Limited Proteolysis of Cloacin DF13 and Characterization of the Cleavage Products†

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ABSTRACT: Limited proteolysis of cloacin with thermolysin or thrombin results in the accumulation of one well-defined cloacin fragment which has an apparent molecular weight of 35 000 and 36 000, respectively. Digestion of cloacin with either trypsin, chymotrypsin, subtilisin, or clostripain yields fragments of various molecular weight. The cloacin is extremely sensitive to these proteases. Several high-molecular-weight fragmentation products, formed by these proteases, are immediately further digested, even before all of the starting material has disappeared. The complex of cloacin and its immunity protein is much less sensitive to proteolytic cleavage. Low-molecular-weight cloacin fragments appear in the incubation mixtures. These low-molecular-weight fragments remain attached to the immunity protein which is not cleaved at the conditions used. If purified preparations of cloacin or cloacin complex are stored in phosphate buffer, considerable fragmentation occurs. The rate and extent of this fragmentation are strongly dependent on temperature and buffer concentration. Cloacin complex is more resistant to this (auto)-digestion than the cloacin itself. The cloacin fragments obtained after cleavage with thermolysin and thrombin have been isolated and purified on a preparative scale together with a "spontaneous" cloacin fragment which accumulates after storage of cloacin in 50 mM phosphate/NaCl buffer at 37 °C. This "spontaneous" fragment has an apparent molecular weight of 21 000. Amino acid analysis has shown that all three isolated fragments are derived from the C-terminal part of the cloacin molecule. This C-terminal part contains almost all the charged amino acid residues present in the intact cloacin molecule, whereas the N-terminal part consists almost exclusively of hydrophobic amino acids. All three isolated fragments are still able to inactivate bacterial ribosomes in vitro. The endoribonucleolytic activity is inhibited by the addition of immunity protein. The fragments obtained after cleavage with thrombin or thermolysin are comparable to intact cloacin with respect to the receptor-binding activity, but the smaller "spontaneous" fragment has lost this property. The significance of these results with respect to the localization of different functions on the cloacin molecule is discussed.

Cells of *Enterobacter cloacae* harboring the bacteriocinogenic plasmid Clo DF13 produce a bacteriocin which possesses a very effective killing activity against *Enterobacter* and *Klebsiella* species (de Graaf et al., 1969). This killing activity in vivo is the result of two events: leakage of potassium ions across the cytoplasmic membrane and inhibition of protein biosynthesis (de Graaf et al., 1971; de Graaf, 1973). The bacteriocin is excreted by cloacinogenic cells as an equimolar complex of two plasmid-specific gene products: the cloacin and its inhibitor the immunity protein (de Graaf and Klaasen-Boor, 1977). The cloacin is characterized by its ability to inhibit protein synthesis by endoribonucleolytic cleavage of the 16S rRNA near its 3′ terminus in vivo as well as in vitro (de Graaf et al., 1973; Oudega and de Graaf, 1976). The immunity protein confers "immunity" to the cloacinogenic cells by inhibiting the enzymatic activity of intracellular cloacin (Oudega et al., 1975a). These findings suggest that the cloacin has to penetrate the cytoplasmic membrane of sensitive cells in vivo to contact the ribosomes and that the immunity protein has to be removed from the complex at some step before penetration. Comparable results have already been described for colicin E3 (Bowman et al., 1971; Jakes et al., 1974; Sidikaro and Nomura, 1974; Jakes and Zinder, 1974).

Since it is not known whether ribosome inactivation requires a penetration of the intact cloacin or possibly only a fragment of these molecules as, for instance, has been described for diphtheria toxin (Collier, 1975), we started to investigate the effect of proteolytic cleavage on cloacin activity by isolation and characterization of proteolytic fragments. A comparable study about the effect of proteolytic modification on colicin E3 activity has been published by Lau and Richards (1976).

Another reason to start this study was the observation that preparations of cloacin show a rather rapid fragmentation upon storage in low concentration phosphate buffer at pH 7.0. The low-molecular-weight fragments which appear after aging of the preparations possess no killing activity but are still able to inhibit protein synthesis in vitro.

In a previous paper we described that limited trypsinolysis of the cloacin–immunity protein complex generates a cloacin fragment–immunity protein complex (Mooi and de Graaf, 1976). Destruction of the activity of the immunity protein in the fragment–immunity protein complex by heat treatment showed that this fragment still inhibits protein synthesis in vitro.

