MASKED PROTEOLYTIC ACTIVITY LOCALIZED IN THE OUTER MEMBRANE OF *ESCHERICHIA COLI*

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Received 30 March 1979
Revised version received 28 April 1979

1. Introduction

Many mechanisms needing proteases have been described in *Escherichia coli*. The general turnover of proteins and the degradation of abnormal proteins need endoproteases and peptidases. An amino-peptidase cleaving the NH_2-terminal methionine of newly synthesized polypeptides is also required [1]. The study of proteolytic enzymes in *E. coli* in many laboratories has led to reports of many distinct enzymic activities: cytoplasmic or periplasmic, endoproteases, carboxypeptidases or aminopeptidases (proteases reviewed in 1). We have reported [2] a proteolytic activity bound to membrane fragments derived from *E. coli* ML 304G. Since the separation of inner and outer membrane in the ML strain was not possible, the localization of the proteolytic activity has been studied with *E. coli* K12 (Ymc). Here we extend our previous observations to this strain of *E. coli* and we show that the membrane-bound proteolytic activity is localized in the cell wall and is unmasked by detergents. Some of the results presented here were summarized in [3].

2. Materials and methods

E. coli K12 Ymc (Su_3^+) was grown on M9 minimum medium supplemented with 1% casamino acids and 0.2% glucose. The cells were collected during exponential growth at $A_{660} = 1$.

Membranes were prepared as in [4] and spheroplats were broken by sonic irradiation. The crude membrane preparation (3 mg protein in 0.7 ml) was sedimented on a 25–55% w/w linear sucrose gradient in 5 mM EDTA (pH 7.5) for 18 h at 38 000 rev./min in an SW 41 rotor. Gradients were fractionated by pumping the solution out through a needle inserted into the bottom of centrifuge tube. The cells were also disrupted in a Ribi cell fractionator and separated by centrifugation into supernatant, 'heavy particles' (HP) and 'light particles' (LP) as in [5]. HP and LP contained, respectively, 89% ketodeoxy octulosonate and 85% of NADH oxidase.

The Sepharose A_{150m} column (1.5 × 25 cm) was equilibrated with Tris–HCl (pH 7.5) 10 mM, the flow rate was 3.5 $ml.h^{-1}$ and 1 ml fractions were collected. A constant pressure was applied to the column (15 cm H_2O) using a Mariotte flask.

Protein concentration was measured according to [6]. Bovine serum albumin was used as standard. The concentrations of 2-keto-3-deoxyoctulosonate (KDO) was measured according to [7].

NADH oxidase was measured by the decrease in A_{340} at 25°C of the following reaction mixture: Tris–HCl (pH 7.5) 40 mM, NADH 0.3 mM.

The proteolytic activity was measured essentially as in [8] with ^{125}I-labelled casein as substrate. The pH and the ionic strength of the incubation mixture are indicated in the figure legends and tables.

3. Results

3.1. *Localization of the proteolytic activity on the membrane*

The casein degrading activity associated with *E. coli* K12 membrane fragments was substantially the same

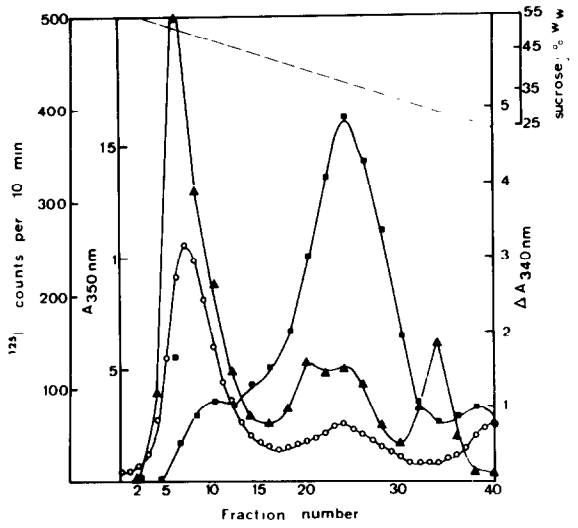

Fig.1. Isopycnic separation of the method [4] membrane fragments. The membrane fragments were prepared and sedimented on a sucrose gradient as in section 2. The incubation medium for proteolytic activity contained Tris–HCl (pH 9) 100 mM, ^{125}I-labelled casein 0.8 mg.ml^{-1}, $MgCl_2$ 20 mM and 50 μl of each fraction in 120 μl. The incubation was 18 h at 37°C. The specific radioactivity of ^{125}I-labelled casein was 130 cpm.μg^{-1}. (▲) proteolytic activity (^{125}I counts/10 min); (■) NADH oxidase (ΔA_{340}); (○) turbidity (A_{350}); (——) sucrose concentration.

