

Detection of lysozyme in llama, sheep, and cattle tears

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Objective—To determine whether the tears of llamas, sheep, and cattle contain lysozyme and compare lysozyme concentrations in tears among these species.

Animals—40 llamas, 5 sheep, and 36 cattle.

Procedure—Electrophoresis, western blot immunoassay for lysozyme, a spectrophotometric assay to detect tear lysozyme by its ability to lyse a suspension of *Micrococcus lysodeiticus*, and a microtiter plate colorimetric assay were performed.

Results—A 13.6-kd protein band was detected by use of electrophoresis and western blot immunoassay in llama and sheep tears but not cattle tears. Results of spectrophotometric assay suggested that llama and sheep tears had high concentrations of lysozyme, whereas cattle tears had low concentrations. Results of the microtiter plate colorimetric assay suggested that llama tears had high concentrations of lysozyme, whereas concentrations in sheep and cattle tears were lower.

Conclusions and Clinical Relevance—Lysozyme concentrations in tears may vary among species and this variability may contribute to differing susceptibilities to ocular diseases such as infectious keratoconjunctivitis. (*Am J Vet Res* 2000;61:1294–1297)

The preocular tear film plays an important role in defense of the ocular surface. Mechanical flushing action by tears helps prevent invasion of the cornea and conjunctiva by microorganisms. In addition, tears contain numerous immunoglobulins, enzymes, and other proteins that provide specific and nonspecific antimicrobial actions.¹

Lysozyme is the most abundant enzyme in human tears, and results of studies indicate that it constitutes 11 to 30% of human tear protein.^{2–5} Tear lysozyme has also been found in high concentrations in nonhuman primates.^{6,7}

Domestic animals also have tear lysozyme. Prieur et al⁸ found variable, but generally high, tear lysozyme concentrations in goats and sheep. In that study, clinically normal cattle had only a small amount of tear lysozyme; however, in cattle with inflamed eyes,

lysozyme concentration was increased.⁸ In a separate study, lysozymal activity was not detected in cattle tears, but moderate amounts of the enzyme were found in sheep and goat tears.⁹ Dogs and rabbits appear to have low tear lysozyme concentration.^{6,10–12} Roberts and Erickson¹⁰ detected lysozyme in some dogs but not in others, whereas Bonavida and Sapse¹² found low concentrations in all the rabbit tears they tested. To our knowledge, the tear lysozyme content of llamas has not been investigated.

Lysozyme is manufactured in acinar cells of the main and accessory lacrimal glands^{3,13,a} and its concentration in tears is independent of the rate of tear production.^{13,14} It has specific antibacterial action against gram-positive bacteria by hydrolyzing the α -1,4 glycosidic bond between N-acetyl muramic acid and N-acetyl glucosamine in the cell walls.⁵ Its main activity is thought to be against nonpathogenic bacteria and chitin-covered fungi.^{7,8,12} The enzyme has no specific activity against gram-negative bacteria.⁸ Although lysozyme probably has no direct action against many bacterial pathogens, it may work synergistically with other tear components, such as β -lysin, IgA, and lactoferrin, to destroy most ocular pathogens.^{14,15}

Results of a retrospective study suggest that llamas and cattle have different susceptibilities to ocular disease.^{16,17} Llamas appear to be resistant to infectious keratoconjunctivitis (“pink-eye”), a disease that is common in cattle. To our knowledge, outbreaks of disease resembling infectious keratoconjunctivitis have not been reported in llamas.

The reason for the apparent different susceptibilities to ocular disease between llamas and cattle is unknown but could be related to species differences in tear constituents. In a separate study,¹⁸ we compared tear protein content between cattle and llamas. In that study, many tear proteins were detected in llamas and cattle, although there were differences in the number and locations of several protein bands detected by use of gel electrophoresis. A 13.6-kd protein was detected in llama tears but not in cattle tears. Because this is the approximate molecular weight of tear lysozyme of other species, it was hypothesized that this protein was lysozyme. Amino acid sequencing failed to adequately confirm this hypothesis. The purpose of the study reported here, therefore, was to determine whether the tears of llamas, sheep, and cattle contain lysozyme and compare lysozyme concentrations in tears among these species.

Materials and Methods

Tear collection—Tears were collected from both eyes of 40 clinically normal llamas, 1 eye of 36 clinically normal cows, and both eyes of 5 clinically normal sheep. Tears were

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collected with a 50- μ l pipette that was placed in the lower conjunctival fornix.¹⁷ Care was taken to cause as little conjunctival trauma as possible during collection. Tears were centrifuged for 2 minutes to remove gross debris and mucus, pooled (by species), divided into 50- μ l aliquots, and frozen at -20 C for later analysis.

