The Study of *Escherichia coli* Proteases. Intracellular Serine Protease of *E. coli* – an Analogue of *Bacillus* Proteases

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Two serine proteases in extracts of *Escherichia coli* grown to stationary phase were purified to homogeneity using affinity chromatography on gramicidin S–Sephrose 4B. One enzyme was closely related to, if not identical with, the ‘trypsin-like’ protease II of *E. coli*. The other was capable of cleaving the subtilisin chromogenic substrate *N*-carbobenzoxy-L-alanyl-L-alanyl-L-leucine-*p*-nitroanilide and resembled the intracellular serine proteases of *Bacillus* spp. The amino acid composition of this *E. coli* protease was similar to that of the *Bacillus licheniformis* enzyme. These data indicate a relationship between proteolytic enzymes of evolutionary distant Gram-negative Enterobacteriaceae and Gram-positive spore-forming *Bacillus*.

INTRODUCTION

The existence of proteolytic activity within bacterial cells has been known for many years, but few studies of purified bacterial intracellular proteases have appeared in the literature. Intracellular serine proteases have been extracted and purified to homogeneity from sporulating cells of certain *Bacillus* species, namely *Bacillus subtilis*, strains A-50 and Marburg 168 (Stepanov *et al.*, 1977*a*; Strongin *et al.*, 1978*a*; Strongin *et al.*, 1978*c*), *Bacillus thuringiensis* var. *berliner* (Lecadet *et al.*, 1977) and *Bacillus licheniformis* (Strongin *et al.*, 1979*b*). The amino acid composition, *N*-terminal sequence [homologous with those of extracellular serine proteases, the subtilisins, of *Bacillus* spp. (Strongin *et al.*, 1978*b*)], cellular distribution (Stepanov *et al.*, 1977*b*; Strongin *et al.*, 1979*a*), involvement in protein turnover and spore-coat processing (Cheng & Aronson, 1977*a, b*), specific action on RNA polymerase (Lecadet *et al.*, 1977) and some other properties and functions were reported. Comparable data are lacking for Enterobacteriaceae proteases, and so the possible relationship between the intracellular serine proteases of these distant bacterial species remains uncertain. Although *Escherichia coli* is the most extensively studied bacterial species, present knowledge of its proteolytic system is also rather limited. The scarcity of biochemical data on the purified *E. coli* enzymes is caused by difficulties in their isolation, the extremely low concentration within the cells and the absence of specific affinity adsorbents suitable for the simultaneous isolation of several proteases. ‘Chymotrypsin-like’ protease I (Pacaud & Uriel, 1971; Pacaud *et al.*, 1976) and ‘trypsin-like’ protease II (Pacaud & Richaud, 1975; Pacaud, 1976) are the only endopeptidases of *E. coli* to have been purified and studied in some detail.

In the present paper we report the isolation of *E. coli* intracellular serine proteases applying the same approach for their purification as previously employed for proteases from *Bacillus* spp. (Strongin *et al.*, 1978*a*). The main purification step used was affinity chromatography

on Sepharose 4B containing covalently bound cyclopeptide antibiotic gramicidin S (Izotova *et al.*, 1978). This adsorbent was satisfactory for the isolation of at least two distinct *E. coli* intracellular serine proteases in high yields and of sufficient purity for their preliminary enzymological characterization and comparison with the *Bacillus* enzymes.

