Antimicrobial Proteins Isolated from the Teat Canal of the Cow

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SUMMARY

Proteins which inhibited the growth of two strains of *Staphylococcus aureus* and one strain of *Streptococcus agalactiae* were isolated from the teat canal keratin of the cow. Electrophoresis of the proteins on polyacrylamide gels suggested that they were basic. They were separated into six bands at pH 3.0 but gave only two bands at pH 4.5 and two lines of precipitin after gel diffusion and immunoelectrophoresis at pH values between 4.5 and 8.5. In the presence of the anionic polymers DNA or heparin, complexes were formed which were not inhibitory of bacterial growth. The isolated proteins and the whole teat canal keratin were completely free from lysozyme. Their role is discussed in relation to the natural defence mechanisms of the teat canal.

INTRODUCTION

The teat canal is generally regarded as a barrier to artificial and natural infections of the mammary gland (Murphy & Stuart, 1953; Fincher, Hodges, Murphy & Morse, 1956; Plastringe, 1958). Pathogens placed beyond the teat canal into the teat cistern invariably lead to infection (Murphy & Stuart, 1954; Newbould & Neave, 1965; Hibbitt & Jones, 1967). The teat canal may function as a mechanical barrier due to its efficient sphincter muscles but, in addition, it has been reported to possess antimicrobial properties which can be attributed to the sebum-like material, called teat canal keratin, which probably originates from the heavily keratinized squamous epithelium (Murphy, 1959). Teat-canal keratin was reported to consist of up to 90% lipid and has been shown to inhibit the growth of streptococci *in vitro* (Adams & Rickard, 1963), but the lipid content of the keratin and its role in natural resistance has now been disputed (Treese, Morse & Levy, 1966). More recently cationic proteins have been isolated from teat-canal keratin and they strongly inhibited the growth of staphylococci and streptococci isolated from mastitic udders *in vitro* (Hibbitt & Cole, 1968).

This paper describes some of the properties of the proteins isolated from teat canal keratin.

METHODS

The isolation of antimicrobial protein fractions from teat-canal keratin. Teat-canal keratin was obtained from lactating and dry cows of the Institute herd by the 'reaming' procedure described by Murphy & Stuart (1953); larger amounts came from cows slaughtered at the abattoir. All keratin samples were stored at -20° until used.

The keratin was extracted by a procedure based on the method of Zubay & Wilkins (1962). Approximately 300 mg. tissue were homogenized for 2 min. in 25 ml. 0.15 M-NaCl in an MSE blender. The homogenate was centrifuged at 12,000 g (av.) for 20 min. and the supernatant fluid discarded. The precipitate was resuspended in 25 ml. glass-distilled water, and an equal volume of 0.5 M-HCl containing 0.2 M-barium acetate was added slowly and mixed thoroughly. The mixture was rehomogenized for 1 min. in the barium acetate solution, stirred for 15 min. and centrifuged at 20,000 g (av.) for 30 min. The supernatant fluid was dialysed against four changes of 5 l. glass-distilled water for 48 h. After dialysis the proteins were freeze-dried and chromatographed on a carboxymethylcellulose column equilibrated with an 0.1 M-acetate buffer (pH 4.2); after exhaustive washing with the buffer they were eluted with 0.2 M-HCl. The eluted proteins which appeared as a single peak were dialysed and freeze-dried. All operations were done at 4° .

Disc electrophoresis. The proteins were separated by electrophoresis in polyacrylamide gels at pH 3.0 and pH 4.5 by the procedure described by Narayan, Narayan & Kummerow (1964). A buffer consisting of 0.1 M-tartaric acid + 0.1 M-formic acid containing 0.01 M-EDTA and 6 M-urea was used for the separation at pH 3.0. For the experiments at pH 4.5 the same concentration of EDTA was added to an 0.1 M-acetate-buffer.

Preparation of proteins for assay. Approximately 2.0 mg. protein were shaken in 4 ml. 0.01 M-citric acid + Na_2HPO_4 buffer (pH 7.0) containing 0.1 M-NaCl. The undissolved protein was sedimented at 1500 g for 10 min. and the protein content of the supernatant determined spectrophotometrically (Layne, 1957). The volume of this solution was finally adjusted to give a protein concentration of 150 $\mu\text{g.}/\text{ml.}$

Organisms. Cultures of two strains of *Staphylococcus aureus* (42D and 52B phage type) were grown in Oxoid nutrient broth and *Streptococcus agalactiae* S13 (Pattison, 1948) in Todd-Hewitt medium. After incubation at 37° for approximately 6 hr they were harvested while still in their logarithmic growth phase and diluted in physiological saline to give a count between 50 and 100 organisms/0.02 ml. This bacterial suspension could be stored at 4° for up to 2 days without deterioration.