Experimental Procedure

Bacterial Strains. The complex of cloacin and immunity protein, designated as cloacin complex, was isolated from the bacteriocinogenic strain *Enterobacter cloacae* (Clo DF13) as described previously (de Graaf and Klaasen-Boor, 1977). Purified cloacin was obtained by dissociation of the cloacin complex (de Graaf and Klaasen-Boor, 1977). Purified immunity protein was prepared from *Escherichia coli* P678-54 (Clo DF13-Rep3) as described previously (de Graaf and Klaasen-Boor, 1974). The cloacin-sensitive strain *Klebsiella edwardsii* var. *edwardsii* was used for determination of killing activity (de Graaf et al., 1969). The sensitive strain *Enterobacter cloacae* 02 was used for the determination of recep-

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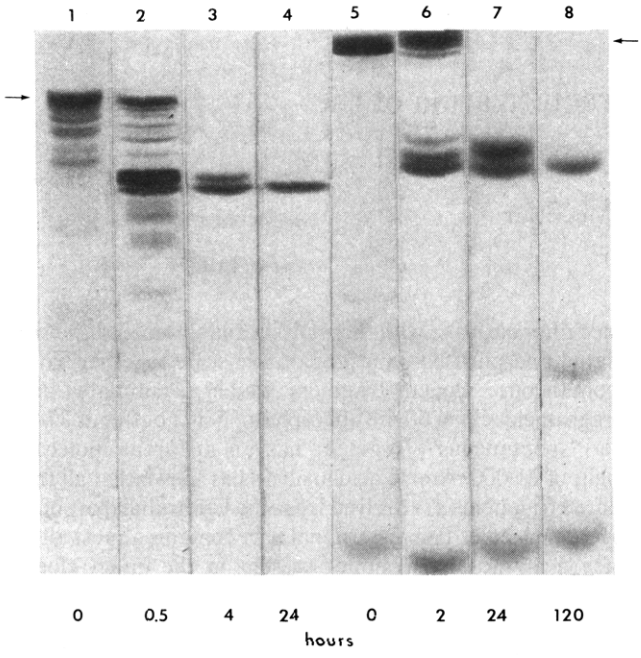


FIGURE 1: Sodium dodecyl sulfate gel electrophoresis of a digestion mixture of cloacin and cloacin complex with thermolysin. One milligram of cloacin or cloacin complex in 1.0 mL of 0.1 M borate buffer (pH 7.2) containing 0.2 mM $CaCl_2$ was incubated with 1.0 μg of thermolysin at 4 °C or 2.0 μg of thermolysin at 30 °C, respectively. Aliquots (50 μL) of the incubation mixtures were taken at timed intervals, supplemented with 2 mM EDTA (final concentration) to stop proteolysis, and subjected to sodium dodecyl sulfate gel electrophoresis. Gels 1–4 represent digestion of cloacin after 0, 0.5, 4, and 24 h, respectively; gels 5–8 represent digestion of cloacin complex after 0, 2, 24, and 120 h, respectively. The arrow indicates the position of intact cloacin in both preparations.

tor-binding activity of cloacin preparations. Cell-free extracts (30 000g supernatants) for protein synthesis in vitro were prepared from *E. coli* MRE 600 (de Graaf et al., 1971).

Enzymes. Trypsin (TPCK treated) was obtained from Worthington and thrombin from Hoffmann-La Roche. The other proteases used in this study were supplied by Boehringer (Mannheim).

Buffer Solutions. Phosphate buffer contains 50 mM phosphate (pH 7.0) unless otherwise stated. Phosphate/NaCl buffer is phosphate buffer containing 0.2 M NaCl.

Determination of Activity of Cloacin and Cloacin Fragments in Vitro. The endoribonucleolytic activity of cloacin or cloacin fragments on 16S ribosomal RNA was assayed by determination of the inhibition of a cell-free poly(U)[1]-dependent polyphenylalanine-synthesizing system as described previously (Oudega and de Graaf, 1976).