METHODS

Materials. Analytical grade reagents were used throughout this work. EDTA, EGTA, diisopropylfluorophosphate, phenylmethylsulphonyl fluoride and *N*-benzoyl-DL-arginine-*p*-nitroanilide (Bz-L-Arg-*p*NA) were purchased from Serva; DEAE-cellulose DE52 was from Whatman; Sepharose 4B was from Pharmacia. *N*-Carbobenzoxy-L-alanyl-L-alanyl-L-leucine-*p*-nitroanilide (Z-L-Ala-L-Ala-L-Leu-*p*NA) was synthesized in our laboratory by Dr L. A. Lyublinskaya as described earlier (Lyublinskaya *et al.*, 1974, 1977). The affinity adsorbent gramicidin S-Sepharose 4B was prepared according to Strongin *et al.* (1978a); as judged by amino acid analysis, it contained 1 μ mol ligand per ml wet adsorbent. Enzymes and proteins were obtained from Serva, except subtilisin BPN' (trade name Nagarse) was from Nagase, Japan, and *Streptomyces* subtilisin protein inhibitor IT-Aj1 (Ikenaka *et al.*, 1974) was isolated from *Actinomyces janthinus* and kindly provided by Dr D. N. Chermensky (Institute of Biochemistry and Physiology of Microorganisms, Academy of Science of the USSR, Poustchino, Moscow Region).

Organism. *Escherichia coli* K12 strain W3350 (*gal*⁻B1⁻), bearing plasmid RSF2124 that determines penicillin resistance, was used. Cultures were grown for 18 h at 37 °C in a 100 l fermenter containing 75 l of M9 minimal medium (comprising 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl and 0.1 g MgSO₄, per litre) supplemented with 10% (v/v) Hottinger broth, 0.8% (w/v) glucose and 100 μ g penicillin ml⁻¹. Stationary phase cells were harvested by centrifugation and frozen at -20 °C until use.

Assay of serine protease. Activity was assayed spectrophotometrically at 410 nm with Bz-L-Arg-*p*NA or Z-L-Ala-L-Ala-L-Leu-*p*NA as the substrate. Reactions were carried out at 40 °C in 50 mM-Tris/HCl buffer, pH 8.5, containing 1 mM-CaCl₂ (buffer A). The reaction mixture contained 2.5 ml of Bz-L-Arg-*p*NA (0.435 mg ml⁻¹ in buffer A supplemented with 2% dimethylformamide) and 10 to 100 μ l of the enzyme preparation, or 1.25 ml of buffer A, 0.25 ml of Z-L-Ala-L-Ala-L-Leu-*p*NA (0.5 mg ml⁻¹ in dimethylformamide) and 10 to 100 μ l of the enzyme preparation. One unit of activity is defined as the amount of enzyme liberating 1 μ mol *p*-nitroaniline min⁻¹ from the substrate under the conditions specified above, using $\epsilon = 8900$ l mol⁻¹ cm⁻¹ for *p*-nitroaniline in all calculations. Protein concentration was calculated from the absorbance at 280 nm.

Acrylamide gel electrophoresis. Electrophoresis was performed in 7.5 and 10% (w/v) acrylamide gels (acrylamide/*N,N'*-methylenebisacrylamide, 19:1) as described by Davis (1964). The mobility (R_F) of protein bands was determined relative to bromophenol blue. Proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Serva) in 7.5% (v/v) acetic acid.

Isolation of the enzymes. All procedures were carried out at 4 °C, unless stated otherwise. Activity was assayed with both *p*-nitroanilide substrates throughout the purification. All buffers used contained 1 mM-CaCl₂ except those for acrylamide gel electrophoresis.

