EFFECT OF LIMITED PROTEOLYSIS ON BACTERIOCIN ACTIVITY IN VIVO AND IN VITRO

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Received 15 December 1975

1. Introduction

Cells of *Enterobacter cloacae* harbouring the bacteriocinogenic factor Clo DF13 produce several plasmid-specific gene products [1]. Two of these products, the cloacin DF13 and its immunity protein form a very tight and stoichiometric complex which protects the producing cells against the lethal effects of their own bacteriocin [2]. After being released from the bacteriocinogenic cells the complex possesses a very effective killing activity towards sensitive cells. This lethal action can be divided into three major phases: (a) adsorption onto specific receptor sites located in the outer membrane; (b) interaction with the cytoplasmic membrane resulting in the leakage of potassium ions: (c) inactivation of the ribosomes by cleavage of the 16 S ribosomal RNA [3–5]. The final step in the interaction with sensitive cells appears to involve a penetration of at least a part of the cloacin molecules into the cytoplasm. Because the cloacin molecules possess no activity towards ribosomes as long as they are bound to the immunity protein, the latter is supposed to be released from the complex at one of the preceeding steps.

At the present time it is not known whether the inactivation of the ribosomes requires a penetration of the intact cloacin molecules or possibly only a fragment of these molecules. A possible approach to this question is the examination of the effect of proteolytic enzymes upon the activity of cloacin DF13. In this communication we describe that limited trypsinolysis of the cloacin-immunity protein complex generates a cloacin fragment-immunity protein complex without loss of in vitro activity. Trypsinolysis of the free cloacin results in a complete digestion of the bacteriocin.

2. Materials and methods

The complex of cloacin DF13 and immunity protein was obtained from *Enterobacter cloacae* (Clo DF13) by induction with mitomycin C and purified by ammonium sulphate precipitation and chromatography on CM-Sephadex. Details of the procedure will be published elsewhere. Analysis of the purified complex by SDS-gelelectrophoresis showed that the preparation contains only two protein components one of which is cloacin (mol. wt. 56 000) and the other is the immunity protein (mol. wt. 10 500).

Preparations of cloacin-immunity protein complex and of free cloacin were stored as lyophilized powders or dissolved in phosphate-NaCl buffers consisting of 10 mM sodium phosphate pH 7.0 and 100 mM NaCl.

The in vivo activity of the preparations was tested on the sensitive strain *Klebsiella edwardsii* [6].

The cloacin-induced inactivation of ribosomes in vitro was tested as described previously [4].

Sodium dodecyl sulphate gelelectrophoresis was performed after the method of Weber and Osborn [7]. The gels were stained with Amido Black followed by destaining in 7% acetic acid. The gels were scanned on a Kipp Lin/Log Densitometer DD2 in order to determine the quantity of the polypeptides. The mole fraction of each polypeptide was calculated from its corresponding area and mol. wt.

Protein determinations were performed as described by Lowry et al. [8].

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The trypsin preparation used was trypsin-TPCK (Worthington); soybean trypsin inhibiter was purchased from Boehringer Mannheim GmbH.

3. Results and discussion

At the present time it is not known whether the immunity protein is involved in the killing activity of bacteriocins upon sensitive cells. We therefore investigated the effect of limited trypsinolysis on both the cloacin–immunity protein complex and the free cloacin. Free cloacin can be prepared from the cloacin–immunity protein complex by gelfiltration in the presence of 0.1% sodium dodecyl sulphate. The best results are obtained with gelfiltration on Sephadex G200 as shown in fig.1. The first peak eluted from the column contains free cloacin and the second peak contains free immunity protein as confirmed by SDS-gelelectrophoresis. The fractions

