PURIFICATION AND CHARACTERIZATION OF THE CLOACIN DF13 IMMUNITY PROTEIN

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Received 25 January 1974

1. Introduction

Cloacin DF13 is a bacteriocin produced by *Enterobacter cloacae* DF13 [1]. Treatment of sensitive bacteria with cloacin DF13 results in leakage of potassium ions and inactivation of ribosomes [2]. This inactivation occurs both in vivo and in vitro and is accompanied by a specific cleavage of 16S rRNA near its 3'-terminus, producing a RNA fragment of 48 nucleotides [3]. In vitro inactivation of ribosomes is only observed if purified preparations of cloacin DF13 are used. Cell-free extracts prepared from cloacinogenic cells inhibit the in vitro activity of purified cloacin, although ribosomes isolated from sensitive as well as bacteriocin-producing cells are susceptible to the bacteriocin. These results suggest the presence of an inhibitor of cloacin DF13-action in cloacinogenic cells and are consistent with the observation that bacteriocinogenic cells are not susceptible to their homologous bacteriocin in vivo [4]. The specific immunity of cells harbouring a bacteriocinogenic factor is believed to be caused by the production of an immunity substance which prevents the inactivation of ribosomes [5, 6].

In this communication we describe the purification and characterization of the cloacin DF13 immunity protein from cloacinogenic cells.

2. Materials and methods

Cloacin DF13 was obtained from *Enterobacter cloacae* DF13 by induction with mitomycin C and purified by precipitation with ammonium sulphate and chromatography on QAE-Sephadex [7].

The sensitive strain *Ent. cloacae* 02 was used for determination of in vivo activity of cloacin DF13 [1].

Cloacin DF13 immunity protein was isolated from the cloacinogenic strain *E. coli* P678-54 (Clo DF13-Rep 3) obtained from A. J. Kool [8]. This strain possesses a three-fold increased number of Clo DF13 plasmids compared to the wild type *Ent. cloacae* DF13.

Cell-free extracts (S-30) were prepared from *E. coli* MRE 600 [2].

Immunity activity was assayed in a cell-free poly U-directed polyphenylalanine synthesizing system [2]. Aliquots of fractions to be tested were mixed with 10 A_{260} units S-30 extract in TMK-buffer (0.01 M Tris–HCl pH 7.8, 0.01 M magnesium acetate and 0.06 M KCl), 80 μl reaction mixture [2] and 5 μg purified cloacin DF13. After 15 min of preincubation at 37°C 20 μg poly U and 0.5 μCi [^{14}C] phenylalanine (477 mCi/mmole) were added and the mixture was incubated for another 20 min at 37°C. The reaction was stopped by addition of 3 ml 5% trichloroacetic acid and the amount of polyphenylalanine synthesis was assayed [2].

3. Results

For the isolation of immunity protein a 10 litre culture of *E. coli* P 678–54 (Clo DF13–Rep 3) in

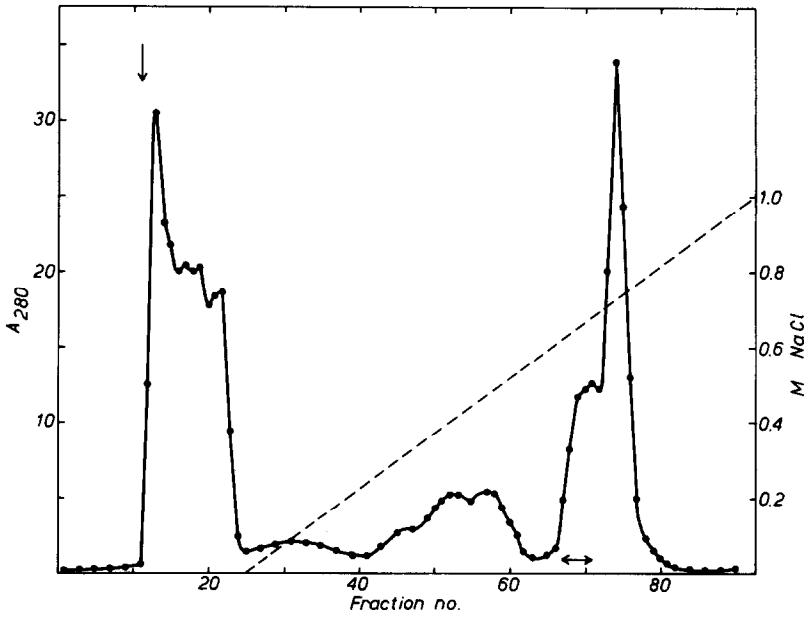

Fig. 1. Column chromatography of dialysed NaCl extracts. The extract was applied to a column (2.5 × 45 cm) of DEAE-Sephadex A50 equilibrated in TMK-buffer and was washed into the column with 75 ml of the same buffer. The banded proteins were then eluted from the column with a 600 ml linear gradient of 0.0 to 1.0 M NaCl in TMK-buffer during 24 hr. Fractions of 9 ml were collected. Fractions above the bracket contain maximum immunity activity. The arrow indicates the void volume of the column (•–•–•) absorbance at 280 nm; (– – – – –) NaCl-gradient.