Sodium Dodecyl Sulfate Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate essentially as described by Weber and Osborn (1969), except that the gels contained 4 M urea. Samples to be analyzed were preincubated for 5 min at 100 °C in the presence of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. After electrophoresis for 6 h at 7 mA per gel, the gels were fixed and stained for 1 h at room temperature in a solution of 0.4% amido black in 7% acetic acid. Destaining was performed by diffusion in 7% acetic acid.

Determination of Receptor-Binding Activity. A 3-mL suspension of *Enterobacter cloacae* 02 containing 6×10^{10} log-phase cells per mL in phosphate/NaCl buffer was incubated with an excess of the protein to be tested at 37 °C. After 5 min of incubation, the nonbound protein was removed by centrifugation. The cell pellet was resuspended in 3 mL of phosphate/NaCl buffer. Two portions of 1.0 mL of this suspension were then incubated with 4.4 μg of ^{14}C-labeled cloacin complex at 37 °C. After 5 min of incubation, the suspensions were centrifuged for 5 min at 20 000g. The supernatant was supplemented with 10 mL of dioxane-based scintillation liquid and the radioactivity of the samples was counted in a liquid scintillation counter. As a control for nonspecific adsorption, a similar experiment was done with a cloacin-resistant mutant of *Enterobacter cloacae* 02. This mutant has lost its receptor functions. The amount of added ^{14}C-labelled cloacin complex was just enough to occupy all receptors of the cells in 1.0-mL suspension (Oudega et al., 1977).

Proteolytic Digestion. Digestion of cloacin and its proteolytic fragments with a mixture of carboxypeptidases A and B was performed at 37 °C for 4 h as described by Ambler (1972). For the other proteases the reaction circumstances are given in the Results section.

Amino Acid Hydrolysis. Amino acid analyses were performed on a Beckman multichrome M amino acid analyzer. Samples were hydrolyzed in 0.4 mL of 6 M HCl at about 110 °C in evacuated sealed glass ampoules for 24 h.

Dansylation and N-Terminal Amino Acids. Dansylation was carried out as described by Gros and Labouesse (1969). Dansyl amino acids were identified by thin-layer chromatography on 5 × 5 cm polyamide sheets (Woods and Wang, 1967). Determination of the N-terminal amino acid with the phenyl isothiocyanate method was performed with the modified Edman degradation described by Tarr (1975). Separation of Pth-amino acids was obtained on silica gel thin layers containing fluorescence indicator in xylene:2-propanol (70:20). Spots were detected under ultraviolet light and by ninhydrin–collidine staining (Roseau and Pantel, 1969).

Results

Digestion of Cloacin with Proteolytic Enzymes. Digestions of cloacin with low concentrations of either trypsin, chymotrypsin, subtilisin, or clostripain result in a gradually increasing fragmentation of the cloacin molecules. Despite the use of very small amounts of these proteolytic enzymes at suboptimal conditions, excessive fragmentation occurs. Analysis of the digestion products of trypsinolysis on sodium dodecyl sulfate gels at several time intervals shows that in the early stages of digestion several high-molecular-weight fragments accumulate in different amounts before all of the starting material has disappeared. This observation indicates a simultaneous digestion of cloacin and intermediate fragmentation products. At no stage the sum of the molecular weights of the fragments equals the molecular weight of the intact cloacin. Considering the extreme sensitivity of cloacin to proteolytic cleavage, these enzymes have proved to be less suitable for the isolation of well-defined cloacin fragments.

More useful results were obtained by digestion of cloacin with thermolysin and thrombin. Limited proteolysis with these enzymes results in the generation of one well-defined cloacin fragment. During digestion with thermolysin, at a weight ratio of cloacin to enzyme of 1000:1 at 4 °C and pH 7.2 in 0.1 M borate buffer, two fragments with a small difference in molecular weight appear in the early stages of digestion (Figure 1). One of these fragments seems to be very susceptible to proteolysis and is completely digested upon further incubation. The final fragment has an apparent molecular weight of 36 000 and accumulates in equimolar amount to the original amount

[1] Abbreviations used are: poly(U), poly(uridylic acid); Pth, phenylthiohydantoin; CM, carboxymethyl.