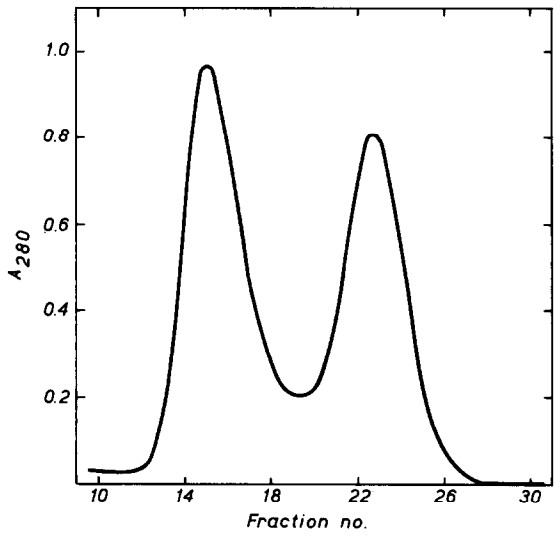

Fig.1. Separation of cloacin and immunity protein by gelfiltration in the presence of sodium dodecyl sulphate. About 7 mg of cloacin–immunity protein complex in phosphate-NaCl buffer supplemented with 1 mM dithiothreitol was treated with sodium dodecyl sulphate (1% final conc.) for 30 min at room temperature. The mixture was then applied to a column of Sephadex G200 (0.9 × 60 cm) equilibrated in phosphate-NaCl buffer containing 0.1% SDS and 1 mM dithiothreitol. The column was eluted with the same buffer and fractions of one ml were collected.

14–17 which contain most of the cloacin were collected and dialysed extensively against phosphate-NaCl buffer to remove the sodium dodecyl sulphate as much as possible.

Both the purified complex of cloacin and immunity protein and the preparation of free cloacin were tested for their killing activity in vivo as well as for the inactivation of ribosomes in vitro. The cloacin–immunity protein complex shows a very strong killing activity towards sensitive cells (4×10^6 killing units/mg protein) but almost no activity towards ribosomes. In order to activate the in vitro activity of the complex, the preparation in phosphate-NaCl buffer was heated for 20 min at 100°C. This procedure inactivates the immunity protein with little or no effect on the nucleolytic ability of the cloacin [9]. Kinetic studies concerning the cloacin-induced inactivation of ribosomes have revealed that one molecule of cloacin can inactivate about 500 ribosomes per min [10].

The in vitro activity of the preparation of free cloacin is comparable to the activity of the heated complex as can be seen from fig.5. In contrast with this very effective ribosome-inactivation the in vivo activity of the free cloacin is only about 1% of the in vivo activity of the cloacin-immunity protein complex. A possible explanation for the loss of in vivo activity is the observation that free cloacin is a very labile protein with a strong tendency towards aggregation and degradation although it still adsorbs to sensitive cells (results not shown). On the other hand the possibility cannot be excluded that some molecules of SDS are still bound to the cloacin molecules which probably prevents the proper folding of the molecules which is required for killing activity.

In order to investigate whether intact cloacin molecules are required for the cloacin-induced inactivation of ribosomes, the complex of cloacin and immunity protein was subjected to limited trypsinolysis under non-denaturing conditions. A time course of the tryptic digestion analyzed by SDS-gelelectrophoresis is shown in fig.2. During the first minutes of incubation a number of larger tryptic peptides is generated which are barely visible in the gels. One terminal fragment appears to accumulate which is not further hydrolysed even after several hours of incubation. The immunity protein seems to be resistant to trypsinolysis under these conditions.

