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Isolation and Properties of a Cytoplasmic Metalloendoprotease in *Escherichia coli*

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大腸菌 細胞質內 Metalloendoprotease의 抽出과 그 性質에 관하여

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요 약

대장균의 세포질내에 존재하는 단백질 분해효소 (protease Ci)를 추출하여 그 성질을 조사하였다. 이 효소는 insulin, glucagon 및 bovine growth hormone을 분해시키나, bovine serum albumin, casein 및 globin 등은 분해시키지 않는다. 이 효소의 native 한 상태에서의 분자량은 120,000이며, 54,000 dalton의 동일한 2개의 subunit로 구성되어 있다. Protease Ci의 최적 pH는 7.5이며 등전점은 5.5이다. 이 효소는 *o*-phenanthroline에 의해 저해되며, Mn^{++}나 Co^{++}와 같은 metal 이온들에 의하여 활성화되므로 metalloprotease임을 알 수 있다. 이 효소는 *p*-hydroxymercuribenzoate에 의해서 크게 저해되나 sulfhydryl protease의 specific한 저해제인 Ep475와 leupeptin에 의해서는 영향을 받지 않는다. 대장균 세포내에는 또 다른 insulin 분해효소(protease Pi)가 존재하는데, 이 효소는 periplasm에 존재하므로 protease Ci와는 다르다.

INTRODUCTION

In recent years, much progress has been made in our understanding of the physiological regulation and selectivity of protein degradation in *E. coli* (Goldberg and St. John, 1976; Hershko and Ciechanover, 1982; Pine, 1972). However, little is known about the pathway of protein degradation and the proteases involved in different stages of this process. A number of soluble (Goldberg *et al.*, 1981) and membrane-associated proteases (Pacaud, 1982; Regnier, 1981) have recently been isolated from *E. coli*. Such enzymes may play

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a role in the degradation of abnormal proteins, such as arised from mutations or biosynthetic errors (Goldberg and St. John, 1976), and of normal cell proteins, which rise during starvation of various essential nutrients (St. John *et al.*, 1978; St. John and Goldberg, 1980; Voellmy and Goldberg, 1980). Aside from their participations in turnover of cell proteins, cellular proteases should also role in many other important physiological processes, such as the processing of membrane and secretory proteins (Chang *et al.*, 1978; Wickner, 1979), viral morphogenesis (Mount, 1980), and the selective hydrolysis of regulatory proteins, including lambda cI repressor (Mount, 1980; Roberts *et al.*, 1978). Further understanding of the mechanism of intracellular protein breakdown and these cellular processes will require detailed characterization of the proteolytic enzymes in *E. coli*.

Goldberg and his colleagues (1981) have recently demonstrated that the soluble extract of *E. coli* contains eight endoproteolytic activities that appear distinct. Two of these named proteases Ci and Pi degrade insulin and auto alpha peptide but not the proteins having higher molecular weights (Cheng and Zipser, 1979; Swamy and Goldberg, 1981, 1982). Six others, named proteases Do, Re, Mi, Fa, So, and La, are serine proteases, and can degrade casein and globin (Swamy *et al.*, 1983; Chung and Goldberg, 1983). One of these enzymes, protease La, requires ATP and Mg^{++} for its activity (Charette *et al.*, 1981; Chung and Goldberg, 1981, 1982; Chung *et al.*, 1983; Larimore *et al.*, 1982), and it appears to be responsible for the rate-limiting step in the degradation of abnormal proteins (Kowit and Goldberg, 1977).

In this communication, we report isolation and various properties of protease Ci, a new cytoplasmic insulin-degrading enzyme that is an endoprotease and requires divalent metal ions for its activity.

MATERIALS AND METHODS

Materials

E. coli K12 cells (NF172) (Voellmy and Goldberg, 1980) were grown overnight with aeration in LB media at 37°C. The cells were then harvested and kept frozen at −70°C until use. All chemicals, except as indicated below, were purchased from Sigma. $Na^{125}I$ and [^{3}H]-formaldehyde were obtained from New England Nuclear; proinsulin, and insulin A and B chains from Eli Lilly; Diisopropylfluorophosphate from Aldrich; DEAE-cellulose (DE52) from Whatman; Ampholytes and DEAE-Sepharose from Pharmacia; Ultrogel AcA34 from LKB; hydroxylapatite from Bio-Rad Laboratories. Antifungal antibiotics used in this study were gift from Dr. Umezawa.

