MEMBRANE ASSOCIATED PROTEASES IN *E. COLI*

Ph. RÉGNIER and M.N. THANG
Institut de Biologie Physico-chimique,
13 rue P. et M. Curie, 75005 Paris, France

Received 26 July 1973

1. Introduction

The study of subcellular distribution of proteases in *E. coli* ML304G [1] showed that 90% of the total proteolytic activity defined by [^{125}I]casein hydrolysis was found to be endocellular and only 10% exocellular. Among the endocellular proteolytic activities, 15% were bound to membrane fragments. The subcellular distribution is independent of the methods with which the cells were broken: sonication, grinding with alumina or spheroplast formation. However, the total amount of casein hydrolysis activity varied with the technique used to open the cells. Thus sonication released 2–3 times more activity than the grinding method although the total protein extracted was the same in the two cases. Moreover, the elution profiles from a DEAE-cellulose column indicated the presence of a basic protease, in sonic acellular extracts which is absent in ground cell extracts.

We thus consider the possibility that sonic treatment might have unmasked proteases from protein complexes and/or solubilized enzymes from membrane structures. In addition, we postulated that activity found in membrane fragments might be artificially low: part of the activity may well be masked by kinds of vesicle structures. The present paper shows that this is indeed the case, high proteolytic activities are in fact associated with the membrane fragments, and can be unmasked by sonication.

2. Materials and methods

The culture of *E. coli* ML 304 G are performed as previously described. Bacteria are washed in 10^{-2} M Tris–HCl buffer, pH 7–5 [1].

After grinding, the extracts are resuspended in 10^{-2} M Tris–HCl buffer, pH 7–5. The suspension is sedimented at 5000 *g* for 5 min. The pellet containing alumina and unbroken cells is discarded. The supernatant is centrifuged once more at 12 000 *g* for 5 min to remove entirely unbroken cells and alumina. The supernatant obtained is centrifuged at 50 000 *g* for 30 min in the rotor 30 of a Spinco L50. The supernatant obtained is called S50. The pellet which contains the membrane fragments is washed twice in 10^{-2} M Tris-HCl buffer, pH 7–5, and finally homogenized in the same buffer. It is called C50. The two supernatants of the washing are kept for proteolytic activity estimation.

Proteolytic activity is measured by the degradation of ^{125}I-labelled casein as previously described [1].

3. Results

When the cells are ground with alumina, about 10% of the total amount of proteolytic activity is associated with membrane fragments (table 1). In this series of experiments, ground acellular extracts have been centrifuged twice at 5000 *g* and 12 000 *g* for 30 min each time instead of once as in the previous work [1]; some of the heavy fragments might be co-sedimented with alumina and unbroken cells. This is one of the reasons that the membrane associated-activity per gram of wet cells is one half of that obtained previously.

The washed membrane fragments are then put into suspension and are sonicated. At various times, aliquots are withdrawn and assayed for casein hydrolysis. The kinetics of acid soluble ^{125}I liberation is shown in fig. 1. One observes first that there is a progressive increase in

Table 1
Distribution of soluble and membrane associated proteases in alumina ground cell extracts.

Total activity	Soluble activity				Activity of C50
	S50	Washes* 1	2	Total	
1500	1100	64	12	1176	160

* These data are obtained from 1 g of wet weight bacteria.

Table 2
Membrane associated protease demasked by sonication.

Initial units 160	Units after 15 min sonication 1250	
	Units associated with fragments*	Solubilized units*
	1050	640

* After 15 min of sonication the suspension is sedimented 30 min at 50 000 *g* as indicated in Materials and methods, and the pellet is homogenized in 10^{-2} M Tris–HCl buffer, pH 7–5.

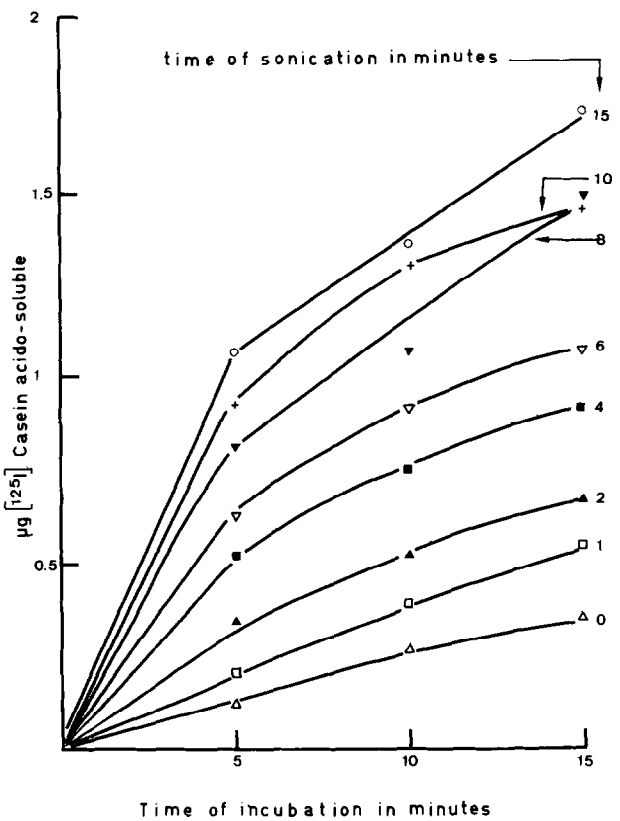

Fig. 1. Activation of the membrane bound proteolytic activity by sonic treatment. 2 ml of C50 are sonicated with a MSE ultrasonic disintegrator (100 W). At each time indicated on the figure – a 90 µl aliquot is withdrawn and its proteolytic activity on [^{125}I]casein is measured by kinetics experiments.