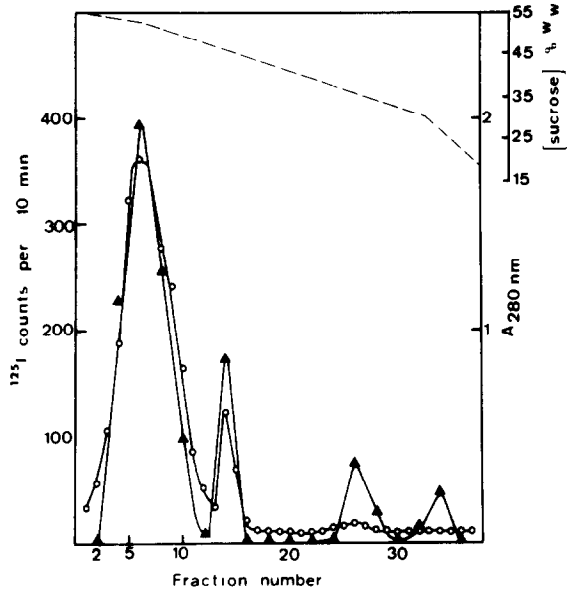

Fig.2. Separation of outer membrane-bound and contaminating proteolytic activity of the HP preparation. HP (600 μl; 2.5 mg protein) prepared as in section 2 were sedimented to equilibrium on a 25–55% w/w sucrose gradient (see fig.1). The proteolytic activity is tested as in fig.1. (▲) proteolytic activity (^{125}I counts/10 min); (○) A_{280}; (- - -) sucrose concentration.

as that found in ML 304G [8]. Figure 1 shows the analysis of envelope fragments obtained by the method in [4]. The outer membrane fragments were well separated from the inner membrane fragments which were located by the NADH oxidase activity. The proteolytic activity was separated into two peaks corresponding to the two membrane fractions. 70% of the activity was associated with the outer membrane (d = 1.238 g.cm^{-3}). The remaining activity was spread over the cytoplasmic membrane region (d = 1.18 g.cm^{-3} and 1.16 g.cm^{-3}) and a part, probably soluble enzyme, was at the top of the gradient.

The HP fraction (see section 2) was also analyzed on an isopycnic sucrose gradient (fig.2). The A_{280} profile confirmed that this preparation was enriched in outer membrane fragments (d = 1.245 g.cm^{-3}) as indicated by the KDO content (table 1). A small part of the activity was found associated with less dense particles which have the same density as cytoplasmic membrane fragments (d = 1.2 g.cm^{-3}) (data not shown).

Table 1
Percent of proteolytic activity and markers in the outer membrane (OM) and inner membrane (IM)

Preparative technique	Percent associated with	
	IM	OM
Method [5]		
KDO[a]	11	89
NADH oxidase[a]	85	15
Proteolytic activity[b]	10[c]	90[a]
Method [4]		
NADH oxidase	88	12
Proteolytic activity[b]	30	70

[a] These results were measured in the crude preparation of HP and LP
[b] The proteolytic activity is measured as described in fig.1
[c] The proteolytic activity associated with inner membrane fragments was estimated to be 23% of the proteolytic activity associated with the LP preparation (see fig.3)
[d] Results from fig.1

When the HP were filtered on a Sepharose A_{150m} column, all the proteolytic activity was found associated with the membrane fragments. The results described above show clearly that:

(i) The proteolytic activity of the HP preparation is essentially associated with the cell wall fragments;
(ii) Very little proteolytic activity is released into the soluble fractions during centrifugation or gel filtration.