[^{3}H]-casein was prepared as described by Rice and Means (1971). Insulin, glucagon, bovine growth hormone, bovine serum albumin and globin were radioactively iodinated by using chloramine T (Greenwood *et al.*, 1963). The specific activity of radioiodinated

proteins was about $1.0\sim1.5\times10^8$ cpm/mg, and that of [^{3}H]-casein was 1×10^7 cpm/mg. Radioactively labeled proteins were kept frozen until use.

Assays

Proteolytic activity against ^{125}I-insulin and other proteins was determined by following the hydrolysis of the substrates to product soluble in 10% (w/v) trichloroacetic acid (TCA). Unless otherwise mentioned, all the assays were performed in 0.5 ml reaction mixtures containing 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 5~25 μg of radioactive substrates, and 0.5 to 100 μg of the enzyme preparation depending on the degree of purification. After incubation at 37°C for 30 to 60 min, 40 μl of 30 mg/ml bovine serum albumin was added as a carrier and 60 μl of 100% (w/v) TCA to precipitate proteins. The assay tubes were kept on ice for 10 min and were spun for 10 min in a typical bench top centrifuge. The supernatant fraction (0.4 ml) was counted in a liquid scintillation counter or in a gamma counter to measure the radioactivity of the acid-soluble materials.

The pH optimum of protease Ci was estimated by using acetate buffer for pH 4.0 to 5.5, 2-(N-morpholino) ethane-sulfonic acid (MES) for 5.5 to 7.0, N-2-hydroxyethyl-pipiperazine-N'-2-ethanesulfonylic acid (HEPES) for 6.5 to 8.0, Tris for 7.5 to 9.0, and glycine for 9.0 to 10.0. These buffers were used at 50 mM and contained 25 mM NaCl to equalize ionic strength of the various buffers.

Protein was assayed by the method of Lowry *et al.* (1951) or by the dye-binding procedure of Bradford (1977). Crystalline bovine serum albumin was used as standard.

Electrophoresis

Disc gel electrophoresis under non-denaturing condition was performed at 4°C at a pH of 8.9 (Swamy *et al.*, 1983). Proteolytic activity was also determined in the gel by slicing them in 2 mm pieces, grinding the pieces, and extracting with assay buffer overnight. An aliquot of the extracted fractions was assayed for proteolytic activity against [^{125}I]-insulin, as described above.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) in 10% slab gels was performed according to the procedure of Laemmli (1970).

Two dimensional electrophoresis of the protein samples was carried out to determine the subunit size of the isolated protease Ci. The first dimension was disc gel electrophoresis under nondenaturing condition that was carried out at 4°C at a pH 8.9. After the run, the gel was sliced, extracted, and assayed for insulin degrading activity. An aliquot (50 μl) of the fraction containing the highest insulin-degrading activity was then electrophoresed in the second dimensional slab gel in the presence of SDS according to the procedure of Laemmli (1970).

Isoelectric focusing of the isolated protease Ci was carried on 10 cm slab gel as described elsewhere (Chung *et al.*, 1983). After the run, final pH gradient was estimated by cutting a gel lane into 0.4 mm pieces and eluting them for 2 hrs into 1 ml of degassed water. Meaurement of pH was carried out on a Radiometer pHM62 pH meter. The other gel

lane was also cut into 0.4 mm slices, and was assayed for protease Ci as described above.

Estimation of Native Molecular Weight

The molecular weight of the protease under nondenaturing condition was determined by gel filtration on a Sephadex G200 column (1×45 cm) equilibrated with 50 mM Tris-HCl, pH 7.8, containing 5 mM $MgCl_2$ and 100 mM NaCl. Makers used were catalase (MW of 240,000), alkaline phosphatase (82,000) and bovine serum albumin (68,000).

Preparation of Crude Extract

E. coli crude extract was prepared as described previously (Goldberg *et al.*, 1981). Frozen cells (100 g) were suspended in 200 ml of 10 mM Tris-HCl, pH 7.8, containing 5 mM $MgCl_2$ and 0.1M NaCl. The cells were then disrupted in a French Press at 14,000 psi. The suspension was spun at 30,000×g for 30 min, and the supernatant was again centrifuged at 120,000×g for 2 hrs. The soluble fraction obtained from the ultracentrifugation was referred as crude extract, and was dialyzed against the same buffer overnight at 4°C. All isolation procedures were carried out at 4°C unless otherwise described.

