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IDENTIFICATION OF PROTEASE IV OF E.COLI : AN OUTER MEMBRANE BOUND ENZYME

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SUMMARY

The proteolytic activity of E.coli measured using ^{125}I-labelled αS1 casein as substrate, is mainly localised in the outer membrane and is due to an intrinsic outer membrane protein which can be solubilized by deoxycholate. This enzyme exhibits maximum activity at pH 7,5 in Tris-HCl buffer, is resistant to thermal denaturation with a half-life of 28 min. at 90°C in deoxycholate-NaCl buffer and is inhibited by ethylene-diamine tetraacetate, high concentrations of p-aminobenzamidine, tosyl-L-lysine chloromethyl ketone, tosyl-L-phenylalaninechloromethyl ketone and by two inhibitors of the processing of the secreted protein precursors, procaine and phenehylalcohol. Whole cells do not exhibit proteolytic activity, nevertheless, some is unmasked when the outer membrane is permeabilized by Tris or ethylenediamine tetraacetate or when vesicles are sonicated. This suggests that the protease is on the inner side of the outer membrane. Because the protease is different from the soluble proteases described in E.coli,and especially from proteases I,II and III, it has been called protease IV.

INTRODUCTION

Some cellular functions in E.coli are mediated by specific proteolytic processes (1-3) and many investigations have attempted to demonstrate a role for particular proteolytic enzymes in such mechanisms.
Several proteases were detected by their ability to cleave synthetic substrates. Protease I and protease II are most active against ester substrates but only weakly against protein substrates (4-6). Amino-peptidase N, although exhibiting a proteolytic activity, is more active against amino-peptidase substrates (7). No physiological functions have been assigned to these enzymes (5,8,9).
Protease III is the only protease of E.coli capable of degrading in vitro

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[*] Abbreviations : DOC,sodium deoxycholate ; TPCK, N-tosyl-L-phenylalanine chloromethylketone ; TLCK, N-tosyl-L-lysinechloremethyl ketone ; PAB, p-aminobenzamidine ; PMSF, phenylmethanesulfonyl fluoride

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the amino-terminal nonsense fragments of β-galactosidase (10). However the degradation of "abnormal proteins" by protease III *in vivo* has not been established (11). In contrast recA gene product has been demonstrated to be a protease responsible for the cleavage of λ repressor (2).

To demonstrate the existence of proteases in *E.coli* we have utilized ^{125}I-labelled casein as substrate. Casein is a mixture of polypeptides whose main components are αSI,β and κ caseins. These polypeptides have loose spatial conformations (12) which probably explains why they are degraded by a great number of proteases with different specificities.Caseins may thus be expected to allow the detection of several *E.coli* proteases. Using a caseinolytic assay, we have demonstrated in our previous work that : i) at least three soluble proteases exist in *E.coli* (13). One of them, protease A, has been partially purified and has several properties in common with protease III (10,14) ; ii) proteases are located in the periplasmic fluid (15) ; iii) a significant fraction of the proteolytic activity is bound to the membrane, particularly to the outer membrane and is activated both by sonicating the membrane fragments and by adding deoxycholate (16,17). In the present paper, I report the identification and the characterization of a membrane bound protease of *E.coli* called protease IV.

MATERIALS AND METHODS

Procaine hydrochloride, PMSF*, TLCK* hydrochloride, PAB*dihydrochloride, TPCK*, and DOC*were from Sigma Chemicals Company (St Louis, Mo, U.S.A.), casein was from Merck-Darmstadt (F.R.G.) ^{125}I was from the Commissariat à l'Energie Atomique (Gif sur Yvette, France),phenethyl alcohol was from Eastman Kodak Company (Rochester, N.Y.,U.S.A.) and αSI casein was the gift of Dr M. Desmazeaud, I.N.R.A. Laboratory (Jouy en Josas, France).*E.coli* K12 Ymc(Su_3^+) was grown as previously described (17). The cells were disrupted in a Ribi cell fractionator and separated into supernatant (soluble enzymes), heavy particles (outer membrane) and light particles (inner membrane) as described by Joseleau-Petit and Kepes (18).Separation of inner and outer membrane vesicles is followed by measuring NADH oxydase and ketodeoxyoctulosonate content (17).

Protease solubilisation was performed by incubating outer membranes in 50 mM Tris-HCl (pH 7,5), 0,2% DOC and 500 mM NaCl (when indicated). The final protein concentration was 1.7 mg/ml. After 1 hour at room temperature the supernatant was recovered by 1 hour centrifugation at 45,000 rpm in the rotor 50 of a Beckman ultracentrifuge. For proteolytic activity measurements the pellet was resuspended by homogeneisation in the original volume of 10 mM Tris-HCl (pH 7.5) buffer.