Cells (150 g wet wt) were thawed, resuspended in buffer A and ultrasonically disrupted in a 150 W MSE sonifier. The homogenate (about 1 l) was centrifuged at 30000 g for 60 min, and to the supernatant (crude cell extract) 10% streptomycin sulphate solution was added to give a final concentration of 1%. After several hours at 0 °C the precipitated nucleic acids were removed by centrifugation (30000 g, 30 min). The supernatant was then treated with solid ammonium sulphate, and the fraction precipitated between 0.35 and 0.75 saturation was collected by centrifugation (30000 g, 60 min), dialysed against buffer A overnight and applied to a DEAE-cellulose DE52 column (5 $\times$ 25 cm) equilibrated with buffer A. The column was eluted with 2 l of buffer A, followed by 600 ml of a Tris/HCl buffer linear gradient (50 to 500 mM, pH 8.5). The active fractions (Fig. 1) were pooled, dialysed against buffer A and loaded on to a gramicidin S-Sepharose 4B column (2 $\times$ 45 cm). The proteins were eluted stepwise with 1 l of buffer A, 300 ml of buffer A containing 1 M-NaCl and 200 ml of buffer A containing 1 M-NaCl and 25% 2-propanol (Fig. 2). The fractions active against Bz-L-Arg-*p*NA and Z-L-Ala-L-Ala-L-Leu-*p*NA were pooled separately and dialysed against buffer A. After concentration by ultrafiltration through a UM-2 Diaflo membrane in a 10 ml Amicon cell, they were subjected to acrylamide gel electrophoresis. For these runs, 6 $\times$ 100 mm tubes with 7.5% gel loaded with 1 mg protein were used, and electrophoresis was carried out at 0 °C in Tris/glycine buffer, pH 8.3 (Davis, 1964). The protein bands possessing protease activity were visualized in ultraviolet light after incubating gels in Bz-L-Arg-*p*NA or Z-L-Ala-L-Ala-L-Leu-*p*NA solutions as described previously (Lyublinskaya *et al.*, 1974; Strongin *et al.*, 1976). The bands were cut out, and the proteins were extracted from the crushed gel with buffer A containing 100 mM-NaCl overnight. Gel particles were removed by filtration through a sintered glass no. 4 filter, and the filtrates were dialysed against buffer A and concentrated by ultrafiltration as above.

Fig. 1. Chromatography of proteases on DEAE-cellulose DE52. Dialysed enzyme preparation obtained after ammonium sulphate fractionation was applied to a DEAE-cellulose column (5×25 cm) equilibrated with buffer A. Fractions of 10 ml were collected at a flow rate of 50 ml h^{-1} . The arrow indicates the beginning of a 600 ml linear gradient of 50 to 500 mM-Tris/HCl buffer, pH 8.5, containing 1 mM- Ca^{2+} . —, Absorbance at 280 nm; ○, activity with Bz-L-Arg-pNA; ●, activity with Z-L-Ala-L-Ala-L-Leu-pNA.

Fig. 2. Chromatography of proteases on gramicidin S-Sepharose 4B. Dialysed enzyme preparation obtained after DEAE-cellulose chromatography was applied to an affinity column (2×45 cm) equilibrated in buffer A. Fractions of 5 ml were collected at a flow rate of 40 ml h^{-1} . The first arrow indicates the beginning of elution with buffer A containing 1 M-NaCl; the second arrow indicates the beginning of elution with buffer A containing 1 M-NaCl and 25% 2-propanol. —, Absorbance at 280 nm; ○, activity with Bz-L-Arg-pNA; ●, activity with Z-L-Ala-L-Ala-L-Leu-pNA.

Amino acid analysis. Before amino acid analysis, the enzyme samples were inactivated with 1 mM-diisopropyl-fluorophosphate for 60 min at 20°C , extensively dialysed against water, freeze-dried and hydrolysed with 5.7 M-HCl in the sealed tubes *in vacuo* for 24 h at 110°C . The amino acid composition of the hydrolysates was determined using a Durrum D500 amino acid analyser.

Gel filtration. The molecular weight of the enzymes was estimated by Sephadex G-100 gel filtration according to Andrews (1964). A column (1.5×150 cm) was run in buffer A containing 100 mM-NaCl at a flow rate of 6 ml h^{-1} ; fractions of 2 ml were collected and assayed for enzyme activity.

Table 1. *Purification of serine proteases ISP-A-Eco and ISP-L-Eco from extracts of E. coli grown to stationary phase*

