Research article

Resistance of rumen bacteria murein to bovine gastric lysozyme

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Abstract

Background: Lysozymes, enzymes mostly associated with defence against bacterial infections, are mureinolytic. Ruminants have evolved a gastric c type lysozyme as a digestive enzyme, and profit from digestion of foregut bacteria, after most dietary components, including protein, have been fermented in the *rumen*. In this work we characterized the biological activities of bovine gastric secretions against membranes, purified murein and bacteria.

Results: Bovine gastric extract (BGE) was active against both G+ and G- bacteria, but the effect against Gram- bacteria was not due to the lysozyme, since purified BGL had only activity against Gram+ bacteria. We were unable to find small pore forming peptides in the BGE, and found that the inhibition of Gram negative bacteria by BGE was due to an artefact caused by acetate. We report for first time the activity of bovine gastric lysozyme (BG lysozyme) against pure bacterial cultures, and the specific resistance of some rumen Gram positive strains to BGL.

Conclusions: Some Gram+ rumen bacteria showed resistance to abomasum lysozyme. We discuss the implications of this finding in the light of possible practical applications of such a stable antimicrobial peptide.

Background

Lysozymes are beta-N-acetyl-muramyl-hydrolase that disrupt bacterial murein [1]. The most common animal lysozyme is the c type, such as the chicken egg white lysozyme (EWL, 14.3 Kda), found in animal, insects and plants [2]. Monogastric animals possess a single gene for lysozyme c expressed in various tissues [3] and is thought to be primarily involved in defence against bacterial infections.

In contrast, ruminants have multiple lysozyme genes [4], and at least four code for a gastric lysozyme that functions as a digestive enzyme. Most dietary components are fermented in the rumen to volatile fatty acids [5], and the ruminant benefits of digesting foregut bacteria as a source of amino acids. Cow gastric lysozyme is a basic enzyme adapted to act in the harsh gastric conditions, with optimal activity at low pH (4.5–5.2), low ionic strength values, and resistant to acid and pepsin [6,7]. We have previously reported that the recruitment of lysozyme as a gastric digestive enzyme has convergently occurred in the

avian foregut fermenter hoatzin [8], also with multiple gene duplication events during its evolution [9]. Amino acid differences between homologous proteins from different species may have adaptive significance, as is the case with gastric lysozymes. Lysozymes from the stomachs of cows and langur monkeys (both with fermentation in the foregut) are subject to positive darwinian selection [10,11].

Rumen bacteria represent an important component of the rumen biomass [5] and is a likely factor exerting evolutionary pressure on gastric lysozyme and other gastric secretions in herbivores with foregut fermentation. This prompted us establish 2 hypotheses: 1 – there might be other abomasum peptides with antimicrobial activity in addition to lysozyme, that would also act against Gram negative bacteria; 2 – since rumen bacteria have been subject to the selective pressure of gastric antimicrobial secretions, rumen bacteria might have developed resistance to these compounds. The objective of this work was to characterize the biological activities of bovine gastric secretions against membranes, purified murein and bacteria.

Results

Cow abomasum mucosa yielded 26.4 mg BGE per g of tissue (1.32 g, of lyophilized extract per 100 ml of acetic extract from 50 g wet mucosa, sd 0.61, N = 20). Protein content of extracts was 60%, equivalent to 16 mg protein per gram of gastric tissue. A total of 5 g of gastric extract was subject to chromatography, and 82,2 mg of active (lytic) protein were obtained. This represents a yield of 1.64% w/w of lysozyme from the crude extract, and 0.43 mg of lysozyme per gram of tissue.

SDS-PAGE of the bovine gastric extract revealed a major protein band of about 15 Kda and two other bands of bigger proteins (30 and 45 Kda). Non denaturing electrophoresis showed the protein band with lytic activity on *M. luteus*-embedded overlay gel, presumably gastric lysozyme. Lysis of *M. luteus* suspension by the gastric extract is shown in Fig. 1. Specific activity (on *M. luteus*) of the extract from the whole gastric mucosa was 3.40 U (sd 1.48, N = 20) at pH 5.5. Specific activity of the gastric extract decreased 32% at pH 6 (2.42 U) and 42% at pH 6.5 (2.04 U). Extract specific activity at pH 5.5 was higher in the extract of gastric fundus (4.39 U) than in that of the antrum and body (2.49 U and 2.34 U, respectively).