The proteolytic activity and the protein concentrations were determined essentially as described previously (15). The standard incubation mixture was 10 mM Tris-HCl (pH 7,5), 330 µg/ml αSI casein. Changes in the composition of the incubation medium are indicated in figure legends. One proteolytic activity unit transforms one µg of ^{125}I labelled casein or αSI casein into acid soluble peptides within an hour at 37°C.
Casein and αSI casein were labelled according to Pommier *et al* (19),

TABLE 1

Localization and solubilization of αS1 casein degrading proteolytic activity
200 µl of different fractions were diluted to a final volume of 1 ml, adjusted to 50 mM Tris-HCl (pH 7.5) and 0.2% DOC, where indicated, and incubated for 1 hour at 25°C. Membrane fragments were then sedimented and the pellets were resuspended in 10 mM Tris-HCl (pH 7.5). The proteolytic activities of resulting pellets and supernatants were measured. Activities were expressed per gram of wet bacteria. The data in brackets represent the percentage of the total activity. n.d. : not determined, I.M. : inner membrane, O.M. : outer membrane

		proteolytic activity (units)		
fraction	DOC	pellet	supernatant	total
OM	-			96
	+	80.5	1610	1690.5(78)
IM	-			0
	+	n.d.	n.d.	363.6(17)
soluble	-			322
	+			116.9(5)

except that lactoperoxidase was used instead of hog thyroid peroxydase. Caseins were then precipitated by 10% (w/v) tricholoroacetic acid, solubilized and dialysed in 8 M urea and finally equilibrated in 10 mM Tris-HCl (pH 7,5).

RESULTS

Unmasking by DOC of an outer membrane bound αSI casein degrading protease

In searching for a substrate specifically degraded by *E.coli* outer membrane proteases, we questioned whether these enzymes preferentially cleave one of the casein components. It appears that αSI casein is a suitable substrate for a specific outer membrane bound protease since, by measuring the proteolytic activity of different fractions, outer membrane, inner membrane and soluble, we found 78% of αSI casein degrading activity in the DOC activated outer membrane fraction (table 1). The activation by DOC is accompanied by the solubilization of the protease(s). The low proteolytic activity associated with the inner membrane fraction is also unmaked by DOC (Table 1) and conceivably results from contamination of this preparation by outer membrane fragments, as we have explained previously (17).

When DOC solubilized proteins are chromatographied on a DE52 column with eluants containing detergents, the total proteolytic activity applied is recovered in one peak indicating that DOC solubilizes only one protease

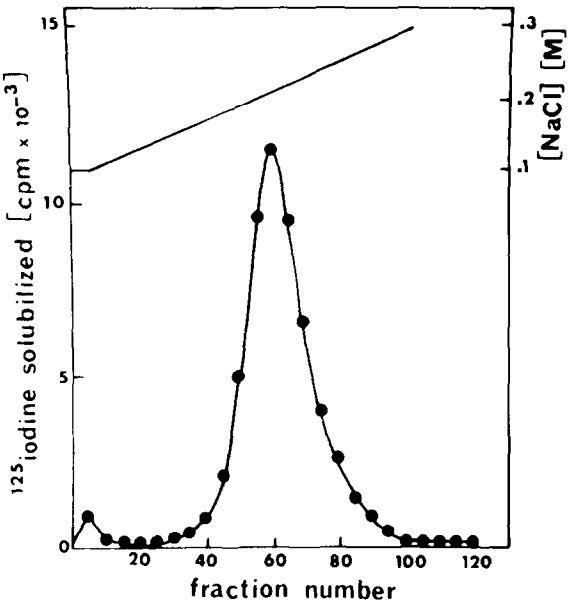

Figure 1. DE 52 chromatography of crude protease IV
After solubilization with DOC and NaCl, 240 µl of the supernatant were equilibrated with 10 mM Tris-HCl (pH 7.5), 0.1% DOC, 75 mM NaCl by dialysis, and then adsorbed on a DE 52 column (1x3cm) equilibrated with the same buffer. The column was washed with 20 ml of this buffer and then eluted with a continuous gradient from 75 to 500 mM NaCl in 20 mM Tris-HCl (pH 7.5), 0.1 % DOC, of total volume 100 ml. The flow rate, controlled by a peristaltic pump, was 0.5 ml/min and 0.9 ml fractions were collected. The proteolytic activity of 15 µl of each fraction was measured by incubation for 8.5 hours with ^{125}I-labelled αS1 casein at 37°C

from the outer membrane (Figure 1). Furthermore, during incubation at 98°C the DOC solubilized proteolytic activity follows a logarithmic inactivation kinetic (figure 3) which confirms that only one αSI casein degrading protease is solubilized by this detergent. It is therefore possible to study the enzyme properties in crude protein fractions solubilized from membrane fragments by DOC.