When the LP were sedimented on an isopycnic sucrose gradient, the proteolytic activity spread over a large number of fractions and only a part of it cosedimented with inner membrane fragments. To better separate the membrane-bound proteolytic activity from contaminating proteases, the LP preparation was filtered on a Sepharose A_{150m} column. The proteolytic activity as well as the A_{280} profile were well separated into two peaks (fig.3). The proteolytic activity eluted at the same time as NADH oxidase represents 23% of the initial activity found in the LP preparation. The main part of the proteolytic activity is eluted from the column at the same time as material identified by sodium dodecylsulfate gel electrophoresis as being ribosomes or ribosomal subunits. The repartition of proteolytic activity, NADH oxydase and KDO in the inner and outer membrane preparation are summarized in table 1.

3.2. *Properties of soluble and membrane-bound proteases*

The soluble enzymes have a maximum activity at pH 9 and the membrane-bound enzyme at pH 7.5. We have previously shown that the proteolytic activity bound to the *E. coli* ML 304G membrane fragments is stimulated by ultrasonic irradiation [2]. The same result was found with the proteolytic activity bound to the outer membrane fragments of *E. coli* K12 (table 2). Proteases of the soluble extract and of the LP preparations are not stimulated by an ultrasonic treatment. When LP and HP preparations are preincubated with deoxycholate (DOC), the proteolytic activity is strongly enhanced (table 2); on the contrary, the soluble proteases are inhibited by DOC. In fact, if the fractions were first preincubated in the presence of DOC and then assayed at the optimal pH for membrane proteases (pH 7.5), it appeared that

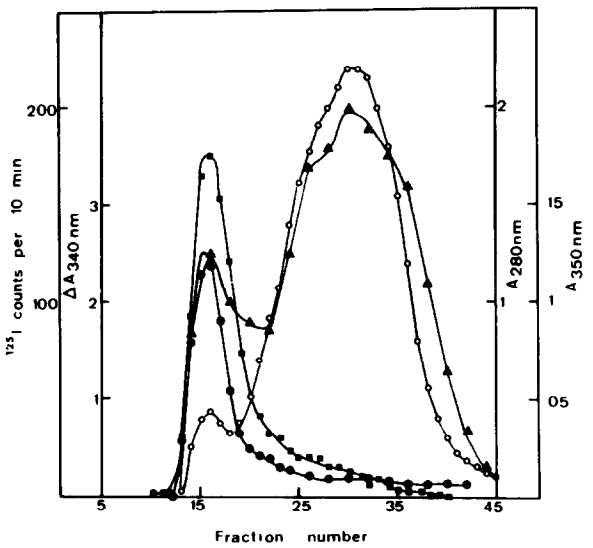

Fig.3. Separation of inner membrane-bound and contaminating proteolytic activity of the LP preparation. Light particles (500 µl; 4.2 mg protein) were filtered on a Sepharose A_{150m} column. The proteolytic activity is tested as in fig.1. (▲) proteolytic activity (^{125}I counts/10 min); (●) turbidity (A_{350}); (■) NADH oxidase (ΔA_{340}); (○) A_{280}.

Table 2
Variations of soluble and membrane-bound proteolytic activity

	Proteolytic activity associated with		
	Soluble fraction	LP	HP
pH 9[a]	100[b]	100	52
pH 7.5[c]	74	100[b]	100[b]
Sonicated[d,e]	100	91	198
+ DOC[e,f]	74	225	280

[a] The proteolytic activity was measured in the following incubation mixtures: Tris–HCl (pH 9) 10 mM, ^{125}I-labelled casein 0.4 mg.ml^{-1}
[b] These measures were taken as 100% proteolytic activity and the other activity was expressed as % of these values
[c] The incubation mixture is essentially as in [a] except that Tris–HCl (pH 7.5) is used
[d] Each preparation (0.25 ml) was sonicated 4 times for 30 s with the small probe of MSE 100 W sonicator
[e] The proteolytic activity of HP and LP preparations was tested at pH 7.5 and the activity of soluble proteases at pH 9
[f] Aliquots of the different preparations were preincubated for 1 h in DOC 0.2% Tris–HCl 10 mM (pH 7.5) for HP and LP and pH 9 for the soluble preparation