Brain Heart Infusion (Oxoid) was grown in a MicroFerm laboratory fermentor (New Brunswick Scientific Co.) to a density of 5×10^8 cells per ml and then induced for 60 min with 0.5 mg per liter of mitomycin C (Sigma). The cells were harvested by centrifugation and washed with saline. The cell pellet was then lysed by freezing and thawing twice. After addition of 1 mg DNase the broken cells were extracted for 20 min with 50 ml 1 M NaCl in TMK-buffer. The extraction was done at 0°C and repeated twice. The pooled extracts (150 ml) contain 950 mg protein including cloacin DF13 and immunity substance. After overnight dialysis against TMK-buffer the immunity protein was separated from the bacteriocin and most of the other material by chromatography on a column of DEAE-Sephadex (fig. 1). Eluted fractions were monitored for absorbance at 280 nm, in vivo cloacin activity and in vitro immunity activity. All cloacin activity elutes virtually in the void volume of the column. The immunity activity binds tightly to the DEAE-Sephadex and was eluted between 0.6 and 0.7 M NaCl. Fractions containing maximum immunity activity were pooled and concentrated by ammonium sulphate precipitation at 100% saturation. The pellet (56 mg protein) was dissolved in 3 ml TMK-buffer and dialysed against the same buffer. The concentrated preparation was further purified by gel-filtration on Sephadex G75 in TMK-buffer (fig. 2). The major peak eluted from this column contains much tRNA, the minor peak contains all the immunity activity. Fractions containing maximum activity were pooled and used without further concentration. The final yield of this purification was 24 mg purified immunity protein.

Purified immunity protein runs as a single band on 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate (results not shown). For determination of the isoelectric point, a sample of purified immunity protein was subjected to isoelectric focusing in a density gradient-stabilized natural pH gradient as described by Vesterberg and

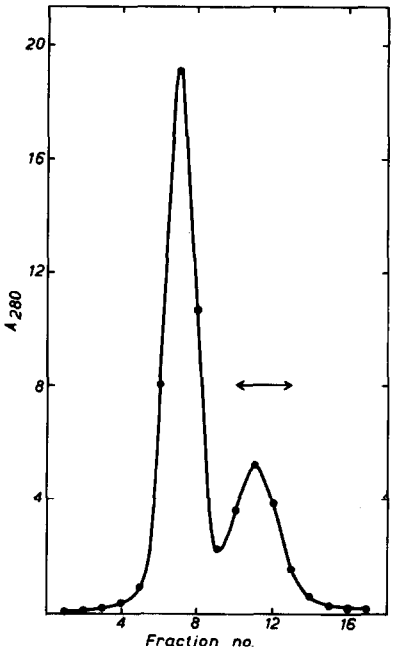

Fig. 2. Gel-filtration of immunity protein. The concentrated preparation from the DEAE-Sephadex column was applied to a column (1 X 60 cm) of Sephadex G75 equilibrated in TMK-buffer. The column was eluted with the same buffer and 5 ml fractions were collected. Fractions under the bracket contain maximum immunity activity. (•–•–•) Absorbance at 280 nm.

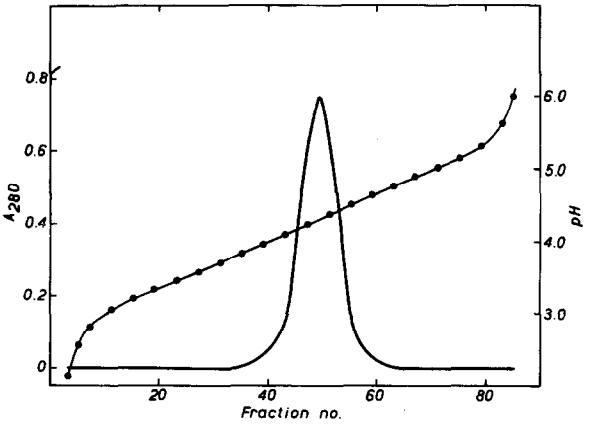

Fig. 3. Isoelectric density gradient electrophoresis of purified immunity protein. Electrophoresis of 4.5 mg protein was performed in a pH 3 to 6 ampholyte preparation (Ampholine, LKB). Electrophoresis was continued for 72 hr. Fractions of 30 drops were collected and monitored for absorbance at 280 nm and pH. (——) Absorbance at 280 nm; (•–•–•) pH gradient.