RESULTS

Purification procedure

The initial purification steps were based on the fractionation procedure described previously (Goldberg *et al.*, 1981). The dialyzed crude extract obtained from 100 g of frozen *E. coli* K12 cells was loaded on a DEAE-cellulose column (4×16 cm) that had been equilibrated with 10 mM Tris-HCl, pH 7.8, containing 5 mM $MgCl_2$ and 100 mM NaCl. After the column was washed extensively, the adsorbed proteins were eluted by a linear gradient of 0.1 to 0.2 M NaCl (1 liter each side). Fractions of 10 ml were collected at a flow rate of 120 ml per hr. The peak of protease Ci was eluted at about 0.16 M NaCl (Fig. 1). Fractions having high [^{125}I]-insulin-degrading activity were pooled and dialyzed against 10 mM Tris-HCl, pH 8.4, containing 5 mM $MgCl_2$ and 100 mM NaCl. After the dialysis, proteins were applied on a DEAE-Sepharose column (1.5×23 cm) equilibrated with the same buffer. Unadsorbed proteins were washed with the buffer, and a linear gradient of 0.1 to 0.25 M NaCl (250 ml each side) was applied on the column. Fractions of 4 ml were collected at a flow rate of 30 ml per hr. Active fractions of protease Ci were pooled, titrated to pH 5.6 by adding 0.1 M NaAcetate, pH 4.5, and dialyzed overnight at 4°C against 10 mM NaAcetate, pH 5.6, containing 5 mM $MgCl_2$. Insoluble proteins were removed by spinning the dialyzed sample at 30,000×g for 30 min. The soluble fraction was adjusted to pH 7.8 by adding 0.2 M Tris-HCl, pH 8.0, and was added with solid ammonium sulfate crystallines to give 65% saturation with stirring. The precipitated proteins were collected by centrifugation, resuspended in 2.5 ml of 10 mM Tris-HCl, pH 7.8, containing 5 mM $MgCl_2$ and 0.1 M NaCl, and dialyzed against the same buffer.

The concentrated protease preparation was loaded on an Ultrogel ACA34 column (2.5

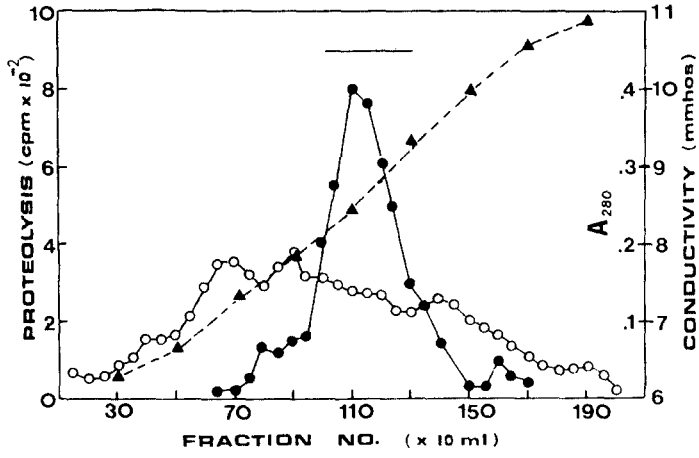

Fig. 1. DEAE-cellulose chromatography of *E. coli* crude extract. The dialyzed crude extract (4.28 g of protein) was chromatographed on a DEAE-cellulose column (4×16 cm) as described in the text. Reaction mixtures (0.5 ml) contained 0.15 ml of each fractions, 5 μg of [^{125}I]-insulin, 50 mM Tris-HCl (pH7.8), and 5 mM $MgCl_2$. Incubations were performed for 30 min at 37°C. Bar indicates the pooled fractions containing high insulin-degrading activity. Absorbance at 280 nm (A_{280}), (○); insulin hydrolysis, (●); salt gradient as shown by increase in conductivity (▲).