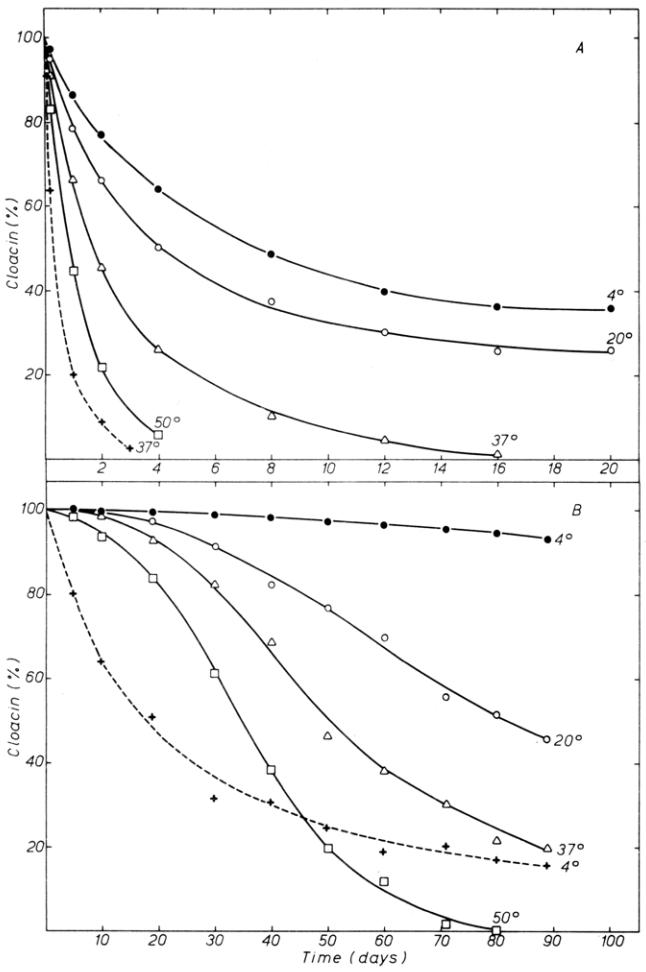


FIGURE 2: Decrease in cloacin concentration after incubation of a cloacin solution at different temperatures. (A) Four solutions of 1.0 mg of cloacin in 1.0 mL of phosphate/NaCl buffer and one solution of 1.0 mg of cloacin in 1.0 mL of 10 mM phosphate buffer (pH 7.0) were incubated at the indicated temperatures. At timed intervals, a sample of 50 μL was taken and analyzed by sodium dodecyl sulfate gel electrophoresis. (B) A comparable experiment with cloacin complex. The dotted lines represent the incubation mixture in 10 mM phosphate buffer (pH 7.0). The cloacin concentration is given as percentage of the initial concentration of intact cloacin.

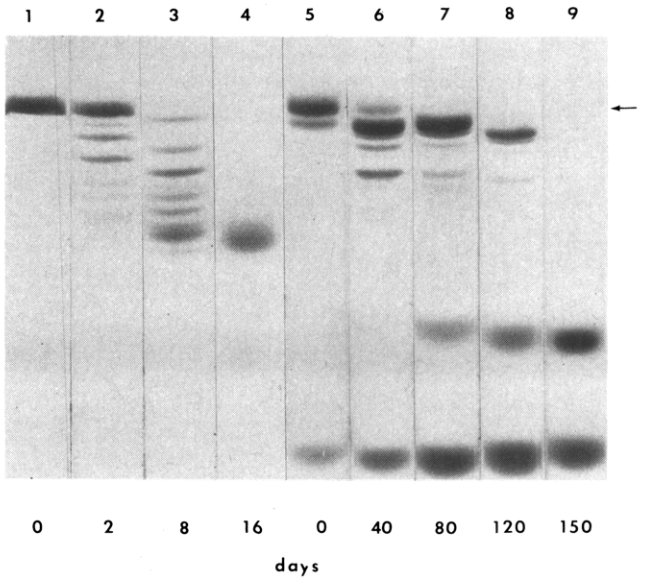


FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of solutions of cloacin and cloacin complex incubated at 37 °C. A solution of 1.0 mg of cloacin or cloacin complex in 1.0 mL of phosphate/NaCl buffer was incubated at 37 °C. At timed intervals, samples of 50 μL were taken and analyzed by sodium dodecyl sulfate gel electrophoresis. Gels 1–4 represent the cloacin preparation after 0, 2, 8, and 16 days of storage, respectively; gels 5–9 represent the preparation of cloacin complex after 0, 40, 80, 120, and 150 days of storage, respectively. The arrow indicates the position of intact cloacin in both preparations.

of intact cloacin. Comparable results were obtained by digestion with thrombin. Incubation of thrombin and cloacin in a weight ratio of 1:50 at 25 °C in phosphate buffer generates a fragment with an apparent molecular weight of 35 000.