Table 3
Solubilization of K12 membrane-bound proteases

Membrane fraction	Incubation[a] medium	Proteolytic activity[b] in		
		Supernatant[c]	Pellet[c]	Total
HP	+ 0.5 M NaCl	42	114	156
HP	+ 0.2% DOC	205	76	281
HP	+ 0.5 M NaCl + 0.2% DOC	236	76	312
IM[d]	+ 0.5 M NaCl + 0.2% DOC	217	22	239

[a] HP (2 mg protein) and inner membrane fragments (0.79 mg protein) were incubated for 1 h at 25°C in 200 μl Tris–HCl (pH 7.5) 50 mM, completed with NaCl and detergent as indicated

[b] The proteolytic activity is expressed as % of the activity associated with membrane fragments before the solubilization. It was measured at pH 7.5

[c] Membrane fragments were sedimented for 1 h at 150 000 × *g* in a Spinco fixed angle 50 or 65 rotor. The supernatants were equilibrated in Tris–HCl (pH 7.5) 50 mM, NaCl 0.1 M buffer and the pellet was suspended and homogenized in the same buffer

[d] The inner membrane (IM) fragments from LP preparation were separated from ribosomes by filtration on a Sepharose A_{150m} column

63% of the proteolytic activity was located in the outer membrane, 14% with LP and 23% with the soluble fraction. On the contrary, when it was measured at pH 9 without previous incubation of the extracts with DOC, 80% of the total proteolytic activity was in the soluble fraction and only 12% was associated with HP and 8% with LP.

3.3. *Solubilization of HP-bound proteolytic activity*

Comparative studies on the solubilization of the membrane-bound proteolytic activity were performed with various detergents. DOC-solubilized part of bound enzymatic activity, while Triton X-100 and SDS were much less efficient. The solubilization efficiency of DOC was enhanced by the presence of 0.5 M NaCl. However, little activity, was solubilized in the presence of 0.5 M NaCl alone (table 3). The best conditions found (0.2% DOC in 0.5 M NaCl) resulted in the solubilization of 90% of inner membrane-bound proteolytic activity and 80% of that from the outer membrane.

3.4. *Aggregation of proteases solubilized from the outer membrane*

When DOC was removed from the solubilization mixture, the outer membrane protease did not reassociate with the membrane fragments and was found in the supernatant after sedimentation of the membrane fragments (data not shown). Nevertheless, after DOC elimination, the proteases of the supernatant aggregated into complexes of higher molecular weight. When filtered on a Sepharose A_{150m} column equilibrated with detergent, the soluble enzymes are eluted in one peak in the presence of DOC, after the peak of alkaline phosphatase used as marker; its apparent molecular weight (M_r) is smaller than that of alkaline phosphatase (86 000) (fig.4). However when the solubilized enzymes were freed of the detergent by dialysis against a low ionic strength buffer and filtered on the same Sepharose A_{150m} column in the absence of detergent, the proteolytic activity was eluted in one peak before catalase (M_r 250 000) (fig.4). The apparent molecular weight of the proteases in this case is ~300 000.

4. Discussion

Here we have demonstrated by two techniques that most of the membrane-bound proteolytic activity (70% and 90%) is located in the outer membrane. Furthermore by studying some properties of soluble and membrane-bound proteases, it was possible to ascertain that membrane-bound proteases are not

