1.1	214.9	86.0	250	4.6
EP II	0.3	667.4	267.0	226	4.2

[a] Estimated by Lowry *et al.* (24).
[b] Units per milligram of protein. One unit was defined as the amount of protein needed to increase an absorbance at 370 nm of 0.1 in 15 min.
[c] Units.

The purified enzymes migrated as single band on a denaturing polyacrylamide gel (Fig. 1). The enzymes were determined to be better than 95% homogeneous after densitometric analysis of the gel stained with CBB.

Molecular properties

The molecular weights of the native EP I and II were similar and estimated to be 23,000 after Sephadex G-75 column chromatography by the method of Andrews (1) with several reference proteins of known molecular weights. Denaturing PAGE of EPs I and II revealed the presence of a single polypeptide in each protease with a molecular weight of 21,000 and 24,000, respectively (Fig. 1), indicating that the purified proteases are monomers.
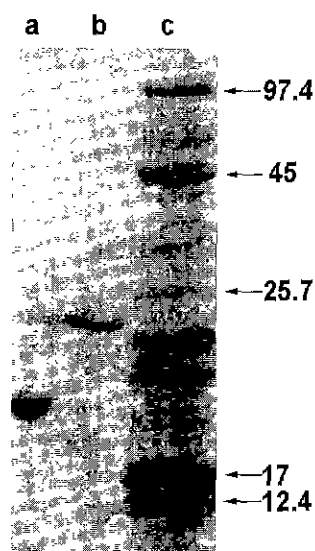

Fig. 1. Purified proteases on a denaturing polyacrylamide gel. Denaturing PAGE (12.5% acrylamide, 0.1% SDS) of EP I (lane a) and EP II (lane b) was carried out together with several reference proteins of known molecular weight (lane c) according to Laemmli (20) and stained with CBB.

Effect of protein modification reagents

Among the eight protein modification reagents tested, phenylmethylsulfonyfluoride (PMSF), diisopropyl fluorophosphate (DFP), and dithiothreitol showed inhibitory effects on the two purified enzymes (Table 2). 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and pyridoxal 5-phosphate (PLP), however, stimulated activities of the two enzymes.

Effect of chelating agents

The effect of several metal chelating agents on the purified proteases was tested. Potassium cyanide and sodium azide did not show a significant effect on the two proteases. EP I was found to be more sensitive to EDTA and EGTA than EP II (Table 2).

Effect of divalent cations

The effect of divalent cations on the activity of purified proteases is shown in Table 2. The enzymes were completely inhibited by 10 mM Fe^{2+}, Hg^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+}. Ca^{2+}, Mg^{2+}, and Ba^{2+} were found to be stimulatory to the EP I, but not to the EP II.

Effect of pH and temperature

The purified enzymes were most active at pH 9.0. EP I and EP II retained over 90% of their original activity when they were incubated for 1 h at pH 8-9 and 8-11, respectively. The optimal reaction temperatures for EP I and EP II were found to be 60 and 50°C, respectively. EP I retained almost 100% of its original activity when it was incubated for 1 h at 50°C, but 50% of the activity was inactivated in 30 min at 70°C. EP II, on the other hand, lost 50% of its enzyme activity in 30 min at 40°C and was completely inactivated in 15 min at 70°C.

Effect of cell-free extract

To test the presence of a cell-associated inhibitor for EP I and II in *O. carboxydovorans*, the enzymes

Table 2. Effect of various chemicals on extracellular protease activity[a]

Chemicals	Concentration (mM)	Relative activity (%)[b]	
		EP I	EP II
None		100	100
Protein modifiers			
DTNB[c]	1	145	142
PCMPS[d]	1	91	98
Iodoacetamide	1	92	110
PLP[e]	1	114	119
Phenyl glyoxal	1	98	103
DEP[f]	1	102	101
PMSF[g]	1	64	70
	10	37	60
DFP[h]	1	21	21
EDC[i]	1	99	97
Dithiothreitol	1	48	87
	10	25	69
Chelating agents			
KCN	10	90	98
NaN₃	10	99	90
EDTA	1	52	101
	10	42	83
EGTA	1	50	97
	10	0	14
Metal ions[j]			
Fe²⁺	10	0	0
Hg²⁺	10	0	0
Cu²⁺	10	69	30
Ca²⁺	10	138	93
Mn²⁺	10	57	37
Co²⁺	10	0	0
Mg²⁺	10	125	107
Zn²⁺	10	0	0
Ba²⁺	10	135	93
Cd²⁺	10	0	0