BGE was bacteriolytic against *M. luteus* in a concentration-dependent manner (Fig 1). BGE also degraded Muramidase *E. coli* murein, producing a different profile to that of the fungal lysozyme cellosyl (Fig 2). Muropeptides have a basic structure consisting of N-acetylglucosaminyl-N-acetylmuramyl-L-Ala-D-Glu-gamma-mDap-R1R2, where R1 and R2 are substituents at the L-carboxy and D-amino

groups. BGE (500 μg/ml) produced different muropeptides to those produced by cellosyl (50 μg/ml), including the lack of a peak at minute 27 (muropeptide with R1 = D-Ala and R2 = -H) that was produced by treatment with cellosyl. This peak was eluted and incubated with both BGE and cellosyl. BGE further digested this muropeptide (Fig 2c) while cellosyl did not (Fig 2d). This suggests hat unlike cellosyl, BGE lysozyme has L, D carboxypeptidase or Nacetyl muramyl L-ala amidase activity.

BGE was active against *P. aeruginosa* and *E. coli*, permeabilized vesicles and depolarized liposomes. These activities were screened in isolated proteins and small peptides yielding no active peptides from the BGE extract, including BGL, inactive against Gram negative bacteria (Table 1). Permeabilizing activity proved to be due to acetate in the gastric extract, since BSA resuspended in acetic acid and lyophilized had similar activity to BGE. BGL not inhibit the growth of some Gram positive rumen bacteria such as *L. acidophilus* and *S. bovis*, which were highly inhibited by EWL, indicating specific resistance of the rumen bacteria murein to BGL (Table 1).

Discussion

Muramidase activity in the acid stomach of a foregut fermenter, receiving a considerable biomass of bacteria, allows the digestion of bacterial cell walls and liberalisation of amino acid-rich cell contents. The activity of BG lysozyme on purified murein was different to that of other c lysozymes in having lower optimal pH and L, D carboxypeptidase or Nacetyl muramyl L-ala amidase activity. It was, like other lysozymes, active only against Gram positive bacteria. Other systems such as *Entamoeba histolytica*, are known to contain lysozyme acting in concert with membrane permeabilizing peptides [12], but attempts to purify active small peptides from BGE have failed, so far.