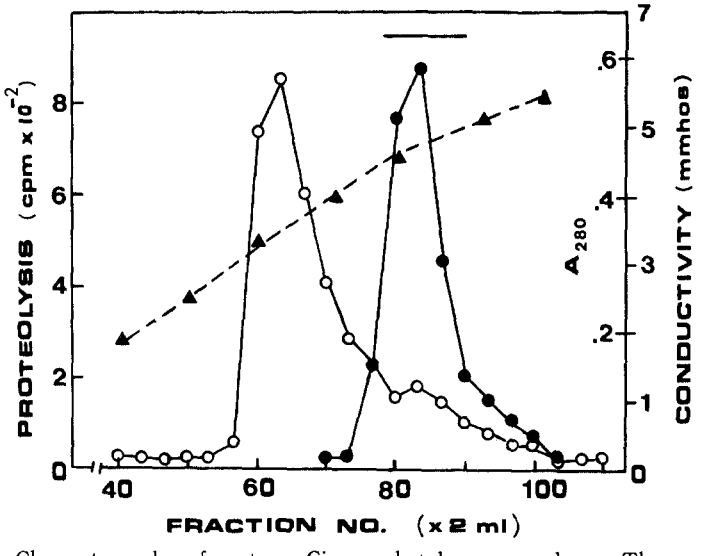

Fig. 2. Chromatography of protease Ci on a butyl-agarose column. The enzyme preparation (20.6 mg) obtained from the Ultrogel AcA34 step was loaded on a butyl-agarose column (1.0×10 cm) as described in the text. Preparation of reaction mixtures and incubations were performed as described in Fig. 1. Bar indicates the pooled fraction. A_{280} (○); insulin degradation (●); conductivity (▲).

×100 cm) eqiulibrated with the same buffer. Fractions of 5 ml were collected at a flow rate of 20 ml per hr. A sharp and symmetric peak of protease Ci eluted from this column was pooled, dialyzed against 10 mM Tris-HCl, pH 7.8, and loaded onto a butyl-agarose column (1.0×10 cm) that had been equilibrated with the same buffer. After the column was washed to remove unadsorbed proteins, the bound materials were eluted with a linear gradient of 0 to 0.15 M NaCl by collecting 2 ml fractions at a flow rate of 15 ml per hr (Fig. 2). A peak of [^{125}I]-insulin-degrading activity was appeared at 0.1M NaCl. Active fractions of protease Ci were pooled, dialyzed against 5 mM sodium phosphate buffer, pH 6.5, and loaded on a hydroxylapatite column (0.7×5 cm) equilibrated with the same buffer. Fractions showing highest activity were pooled, dialyzed against 20 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, and 10%(v/v) glycerol, and concentrated by ultrafiltration through a PM10 membrane. The purified protease Ci preparation was kept frozen at

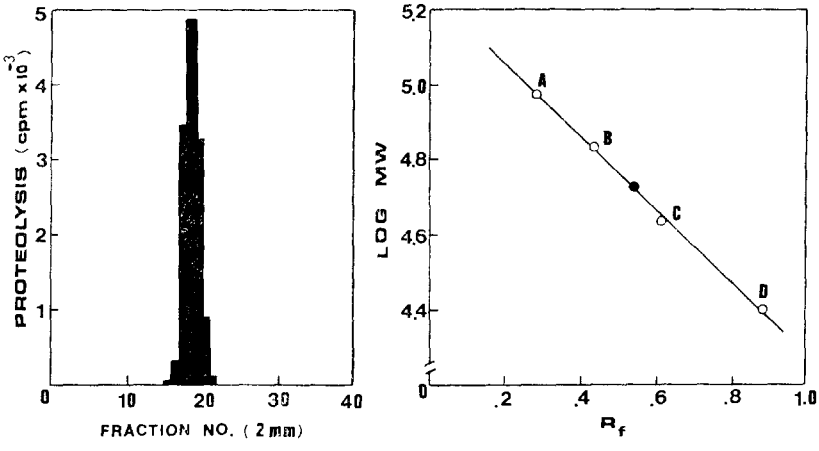

Fig. 3. Two dimensional polyacrylamide gel electrophoresis of protease Ci.

(A) The first dimensional disc gel electrophoresis was carried out by loading 0.15 mg of protease Ci on a tube gel (1×8 cm seperating gel with 1×2 cm stacking gel) as described in the *Materials and Methods*. After the run, the gel was sliced in 2 mm pieces and extracted with 50 mM Tris-HCl, pH 7.8, containing 5 mM $MgCl_2$ at 4°C overnight. The proteolytic activity of each fractions extracted was assayed for 2 hrs at 37°C by incubating the reaction mixtures containing the same buffer, 20 μl of the extracts, and 5 μg of [^{125}I]-insulin.

(B) The second dimensional slab gel electrophoresis in the presence of SDS was performed according to the Laemmli's procedure (1970). The extracted fraction (fraction numbers 17 to 19) containing highest insulin-degrading activity were pooled, and an aliquot (50 μl) of the pool was incubated with 2.0% SDS, 0.05% bromophenol blue, 10% glycerol, and 100 mM Tris-HCl (pH 6.8). After the electrophoresis of the sample, the gel was stained with Coomassie blue R250. Markers used for the estimation of molecular weight were phosphorylase b(A; M_r of 94,000), bovine serum albumin (B; 68,000), ovalbumin (C; 44,000) and chymotrypsinogen A (D; 25,000). The closed circle indicates where the subunits of protease Ci migrated.