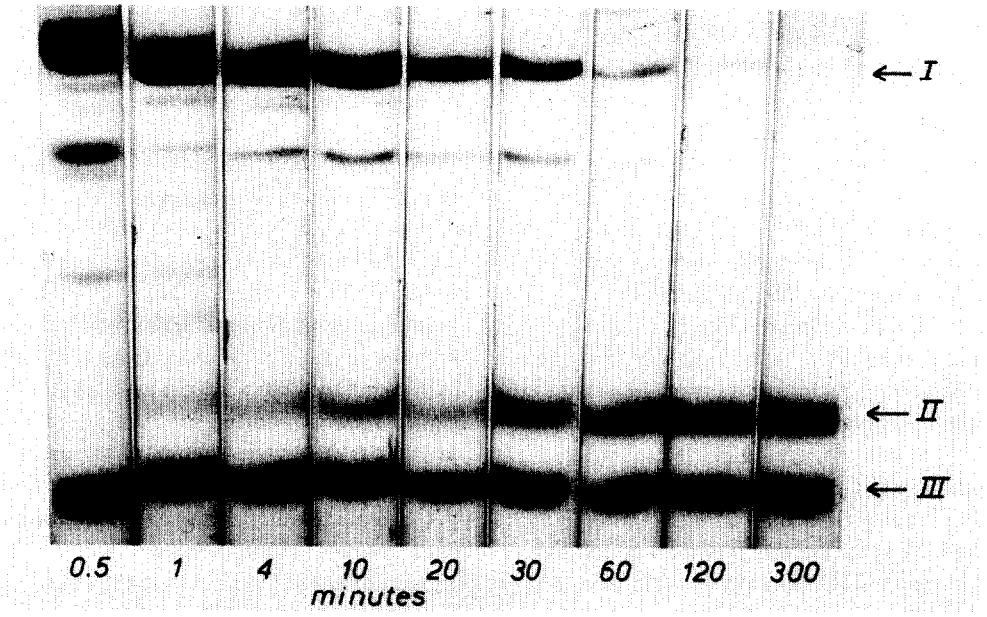

Fig.2. Limited trypsinolysis of the cloacin-immunity protein complex. Two mg of cloacin-immunity protein complex in 1.0 ml phosphate-NaCl buffer, adjusted to pH 8.0 were incubated with 40 μg of trypsin at 30°C. Aliquots (50 μl) of the incubation mixture were taken at timed intervals, supplemented with 4 μg of trypsin inhibitor to terminate the reaction, and subjected to SDS-gelelectrophoresis (40 μg sample per gel). Arrow I indicates the cloacin: arrow II indicates the cloacin fragment; arrow III indicates the immunity protein.

The mol. wt. of the terminal fragment as determined by comparison with standard proteins was found to be about 12 000 (data not shown). Using the mol. wt. of intact cloacin, terminal fragment and immunity protein the mole fractions of these three products were calculated and plotted as a function of time (fig.3). The results presented in figs.2 and 3 suggest that limited trypsinolysis of the cloacin–immunity protein complex finally results in the generation of a smaller complex which contains a cloacin fragment and the immunity protein in equimolar amounts.

In an additional experiment we incubated a preparation of free cloacin with trypsin under the same conditions as described in the legend of fig.2. The time course of the digestion analysed by SDS-gelelectrophoresis showed that the free cloacin is cleaved within 15 min into tryptic peptides which are too small to be detected by gelelectrophoresis (results not shown). From these experiments it may be concluded that the presence of cloacin-bound immunity protein protects the bacteriocin against a complete digestion.

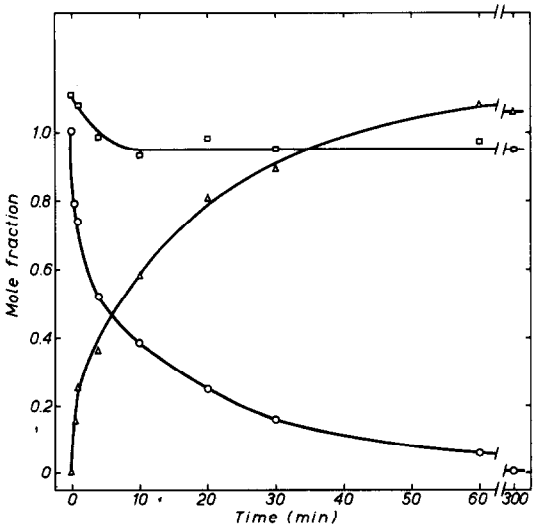

Fig.3. Kinetics of the generation of the terminal tryptic peptide. The mole fractions of cloacin (○-○), terminal fragment (△-△), and immunity protein (□-□) at a given time were calculated from the densitometer tracings of the SDS-gelelectrophoresis described in fig.2 and related to the mole fraction of the cloacin at t=o.

