Antibacterial and antiviral activity of camel milk protective proteins

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SUMMARY. Lysozyme (LZ), lactoferrin (LF), lactoperoxidase (LP), immunoglobulin G and secretory immunoglobulin A were extracted from camel milk. The activity of these protective proteins was assayed against *Lactococcus lactis* subsp. *cremoris*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and rotavirus. Comparative activities of egg white LZ, bovine LZ and bovine LF are also presented. The antibacterial activity spectrum of camel milk LZ was similar to that of egg white LZ, and differed from bovine milk LZ. Bovine and camel milk LF antibacterial activity spectra were similar. The camel milk LP was bacteriostatic against the Gram-positive strains and was bactericidal against Gram-negative cultures. The immunoglobulins had little effect against the bacteria but high titres of antibodies against rotavirus were found in camel milk. The LP system was ineffective against rotavirus.

In 1984 camel milk production in Saudi Arabia alone reached 6400 tons (Barbour *et al.* 1984). Camel milk is consumed in the raw state by desert nomads. Since no refrigeration is possible under these conditions, it is of interest to determine the activity of natural antimicrobial proteins in camel milk, for conservation or fermentation purposes. Moreover, there are reports that camel milk could have medicinal properties (Shiller, 1990), which suggests that this milk contains antimicrobial components.

Various protective proteins are found in milk: immunoglobulins, lysozyme (LZ), lactoferrin (LF) and lactoperoxidase (LP) (Reiter, 1985). There are considerable variations in the content of protective proteins of milks of different species. Thus human milk contains almost 1000 times more LZ than bovine milk, while bovine milk is much richer in LP than human milk (Reiter, 1985). Studies on protective proteins of camel milk have been performed on LZ (Barbour *et al.* 1984; Duhaiman, 1988), but no global study of the activity of the other protective proteins on bacteria and rotavirus has been done.

The aim of this work was thus to evaluate the activity of camel LZ, LF, LP and immunoglobulins on five bacterial cultures and rotavirus.

§ For reprints.

Milk

Milk was collected from 90 camels in the El-Amyria and Abou-El-Matamir areas near Alexandria, skimmed, and then lyophilized at Alexandria University. Bovine milk samples were collected from Holstein cows in Québec.

Bacterial strains

Escherichia coli ATCC 25922, *Staphylococcus aureus* B4 and *Lactococcus lactis* subsp. *cremoris* CRA-1 were obtained from Agriculture Canada. *Salmonella typhimurium* ATCC 14028 and *Esch. coli* O157 H7 were supplied by the Faculty of Veterinary Medicine, University of Montreal. The lactococci were maintained at 30 °C on Elliker agar (Difco, Detroit, MI, USA) slants, while the other bacterial strains were kept on brain–heart infusion agar (Difco) and incubated at 37 °C.

Rotavirus stock preparation and titre determination

Bovine rotavirus NCDV; ATCC VR452 was supplied by Dr Serge Dea, Institut Armand Frappier. MA-104 rhesus monkey kidney tissue was obtained from Microbiological Associates.

Confluent monolayers of MA-104 cells previously grown in 75 cm^2 culture bottles were washed with Dulbecco phosphate buffered saline, pH 7·0 and inoculated with 1 ml bovine rotavirus containing 10 μg trypsin. After 1 h viral adsorption at 37 °C, the culture was overlaid with Eagle's minimum essential medium (MEM) with Eagle's salt and glutamine (Gibco) and incubated at 37 °C. When maximal cytopathic effect was reached, infected cells were frozen (-70 °C) and thawed three times to liberate all viral particles from infected cells. The viral suspension was then centrifuged and the supernatant containing the virus was stored at -70 °C until needed.

Prior to inhibition trials with purified constituents of camel whey, a small volume of the rotavirus was titrated by serial dilutions from 1:10 to 1:10000 in MEM containing 0·1 % bovine serum albumin (BSA), 10 μg trypsin/ml, 100 U penicillin/ml and 100 μg streptomycin/ml. The viral titre was expressed according to the method of Reed & Muench (1938).

Enzymes and chemicals

Bovine lactoperoxidase (EC 1.11.1.7) 80 U/mg protein, bovine LF and glucose oxidase (EC 1.1.2.4) were purchased from Sigma Chemical Co. (St Louis, MO 63178, USA). Egg white LZ (EC 3.2.1.17) was purchased from Boehringer-Mannheim (Germany). Hydrogen peroxide (H$_2$O$_2$, 50%) was obtained from Fisher Chemical Co.