Unmasking of the αSI casein degrading protease associated with whole cells

Although the αSI casein degrading protease is associated with *E.coli* outer membrane, very little proteolytic activity is detected when cells are incubated with ^{125}I-labelled αSI casein in 10 mM Tris-HCl buffer (Table 2). A possible explanation may be that the active site of the enzyme is located on the inner surface of the outer membrane. If this is so, αSI casein will have access to the active site of the protease when the outer membrane is made permeable to macromolecules by incubating cells

TABLE 2

Permeabilisation of whole cells and outer membrane vesicles :

E.coli cells collected at log phase were washed in 10 mM Tris-HCl (pH 7.5) and then suspended in the samme buffer. Bacteria and vesicles were preincubated in either DOC-NaCl buffer as indicated for solubilisation or 10 mM Tris-HCl (pH 7,5). Final concentrations were 1,7mg/ml membrane protein and 0,33 g/ml wet cells.
After one hour preincubation at 25°C the proteolytic activity was measured by diluting 10 µl of cells or outer membrane preparations in 120 µl of either 10 mM Tris-HCl (pH 7,5) ; 10 mM Tris-HCl (pH 7.5), 500 mM NaCl; 100 mM Tris-HCl (pH 7.5) or 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, all containing 40 µg of ^{125}I labelled casein. 100% relative activity refers either to 1950 proteolytic acitivity units, unmasked by DOC-NaCl buffer from 1g wet bacteria, or to 2561 units associated with outer membrane fragments prepared from 1 g wet bacteria.

	preincubation	incubation	relative activity (%)
Bacteria	DOC-NaCl	10mM Tris	100
	10 mM Tris	10mM Tris	5.6
	10 mM Tris	100mM Tris	30.5
	10 mM Tris	10mM EDTA	25.8
outer membrane vesicles	DOC-NaCl	10mM Tris	100
	10 mM Tris	10mM Tris	6.2
	0.5 M NaCl	10mM Tris	5
	10 mM Tris	100mM Tris	64
	10 mM Tris	10mM EDTA	28
	DOC-NaCl	100mM Tris	92

with either Tris or EDTA as is usually done to allow lysozyme to penetrate into the periplasmic space (20). Data of Table 3 demonstrate that incubation of E.coli cells with either 100 mM Tris or 10 mM EDTA unmasks some proteolytic activity. However, in these conditions, other proteases

TABLE 3

Activation of membrane bound proteolytic activity by sonication and DOC

250µl of outer membrane preparation was sonicated 4 times for 30 seconds with an MSE 100 watts sonicator. Incubations with DOC were performed, without NaCl, as indicated in Materials and Methods for the solubilization of protease IV. Membrane preparations which were not preincubated with DOC were diluted in 10 mM Tris-HCl (pH 7.5) to the same final protein concentration (1.74 mg/ml) and incubated for 1 hour at 25°C. The proteolytic activity of 10 µl aliquots of diluted preparations was measured

Activation		relative activity(%)
first step	second step	
DOC	none	100
none	none	4
sonication	none	30
DOC	sonication	100
sonication	DOC	97

TABLE 4

Effect of protease inhibitors

Crude protease IV was usually preincubated for 30 minutes at 4°C with inhibitor at the indicated concentration in 10 mM Tris-HCl (pH 7.5) prior to the addition of 10 µl (40 µg) of ^{125}I-labelled αS1 casein. When protease IV was preincubated with 0.1 mM TPCK or TLCK, 10 µl of the preincubation mixture was diluted in 120 µl of incubation mixture for proteolytic activity measurement. Relative activity refers to controls with solvents only.

Inhibitor	concentration (mM)	relative activity
none	-	100
Soybean trypsin inhibitor	4	121
PMSF	0.33	100
N-ethylmaleimide	8.3	100
EDTA	0.83	35
TPCK	0.1	100
	1[a)]	73
TLCK	0.1	100
	1	62
	5	24
PAB	1	100
	20	20
phenethyl alcohol	32	50
procaine	30	35

a) when 4 µl of 27.5 mM inhibitor in methanol were added in 110 µl of the preincubation mixture it precipitates. Therefore the concentration of soluble inhibitor is much smaller than 1 mM.

localized on the outer surface of the inner membrane (21) and in the periplasmic space (15) may also be active.