[a] Protease activity was measured after 10 min of incubation of the purified enzymes at 20°C with various chemicals as described in Materials and Methods.
[b] Activity in the absence of chemicals was taken as 100%.
[c] 5,5'-dithio-bis(2-nitrobenzoic acid).
[d] p-chloromercuriphenylsulfonic acid.
[e] Pyridoxal 5-phosphate.
[f] Diethyl pyrocarbonate.
[g] Phenylmethylsulfonyl fluoride.
[h] Diisopropyl fluorophosphate.
[i] 1-ethyl-3(dimethyl aminopropyl) carbodiimide.
[j] Divalent cations were added as chloride salts except for Fe²⁺ and Cu²⁺ which were added as sulfate salts.

were assayed after incubation for 10 min at 20°C with cell-free extracts prepared from cells grown in nutrient broth. It was found that the cell-free extracts exhibited no effect on the two purified enzymes.

Substrate specificity

Purified EP I and II hydrolyzed casein, azocasein, azocoll, and bovine serum albumin (Table 3). The en-

Table 3. Substrate specificity of purified extracellular proteases

Proteins	Specific activity (%)[a]	
	EP I	EP II
Casein[b]	101.4	435.1
Bovine serum albumin[b]	19.1	7.6
Azocasein[c]	214.9	667.4
Azocoll[d]	6,695.1	14,372.1

[a] Units per milligram of protein.
[b] One unit of activity was defined as the amount of enzyme needed to increase an absorbance at 280 nm of 0.1 in 30 min.
[c] One unit of activity was defined as the amount of enzyme needed to increase an absorbance at 370 nm of 0.1 in 15 min.
[d] One unit of activity was defined as the amount of enzyme needed to hydrolyze 1 μg of azocoll per min.

zymes were most active in the presence of azocoll. EP II showed higher specific activity than EP I to all substrates tested except for bovine serum albumin.

It was found that the purified enzymes inhibit CO-DH activity (Table 4). The inhibition was found to result from degradation of CO-DH by the purified enzymes since the three CO-DH subunits hydrolyzed upon incubation with the two enzymes (Fig. 2). The degree of CO-DH hydrolysis by the purified extracellular proteases was proportional to the length of incubation and the amount of proteases present in the reaction mixture (data not shown).

Discussion

It has been reported that several carboxydobacteria such as *P. carboxydohydrogena* (21) and *Acinetobacter* sp. strain JC1 (14) produce no extracellular proteases during growth in nutrient broth. We, however, observed in this study that *O. carboxydovorans* growing in nutrient broth produces extracellular proteases, in addition to intracellular proteases (2, 22), indicating that the inability to produce extracellular proteases is not a common property in the carboxydobacterial

Table 4. Effect of extracellular proteases on CO-DH activity[a]

Proteases	Relative CO-DH activity (%)[b]	
	4°C	40°C
None	100	100
EP I	99.5	50.9
EP II	101.5	16.9

[a] CO-DH activity was measured after 30 min of incubation at different temperatures of cell-free extracts prepared from CO-grown cells with purified proteases as described in Materials and Methods.
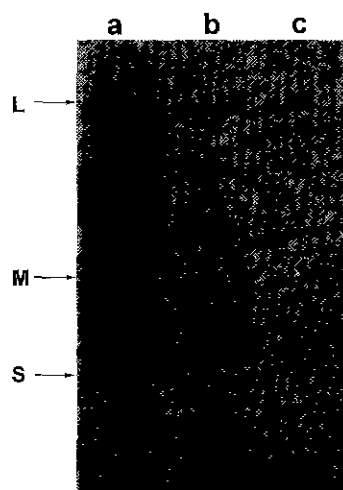[b] Activity of protease-untreated extracts was taken as 100%.