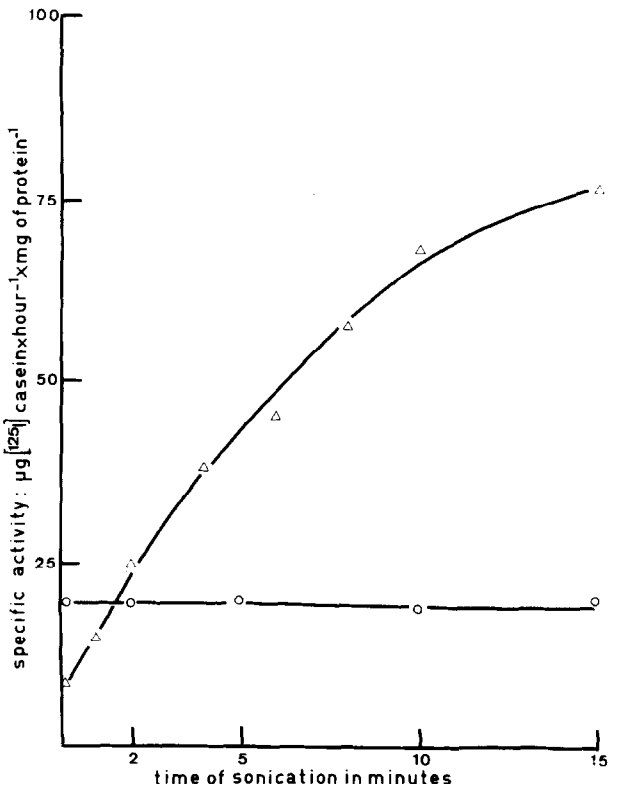

Fig. 2. Proteolytic activity of membrane bound and soluble proteolytic activity after sonic treatment. 2 ml of S50 are sonicated as indicated in fig. 1 for the C50 suspension. Specific activity of S50 (○) and C50 (△) is plotted as a function of the time of sonication. For C50 specific activity is calculated from the data of fig. 1. The ^{125}I, soluble in trichloroacetic acid after 5 min of incubation is used for the estimation of the initial rate of activity.

proteolytic activity with increasing time of sonication. Secondly, the hydrolysis kinetics become less linear (over a 15 min incubation) the longer the sonication had been carried out. If one takes arbitrarily the rates at 5 min of incubation as initial rates for each fraction, one obtains proportionality between augmentation of specific activity and time of sonication for the first 4 min of sonic treatment (fig. 2). Then, the increase of specific activity slows down as the sonic treatment continues. This suggests strongly that sonication affects the proteolytic activity expression by altering either the protease or an enzymes–membrane complex.

After 15 min of sonication, there is an 8-fold increase of the original activity (table 2). The total units unmasked by sonic treatment are now equal to those found in the soluble fraction (table 1). Consequently, distribution of endocellular activity is found to be one half soluble, one half associated with membrane.

When the suspension that had been sonicated for 15 min is centrifuged at 50 000 *g* as in the preparation of the C50, 70% of the activity remains associated with membrane fragments; only 30% is released into the supernatant fraction (table 2). The protease activity seems to be tightly linked to the membrane fragments obtained by cell grinding and is hardly solubilized by sonication.

It is important to notice that application of sonic treatment to the corresponding soluble fraction (S50) does not result in any change of the initial activity (fig. 2).

4. Discussion

It is clearly shown that a large amount of endocellular proteolytic activity in *E. coli* (at least 50% of total as measured by [^{125}I]casein hydrolysis) is associated with the cell membrane.

This estimation, appears to be a minimum since the unmasked activities are not yet at a plateau after 15 min of sonication.

On the other hand, the association of the protease to the ground membrane fragments is stable, since after 15 min of sonication, only 30% of the total unmasked activity is solubilized. This indicates that the 8-fold increase seen after sonication is not mainly due to a solubilization of the protease. It is significant that no increase occurs in the soluble protease fraction after sonication (fig. 1). The result supports the idea that the large amount of proteolytic activity found in sonicated soluble extracts [1] does not result from an activation of either an enzymatic precursor or a protease–protein complex, but rather from a more efficient release of membrane proteases when whole cells are sonicated. It is interesting to relate the release of a membrane protease by sonication of whole cells to an observation on the *E. coli* methionyl-tRNA synthetase. This enzyme has a mol. wt. of 172 000–180 000 in the native form [2, 3]. A light species of this enzyme with a mol. wt. of 96 000 has been isolated [4] from *E. coli* CA244. This is in fact a degraded form of the native enzyme. When the enzyme is prepared from *E. coli* CA244 without the autolysis step, only the native form is present [J.P. Waller, personal communication]. Nevertheless, if the cells are broken by sonication instead of using the Manton–Caulin mill, there is occasionally the appearence of the 96 000 species [J.P. Waller, personal communication]. It is tempting to suggest that the cleavage of the native met-tRNA synthetase to the 96 000 dimeric enzyme is due to a specific membrane protease. Experiments are under way to verify the hypothesis.

Acknowledgements

We thank Dr. Pommier from Unité de Recherche surla glande Thyroide et la Régulation hormonale. INSERM Laboratory in Bicêtre for the preparation of [^{125}I]casein. The work benefits a grant from Centre National de la Recherche Scientifique (groupe no. 5).

References

[1] Régnier, Ph. and Thang, M.N. (1972) Biochimie 54, 1227.
[2] Lemoine, F., Waller, J.P. and Rapenbusch, R. van (1968) European J. Biochem. 4, 213.
[3] Heinrikson, R.L. and Hartley, B.S. (1967) Biochem. J. 105 17.
[4] Bruton, C.J. and Hartley, B.S. (1968) Biochem. J. 108, 281.