Isolation of the protective proteins

Isolation of IgG$_1$ and IgG$_2$ from camel serum and milk was performed as described by Gray *et al.* (1969). The peaks were analysed by immunoelectrophoresis and SDS-PAGE as described by Axelsen *et al.* (1973) and Hames (1981). The preparation of immunoglobulin A (IgA) from serum and secretory immunoglobulin A (sIgA) from milk was based on the method of Butler *et al.* (1980).

The isolation of LP, LF and LZ from camel milk was carried out with the methods developed for bovine milk by Ekstrand & Björck (1986). Purity was determined by measuring the absorbance at 412 and 280 nm (Morrison & Hultquist, 1963).

Activity assays

The activity of camel LP was measured according to the method described by Shindler & Bardsley (1975). The oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma) by LP was performed in 0·1 M-acetate buffer, pH 5·2 at 20 °C containing appropriate concentrations of TMB, H_2O_2 and LP in a volume of 1 ml and was measured spectrophotometrically at 413 nm using a Pye Unicam SP6-550 spectro-photometer. The lysoplate assay was used for LZ activity as described by Lie *et al.* (1986).

The inhibition assay of microorganisms by the LP–SCN–H_2O_2 system was carried out with different bacterial strains and different media according to the method described by Reiter *et al.* (1976). Media were incubated at 37 °C for all strains except for *Lc. cremoris*, which was kept at 30 °C. Viable bacteria were estimated by plating on Elliker, violet red bile, mannitol salt and Mueller–Hinton agars (all from Difco), which were respectively used for *Lc. cremoris*, *Esch. coli*, *Staph. aureus* and *Sal. typhimurium*.

For IgG and sIgA inhibition assays, the cultures, media and enumeration procedures were the same as with the LP inhibition assays. The method of Spik *et al.* (1978) was used with concentrations of 2 mg/ml of both IgG and sIgA.

The susceptibility of the bacterial cultures toward LZ and LF was estimated with a disc-assay technique described by Vakil *et al.* (1969). The plates were preincubated at 10 °C for 2 h to allow the diffusion of LZ or LF into the agar and then incubated for 16–18 h for LZ and 24–36 h for LF at 30 °C for *Lc. cremoris* and at 37 °C for *Esch. coli*, *Sal. typhimurium* and *Staph. aureus*. Thereafter the diameters of the inhibition zones were measured.

Titration of neutralizing antibodies in IgG and sIgA and study of antiviral activity of LP system were carried under the following procedures. Two-fold dilutions (1:2 to 1:1024) of purified IgG (8 mg/ml), sIgA, (8 mg/ml) as well as solutions of LP alone (12 U/ml), LP with KSCN (0·450 mM), glucose (0·3%) with glucose oxidase (0·4 μg/ml) and the whole system (12 U LP/ml–0·450 mM-KSCN–0·3% glucose–0·4 μg glucose oxidase/ml) were prepared. Dilutions were made in 0·01 M-phosphate buffered saline, pH 7·2 supplemented with 0·1% BSA; 100 μl of a rotavirus dilution containing 300 TCID 50 was added to 100 μl of each diluted protein. These mixtures were shaken and allowed to remain at 37 °C for 1 h. Thereafter 0·05 ml of each mixture was inoculated into each of three wells of a 96-well Microtest culture plate (Falcon) previously seeded with MA-104 cells. The plate was then incubated at 37 °C in 5% CO_2 in a humidified incubator. This culture was examined for evidence of cytopathic effect 3–5 d post infection. The titres were expressed as the reciprocal of the highest neutralizing dilution.