Presence of Gram negative bacteria in the rumen does not seem to have driven the evolution of gastric peptides capable of degrade them. This would limit the efficiency of the system, since only Gram positive bacteria are amenable to digestion, and Gram negative bacteria represent a considerable proportion of rumen bacterial biomass. Furthermore, some rumen Gram positive bacteria seem to have developed resistance to the action of BG lysozyme. Rumen bacterium *Streptococcus bovis* can develop resistance to the pore-forming peptide nisin, by modifying cell lipoteichoic acids. Acquisition of nisin resistance also conferred lysozyme resistance [13]. Little is known about bacteria developing resistance to lysozymes from the host they colonize, as in the case of rumen bacteria and gastric bovine lysozyme. The phenomenon might reflect the selective pressure of the gastric enzyme on rumen bacteria, since BGL-resistant bacteria will be more likely to colonize
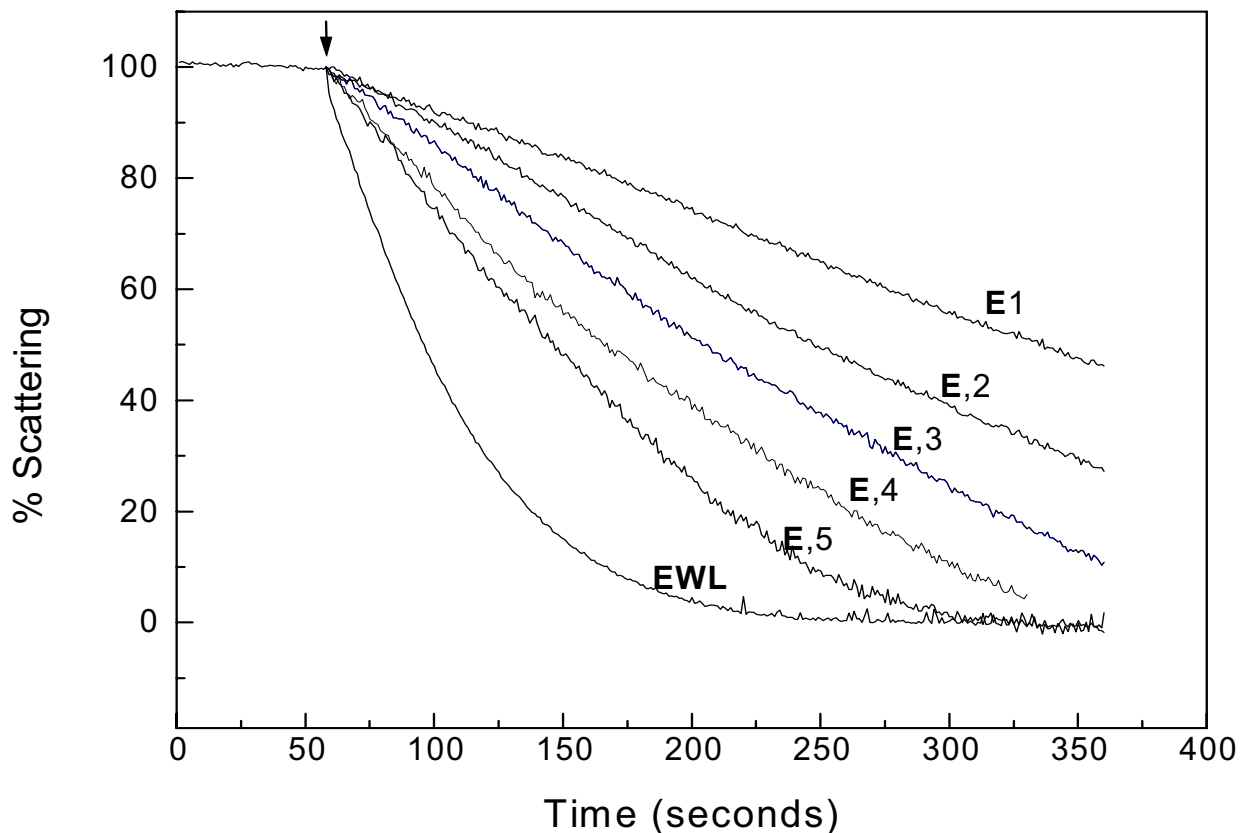
Figure 1
Bacteriolytic activity of gastric extract against *M. luteus*. Lytic activity shown as % of initial scattering of a *M. luteus* suspension (0.25 mg/ml in acetate buffer pH 5.5) treated (indicated by arrow) with gastric extracts of whole abomasum mucosa (E1, E2, E3, E4 and E, corresponding to extract concentrations of 42, 133, 233, 417 and 554 µg/ml, respectively) and EWL (5 µg/ml).

the lower gut and the rumen of young animals, than BGL-sensitive strains.

The search for new antibacterial drugs has lead to explore more speculative approaches to destroy or inhibit pathogen microorganisms and viruses. It is tempting to optimistically predict that highly stable peptides that naturally evolved as gastric antimicrobials may have a high potential for clinical applications. Human lysozyme (secreted by monocytes), while playing a protective role against infections, depresses superoxide generation of neutrophyls and enhances lymphocyte proliferative response [14-17]. EWL has been applied against bacterial and viral infections, based on its immune-stimulation and anti-inflammatory properties [18].