Antimicrobial assay. The teat-canal proteins were assayed for inhibition of the test organisms by the procedure described by Hirsch (1958), with the exception that the colonies were counted in a Hannay counting chamber.

Lysozyme assay. Lysozyme was assayed by the procedure describe by Shugar (1952).

Preparation of antisera. Rabbits were immunized by a course of four intramuscular injections, given at weekly intervals, and consisting of 10 mg. of antimicrobial protein extracted from teat-canal keratin in 0.5 ml. of complete Freund's adjuvant (Difco). Three weeks after the last inoculation the sera were harvested.

Immuno-electrophoresis. The micro method of Scheidegger (1955) was used with 0.02 M-veronal, 0.004 M-EDTA buffer (pH 8.6) or 0.05 M- KH_2PO_4 buffer (pH 4.5). The applied potential was 75 V/cm. for 1 hr.

RESULTS

The antimicrobial proteins extracted in this study constituted approximately 4–5 % (wet weight) of the teat canal keratin. These proteins which are soluble in distilled water and dilute acids were subjected to polyacrylamide gel disc electrophoresis. At pH 4.5 the proteins were resolved into two principal bands which moved towards the cathode. At pH 3.0, however, the proteins moved more rapidly towards the cathode and were resolved into 6 bands. The ultraviolet absorption curve (Fig. 1) of the extracted proteins showed a characteristic pattern for material with minimal nucleic acid contamination, a major absorption peak occurring at 276 m μ .

Fig. 1

Fig. 2

Fig. 1. The ultraviolet absorption spectrum of antimicrobial proteins isolated from teat-canal keratin.

Fig. 2. The inhibitory effect of proteins isolated from teat canal keratin on the growth of micro-organisms. ○—○, *Staphylococcus aureus* 42D; ●—●, *Staphylococcus aureus* 52B; △—△, *Streptococcus agalactiae* s13.

Inhibition of staphylococci and streptococci by teat canal proteins. The effect of various concentrations of the teat canal proteins on the growth of the test organisms is shown in Fig. 2. The growth of the two strains of *Staphylococcus aureus* was inhibited by 50% at a concentration of 2–5 μ g. protein/ml. while *Streptococcus agalactiae* was inhibited at 11.5 μ g./ml.

Removal of inhibition by anionic polymers. Electrophoresis had indicated that the proteins were basic; it was considered, therefore, that anionic polymers would combine with the teat-canal proteins and destroy their biological activity. The proteins were incubated at 37° for 15 min. with the anionic polymers DNA and heparin and assayed for their inhibitory activity. Table 1 shows that the inhibition of staphylococcal growth by the teat canal proteins at a concentration of 8 μ g./ml. was progressively destroyed by increasing concentrations of both anionic polymers with complete destruction occurring at 3.2 μ g./ml.

Absence of lysozyme in the teat canal protein preparations. Although staphylococci and streptococci are not susceptible to lysozyme (also a basic protein) in the sense that the cells are lysed, it is known that lysozyme can decrease the viable count of staphylococci (Kern, Kingade, Kern & Behrens, 1951). As this enzyme is known to be widely distributed in biological fluids and tissues it was thought to be of some interest to test the protein preparations from the teat canal for their lysozyme content. There was no lysozyme activity in the teat canal protein preparation or in the original keratin (Table 2).

Table 1. *The effect of DNA and heparin on the antibacterial activity of the teat canal proteins*

Organism:	Treatment	Growth (%)
<i>Staphylococcus aureus</i> .*	Nil	100.0
	Incubated† with cationic proteins	0.0
	Incubated with cationic proteins complexed‡ with:	
	0.4 µg. DNA	0.0
	0.8 µg. DNA	0.7
	1.6 µg. DNA	49.2
	3.2 µg. DNA	100.0
	0.2 µg. heparin	0.0
	0.4 µg. heparin	2.1
	0.8 µg. heparin	5.0
	1.6 µg. heparin	69.2
	3.2 µg. heparin	100.0

* Approximately 70 colony forming units of *Staphylococcus aureus* were incubated for 30 min. in 1 ml. 0.01 M-citric acid + Na₂HPO₄ buffer (pH 7.0) then grown in Oxoid nutrient broth containing 1 % Davis agar.