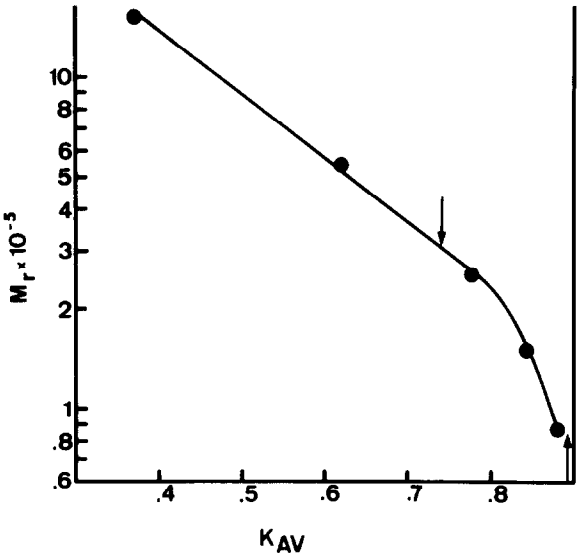

Fig.4. Aggregation of the outer membrane proteins. Heavy particles of *E. coli* K12 strain (2 mg protein) were solubilized in 400 μl DOC NaCl buffer. The membranes were sedimented and the supernatant was removed (see table 4). The supernatant was then filtered on a Sepharose $A_{150}m$ column (see section 2), equilibrated in Tris–HCl (pH 7.5) 100 mM, DOC 0.1%, NaCl 0.25 M. In another experiment the supernatant was equilibrated in Tris–HCl (pH 7.5) 10 mM by dialysis and filtered on the same Sepharose $A_{150}m$ column equilibrated in Tris–HCl (pH 7.5) 10 mM buffer. The following M_r standards were filtered on the column equilibrated with Tris–HCl (pH 7.5) 10 mM. *E. coli* 50 S ribosomes (M_r 1.5 × 10^6), *E. coli* β-galactosidase (M_r 540 000), beef liver catalase (M_r 250 000), yeast alcohol:NAD oxydoreductase (M_r 150 000) and *E. coli* alkaline phosphatase (M_r 86 000). The activities were measured as in section 2. When the filtration was performed with DOC in the buffer the proteolytic activity was measured after removing the detergent by dialysis in Tris–HCl (pH 7.5) 10 mM. Arrows indicate the K_{av} values of two forms of protease.

soluble proteins adsorbed to the outer membrane. In addition the soluble and membrane-bound proteases hydrolyse different polypeptides among those found in casein (in preparation).

The masked proteolytic activity is activated by solubilization with DOC and by sonic irradiation. No additional latent proteolytic activity is unmasked, when outer membranes previously depleted of proteases by DOC are sonicated (data not shown). It is likely that the proteolytic activity unmasked by DOC or by sonication corresponds to the same enzyme.

When DOC is eliminated by dialysis, the proteases aggregate but do not bind to the membrane fragments. Nevertheless this data indicating the hydrophobic nature of the molecules, is in agreement with their location in the membrane.

Proteolytic activity was also found associated with LP preparations. The contamination of LP by outer membrane fragments was demonstrated by the presence of KDO in the LP preparation. The proteolytic activity of LP unmasked by DOC is probably bound to outer membrane fragments.

Several enzymes of the cytoplasmic membrane, nitrate reductase [9], adenosine triphosphatase [10] and ferric enterobactin receptor [11] are solubilized or degraded by proteases present in membrane preparations. Nitrate reductase is solubilized by an enzyme localized in the outer membrane [12]. These degradative processes are probably not the in vivo function of the proteases. Membrane-bound proteases could perform the maturation step occurring during the secretion of bacteriophage, periplasmic and membrane proteins [13–18]. The precursor of alkaline phosphatase is matured by an outer membrane-bound protease [15]. The precursor of f1 coat protein, arabinose-binding protein and maltose-binding protein are processed in vitro by membrane bound proteases only when detergent is added to the protein synthesis medium [14,19].

The proteases of the outer membrane described here and especially the enzyme unmasked by detergent could be implicated in the splitting of secreted proteins. The protease unmasked and solubilized by DOC has been further studied and we are about to report its characterization.

Acknowledgements

We are very grateful to Dr Jacques Pommier for labelling the casein, to Drs Danièle Joseleau-Petit and Claude Burstein for the use of the Ribi Cell fractionator and for valuable advice and to Dr Carolyn MacGregor for communicating a preprint. This work was supported by the Centre National de la Recherche Scientifique (GR no. 18), the Délégation Générale à la Recherche Scientifique et Technique (convention GBM 78.7.0693), the Institut National de la Santé et de la Recherche Médicale (CRL 77.04.0741), the Commissariat à l'Energie Atomique and the Fondation pour la Recherche Médicale Française.

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