RESULTS AND DISCUSSION

Biological activity of camel milk proteins against bacteria

The inhibitory effect of LZ varied according to origin and test organism of the strains tested. Camel milk LZ was only inhibitory to *Sal. typhimurium* and had the same activity spectrum as that of egg white. Our results thus agree with those of Vakil *et al.* (1969) who showed that bovine milk and egg white LZ had no lytic effect toward both *Esch. coli* and *Staph. aureus*. They also agree with those of Barbour *et al.* (1984) who had found that camel milk LZ was effective against *Sal. typhimurium*, but ineffective toward *Staph. aureus*. As opposed to Duhaiman (1988), camel milk LZ
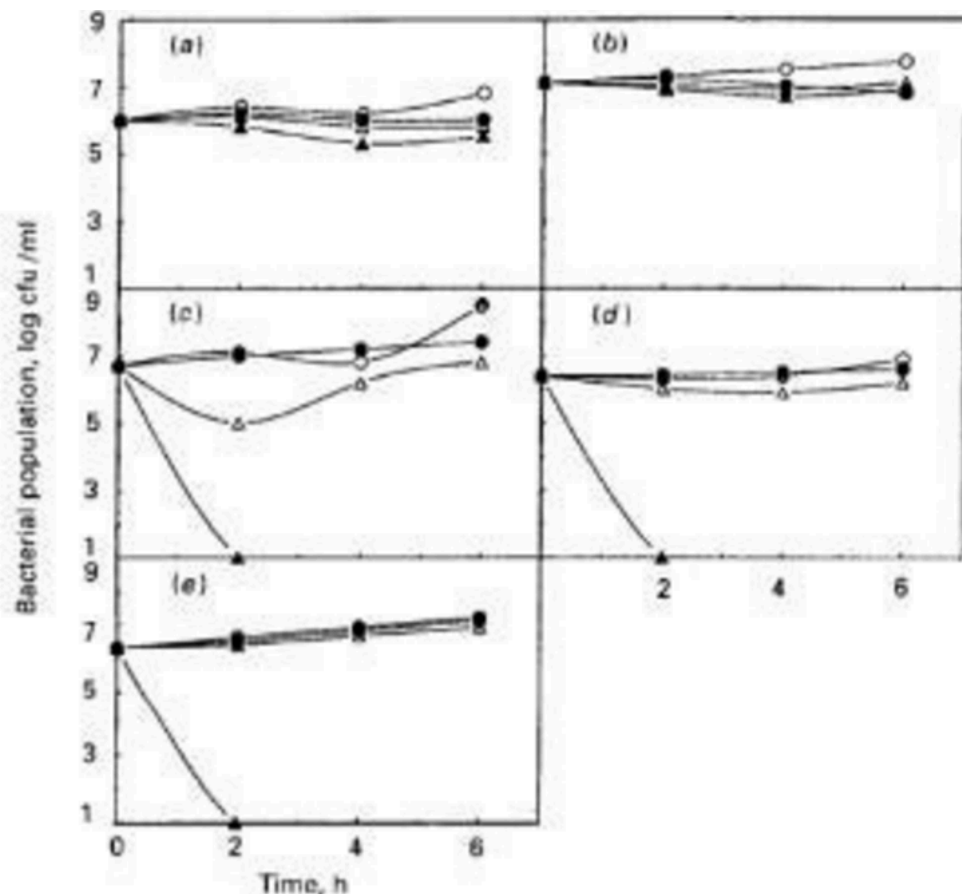
Fig. 1. Effect of lactoperoxidase (LP, 1·5 U/ml), thiocyanate (SCN⁻, 0·225 mM), glucose (Glu, 0·3%), glucose oxidase (GO, 0·1 µg/ml), immunoglobulin G (IgG, 2 mg/ml) and secretory immunoglobulin A (sIgA, 2 mg/ml) in a synthetic medium (ammonium salts, pH 5·5) on (a) *Lactococcus lactis* subsp. *cremoris* CRA-1, (b) *Staphylococcus aureus* B4, (c) *Escherichia coli* ATCC 25922, (d) *Escherichia coli* O157:H7 and (e) *Salmonella typhimurium* ATCC 14028. ○, Control; ▲, whole LP system (LP, SCN⁻, Glu and GO); △, whole system without LP; ●, immunoglobulins.

was ineffective against our *Staph. aureus* B4 strain. Bovine milk LZ demonstrated a different spectrum from camel LZ, since it was ineffective against *Sal. typhimurium* and was inhibitory to *Lc. cremoris*.

The activity spectrum of camel milk LF was similar to that of bovine milk LF. Of the bacterial strains tested, only *Sal. typhimurium* was affected by these protective proteins. It is generally thought that the LF inhibition effect is related to Fe requirements of microorganisms. This might explain why *Sal. typhimurium* was more sensitive to LF than *Staph. aureus*. Although *Esch. coli* has been shown to be more sensitive to LF than the lactic acid bacteria (Reiter & Oram, 1968), we did not find any lysis zone with our *Esch. coli* strains. Thus, as suggested by Reiter (1985), the Fe requirement explanation appears insufficient. It has been shown that some *Esch. coli* strains produce siderophores that help them counter the Fe-binding activity of the LF (Rogers & Synge, 1978; Griffiths & Humphreys, 1977). Citrate ions compete for Fe with LF and high levels of citrate in milk reduce the inhibitory effect of LF (Reiter, 1985). Since camel milk contains less citrate than does cows' milk (El Agamy, 1983), the *in vivo* activity of camel LF would be expected to be high.