−20°C. All subsequent experiments in this paper used this purified material.

The purification steps were summerized in Table 1. Because the crude extract of *E. coli* contained another protease active against insulin (protease Pi) (Cheng and Zipser, 1979; Swamy and Goldberg, 1981), the specific activity and the recovery at each step cannot be calculated for protease Ci until after the DEAE-cellulose step.

Purity

The purity of the protease obtained by these procedures was analyzed by electrophoresis on polyacrylamide gels under nondenaturing condition. When analyzed on 8.75% gels at pH 8.9, two major bands (Rf's of 0.34 and 0.49) and two minor bands (those of 0.47 and 0.57) were appeared after staining the gel with Coomassie blue R250. When a duplicate gel was analyzed for insulin-degrading activity, there was a peak of the proteolytic activity which coincided with the protein band with a relative mobility of 0.49 under the conditions described above (Fig. 3A). No other protein bands showed the activity against insulin, and therefore protease Ci appeared to have Rf value of 0.49. Further attempts on the purification of protease Ci to homogeneity was so far unsuccessful due to instability of the enzyme and to its existence in low amount in the cell (data not shown).

Molecular weight and subunit size

The native size of protease Ci was estimated to be 120,000 by gel filtration on a Sephadex G200 column. To determine the subunit size of the protease, two dimensional gel electrophoresis was performed as described in the Materials and Methods, since the enzyme preparation obtained after the final purification step was not homogeneous (Table 1). The subunit size of protease Ci was estimated to be 54,000 daltons by second dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate after the first dimensional gel electrophoresis under nondenaturing condition (Fig. 3B). Thus,

Table 1. Summary of purification.

Steps	Total protein (mg)	Total* units	Specific activity (units/mg/h)	Yield (%)	Purification fold
Crude extract	4,280	—**	—	—	—
DEAE-cellulose	208.8	617.6	3.0	100	1.0
DEAE-Sepharose	95.0	690.4	7.3	112	2.4
pH precipitation	81.0	571.6	7.1	93	2.4
Ammonium sulfate precipitation	70.4	597.6	8.5	97	2.8
Ultrogel AcA34	20.6	362.0	17.6	59	5.9
Butyl-agarose	3.6	204.8	56.9	33	19.0
Hydroxylapatite	0.9	144.0	160.0	23	53.3

Determination of insulin-degrading activity at each step was carried out under linear assay conditions as described in the *Materials and Methods.*

* One unit was defined as 1 μg of insulin hydrolyzed per mg protein per hr.

** Not estimatable (see the text).

protease Ci appeared to be consisted of two identical subunits.

pH optimum and isoelectric point

The effect of varying pH on the insulin-degrading activity of protease Ci was examined by using various buffers. The enzyme was maximally active at pH 7.5, but was nearly inactive at pH values below 6.0 and above 8.5. Isoelectric point of protease Ci was estimated to be about 5.5 by isoelectric focusing gel electrophoresis using polyacrylamide.

Effect of inhibitors of protease and divalent cations

The effect of various protease inhibitors and site-specific reagents were tested by pre-incubating them with the enzyme for 20 min at 37°C and then measuring [^{125}I]-insulin hydrolysis for 1 hr at the same temperature. As shown in Table 2, *o*-phenanthroline at 1 mM markedly inhibited protease Ci while little effect was shown by EDTA at the same concentration. Thus, protease Ci appeared to be a metalloprotease.

While phenylmethylsulfonylfluoride showed no effect on protease Ci, diisopropylfluorophosphate at 1 mM inhibited by about 30% of its activity. The specific chloromethyl

Table 2. Effect of inhibitors on protease Ci.

