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Purification, characterization, antibacterial activity and N-terminal sequencing of buffalo-milk lysozyme

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Summary. Lysozyme from buffalo milk was purified to homogeneity and its N-terminal amino acid sequence, biochemical properties and antibacterial spectrum were determined. The purification procedure, comprising ion-exchange chromatography using CM-cellulose and size-exclusion chromatography using Sephadex G-50, conferred 8622-fold purification and 39·3% recovery of lysozyme. The purified enzyme migrated as a single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE. Immunological purity of lysozyme preparation was confirmed by immuno-electrophoresis. Molecular weight of buffalo-milk lysozyme as determined by SDS-PAGE was 16 kDa and its amino acid composition was determined by reverse phase high performance liquid chromatography (HPLC). The sequence of 23 amino acid residues at the N-terminal end showed 56·5% homology with bovine milk lysozyme and 30·4% with equine milk lysozyme. The specific activity of buffalo milk lysozyme was ten-times that of bovine milk lysozyme. Buffalo-milk lysozyme was active over a wide range of pH and its activity was strongly influenced by molarity of the medium. Antibacterial activity of buffalo-milk lysozyme was determined against 11 species of bacteria; out of seven Gram-positive bacteria tested, four were inhibited, while Gram-negative bacteria were resistant.

Keywords: Buffalo-milk lysozyme, purification, antibacterial activity, N-terminal sequence

Milk provides the neonate not only with nutrients but also with a host of defence factors such as antibacterial, anti-inflammatory and immuno-modulatory agents (Goldman & Goldlum, 1995). Antimicrobial agents in milk include immunoglobulins, lactoferrin, antiviral lipids, vitamin-binding proteins and the enzymes, lysozyme, lactoperoxidase and xanthine oxidase. Lysozyme is an important antimicrobial agent in milk, which kills bacteria by cleaving the β-1,4-glycosidic bond between N-acetyl muramic acid and N-acetyl glucosamine residues of the peptidoglycan in the bacterial cell wall. Lysozyme content in milk and its physicochemical and enzymatic properties vary widely among species. Human and equine milks are very rich in lysozyme (Chandan *et al.* 1964; Jaurequi-Adell *et al.* 1972), while milk of many other species contains only low concentrations (Chandan *et al.* 1965; McKenzie & White, 1986; Elagamy *et al.* 1996).

Buffalo milk is relatively more resistant to microbial spoilage than bovine milk.

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Antimicrobial properties of buffalo milk are not fully understood; in particular no information exists about lysozyme. In this paper we report information on purification, characterization, N-terminal amino acid sequencing and antibacterial activity of buffalo-milk lysozyme.

materials and methods

Chemicals and bacterial cultures

Egg-white lysozyme, lysophilized cells of *Micrococcus lysodeikticus*, CM-cellulose, Sephadex G-50, bicinchoninic and barbital were obtained from Sigma (St Louis, MO 63178, USA). Amino acid standards were from Pierce (Rockford, IL 61105, USA). Protein molecular weight markers were from Bangalore Genei (Bangalore 560058, India). Other chemicals were of analytical grade. Bacterial cultures were from NCDC, National Dairy Research Institute, Karnal, India.

Determination of lysozyme activity

The assay of lysozyme was as described by Selested & Martinez (1980) with some modifications. A suspension of *Mc. lysodeikticus* (350 mg/l) was prepared in 0·05 M-potassium phosphate buffer, pH 7·4. The reaction mixture contained 2·1 ml cell suspension, 0·3 ml bovine serum albumin (1 g/l), 0·3 ml sodium azide, a source of lyzozyme and 0·05 M-potassium phosphate buffer to a final volume of 3 ml. Absorbance was read at 450 nm before and after incubation at 37 °C with mild agitation. Reduction in absorbance relative to control (without enzyme) was taken as a measure of lysozyme activity. Reaction time was kept at 6 h instead of the 18 h used by Selested & Martinez (1980). Change in absorbance was linear with respect to enzyme concentration, providing sensitivity of 10–50 ng egg-white lysozyme, sufficient for the assay of lysozyme in diluted milk samples. The unit of enzyme activity was defined as change in unit absorbance per minute per millilitre reaction mixture, measured at 450 nm using a Spectronic 601 Spectrophotometer (Milton Roy, Rochester NY 14625 USA).