The LP system influenced to various degrees both growth and activity of the cultures. Camel LP was only bacteriostatic against Gram-positive cultures, but was highly bactericidal against Gram-negative strains (Fig. 1). The higher sensitivity of Gram-negative strains has already been demonstrated with bovine LP (Reiter, 1985), and camel LP demonstrated the same activity pattern as that of bovine LP. The

Table 1. *Effect of camel lactoperoxidase (LP), thiocyanate and hydrogen peroxide (whole LP system), of LP system without LP, and of immunoglobulin G (IgG) and secretory immunoglobulin A (sIgA) on the acidifying activity of* Lactococcus lactis subsp. cremoris *CRA-1, expressed as % lactic acid†*

Treatment	Time, h	
	0	2
Control	0·13	0·18
LP system without LP	0·13	0·18
Whole LP system	0·13	0·14
IgG with SIgA	0·13	0·18

† For method of assessment, see p. 171.

Table 2. *Antiviral activity of camel milk immunoglobulins and lactoperoxidase system against rotavirus†*

Purified proteins	Neutralizing titre or inhibition
Secretory immunoglobulin A	1 : 256
Immunoglobulin G	1 : 32
Lactoperoxidase (LP)	None
Lactoperoxidase + thiocyanate (SCN⁻)	None
Glucose (Glu) + glucose oxidase (GO)	None
Lactoperoxidase system (LP + SCN⁻ + Glu + GO)	None

† For method of assessment, see p. 171.

bovine LP system appears to have a chaotropic effect on the inner membrane of bacteria, and various compounds leak into the medium (Reiter, 1985).

The effect of camel LP on the *Lc. cremoris* population was confirmed by activity tests. Thus, very little lactic acid was produced during the 2 h fermentation when the lactococci were exposed to the complete LP system (Table 1). Presence of the enzyme was necessary to inhibit lactic acid production. The activities of LZ and LP on *Lc. cremoris* suggest that fermentation of raw camel milk would be slower than that of heat-treated milk. Thus, as for bovine milk (Feldstein & Westhoff, 1979), the production of cultures or fermented products could more easily be done in heat-treated camel milk.

The various immunoglobulins did not show any bactericidal effect on the bacterial cultures (Fig. 1, Table 1).

Antiviral activity of camel milk protective proteins

Rotaviruses are the most frequent cause of non-bacterial enteritis in neonates in most parts of the world (Kapikien *et al.* 1976). To study the antiviral activity of both specific and non-specific minor protective proteins in camel milk, bovine rotavirus was used as a viral model. This choice was based on the fact that rotaviruses from several animal species and man share a common antigen so that one member of the group can be used in serological tests for infection with any of the others (Flewett & Woode, 1978).

Table 2 shows the results of the activity of camel milk protective minor proteins against bovine rotavirus. Results obtained indicate that LP as one of non-specific protective minor proteins did not inhibit the rotavirus. However, the specific minor protective proteins such as IgG and sIgA did neutralize the virus at a relatively high

titre. The fact that these two specific proteins were inhibitors of the bovine rotavirus indicates that camels are also subject to rotavirus infection and that this virus shares a common antigen with other rotaviruses. Moreover, the fact that these protective proteins originated from camel milk collected from areas where no bovines were present leads one to believe that this rotavirus could be specific to the camel, a hypothesis that should be investigated further. The presence of sIgA in high titres against rotavirus reflects that the camel mammary gland produces a high concentration of this local secretory immunoglobulin (sIgA) as a defence factor.

We conclude that camel milk contains a variety of protective proteins that contribute to bacterial growth inhibition. Camel milk LZ seemed to have an activity spectrum more closely related to egg white LZ than that of bovine milk. On the other hand, the inhibition pattern of camel milk LF seemed similar to that of bovine milk. Camel milk LP demonstrated the same antimicrobial mechanism as that of bovine milk, being active in presence of H_2O_2 and SCN^-. In addition to the inhibitory effect of camel LZ and LF on *Sal. typhimurium*, as well as the bactericidal effect of the camel LP system on Gram-negative bacteria, the high immunoglobulin titres against rotaviruses may also explain why there are reports of medicinal properties (Shiller, 1990), and why some nomads use camel milk as a remedy against diarrhoea.

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