Purification step	Protein (mg)	Total activity* (units)	Specific activity (units mg ⁻¹)	Yield (%)	Purification factor
Crude cell extract	—				
ISP-A-Eco		26	—	100	—
Z-L-Ala-L-Ala-L-Leu-pNA-cleaving activity†		8	—	100	—
Streptomycin sulphate	15000				
ISP-A-Eco		25	0.00165	96	1
Z-L-Ala-L-Ala-L-Leu-pNA-cleaving activity		7.5	0.00050	94	1
Ammonium sulphate	10000				
ISP-A-Eco		24	0.0024	92	1.45
Z-L-Ala-L-Ala-L-Leu-pNA-cleaving activity		7.5	0.00075	94	1.5
DEAE-cellulose DE52	1700				
ISP-A-Eco		21.5	0.0126	83	7.5
Z-L-Ala-L-Ala-L-Leu-pNA-cleaving activity		7.0	0.0041	88	8.0
Affinity chromatography on gramicidin S-Sepharose 4B					
ISP-A-Eco	16	13.0	0.81	50	490
ISP-L-Eco	6	1.6	0.14	20	280
Acrylamide gel electrophoresis‡					
ISP-A-Eco	0.17	1.3	7.5	5	4550
ISP-L-Eco	0.20	0.28	1.4	3.5	2800

* Activity of ISP-A-Eco was determined with Bz-L-Arg-pNA and ISP-L-Eco with Z-L-Ala-L-Ala-L-Leu-pNA as substrates.

† Indicated as Z-L-Ala-L-Ala-L-Leu-pNA-cleaving activity since up to the affinity chromatography step this activity was due to at least two proteases.

‡ At the last preparation step there was a marked inactivation of ISP-A-Eco, presumably due to autolysis. A similar, but not so pronounced, effect was observed for ISP-L-Eco.

RESULTS

Isolation of two E. coli intracellular proteases

Extracts of *E. coli* contained two types of proteolytic activity, one active with the subtilisin chromogenic substrate Z-L-Ala-L-Ala-L-Leu-pNA and the other capable of hydrolysing the trypsin chromogenic substrate Bz-L-Arg-pNA. The activity towards Z-L-Ala-L-Ala-L-Leu-pNA was due to at least two enzymes (see below) and was two orders of magnitude lower than the corresponding activity in crude extracts of *B. subtilis* A-50 (Strongin *et al.*, 1978*a*). The activity towards Bz-L-Arg-pNA behaved as a single protease and was termed intracellular protease-A-Eco (ISP-A-Eco).

Activities towards both substrates were purified from the crude cell extracts of *E. coli*. A key step in the purification procedure was affinity chromatography on gramicidin S-Sepharose 4B. ISP-A-Eco was retained on this adsorbent and was quantitatively eluted as the ionic strength of the eluting buffer was increased by adding 1 M-NaCl (Fig. 2). About 80% of the total activity with Z-L-Ala-L-Ala-L-Leu-pNA passed freely through the affinity column. The remaining 20% of the total Z-L-Ala-L-Ala-L-Leu-pNA-cleaving activity applied to the column was tightly bound to the adsorbent and required high ionic strength plus 25% 2-propanol for elution (Fig. 2). This activity was termed intracellular protease-L-Eco (ISP-L-Eco). Since the capacity of the column was sufficient to bind the total amount of Z-L-Ala-L-Ala-L-Leu-pNA-cleaving activity applied, it seems that *E. coli* possesses at

Fig. 3. Acrylamide gel electrophoresis pattern of purified proteases ISP-A-*Eco* and ISP-L-*Eco*. A, Freshly prepared ISP-A-*Eco*; B, ISP-A-*Eco* after 3 d storage in buffer A at 4 °C; C, ISP-L-*Eco*. The samples (20 to 30 µg) were run in 7.5% acrylamide gels; migration was from top to bottom.

least two proteases active against this substrate. Only the activity which was tightly bound to gramicidin S-Sepharose 4B was studied further.

Pacaud *et al.* (1976) found that *E. coli* contained protease I, an enzyme capable of cleaving *p*-nitrophenyl esters of *N*-substituted amino acids and possessing a 'chymotrypsin-like' substrate specificity. It may well be that this enzyme could cleave Z-L-Ala-L-Ala-L-Leu-*p*NA and possibly the activity towards this substrate that did not bind to gramicidin S-Sepharose 4B was protease I.