The fragments obtained after cleavage of cloacin with thermolysin or thrombin were isolated on a preparative scale by gel filtration of digestion mixtures on a column of Sephacryl S-200. Further purification was achieved by ion-exchange chromatography on CM-Sephadex.

Digestion of Cloacin Complex with Proteolytic Enzymes. Digestion of the cloacin complex with proteolytic enzymes has shown that this complex is much less sensitive to proteolytic attack when compared with cloacin itself. With none of the proteases used did we observe a detectable fragmentation of the immunity protein and fragmentation of the cloacin moiety of the complex could only be achieved at higher concentrations of proteolytic enzymes and optimal conditions. The characteristics of the fragmentation of the cloacin bound to the immunity protein are, however, comparable to the fragmentation of "free" cloacin. Several high-molecular-weight intermediates appear and are digested before all of the starting material has disappeared. As an example, Figure 1 shows the analysis of the fragmentation products in a digestion mixture of cloacin complex with thermolysin. Thrombin could not digest the complex even after a long period of incubation at optimal conditions.

Analysis of the final stage of proteolysis on polyacrylamide gels without sodium dodecyl sulfate shows that the fragments obtained are still bound to the immunity protein since only one single protein band was detected. These results suggest that the immunity protein probably protects the part of the cloacin molecule to which it is attached from proteolytic cleavage while the other part of the molecule is digested rapidly into smaller fragments which could not be detected on the gels. A comparable situation has already been described for the limited trypsinolysis of cloacin complex (Mooi and de Graaf, 1976).

Fragmentation of Cloacin and Cloacin Complex upon Aging. As can be seen from Figure 1 freshly prepared cloacin shows some minor additional protein bands with a molecular weight slightly less than the intact cloacin. These high-molecular-weight fragments are absent in the original purified cloacin complex but appear during the isolation of cloacin from the complex (see Materials and Methods). If a preparation of cloacin is stored in phosphate buffer this fragmentation gradually increases concomitant with a decrease in killing activity. Figure 2A shows the percentage of the initial amount of cloacin which is left after storage of a solution of cloacin at different temperatures at pH 7.0. About 50% of the cloacin has been lost after 1 day of incubation at 50 °C, 2 days at 37 °C, 4 days at 20 °C or 8 days at 4 °C, respectively. The killing activity of the samples is directly proportional to the amount of intact cloacin. It can be observed that fragmentation is not only dependent on the temperature but also on the concentration of the buffer solution. At low buffer concentration (10 mM phosphate) fragmentation proceeds very rapidly and after 3 days at 37 °C all of the intact cloacin has disappeared. In low concentration buffers the cloacin molecules show a strong tendency to aggregation, finally resulting in precipitation of the protein after about 5 days. Figure 3 shows an analysis of

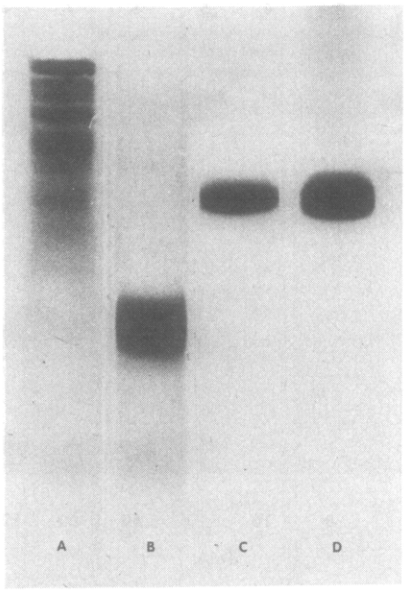


FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of cloacin fragments. Gel A represents intact cloacin and its high-molecular-weight fragmentation products; gel B represents the cloacin fragment isolated after incubation of cloacin for 5 days in phosphate/NaCl buffer at 37 °C; gel C represents the purified fragment isolated after cleavage with thermolysin; gel D represents the purified fragment isolated after cleavage with thrombin.

TABLE I: Amino Acid Analysis of Cloacin and Its Proteolytic Fragments.