Fig. 2. Hydrolysis of CO-DH by purified proteases. Denaturing PAGE (12.5% acrylamide, 0.1% SDS) of crude cell extracts prepared from cells grown in CO (lane a) was performed together with the extracts treated with EP I (lane b) and EP II (lane c) for 30 min at 40°C. L, M, and S denote large, medium, and small subunits of CO-DH, respectively.

group. The ability to produce extracellular protease of *O. carboxydovorans* growing in nutrient broth containing peptone is interesting since *S. marcescens* growing in a medium containing peptone produced no extracellular proteases (4).

O. carboxydovorans produced at least two different kinds of extracellular proteases, EP I and EP II, which have relatively broad substrate specificity compared to intracellular proteases found in several carboxydobacteria (2, 14, 21, 22). The purified enzymes exhibited higher specific activities in the presence of casein, azocasein, and azocoll than several intracellular or extracellular proteases studied to date (2, 5, 10, 11, 13, 14, 21, 22). The ability of EPs I and II to hydrolyze CO-DH suggests that the reaction mechanism of the two enzymes may be different from those of carboxydobacterial intracellular proteases which are inactive on CO-DH (2, 14, 21, 22).

As expected from the extracellular nature of EP I and II, the two enzymes were quite small. The elution patterns obtaind from a Sephadex G-75 column of native proteins were found to be identical. The migration rates observed from polyacrylamide gel of denatured enzymes, however, did not coincide with each other, implying that EP I may be less globular than EP II in the native state.

The EP I and II, like intracellular proteases studied in carboxydobacteria (2, 14, 21, 22), seem to be serine-type proteases since the enzymes were strongly inhibited by PMSF and DFP, the serine-type pro-

tease inhibitors (3, 19, 32). PMSF has also been known to inhibit several cysteine-type proteases (3). The enzymes, however, were not considered cysteine-type proteases since well-known cystein-type protease inhibitors such as DTNB, PCMPS, and iodoacetamide did not inhibit activities of the two enzymes. DTNB was found stimulatory rather than inhibitory to the purified enzymes. The inhibition of the two enzymes by dithiothreitol, like the two intracellular proteases from *Acinetobacter* sp. strain JC1 (14), may be due to reduction by the strong reducing agent of critical disulfide bond(s) required to keep the enzymes in their active form.

Several divalent cations and metal chelating agents are known to affect bacterial proteases including intracellular proteases from several carboxydobacteria (2, 12, 14, 18, 21, 22, 39). Complete inhibition by Fe^{2+}, Hg^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} and strong inhibition by Mn^{2+} and Cu^{2+} of the purified enzymes are similar to those of other proteases. Strong inhibition of EP I and II by 10 mM EGTA indicates that Ca^{2+} is required to retain activities of the enzymes. Thus, it seems that Mg^{2+} and Ba^{2+} are involved in the stabilization of EP I.

Extracellular proteases may be toxic to the cell if they are active before excretion into the environment. It has been reported that *Erwinia chrysanthemi* produces certain inhibitors to repress extracellular proteases in cell (25, 36, 37). The observation that the cell-free extracts prepared from *O. carboxydovorans* grown in nutrient broth did not inhibit the purified extracellular proteases indicates that the bacterium does not produce any inhibitors for the two extracellular proteases. The two proteases may be present as inactive precursors in the cell and transform to active molecules during secretion into the environment (6, 34).

The optimal pH (pH 9.0) for the two extracellular proteases is higher than those of carboxydobacterial intracellular proteases (pH 8.0) (2, 14, 21, 22). The optimal pH and pH stabilities of EP I and II indicate that the two enzymes are alkaline proteases as are carboxydobacterial intracellular proteases. The extracellular proteases, however, may be weakly alkaline enzymes since several serine-type alkaline proteases are most active at pH 10~12 (33, 35).

In sum, the high specific activities in the presence of various substrates, the extracellular nature, the optimal pH and temperatures together with the pH and temperature stabilities of the two extracellular proteases of *O. carboxydovorans* suggest that EP I and EP II may be used as additives in the production of alkaline detergents working in hot and warm temperatures, respectively.

Acknowledgment

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