After the affinity chromatography step in the purification procedure, ISP-A-*Eco* and ISP-L-*Eco* appeared to be about 10% pure, as judged by acrylamide gel electrophoresis followed by protein staining with Coomassie Brilliant Blue. The bands corresponding to ISP-A-*Eco* and ISP-L-*Eco* were visualized by incubating half of the gel in the corresponding chromogenic substrate solution.

The final purification of the enzymes was achieved by acrylamide gel electrophoresis and resulted in electrophoretically pure preparations of ISP-A-*Eco* and ISP-L-*Eco* (Fig. 3). The purification procedure is summarized in Table 1.

Properties of ISP-A-Eco

The enzyme had an approximately 10-fold higher specific activity than commercially available trypsin with Bz-L-Arg-*p*NA (a commonly used trypsin substrate) but was completely inactive with Z-L-Ala-L-Ala-L-Leu-*p*NA, a substrate specific for subtilisins and 'subtilisin-like' intracellular serine proteases of *Bacillus* spp. (Strongin *et al.*, 1978*a*). When incubated with 1 mM-diisopropylfluorophosphate in buffer A for 60 min at 20 °C, ISP-A-*Eco* was completely inactivated, whereas under comparable conditions 1 mM-phenylmethylsulphonyl fluoride (another common serine protease inhibitor) was without any inhibitory effect. Similar behaviour has been reported for proteases I and II of *E. coli* and for acetylcholinesterase (Pacaud & Uriel, 1971; Pacaud & Richaud, 1975).

Chelating agents [EDTA and EGTA (5 mM)] and a 50-fold excess of soybean trypsin inhibitor did not affect the activity of ISP-A-*Eco*. In contrast, subtilisin protein inhibitor IT-Ajl in a 30 to 50-fold molar excess inhibited ISP-A-*Eco* completely.

Table 2. *Properties of intracellular serine proteases of E. coli and B. subtilis A-50*

The properties of protease I of *E. coli* are from Pacaud & Uriel (1971) and Pacaud *et al.* (1976), protease II of *E. coli* from Pacaud & Richaud (1975) and intracellular serine protease of *B. subtilis* A-50 (ISP-*Bsu* A-50) from Strongin *et al.* (1978*a*).

Property	Protease I	Protease II	ISP-A- <i>Eco</i>	ISP-L- <i>Eco</i>	ISP- <i>Bsu</i> A-50
Activity with Bz-L-Arg-pNA*	0	14.1	7.5	0	0
Activity with Z-L-Ala-L-Ala-L-Leu-pNA*	—	—	0	1.4	18
Percentage of activity retained after treatment with:					
Diisopropylfluorophosphate	0	0	0	0	0
Phenylmethylsulphonyl fluoride	100	100	100	0	0
EDTA	100	100	100	0	0
Soybean trypsin inhibitor	100	100	100	100	100
Subtilisin inhibitor IT-Aj1	—	—	0	0	0
Electrophoretic mobility in 7.5% acrylamide gel†	—	—	0.4	0.7	0.7
Molecular weight					
From Sephadex G-100 gel filtration in the presence of 0.1 M NaCl or KCl	43 000	75 000	75 000	55 000	55 000
From SDS-gel electrophoresis	21 000	60 000‡	—	—	30 000
Stability in buffer at 4 °C§	S	U	U	S	S

* Specific activity (units mg⁻¹) under the standard conditions.

† Data for proteases I and II electrophoretic mobilities are not comparable with ours.

‡ Polypeptide chain molecular weight of protease II was estimated (presumably) in the absence of reducing agents, such as 2-mercaptoethanol (Pacaud & Richaud, 1975).

§ S, Stable; U, unstable.

The molecular weight of the protease estimated by Sephadex G-100 gel filtration in buffer A containing 100 mM-NaCl, pH 8.5, was 75 000 ± 2000.

The enzyme was unstable in buffer A at 4 °C and was degraded over several days, presumably due to autolysis. Only fresh preparations of the enzyme gave a single protein band (R_F 0.4) when subjected to electrophoresis in 7.5% acrylamide gels. After storage in buffer A at 4 °C, a second rather diffuse band (R_F near 0.45) appeared in the protease preparation (Fig. 3). The properties of the enzyme are summarized in Table 2.