Gel electrophoresis—Tear protein separation was completed by use of **sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**. To control for experimental error, llama, sheep, and cattle tears were electrophoresed on the same gels. Whole tears were placed on precast 11% agar running gels with 5% stacking gels. Gels were loaded with 20-ml of tears (25 ml of tears diluted with 175 ml of 2 X SDS). Gels were run in 1X SDS running buffer at 20 V for approximately 35 minutes. Known molecular weight standards^b were run with each gel. Protein bands were then transferred to nitrocellulose membranes^c by use of a semidry electrophoretic transfer cell.^d After transfer, gels were soaked away from the membrane in transfer buffer. The membranes were dried and saved for immunoblotting. Gels were stained with silver stain^e or blue stain reagent^f to ensure proper transfer of proteins.

Western blot immunoassay—Western blot immunoassays were performed to identify lysozyme. The nitrocellulose membranes were transferred from the SDS-PAGE gels and rinsed twice in **phosphate buffered saline solution (PBSS)** with 0.05% Tween 20 for 10 minutes. Nonspecific binding sites were blocked with PBSS containing 5% **bovine serum albumin (BSA)** for 2 hours. Blots were incubated with primary antibody (goat anti-rabbit lysozyme^g) in PBSS containing 5% BSA and sodium azide for 1.5 hours, washed 3 times in PBSS with Tween 20 for 10 minutes, incubated with horseradish peroxidase-labeled anti-goat IgG in PBSS containing Tween 20 for 1 hour, and incubated for 5 minutes in working solution.^h Blots were removed from solution and placed in a plastic sheet protector. Each blot was placed against radiographic film and exposed until a distinct band could be seen.

Enzymatic assay—An enzymatic assay for lysozyme was conducted on tears and was performed by use of a standard method based on the ability of lysozyme to lyse the gram-positive bacterium *Micrococcus lysodeikticus*.^{14,8} A 0.015% suspension of *M lysodeikticus*^g was incubated with 0.10 ml of whole tears at 25 C at pH 6.24. The decrease in light absorption attributable to lysis of the bacteria by lysozyme was recorded on a spectrophotometerⁱ at an absorbance of 450 nm every 30 seconds for 5 minutes. A solution containing 0.10 ml of a **hen egg lysozyme (HEL)** standard^g (200 U/ml) was used as a positive control and a solution containing only bacteria was used as a negative control. Changes in light absorbency were plotted and the HEL equivalent units of lysozyme per ml of tears were calculated, using the value of the maximum rate of change in absorbency.

Colorimetric assay—A microtiter plate colorimetric assay,⁵ developed to measure human tear lysozyme, was conducted. This assay is based on the ability of lysozyme to selectively cleave the synthetic substrate **p-nitrophenyl penta-N-acetyl b-chitopentaside (PNP)**, resulting in an intermediate product, p-nitrophenyl N-acetyl B-D-glucosaminide, which may be coupled to a reaction with **b-N-acetylhexosaminidase (NAHase)** to liberate p-nitrophenol. The free p-nitrophenol is yellow and can be accurately quantified with a colorimeter. In the colorimetric assay, 100 ml of PNP reagentⁱ was incubated with 5 ml of whole tears from each species (pooled tears) and 10 ml of NAHase at 37 C for 1 hour.⁵ The reaction was stopped with 100 ml of sodium carbonate and immediately read (as **optical density [OD]**) on a spectrophotometric microplate reader at absorbance of 405 nm. Known dilutions of HEL were used as positive controls.⁵

Statistical analyses—Means and standard deviations were calculated from 2 separate runs of the *Micrococcus* assay for each species. For the PNP analysis, 3 absorbency readings were taken on each pooled tear sample (1 from each species) and mean and SD of optical density were calculated. Mean OD of each unknown was plotted against known lysozyme concentrations to produce a reference curve.⁵ Values of unknown lysozyme concentrations from the tears were extrapolated from the reference curve by use of a computer software program.^j

Results

Results of SDS-PAGE of llama, sheep, and cattle tears confirmed that numerous protein bands were present in all 3 species (Fig 1). Llama tears had a distinct, wide band of approximately 13 kd, whereas sheep tears had a faint 13-kd band and a similar band was not detected in cattle tears. Western blot immunoassay revealed a large amount of antilysozyme antibody bound to the 13-kd band in llama tears and a smaller, but distinct area of binding in sheep tears (Fig 2); antibody binding was not detected in cattle tears. These results supported the hypothesis that the 13-kd protein in llama and sheep tears was lysozyme and that this protein was absent or in low concentration in cattle tears.