Amino acid residue	Number of residues			
	Cloacin	"Thrombin" fragment	"Thermolysin" fragment	"Spontaneous" fragment
Asp	40.12	33.75	40.75	22.01
Thr	22.56	13.05	12.44	7.48
Ser	24.38	9.40	8.77	8.82
Glu	55.11	50.46	54.74	25.41
Pro	40.00	17.78	17.55	11.76
Gly	80.49	29.40	32.28	29.80
Ala	50.29	36.71	34.35	18.58
Cys	–	–	–	–
Val	33.73	12.44	10.75	7.51
Met	+	+	+	–
Ile	18.07	8.62	9.26	4.71
Leu	33.74	18.80	18.12	9.15
Tyr	11.27	8.92	9.15	4.09
Phe	11.52	3.99	4.68	3.92
Lys	67.93	52.75	53.23	27.22
His	10.20	6.38	7.02	4.19
Arg	24.86	17.58	18.05	8.40
Trp	Present	Present	Present	–

[a] + means present as methionine sulfoxide and methionine sulfone.

the fragmentation products of cloacin obtained in phosphate/NaCl buffer at 37 °C on sodium dodecyl sulfate gels. Several high-molecular-weight fragmentation products can be observed. Finally, a polypeptide with an approximate molecular weight of 21 000 accumulates. This cloacin fragment seems to be rather resistant to further proteolytic cleavage but after long periods of incubation it is also digested and no polypeptides are detectable anymore with our electrophoresis system.

Similar phenomena as described for cloacin can be observed upon aging of purified cloacin complex (Figure 2B). The cloacin moiety of the complex is also cleaved but at a much reduced rate when compared with "free" cloacin. At 37 °C about 50% of the initial amount of complex is lost in 50 days, but at 4 °C we observed about 10% loss of intact cloacin after 3 months of storage. These findings confirm the results obtained with proteolytic enzymes which also demonstrate that the complex is much less sensitive to proteolytic cleavage than cloacin itself. Figure 3 shows an analysis of the cloacin complex which was stored in phosphate/NaCl buffer at 37 °C. The immunity protein seems to be resistant to proteolysis since the amount of this protein does not change upon storage.

The observed proteolytic activity of the cloacin preparations is very weak and we did not succeed in estimating this activity with the aid of the usual synthetic substrates for proteolytic enzymes. Inhibitors of proteolytic activity like *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) or *p*-chloromercuriphenylsulfonic acid do not prevent this fragmentation.

The low-molecular-weight fragment which accumulates upon aging of the cloacin in phosphate/NaCl buffer at 37 °C was isolated on a preparative scale by gel filtration on a column of Sephacryl S-200. Sodium dodecyl sulfate gel electrophoresis of the purified cloacin fragments is shown in Figure 4. As can be seen the isolation of the fragments is clean and unambiguous.

Analysis of the Isolated Cloacin Fragments. The amino acid composition of cloacin and its proteolytic fragments is given in Table I. It is remarkable that except for some lysine and arginine residues the two larger proteolytic fragments almost exclusively contain the charged residues present in the cloacin molecule and that the part removed from the cloacin by proteolysis consists mainly of hydrophobic amino acid residues. The fragment which appears upon "aging" of cloacin also consists of 50% of basic and acidic amino acid residues.

The N-terminal amino acids of the "thermolysin" fragment (Val) and of the "thrombin" fragment (Gly) are in agreement with the specificity of these enzymes. No N-terminal amino acid could be determined for cloacin itself, whereas several N-terminal amino acids were found for the "spontaneous" fragment. Analysis of the C-terminal amino acids of the "thrombin" and "thermolysin" fragment shows that both are derived from the same, namely, the C-terminal part of the cloacin determined as Thr, Leu, Ser, $(Lys)_2$, Tyr, Arg, Ile, –COOH. Analysis of the C-terminal amino acids released from the "spontaneous" fragment upon the interaction of carboxypeptidases A and B indicates that this fragment has lost some residues at the C-terminal part of the intact cloacin molecule.