Ruminant BG lysozymes, having evolved in a very hostile environment such as the stomach, have developed resistance to acid and proteolysis, and may hold promising uses in treating infections, and in the food industry. In the animal production industry, the uses of antibiotics and hormones as growth promoters has posed health threats to humans and animals, and feed supplementation with some growth promoters is now illegal. The use of new growth promoters that don't pose problems of resistance is a priority, and antimicrobial peptides are of course good candidates. Indeed, lysozyme has been found to be as effective as conventional antibiotics, in promoting growth of poultry [19]. However, the discovery of the capacity of bacteria to presumably modify its bacterial murein and become resistant to the lytic action of lysozymes, will
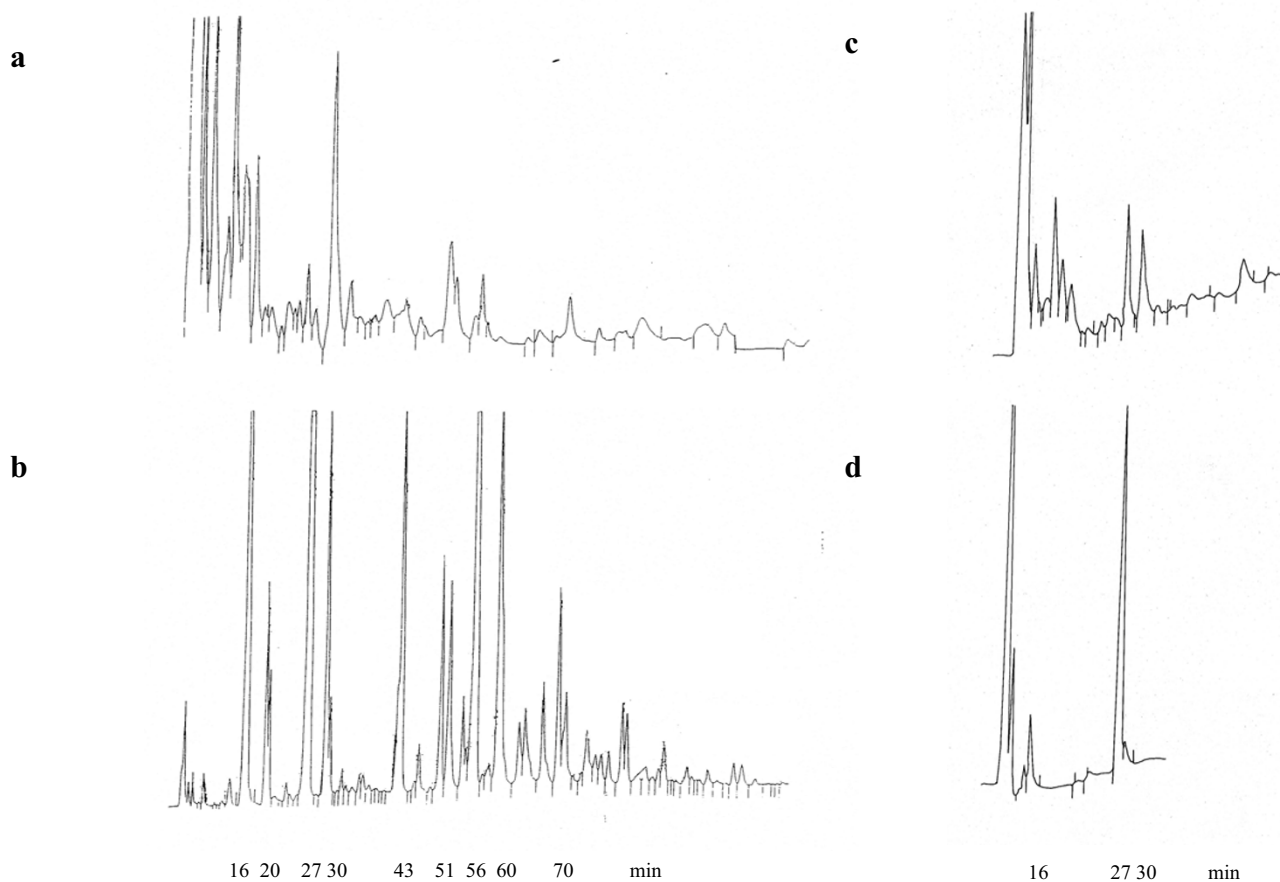
Figure 2
Muramidase activity of gastric extract on *Echerichia coli* purified murein. Muropeptide profile after digestion of murein with gastric extract (500 μg/ml, a) or with the fungal muramidase cellosyl (50 ug/ml, b). Cellosyl produced a tetrapeptide at 27 min corresponding to M4 (having R1 = D-Ala and R2 = -H). This was further digested by gastric extract (500 ug/ml, c) but not by cellosyl (50 ug/ml, d).

Table 1: Percentage of growth of control cultures at 6 hours, of Gram+ and Gram- bacteria grown in the presence of EW lysozyme, BG lysozyme and BGE.

ORIGIN	BACTERIUM		Egg White Lysozyme 150 ug/ml		Bovine Gastric Lysozyme 150 ug/ml		Bovine Gastric Extract 10 mg/ml	
			pH 5.5	pH 7.0	pH 5.5	pH 7.0	pH 5.5	pH 7.0
ENVIRONMENTAL, INTESTINAL	Gram positive	*M. luteus*	*lysed[1]*	*lysed[1]*	*lysed[1]*	*lysed[1]*	*lysed[1]*	*lysed[1]*
	Gram negative	*P. aeruginosa*	106	173	142	182	*0*	90
		E. coli	103	91	86	124	*15*	*44*
RUMEN	Gram positive	*L. vitulinus*	*1*	*4*	*81*	*69*	*10*	*8*
		L. acidophilus	*0*	NG	*64*	NG	168	NG
		S. bovis	101	*6*	108	114	98	111
	Gram negative	*S. ruminantium*	109	112	130	96	*89*	*87*
		P. ruminicola	NG	*24*	NG	113	NG	208

[1] = cells lysed by treatment in scattering assay. Italic bold numbers = growth inhibited. *NG* = no growth of culture at this pH.

surely be of concern in practical applications, as has bacterial antibiotic resistance.