Inhibitors	Concentration	% Activity
None	—	100
EDTA	1 mM	87
o-phenanthroline	1 mM	23
m-phenanthroline	1 mM	69
N-tosyl-L-phenylalanine-chloromethylketone	1 mM	85
N-tosyl-L-lysine-chloromethylketone	1 mM	76
Phenylmethylsulfonylfluoride	1 mM	100
Diisopropylfluorophosphate	1 mM	67
N-ethylmaleimide	1 mM	68
Iodoacetamide	1 mM	98
p-Hydroxymercuribenzoic acid	1 mM	0
Ep475	50 μM	101
Leupeptin	5 μg/ml	89
Pepstatin	5 μg/ml	100
Bestatin	5 μg/ml	94
Bacitracin	5 μg/ml	60

Protease Ci was dialyzed against 50 mM Tris-HCl, pH 7.8, containing 0.1 mM EDTA for 6 hrs during which period the dialysis buffer was changed every 2 hrs. Reaction mixtures (0.5 ml) containing 0.5 μg of the enzyme, the indicated inhibitors, 50 mM Tris-HCl (pH 7.8), and 5 mM $MgCl_2$ were incubated for 10 min at 37°C prior to the addition of [^{125}I]-insulin. When metal chelating agents were tested, the addition of $MgCl_2$ was omitted. Some reagents that could not be solubilized in H_2O were dissolved either in ethanol or dimethylsulfoxide before being added to the reaction mixtures. When such reagents were used, the control mixtures contained equal amounts of the solvent only. Incubations were then performed at 37°C for 1 hr by adding 5 μg of [^{125}I]-insulin. Insulin hydrolysis in the absence of inhibitor is expressed as 100% activity.

ketone inhibitors of trypsin (N-tosyl-L-lysine-chloromethylketone) and of chymotrypsin (N-tosyl-L-phenylalanine-chloromethylketone) showed little effect on the enzyme, nor did the antifungal peptide aldehydes (Aoyagi and Umezawa, 1975), except bacitracin which showed inhibition by about 40% at 5 μg per ml concentration (Table 2).

At 1 mM concentrations, N-ethylmaleimide inhibited the insulin-hydrolysis by about 30 % and *p*-hydroxymercuribenzoic acid abolished the activity, but iodoacetamide showed little or no effect. Since the specific inhibitors of sulfhydryl enzymes including Ep475 and leupeptin exhibited no effect on protease Ci, the inhibitory effect by N-ethylmaleimide and *p*-hydroxymercuribenzoic acid appeared to be due to their nonspecific interaction with the enzyme (Table 2).

The influence of divalent metal ions on the enzyme activity was also tested after dialyzing protease Ci against 50 mM Tris-HCl, pH 7.8, containing 0.1 mM EDTA. While $ZnCl_2$ showed strong inhibitory effect, the other metal ions tested were all stimulatory. Furthermore, Co^{++} and Mn^{++} increased the insulin-degrading activity of protease Ci by

Table 3. Effect of divalent metal ions on protease Ci.

Cations	Concentration(mM)	% Activity	Cations	Concentration(mM)	% Activity
None	—	100	$MnCl_2$	1	196
				10	384
$MgCl_2$	1	111	$ZnCl_2$	0.1	16
	10	160		1	4
$CaCl_2$	1	113	$CoCl_2$	0.1	245
	10	189		1	326

Protease Ci was dialyzed in the same buffer used in Table 2. Reaction mixtures contained 0.5 μg of the enzyme, 50 mM Tris-HCl, and the indicated cations. Incubations were performed at 37°C for 1 hr by adding 5 μg of [^{125}I]-insulin. Realtive activities in the presence and absence of the cations were expressed as described in Table 2.

Table 4. Substrates of protease Ci.

Substrates	Concentrations* (μg)	Relative Activity (μg hydrolyzed/mg/h)
Insulin	5	153.4
Glucagon	25	1,476.4
Bovine growth hormone	12.5	22.2
Bovine serum albumin	25	0.0
Casein	25	9.7
Globin	25	3.9

Reaction mixtures contained 0.5 μg of protease Ci, protein substrates, 50 mM Tris-HCl (pH 7.8), and 5 mM $MgCl_2$, and were incubated for 1 hr at 37°C. The hydrolysis of the radioactively labeled protein substrates was determined as described in *Materials and Methods.*

* Concentrations indicated are the amounts of protein added to the 0.5 ml reaction mixtures.

about 3 to 4 fold at their concentrations of 1 and 10 mM, respectively (Table 3). These data further indicate that protease Ci is a metalloprotease, in addition to the fact that *o*-phenanthroline is also inhibitory (see above). The activity of protease Ci in the absence of divalent cations probably due to tight binding of protease Ci with contaminated metal ions during purification procedure, which is typical in most of metalloproteases (Kirschner and Goldberg, 1981).

Substrate specificity

In addition to insulin, protease Ci hydrolyzed glucagon. The enzyme also degraded bovine growth hormone but at a much slower rate. Little or no degradation was observed against globin, casein and bovine serum albumin (Table 4).