The most characteristic feature of the amino acid composition of ISP-A-*Eco* was the high content of dicarboxylic amino acids, especially Glx (Table 3). It also contained rather high concentrations of Lys, Leu, Phe and Arg and low concentrations of Val and Ala. Based on the amino acid composition, a minimum molecular weight of approximately 30 000 was calculated (Table 3), a value in agreement with those for intracellular serine proteases from *Bacillus* spp. (Strongin *et al.*, 1978*a*).

Properties of ISP-L-Eco

This enzyme was clearly different from ISP-A-*Eco* in several respects. No activity was observed with the trypsin substrate Bz-L-Arg-pNA, but Z-L-Ala-L-Ala-L-Leu-pNA was rapidly hydrolysed. Its activity against the latter substrate was similar to that of subtilisin BPN' (Nagarse) but substantially lower than that of intracellular serine protease from *B. subtilis* A-50 (Strongin *et al.*, 1978*a*). The enzyme was completely inactivated by 1 mM-diisopropylfluorophosphate, 1 mM-phenylmethylsulphonyl fluoride and by 2 mM-EDTA or EGTA (a consequence of a strict Ca²⁺-dependence). In addition, the enzyme was completely inhibited in the presence of a 3 to 5-fold molar excess of subtilisin protein inhibitor IT-Aj1, but was not inhibited by a 50-fold molar excess of soybean trypsin inhibitor. ISP-L-*Eco* was stable when stored in buffer A at 4 °C for several weeks.

Table 3. Amino acid composition of intracellular serine proteases of *E. coli*, *B. subtilis* and *B. licheniformis*

The amino acid composition of protease I of *E. coli* is from Pacaud *et al.* (1976), intracellular serine protease of *B. subtilis* 168 (ISP-*Bsu* 168) from Strongin *et al.* (1979*a*), intracellular serine protease of *B. subtilis* A-50 (ISP-*Bsu* A-50) from Strongin *et al.* (1978*a*) and intracellular serine protease of *B. licheniformis* 1001 (ISP-*Bli*) from Strongin *et al.* (1979*b*). For each enzyme, the analytical data show the percentage composition (*b*) and the nearest integer values calculated from the molecular weight (*a*). The molecular weights of the ISP-A-*Eco* and ISP-L-*Eco* polypeptide chains were assumed to be about 30000, to allow ready comparison of their composition with the *Bacillus* enzymes.

Amino acid	Protease I		ISP-A- <i>Eco</i>		ISP-L- <i>Eco</i>		ISP- <i>Bli</i>		ISP- <i>Bsu</i> 168		ISP- <i>Bsu</i> A-50	
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
Lys	12	7.1	14	4.7	13	4.5	12	4.0	17	5.8	20	6.8
His	2	1.2	7	2.4	10	3.55	10	3.4	10	3.4	6	2.0
Arg	10	5.9	14	4.7	7	2.4	5	1.7	6	2.0	6	2.0
Asx	18	10.7	30	10.1	27	9.3	33	11.1	36	12.1	36	12.1
Thr	4	2.0	14	4.7	14	4.7	13	4.4	13	4.4	13	4.4
Ser	11	6.5	24	8.1	40	13.6	34	11.5	23	7.8	25	8.4
Glx	21	12.4	40	13.5	43	14.7	42	14.2	38	12.8	33	11.1
Pro	9	5.3	13	4.4	13	4.5	13	4.4	13	4.4	13	4.4
Gly	11	6.5	39	13.1	36	12.4	42	14.0	40	13.5	36	12.2
Ala	13	7.7	26	8.8	30	10.6	28	9.5	26	8.8	32	10.8
Val	17	10.0	15	5.1	12	4.15	15	5.1	19	6.4	19	6.4
Met	4	2.4	7	2.35	ND	ND	5	1.7	5	1.7	5	1.7
Ile	10	5.9	12	4.0	9	3.1	11	3.7	13	4.4	13	4.4
Leu	15	8.9	23	7.8	20	6.9	19	6.4	22	7.4	25	8.4
Tyr	6	3.6	7	2.35	6	2.05	6	2.0	7	2.35	7	2.35
Phe	6	3.6	12	4.0	10	3.55	8	2.7	8	2.7	7	2.35
Total	169	100	297	100	290	100	296	100	296	100	296	100

ND, Not determined.