Properties of the Cloacin Fragments. None of the purified cloacin fragments possesses any killing activity toward sensitive cells in vivo. The activity of the fragments in vitro was tested as described under Experimental Procedure. Figure 5 shows that all isolated fragments are still able to inhibit protein synthesis. The efficiency of the ribosome inactivation seems comparable for the different fragments but is slightly enhanced when compared with the activity of intact cloacin. In an additional experiment we observed that the endoribonucleolytic activity of the cloacin fragments can be inhibited by the addition of purified immunity protein.

Another essential property of the cloacin is the binding to cell surface receptors. This receptor-binding activity was tested for the purified fragments as described under Experimental Procedure. The fragments obtained after cleavage by thrombin or thermolysin are still able to bind to the receptors since the maximum amount of ^{14}C-labeled cloacin complex which can be bound to the cells is reduced to about 15% after preincubation of the cells with the particular fragments. The same

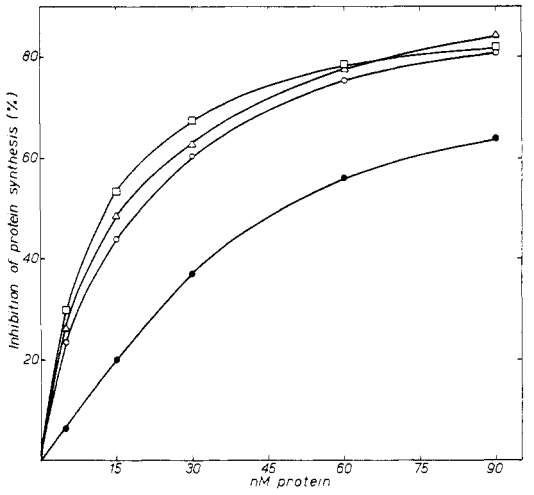


FIGURE 5: Assay of endoribonucleolytic activity of cloacin fragments. Different amounts of cloacin or cloacin fragment were added to 60 μL of cell-free extract. The incorporation of [^{14}C]phenylalanine was then assayed (see Experimental Procedure). (●—●) Intact cloacin; (○—○) cloacin fragment obtained after cleavage with thermolysin; (△—△) cloacin fragment obtained after cleavage with thrombin; (□—□) cloacin fragment obtained after storage of a cloacin preparation in phosphate/NaCl buffer at 37 °C. Inhibition of protein synthesis is given as a percentage of the incorporation without cloacin.

result was obtained by preincubation of the cells with nondigested cloacin. However, the smaller fragment isolated after "aging" of the cloacin at 37 °C in phosphate/NaCl buffer does no longer possess this function since in this case the cells adsorb almost all of the added cloacin complex.

Discussion

The present study was initiated by the observation that preparations of cloacin in phosphate buffer rather rapidly lose their killing activity but retain their capacity to inactivate bacterial ribosomes in vitro after a longer period of storage. Preliminary analysis of "aged" preparations by sodium dodecyl sulfate gel electrophoresis has shown that the decrease in killing activity most probably must be considered as a result of proteolytic cleavage of the cloacin molecules since an extensive fragmentation was observed on the gels.

Based on these previous observations this paper describes the decrease in the concentration of intact cloacin both in the presence and in the absence of immunity protein in different buffers and at several temperatures. Analysis of the incubation mixtures by sodium dodecyl sulfate gel electrophoresis shows a gradually increasing proteolytic cleavage of the cloacin molecules which proceeds faster at higher temperature or at lower buffer concentration. The complex of cloacin and immunity protein was found to be more resistant to proteolytic cleavage than the cloacin itself. A solution of cloacin complex in phosphate/NaCl buffer can be stored for months without a considerable loss of killing activity. The killing activity of the preparations is directly proportional to the amount of intact cloacin. Apparently the cleavage of one or a few peptide bonds results in a dramatic loss of in vivo activity.