To test if protease Ci is an endoprotease or exopeptidase, the reaction product against [^{125}I]-insulin (i.e., acid-soluble materials) were analyzed on a Sephadex G15 column. As shown in Fig. 4, nearly all of the radioactivity was eluted near to the bed volume (30 ml), suggesting that the size of the reaction product is smaller than a single amino acid. This phenomenon is known to be due to nonspecific interaction of iodinated materials with the

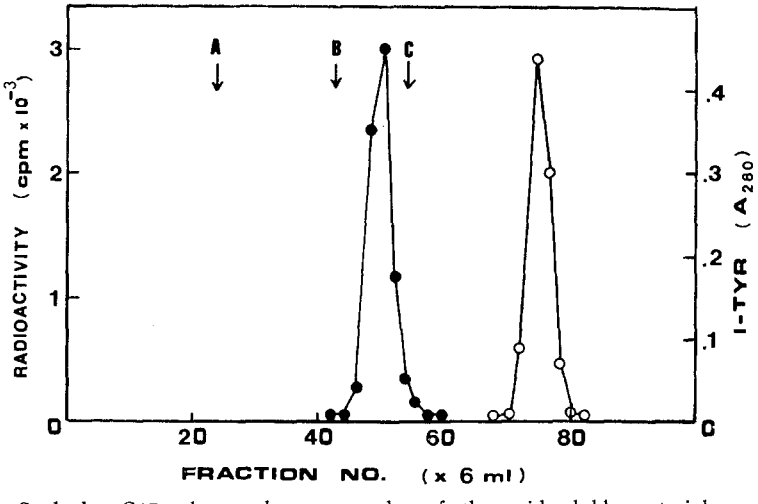

Fig. 4. Sephadex G15 column chromatography of the acid-soluble materials produced by protease Ci.
Protease Ci (2 μg) was incubated with 10 μg of [^{125}I]-insulin for 2 hrs at 37°C. The proteins were then precipitated by addition of trichloroacetic acid as described in the *Materials and Methods*, and the supernatant was applied to a Sephadex G15 column (1×40 cm) equilibrated with 0.2 M acetic acid. Fractions of 0.6 ml were collected at a flow rate of 5 ml per hr and 0.4 ml of each fraction was counted in a Gamma counter (●). After washing the column extensively with the same buffer, 1 mg/ml of unlabeled monoiodo-tyrosine in the buffer was loaded on the same column. Fractions were collected as described above, and the elution profile of the amino acid was monitored by measuring absorbance at 280 nm (○). The void volume of the column was indicated by A, and the bed volume by C. B represents where [^{3}H]-leucine was eluted.

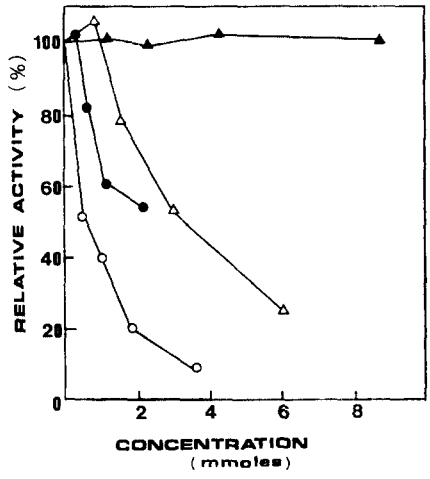

Fig. 5. Effect of unlabeled insulin, proinsulin and insulin A and B chains on the degradation of [^{125}I]-insulin by protease Ci.

Reaction mixtures contained 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 5 μg of [^{125}I]-insulin, and various concentrations of unlabeled insulin (○), proinsulin (●), insulin A chain (▲), or B chain (△). Incubations were performed by adding 5 μg of protease Ci at 37°C for 1 hr. The proteolysis occurred in the absence of added unlabeled proteins was expressed as 100% activity.

Sephadex matrix. Such an interaction was further confirmed by applying nonradioactive monoiodotyrosine to the same colume from which the amino acid was eluted far after the bed volume (45 ml). Therefore, it is at least certain that the reaction products have greater molecular weights that monoiodo-tyrosine has, although the relative size of the products are not estimatable. Furthermore, prolonged incubation with increased amounts of the enzyme did not generate any radioactive products of similar size with monoiodo-tyrosine. Thus, protease Ci appears to be an endoprotease that carries out limited number of cleavage.