Conclusions

This work shows that gastric bovine lysozyme is active against Gram positive bacteria but that some rumen strains are unaffected by the enzyme. Possible practical applications of this antimicrobial peptide as an alternative to antibiotics may be limited by the development of bacterial resistance to the lytic action of lysozymes.

Methods

Gastric extract and lysozyme purification

Acetic acid extract from abomasal mucosa was prepared as described by Dobson et al. [7]. Bovine gastric extracts (BGE) were lyophilised and kept frozen. BGE proteins were separated by denaturing and non-denaturing PAGE electrophoresis. Band lytic activity was revealed on an overlaying agarose gel embedded with *M. luteus*. Purification of BG lysozyme was performed re-dissolving lyophilised BGE in 10 vol. of 10% acetic acid, centrifuging at $150,000 \times g$ at $4°C$ for 1 h. The resulting supernatant, was diluted 1:2.5 with 20% acetonitrile/0.08% trifluoroacetic acid. The material was passed in 0.5 ml batches over a Superdex Pep 10/30 column (Pharmacia LKB) equilibrated with 20% acetonitrile/0.08% trifluoroacetic acid at $20°C$ and a flow rate of 0.1 ml/min. Active fractions were pooled, diluted 1:2 with starting buffer (50 mM sodium-acetate, pH 4.5) and loaded onto a Mono S 5/5 cation-exchange column (Pharmacia LKB) equilibrated with starting buffer. Adsorbed protein was eluted by washing the column with the same buffer (5 ml) and by use of a 0–500 mM NaCl gradient (15 ml) and a final wash of 1 M NaCl at $10°C$. Active material was diluted 1:13 with starting buffer (50 mM sodium-acetate, pH 7.8) and applied again to a Mono S HR 5/5 column (Pharmacia LKB) equilibrated with starting buffer. The column was washed with the same buffer (5 ml) and developed with a 15-ml gradient of 0–500 mM NaCl at $10°C$. Active fractions were pooled and stored at $-20°C$. Reversed-phase HPLC was performed on an Aquapore Butyl 300 column (2.1×30 mm; Brownlee Labs) connected with a 130 A separation system (Applied Biosystems). Elution was done with a linear gradient of 0–84% acetonitrile in 0.1% trifluoroacetic acid at $30°C$ over 45 min. A flow rate of 0.2 ml min-1 was applied, the effluent was monitored by absorbance at 214 nm. Peak fractions were collected manually and tested immediately for lysozyme activity using the lysoplate technique [20]. Tricine-SDS electrophoresis was performed in 13% separation gels [21].

Antimicrobial tests

Lytic activity against *M. luteus* was determined by scattering changes in a suspension of *Micrococcus luteus* (0.25 mg/ml in acetate buffer) [8]. Egg white (EW) lysozyme

(Sigma) was used as a control. Specific activity per ug of gastric extract was defined as units of activity of 1 ng of EWL on *M. luteus*, at pH 5.5. Inhibitory effect on bacterial growth was determined on broth and agar lysoplates [20]. Microdilution susceptibility test [22] was used to determine minimal inhibitory concentrations. Effect of BGE or BG lysozyme on bacterial growth was determined in broth cultures in which growth was monitored turbidimetrically (A600 nm).

Muramidase activity of BGE was assayed on purified *E. coli* murein [23] with cellosyl (Hoechst, Frankfurt am Main; 50 μg/ml) and gastric extract (500 μg/ml), determining by HPLC the production of soluble low molecular weight muropeptides [24]. A control without murein was included in the runs and gave no peaks. When required, produced muropeptides were collected individually at the UV detection outlet, vacuum dried, resuspended in MilliQ water and desalted as previously described [23].

Membrane-permeabilyzing activity of gastric extracts was determined fluorimetricaly, by the liberation of carboxyfluorescein (CF) from brush border membrane vesicles from pig intestinal brush border [25] and by dissipation of a valinomycin-induced diffusion potential in liposomes [26].

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