† *Staphylococcus aureus* was incubated for 30 min. in 1 ml. of the buffer described above containing 8 µg. of the cationic proteins.

‡ The protein complex was prepared by incubating 8 µg. of the cationic proteins with DNA or heparin in 1 ml. of the buffer described above at 37° for 15 min.

Table 2. *Test for lysozyme content of whole teat canal keratin and partially purified antimicrobial proteins*

	Lysozyme (units/mg. protein*)
Standard lysozyme	8600
Whole teat canal keratin	0
Antimicrobial proteins from teat canal keratin	0

* One unit of activity is equivalent to a decrease in absorbency at 450 mµ of 0.001 per min. at pH 7.0 and 25°.

Gel-diffusion tests. The agar gel-diffusion test which is normally performed at pH 7.0 suggested initially some degree of homogeneity of the protein since it showed only one line of identity at a concentration of 2.5 mg./ml. against homologous antiserum. When the concentration of the protein was decreased to 1.25 mg./ml. there was an indication that two precipitin lines may have been present. This result was not sufficiently clear to record; on the other hand two lines could be expected since two bands were also identified in the electrophoresis studies at pH 4.5.

Immunoelectrophoresis. The double line which was thought to be present in the gel diffusion test at the lower concentration was confirmed by immunoelectrophoresis at pH 8.6 (Fig. 3) since two arcs of precipitation were formed against the antiserum. Immunoelectrophoresis is normally not performed at a low pH because of the danger of precipitating the serum proteins and the possible streaking of the protein. Therefore it was impossible to study the effects of the antisera on the protein fractions separated at pH 3.0. On the other hand, at pH 4.5, the pH value which separated the proteins into two principal bands on polyacrylamide gel, two arcs were formed which moved towards the cathode (Fig. 4).

Fig. 3. Immunoelectrophoresis of teat canal antibacterial protein at pH 8.6.

Fig. 4. Immunoelectrophoresis of teat canal antibacterial protein at pH 4.5.

DISCUSSION

An investigation of the natural methods of protecting the bovine mammary gland against the invasion of micro-organisms may lead to a better understanding of the reasons why animals vary in their susceptibility to mastitis. The bacteriostatic activity of the lipids in the teat-canal keratin (Adams & Rickard, 1963) is possibly only one of the many factors involved in natural defence since cationic proteins isolated from various tissues have been shown to possess bactericidal properties *in vitro* (Scarnes & Watson, 1957; Hirsch, 1958; Zeya & Spitznagel, 1966). It is therefore conceivable that the cationic proteins isolated from the teat canal keratin may act *in vivo* as part of the protective mechanism against invasion of pathogens.

The behaviour of the isolated proteins during electrophoresis on polyacrylamide gels suggested that they contain a predominating number of positively charged groups. This possibility was further supported in the experiment, where it was shown that the antimicrobial activity of the proteins was lost when they were pretreated at pH 7.0 with negatively charged substances such as DNA or heparin. The positively charged proteins of teat-canal keratin would in all probability also be linked electrovalently to negatively charged components such as other proteins or nucleic acids, with a consequent loss of activity. This was borne out in a single experiment, not recorded in this paper, when it was found that isolated whole keratin had no anti-

microbial activity. However, in a living teat canal, proteins are being synthesized continuously and would be free to bind to any negatively charged material including micro-organisms.

The complete absence of lysozyme in the isolated proteins and in whole teat-canal keratin was not surprising since Padgett & Hirsch (1967) were unable to demonstrate its presence in tears, saliva, nasal exudates and peritoneal leucocytes obtained from a group of ten cattle. Furthermore, the morphological changes produced by lysozyme on *Staphylococcus aureus* were markedly different from those produced by the proteins isolated from teat-canal keratin (McMillan & Hibbitt, 1969).

Although six bands of protein were separated on polyacrylamide gels at pH 3.0, at pH 4.5 only two principal bands were observed. The resolution of closely related positively charged proteins would be less marked at higher pH values as their isoelectric points are approached. In the immunological studies therefore, which were necessarily carried out on the teat canal proteins at higher pH values, the separation of only two antigens is understandable.

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