Since outer membrane vesicles, like whole cells, exhibit a very low proteolytic activity (Table 2), it seems likely that they retain the orientation of the original outer membrane. Incubation of these vesicles with 100 mM Tris unmasks up to 62% of the total activity of the αSI casein degrading membrane bound protease (Table 2). The apparently lower proteolytic activity unmasked by EDTA (28%) is probably because EDTA itself inhibits the protease (Table 4). Moreover we verified that Tris does not solubilize any proteolytic activity from membrane and that increasing the ionic strength with NaCl do not unmask proteases.

Because sonication induces invertion of right-side-in inner membrane vesicles (22), this procedure may also be expected to induce the same invertion in outer membrane vesicles thus unmasking the membrane bound protease. We had already observed that sonicating crude membrane (16) and outer membrane (17) preparations enhanced the proteolytic activity as measured by the degradation of total casein. We have now found that

a 2 minute sonication of inactive outer membrane vesicles unmasks up to 30% of the total αSI casein degrading activity obtained after preincubation with DOC (Table 3), and that the protease remains bound to membrane fragments. In order to verify that it is the same protease that is unmasked by sonication, by 100 mM Tris incubation or by DOC solubilization we checked that no additional proteolytic activity is unmasked when after preincubation with 0,2% DOC , membrane vesicles were either sonicated or incubated with 100 mM Tris (Table 2 and 3).

Action of protease inhibitors

Soybean trypsin inhibitor, N-ethylmaleimide and PMSF are unable to inactivate the protease (Table 4). Neither is it inhibited by preincubation with low concentration of TLCK nor TPCK. However, at higher concentrations, these competitive inhibitors and also PAB, all inhibit the protease. EDTA is the most effective inhibitor of the enzyme. The local anesthetics, procaine and phenethyl alcohol, inhibit the outer membrane protease at the concentrations described as inhibiting the processing of secreted protein precursors (23,24)(Table 4).

pH optimum

The outer membrane protease exhibits maximal activity at pH 7. The enzyme is much more active in Tris-HCl buffer than in potassium phosphate buffer. It is completely inactive above pH 9, in glycine-NaOH buffer (figure 2). Glycine does not inhibit the enzyme at pH 7,5 in Tris-HCl buffer.

Thermal denaturation

The logarithmic kinetic plot obtained at 98°C in DOC-NaCl buffer is characteristic of the inactivation of a single molecular species. At this temperature the half life of the enzyme is 28 minutes (Figure 3).

Inhibition by non-ionic detergents

We failed to unmask the protease with Triton X100, Brij 58, and sodium cholate. Moreover, we observed that the protease is inhibited by Brij 58 and by Triton X100 above its micellar concentration (0,016%), after being solubilized and unmasked by DOC.

DISCUSSION

In the present report we demonstrate that an endoproteolytic enzyme, with the characteristics of an intrinsic membrane protein, is localized in the outer membrane of E.coli.

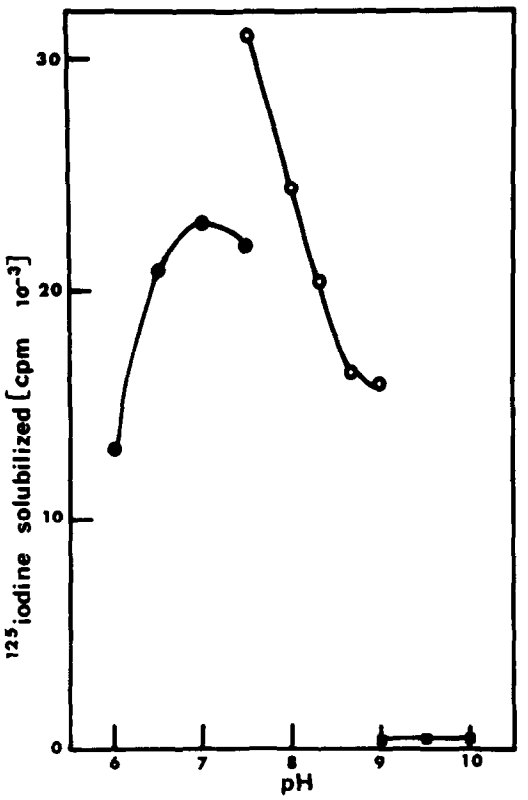

Figure 2. Variation of the activity of the solubilized protease with pH. Buffers (50 mM) used were : potassium phosphate (-•-), Tris-HCl (-○-) and Gly-NaOH (-■-). The enzyme, solubilized from 17.5 µg of outer membrane protein, was incubated with ^{125}I-labelled αS1 casein for 15 minutes at 37°C at the various pH values.