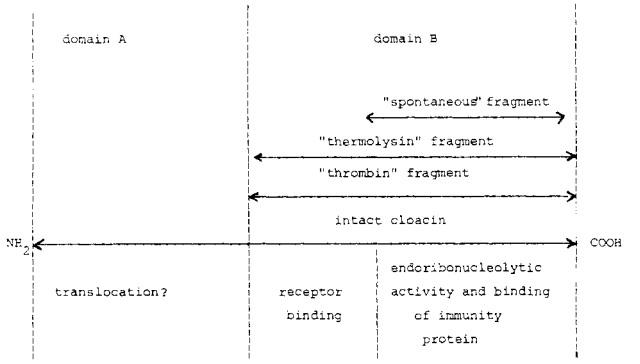
The remarkable difference in the rate of proteolytic cleavage of cloacin and cloacin complex upon storage may indicate that the proteolytic activity of the preparation is not a result of contamination by some unknown protease but possibly an intrinsic enzymatic activity of the cloacin molecules. Binding of the immunity protein inhibits this activity like it inhibits the endoribonucleolytic activity of the cloacin. An argument in favor of this assumption comes from the observation that the purified low-molecular-weight fragment which was separated from the cloacin after incubation in phosphate/NaCl buffer at 37 °C is also cleaved upon further incubation at 37 °C under the same conditions. However, despite the fact that some experimental results may support the hypothesis of (auto) proteolytic activity for the cloacin the data obtained with commercially available proteases show that the cloacin is extremely sensitive to proteolytic cleavage and only a very small contamination is sufficient for the degradation of the molecule. Furthermore, the experiments performed with these enzymes also show that binding of the cloacin to its immunity protein renders the bacteriocin more resistant to proteolytic attack.

Previous experiments on the effect of limited trypsinolysis of cloacin complex have revealed that this treatment results in the generation of a "stable" cloacin fragment–immunity protein complex (Mooi and de Graaf, 1976). This fragment complex is inactive in vitro, but, after destroying the inhibiting activity of the immunity protein by heat treatment (Oudega et al., 1975b), the cloacin fragment is normally active in ribosome inactivation. Unfortunately, low-molecular-weight cloacin fragments cannot be separated properly from the immunity protein and therefore the limited proteolysis of cloacin complex is less suitable for the isolation of purified cloacin fragments.

This paper describes the isolation of cloacin fragments obtained by limited proteolysis of "free" cloacin. Due to the sensitivity of the cloacin for proteolysis most of the available enzymes were found to be less suitable for the preparation and subsequent isolation of fragments but after cleavage with thermolysin or thrombin a "stable" well-defined fragment could be isolated and purified. The characteristics of the gradual fragmentation of the cloacin either "spontaneous" or after incubation with proteases together with the observation that two of the isolated fragments possess the same C-terminal amino acid residues as intact cloacin suggest that the cloacin appears to have a domain-type structure. The N-terminal part of the cloacin molecules consists mainly of hydrophobic amino acids and is highly susceptible to proteolytic cleavage. Probably only one cleavage close to the N terminus completely destroys the killing activity of the cloacin. The hydrophilic C-terminal part of the molecule is rather resistant to proteolytic attack and can only be cleaved after longer periods of digestion or at much higher enzyme/substrate ratios. These two domains can also be functionally distinguished from each other. Properties like endoribonucleolytic activity, affinity for cell-surface receptors, and binding of immunity protein seem to reside in the C-terminal part of the molecule. At the moment we do not know which functions of the intact cloacin molecule reside in the N-terminal part of the molecule. Preliminary experiments with cloacin mutants most probably located in this part have shown that the transfer of the cloacin through the outer membrane is inhibited. This observation might explain why proteolytic cleavage of the N-terminal part of the cloacin results in a loss of killing activity. Based on these observations the following simple schematic drawing (Scheme I) represents what is known about the localization of different functions on the cloacin molecule. With respect to the maximum sequence necessary for the endoribonucleolytic activity, it should be mentioned that the limited trypsinolysis of cloacin complex has shown that a fragment with a molecular weight of 12 000 representing about 20% of the entire molecule still possesses this activity.

Lau and Richards (1976) have studied the effect of proteolytic modification on colicin E3 activity. With colicin E3 cleavage with either trypsin or subtilisin also results in a complete loss of in vivo activity. In contrast to our results with cloacin, the described colicin E3 fragments have lost the ability

SCHEME I



to bind to the receptors. The endoribonucleolytic activity of colicin E3 is 20–30-fold increased in the smaller fragments of both digests which might indicate that the colicin E3 preparation still contains immunity protein. Another remarkable difference between colicin E3 and cloacin DF13 is that the latter shows a much higher susceptibility for proteolytic attack.

Since colicin E3 and cloacin DF13 are entirely different with respect to their affinity to particular cell surface receptors, but also very similar with respect to their endoribonucleolytic activity, it will be of interest to elucidate and compare the structural relationship between both bacteriocins.

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