To determine the cleavage sites on insulin by protease Ci, unlabeled insulin A or B chain or proinsulin was added to the reaction mixtures containing the enzyme and [^{125}I]-insulin. Fig. 5 shows that addition of cold-insulin A chain competed for the [^{125}I]-insulin-degrading activity by protease Ci but B chain did not. Thus, the proteolytic activity of the enzyme appears to be specific on insulin A chain. Both the unlabeled insulin and proinsulin inhibited the activity against the radioactive insulin, since both the polypeptides contained the A chain. However, intact insulin appeared to be more susceptible to protease Ci than proinsulin did, probably due to its native conformation.

DISCUSSION

In this paper, we report the purification and characterization of a new metalloendoprotease from *E. coli* that hydrolyzes insulin, glucagon and bovine growth hormone. This enzyme has been reported to be localized to the cytoplasm (Swamy and Goldberg, 1982). By many criteria, protease Ci appears distinct from protease Pi that also degrades insulin (Swamy and Goldberg, 1982; Goldberg *et al.*, 1981). In contrast to the former, the enzyme is localized to the periplasm and is inhibited by EDTA in addition to by *o*-phe-

nanthroline. Protease Ci also differs from other soluble *E. coli* proteases (proteases Do, Re, Mi, Fa, So, and La) that degrade casein but not insulin (Goldberg *et al.*, 1981).

Several peptide-degrading enzymes have previously been isolated from *E. coli*, but they also differ from protease Ci. Protease I (Pacaud and Ureil, 1971) and protease II (Pacaud and Richard, 1975) hydrolyze specific amino acid esters, but show little or no activity against proteins, such as insulin, casein and globin (Kowit *et al.*, 1976). ISP-L-ECO (Strongin *et al.*, 1981) is an enzyme resembling subtilisin in cleaving the peptide substrate N-carbobenzoxy-L-alanine-L-alanine-L-leucine-p-nitroanilide; however, protease Ci does not hydrolyze this peptide. Protease IV and V (Pacaud, 1982; Regnier, 1981), which have recently been isolated from *E. coli* membrane, can only be solubilized in the presence of detergents.

Further purification of protease Ci to homogeneity and the isolation of mutant cells lacking this enzyme is essential for the elucidation of the pathway of intracellular protein degradation and the physiological role of protease Ci. Protease Ci may play a role in the degradation of abnormal proteins (Goldberg and St. John, 1976) that takes place subsequent to the initial ATP-dependent hydrolysis that are catalyzed by protease La (Charette *et al.*, 1981; Chung and Goldberg, 1981, 1982; Larimore *et al.*, 1982). In addition, Talmadge and Gilbert (1982) have recently reported that overproduced human proinsulin by gene manipulation in *E. coli* is rapidly degraded in the cytoplasm perhaps by protease Ci. These data are in consistent with our data showing that the hydrolysis of [^{125}I]-insulin is competed by addition of unlabeled proinsulin at low concentrations (Fig. 5). Thus, the isolation of mutant cells lacking protease Ci appears to be essential for the overproduction of foreign eukaryotic gene product, at least in case of human insulin or proinsulin by the genetic technology. Therefore, it is certainly of worth to perform such studies (i.e., the purification of protease Ci and isolation of mutants lacking protease Ci) to raise antibody against the enzyme, to develop a sensitive assay that would readily distinguish it from other proteases in *E. coli*, and consequently to elucidate the physiological role of protease Ci.

SUMMARY

A cytoplasmic endoprotease, named protease Ci, has been partially purified by classical chromatographic procedures. This enzyme degrades insulin, glucagon and bovine growth hormone to trichloroacetic acid-soluble materials, but shows little or no hydrolysis of bovine serum albumin, casein or globin. It has a molecular weight of about 120,000 as determined by gel filtration on Sephadex G200, and it appears to be consisted of two identical subunits having molecular weight of 54,000 when estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Protease Ci has an optimum pH of 7.5, and has an isoelectric point of 5.5. This enzyme is a metallopro-

tease, since it is inhibited by *o*-phenanthroline and can be activated by the addition of divalent metal cations, such as Mn^{++} and Co^{++}. Protease Ci is inhibited by *p*-hydroxymercuribenzoic acid, but not by either of leupeptin or Ep475 which are specific inhibitors of sulfhydryl protease. It is distinct from protease Pi, a periplasmic insulin degrading enzyme, since protease Ci is localized to the cytoplasm. The physiological function of protease Ci is presently unknown.

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