The molecular weight of the native enzyme was 55000 ± 2000 in buffer A containing 100 mM-NaCl, pH 8.5, when measured by gel filtration in Sephadex G-100. Electrophoresis in 7.5% acrylamide gel showed ISP-L-*Eco* to have a high electrophoretic mobility (R_F near 0.7) (see Fig. 3 and Table 2).

The amino acid composition of ISP-L-*Eco* was characterized by high concentrations of Glx, Lys, Leu, Phe and Ser and low concentrations of Val and Ala (Table 3). Based on amino acid analysis, a minimum molecular weight of about 30000 was calculated. Native protease I of *E. coli* has been reported to be a dimer of molecular weight 43000, and 21000 under denaturing conditions. Serine proteases of *Bacillus* spp. have also been found as dimers (Strongin *et al.*, 1978*a*). Presumably, ISP-L-*Eco* might also form a dimer having a molecular weight of about 55000.

DISCUSSION

Although the two isolated intracellular proteases are both serine proteases, they are nevertheless quite distinct, having different stabilities, electrophoretic mobilities, substrate specificity and responses to various inhibitors. Furthermore, ISP-L-*Eco* had a strict dependence on Ca^{2+} , whereas ISP-A-*Eco* was independent of Ca^{2+} . The two proteases could also be distinguished by their amino acid compositions.

Of the two serine proteases found by Pacaud *et al.* (1976) in *E. coli* strain B, the 'trypsin-like' protease II was similar in properties to ISP-A-*Eco*, suggesting that protease II and ISP-A-*Eco* are closely related, if not identical. Our second *E. coli* protease, ISP-L-*Eco*, was quite distinct from the previously described proteases I and II of *E. coli* (Pacaud & Uriel,

1971; Pacaud *et al.*, 1976; Pacaud & Richaud, 1975) but had similar properties to the protease from *B. subtilis* A-50. In amino acid composition, ISP-L-*Eco* was similar to the enzymes from *B. subtilis* A-50, *B. subtilis* 168 and, particularly, *B. licheniformis* 1001. To our knowledge this is the first indication of a relationship between proteolytic enzymes of evolutionary distant bacterial species, namely Gram-negative Enterobacteriaceae and Gram-positive spore-forming *Bacillus*.

Thus, cultures of *E. coli* contain at least three intracellular serine proteases: protease I (Pacaud *et al.*, 1976), protease II (Pacaud & Richaud, 1975) (corresponding to ISP-A-*Eco* according to our nomenclature) and ISP-L-*Eco*. Indeed three bands of activity with acetyl-phenylalanine naphthyl ester as substrate were previously seen after gel electrophoresis of crude cell extracts of *E. coli* (Pacaud & Uriel, 1971) and *Salmonella typhimurium* (Miller *et al.*, 1976). Presumably, the three enzymes corresponding to these bands have now been purified. Nevertheless, it should be stressed that components involved in the protein degradation system in bacteria (*deg* system) were not precisely identified. Degradation of *E. coli* β -galactosidase fragments in *S. typhimurium* mutants lacking proteases I and II was not impaired, suggesting that these serine proteases are not directly involved in the *deg* system (Miller & Zipser, 1977). It is tempting to suppose that ISP-L-*Eco*, by analogy with the intracellular serine proteases in *Bacillus* spp. which are involved in protein processing and turnover (Hageman & Carlton, 1973; Szulmajster & Keryer, 1975; Cheng & Aronson, 1977a, b), might be a component of the *deg* system of Enterobacteriaceae.

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