The protease is responsible for 90 to 96% of the proteolytic activity measured with ^{125}I-labelled αS1 casein as substrate. It is thus possible to measure this protease in crude extracts of E.coli.

This protease activity in the cell is rather high : the enzyme contained in one gram of exponentially growing bacteria can cleave in one minute 0,33 mg of ^{125}I-labelled αSI casein. During the same period, the bacteria synthesizes 2.3 mg of protein.

The properties of the αSI casein degrading protease characterized in this report allow us to conclude that it is distinct from proteases I, II, III (4,6,10) and all other soluble proteases identified in E.coli (14,25). Consequently we have named this enzyme protease IV.

Unmasking of the proteolytic activity bound to the outer membrane both by sonication and by incubations with Tris and EDTA strongly suggested

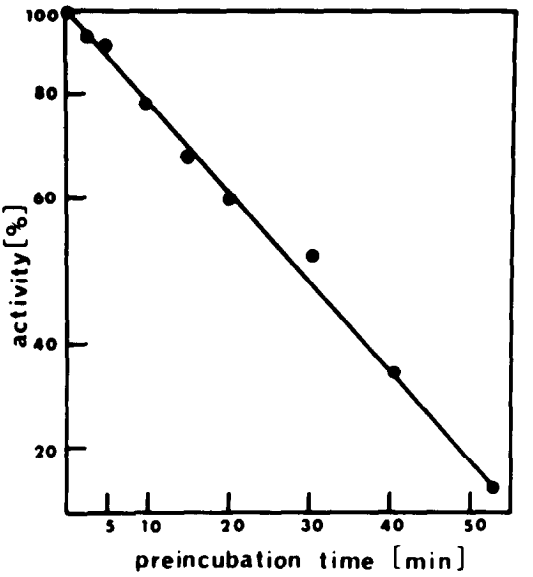

Figure 3. Thermal denaturation of the solubilized protease
The protease solubilized in 50 mM Tris-HCl (pH 7.5), 0.2% DOC, 500 mM NaCl was introduced into capillary tubes, the two extremities of which were then sealed. The tubes were preincubated at 98°C and the proteolytic activity was then measured.

that protease IV is located on the periplasmic face of the outer membrane. Nevertheless, it cannot be excluded that the enzyme is buried in the outer membrane and may be unmasked by structural changes. Assuming that protease IV is localized on the inner surface of the outer membrane it will be possible to follow the permeabilization of the outer membrane and the invertion of right-side-out outer membrane vesicles by measuring proteolytic activity against αSI casein.

Protease IV is inactivated by high concentrations of the trypsin inhibitors (PAB,TLCK), the chymotrypsin inhibitor (TPCK) and by EDTA. It is also inhibited by two local anesthetics : procaine and phenethyl alcohol, which are competitive inhibitors of trypsin and chymotrypsin, respectively (26-27) .

Protease IV may be related to the various proteolytic activities described in the membrane of E.coli. These include the solubilisation of nitrate reductase (28) and of ATPase (29), the cleavage of colicins (30) and the maturation of alkaline phosphatase (I). Furthermore, changes occuring in the protein content of the outer membrane after addition of PAB (28) and TLCK (31) in the E.coli culture medium could be due to the inhibition of protease IV.

It has been established that protein a, a major outer membrane protein, is responsible for a structural modification of the ferric enterobactin receptor and that it is probably a protease (32). This modification occurs in the protein mixture extracted from the outer membrane with 2% Triton X100 and 15 mM EDTA (32). Since it is inhibited by Triton X100 and by EDTA, protease IV appears to be different from protein a.

The probable location of protease IV on the inner surface of the outer membrane, its endoproteolytic activity (33) and the fact that it is inactivated by inhibitors of the processing of secreted proteins (procaine, phenethyl alcohol, TLCK, PAB) (20,21,26) suggest that this enzyme could be a leader peptidase. Nevertheless with respect to its inhibition by Triton X100 and its molecular weight (33) protease IV is different from the leader peptidase purified by Zwizinsky (34). Furthermore, while leader peptidases are expected to cleave peptide bonds between small uncharged aminoacids (35) protease IV was found to split the Phe23-Phe24 bond of αSI casein (33).

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