Svensson [9]. As shown in fig. 3 the immunity protein was banded as a single peak with an isoelectric point of 4.30. The sedimentation coefficient of purified immunity protein was determined by sedimentation velocity experiments in an analytical ultracentrifuge (Beckman-Spinco, model E). The preparation appears to be homogeneous and the $S^0_{20,w}$ could be calculated as 1.7 S. Molecular weight analysis was performed by the sedimentation equilibrium method described by Schachman and Edelstein [10] in an analytical ultracentrifuge equipped with a photoelectric scanner and using the multichannel short column equilibrium centerspiece SP 350.024. The analysis was performed by Dr. J. van 't Riet with three different concentrations of immunity protein in TMK-buffer at 24.630 rpm and 0°C. The molecular weight as determined by this technique is 10 500 assuming a partial specific volume of 0.725 ml per g. Chemical analysis of

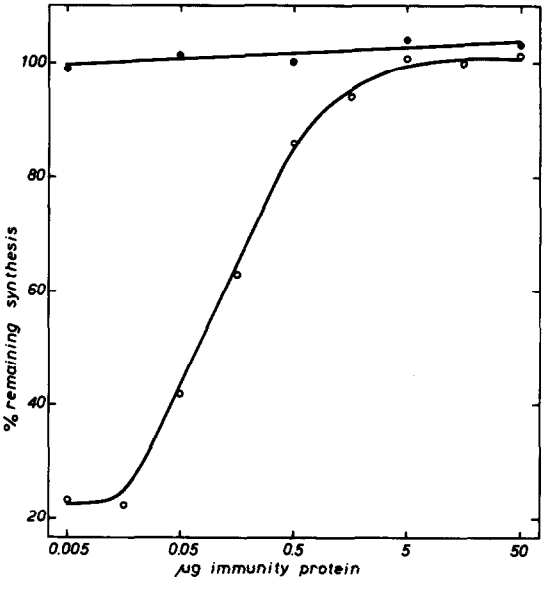

Fig. 4. Titration of purified immunity protein against cloacin DF 13. Various amounts of purified immunity protein were added to in vitro protein synthesizing systems containing 5 µg purified cloacin DF13 (o–o–o) titration curve with cloacin; (•–•–•) control experiment without cloacin. 100% Synthesis in control experiments stands for the incorporation of 2 nmole [^{14}C]phenylalanine into polyphenylalanine. In the presence of 5 µg purified cloacin DF13 this incorporation is reduced to 0.45 nmole [^{14}C]phenylalanine.

purified immunity protein showed that the preparation contained no detectable amount of nucleic acid or carbohydrate.

The potency of purified immunity protein to neutralize the in vitro activity of purified cloacin DF13 was determined by adding various amounts of immunity protein to assay systems containing a given amount of cloacin (fig. 4). From this titration it could be calculated that the neutralization of one μg clacin DF13 requires no more than one μg immunity protein.

If a mixture of purified cloacin and immunity protein was added to exponentially growing cells of sensitive bacteria the immunity protein could not prevent the lethal action of the cloacin even in a 100-fold excess (table 1).

Table 1
Effect of purified immunity protein on the killing action of cloacin DF13 in vivo

Cloacin (μg)	Immunity protein (μg)	% Surviving cells
0	0	100
5	0	2
5	500	2

8×10^7 Exponentially growing cells of *Enterobacter cloacae* 02 were mixed with 500 μg purified immunity protein and/or 5 μg purified cloacin DF13. After incubation for 20 min at 37°C the mixtures were diluted and spreaded on broth–agar plates. After overnight incubation at 37°C the surviving colonies were scored.

No immunity protein could be detected if a parallel purification was carried out on an extract of *E. coli* P678–54 which means that the immunity protein is specific for cells harbouring the cloacinogenic factor.

4. Discussion

Bacterial cells harbouring the cloacinogenic factor DF13 appear to produce a small acidic protein which completely blocks the in vitro inactivation of ribosomes by cloacin DF13. However, this so-called immunity protein cannot protect sensitive cells from being killed in vivo by the bacteriocin. Obviously the immunity protein cannot penetrate into sensitive cells nor prevent the effective adsorbtion of bacteriocin molecules to the specific cell envelope receptors. Therefore the immunity protein is only active in the cytoplasm of bacteriocin producing cells or in in vitro systems. From these results two possibilities might be predicted with respect to its mechanism of action. Either the immunity protein interacts directly with the bacteriocin or it protects the ribosomes from inactivation. Binding of immunity protein with bacteriocin might neutralize the affinity of the bacteriocin molecules to the ribosomes without any effect on their affinity to the cell surface receptors. Binding of immunity protein to the ribosomes should allow protein synthesis while preventing the cleavage of 16 S rRNA by cloacin DF13.

Acknowledgements

The present investigation was sponsored in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.).

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