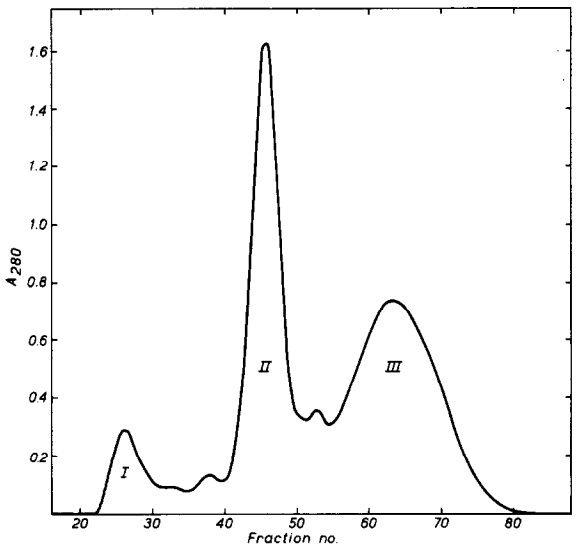

Fig.4. Isolation of the cloacin fragment–immunity protein complex. About 40 mg of cloacin-immunity protein complex in phosphate-NaCl buffer, adjusted to pH 8.0, was incubated with 0.8 mg of trypsin for 5 h at 30°C. Then 1.8 mg of trypsin inhibitor was added to terminate the reaction and the mixture was applied to a column of Sephadex G100 (1.5 × 100 cm) equilibrated in phosphate-NaCl buffer pH 7.0 at 4°C. The column was eluted with the same buffer and fractions of 5 ml were collected.

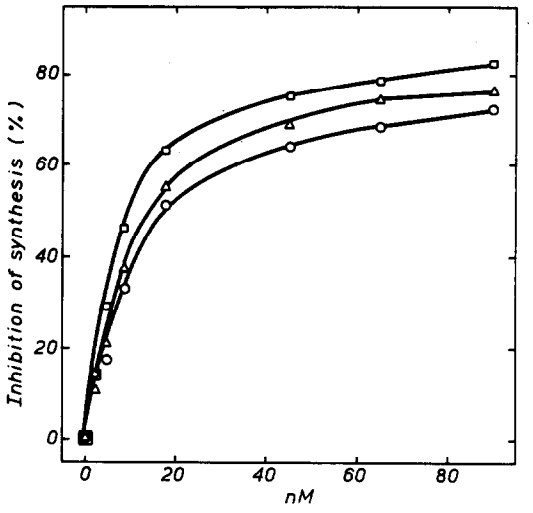

Fig.5. Inactivation of ribosomes by the cloacin fragment in vitro. The in vitro activity of the cloacin fragment was determined after heating the complex of cloacin fragment and immunity protein for 20 min at 100°C. The inhibition of polyphenylalanine synthesis, assayed as described under Materials and methods, was plotted as a function of the concentration of heated complex (○-○). The other lines represent the in vitro activity of the heated cloacin-immunity protein complex (△-△) and of the free cloacin (□-□) at the same concentrations.

In order to prove that the cloacin fragment is still bound to the immunity protein the cloacin–immunity protein complex was incubated with trypsin and subjected to gelfiltration on a column of Sephadex G100 equilibrated with phosphate-NaCl buffer. The mixture is separated into three peaks (fig.4). Peak I contains the complex of trypsin and trypsin inhibitor, peak II contains cloacin fragment and immunity protein, while peak III represents most probably the smaller digestion products which could not be detected by gelelectrophoresis. Cloacin fragment and immunity protein move as a single protein band on polyacrylamide gels without SDS. Any attempt to separate both polypeptides by ion-exchange chromatography have failed. The experimental observations strongly suggest that the cloacin fragment is still bound to the immunity protein. Unfortunately the difference in molecular weight between cloacin fragment and immunity protein is too small to achieve separation of both proteins by gelfiltration in the presence of SDS.

The isolated complex of cloacin fragment and immunity protein was used for the determination of in vivo and in vitro activity. We found that the complex did not possess any activity towards sensitive cells. For determination of in vitro activity the complex was heated for 20 min at 100°C to inactivate the immunity protein. As described in fig.5 the inactivation of ribosomes by this heated preparation was compared with the in vitro activity of a heated cloacin–immunity protein complex and of a preparation of free cloacin. The results indicate that all three preparations possess about the same in vitro activity.

Although the question still has to be answered whether proteolysis of free- or immunity protein-bound cloacin is involved in the interaction with sensitive cells in vivo, the experiments described above clearly demonstrate that intact cloacin molecules are not required for inactivation of bacterial ribosomes.

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