Identification of lysozyme in llama and sheep tears

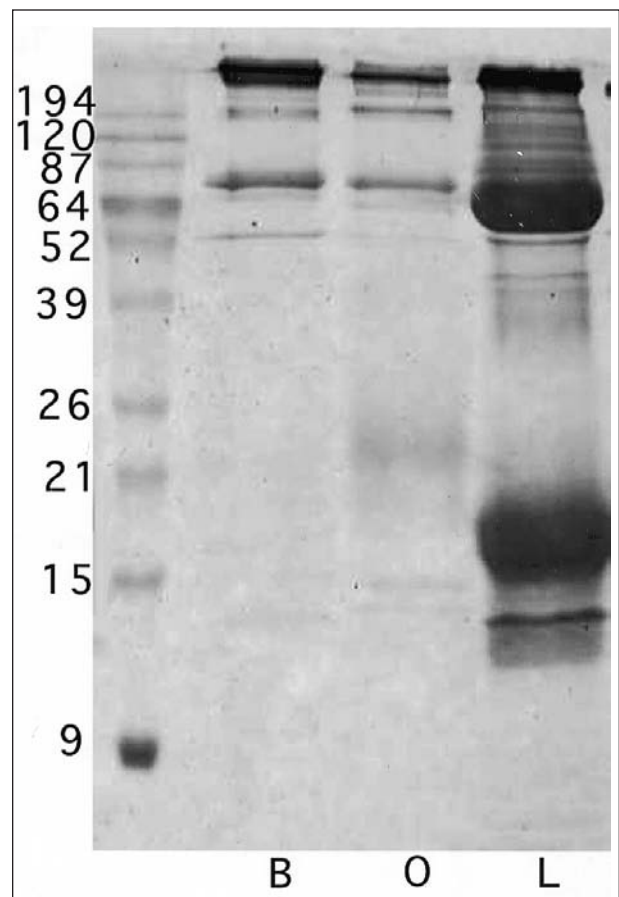


Figure 1—Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of llama (L), cattle (B), and sheep (O) tears. Numbers to the left of the figure indicate molecular weight (kd) standard markers. Notice the protein band, suspected to be lysozyme, at approximately 13 kd in llama tears, which is not evident in cattle tears and faintly visible in sheep tears.

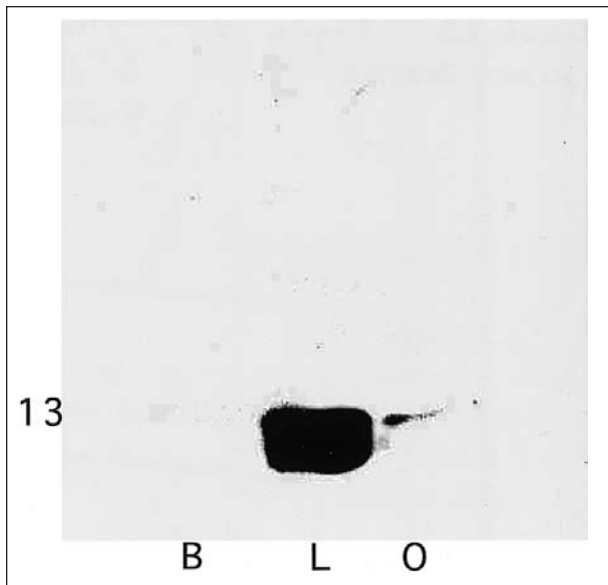


Figure 2—Results of western blot immunoassay of llama (L), cattle (B), and sheep (O) tears. The number to the left of the figure represents the migration of a 13-kd protein. Binding of antilysozyme antibody indicates detection of lysozyme in llama and sheep tears, whereas binding is not apparent in cattle tears.

was further confirmed by results of the enzymatic and colorimetric assays. Results of the enzymatic assay that used *M lysodeikticus* indicated that llama and sheep tears had high concentrations of lysozyme ($2,760 \pm 28.3$ and $2,199 \pm 26.9$ U/ml, respectively), whereas cattle had low concentrations (10 ± 14.1 U/ml). Results of the colorimetric assay also indicated that llamas had high tear lysozyme concentrations (1.01 mg/ml; mean OD, 1.082 ± 0.10 A), whereas sheep and cattle had low, but similar, tear lysozyme concentrations (0.6 mg/ml and 0.58 mg/ml, respectively; mean OD, 0.32 ± 0.02 A and 0.30 ± 1.022 A, respectively).

Discussion

Results of the 3 assays used in the study reported here confirm that llamas have large amounts of tear lysozyme. Quantity of tear lysozyme in llamas was comparable to mean values in humans ($1,483$ U/ml) determined by use of an enzymatic assay using *Micrococcus* sp.,¹⁸ which was similar to the assay used in our study. By use of an immunoturbidimetric rate analysis, human tear lysozyme values range from 750 to $3,300$ mg/L of tears⁴; by use of colorimetric analysis using PNP, human tears contain 0.3 to 2.0 mg of lysozyme/ml.⁵ Llama tear lysozyme content was similar to that determined in goats by use of a *Micrococcus* lysoplate assay ($2,100$ mg/ml of tears).⁸ Our results are similar to those obtained by use of various *Micrococcus* lysing assays,^{8,9,19} which indicate that sheep have moderate amounts of tear lysozyme, whereas cattle have little or none. By use of a *Micrococcus* lysoplate assay, Prieur⁸ detected 366 mg of lysozyme/ml (range, 100 to $1,000$ mg/ml) in the tears of sheep, whereas Padgett and Hirsch¹⁹ detected $1,140$ mg of lysozyme/ml. Neither study detected any lysozymal activity in the tears of clinically normal cattle.

Results of the 3 assays used in the study reported

here were comparable except that the colorimetric assay that used PNP detected larger amounts of tear lysozyme in clinically normal cattle and smaller amounts of lysozyme in sheep tears than did the enzymatic assay. Lysozyme concentrations determined in sheep tears by the colorimetric assay were comparable to those of other studies that used different methods.^{8,9} The reason for the large amount of lysozyme found in cattle tears by use of the colorimetric assay is unknown; a possible explanation is that the colorimetric assay is more accurate and sensitive than the enzymatic or western blot assays for quantifying tear lysozyme activity.⁵ The assay is based on a completely different chemical reaction than other assays and may be able to detect different isomeric forms of lysozyme with different lysing activities; such isomeric forms may have been present in cattle tears.

Although goat anti-rabbit lysozyme was used for the western blot analysis (because commercial antibodies to llama proteins were not available), the amino acid structure of llama tear lysozyme may be similar to that of rabbit lysozyme. Binding of the goat anti-rabbit lysozyme to the suspected lysozyme band on the gels was excellent. Lack of binding to any bands on the cattle tear gels may indicate that lysozyme was truly not present in cattle tears or that cattle tear lysozyme is sufficiently different from rabbit lysozyme that the antibody does not bind to it.

Several different lysozymes may be in tears of llamas, sheep, and cattle. In a recent study, 3 goat tear lysozymes (1, 2a, and 2b) were isolated and their N-terminal amino acid sequences were determined.²⁰ Two of these lysozymes (2a and 2b) differed from sheep kidney lysozymes at only 1 and 2 sites, respectively, in the N-terminal residues and from cow kidney lysozymes at 4 and 5 sites, respectively.²⁰ However, goat tear lysozyme-1 was different from sheep and cow kidney lysozymes and had the highest similarity to cow milk lysozyme.²⁰ It is possible that cattle have a form of tear lysozyme that does not lyse *Micrococcus* organisms but does react with PNP and thus may be detectable by the colorimetric PNP assay.

Whether the high concentration of lysozyme in llama tears is responsible for the apparent low susceptibility of this species to ocular diseases, compared with cattle, is unknown. Padgett and Hirsch¹⁹ reported that whole cattle tears were bacteriostatic but not bacteriolytic for *M lysodeikticus* and *Bacillus megaterium*. Whether lysozyme is protective against invasion of the ocular surfaces by *Moraxella* spp is unknown.

⁴Atkins AM, Barr HA, Schofield GC. Localization of immunoglobulins and lysozyme in sheep lacrimal gland (abstr). *J Anatomy* 1971;110:494.

⁵BenchMark, prestained protein ladder, Life Technologies, Rockville, Md.

⁶Bio-Rad Laboratories, Hercules, Calif.

⁷Bio-Rad trans-blot SD, Bio-Rad Laboratory, Hercules, Calif.

⁸Sliver stain plus, Bio-Rad Laboratories, Hercules, Calif.

⁹Gelcode blue stain, Pierce Chemical Co, Rockford, Ill.

¹⁰Sigma Chemical Co, St Louis, Mo.

¹¹Pierce Chemical Co, Rockville, Md.

¹²Ultrospec R, Protein Solutions Inc, Charlottesville, Va.

¹³Seikaga America Inc, Rockville, Md.

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