Isolation and purification of lysozyme

Activated CM-cellulose was equilibrated for 24 h with 0·05 M-sodium phosphate buffer (pH 6·5) containing 0·2 M-NaCl. It was then stirred gently for 4 h with 1:1 diluted non-fat buffalo milk (1 part CM-cellulose and 20 parts diluted milk). The ion-exchanger was then washed with distilled water followed with 0·2 M-NaCl in 0·05 M-sodium phosphate buffer (pH 6·5), until the absorbance of washings at 280 nm was negligible. Resin was packed into a glass column and lysozyme eluted with 1 M-NaCl in 0·05 M-sodium phosphate buffer (pH 6·5). Lysozyme-active fractions were pooled and then concentrated by ultra-filtration using a Millipore-Minitan S ultra-filtration unit (Massachusetts 01730, USA) and cellulose membrane. Concentration of NaCl in the retentate was brought down to 0·02 M during ultra-filtration. The retentate was subjected to gel-filtration using a Sephadex G-50 column. Lysozyme was eluted with 0·2 M-NaCl in 0·05 M-sodium phosphate buffer containing sodium azide (0·2 g/l) Lysozyme-active fractions were pooled and then concentrated by ultra-filtration and then lyophilized.

Purity of lysozyme was tested by native-PAGE (Gabriel, 1971) and SDS-PAGE (Laemmli, 1970). Molecular weight was determined by SDS-PAGE using standard molecular weight markers. Protein was estimated by the bicinchoninic acid method (Smith *et al*. 1985).

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Immunological studies

Adult male mice were immunized with purified buffalo-milk lysozyme. Immunological purity of buffalo-milk lysozyme and cross-reactivty with egg-white lysozyme was tested by immuno electrophoresis (Bog-Hansen, 1990).

Amino acid analysis

Amino acid composition was determined by reversed-phase-HPLC (Dupont *et al.* 1989) using a Waters HPLC system (Model 680 Automatic Gradient Controller, Pumps 510, Tunable Absorbance Detector 486; Milford, MA 01757, USA). Lysozyme protein was hydrolysed with 6 M-HCl and amino acids were converted to phenyl isothiocynate derivatives and separated on a PICO TAG amino acid analysis-C_{18} column ($3{\cdot}9 \times 150$ mm). Standard amino acids were also derivatized and separated under similar conditions. Retention time, peak area and amount of each amino acid were measured using Millennium Chromatography Manager (Waters, Milford, MA 01757, USA). Number of each amino acid residue in a molecule of lysozyme was calculated from its molecular weight (16 kDa) determined by SDS-PAGE.

Sequencing of lysozyme

The N-terminal amino acid sequence was determined by Edman's degradation method (Edman, 1970) using a Shimadzu PPSQ-21 protein sequencer (Kyoto 604, Japan). Phenylisothiocyanate was reacted with the amino acid residue at the amino terminus under basic conditions (provided by N-methylpiperidine/methanol/water) to form a phenylthiocarbamyl derivative (PTC-protein). Trifluoroacetic acid then cleaved off the first amino acid as its anilinothialinone derivative (ATZ-amino acid), exposing the new amino terminus for the next degradation cycle. The ATZ amino acid was then removed by extraction with N-butyl chloride and converted into a phenylthiohydantoin derivative (PTH-amino acid). The PTH-amino acid was transferred to a reverse-phase C_{18} column for detection at 270 nm. A standard mixture of 19 PTH-amino acids was injected onto the column for separation as the first cycle of the sequencing run. This chromatogram provided standard retention times of the amino acids for comparison with each Edman degradation cycle chromatogram. HPLC chromatograms were collected using a computer data analysis system. The process was repeated sequentially to provide the N-terminal sequence of the protein.

Antibacterial activity

Antibacterial activity of lysozyme was determined using the agar-well method of Ten-Brink *et al.* (1994).

RESULTS

Normal animals with milk having a somatic cell count $< 5 \times 10^5$ cells/ml, were selected for determination of milk lysozyme activity. Mean ($\pm$SE) lysozyme activity in buffalo milk was $60 \pm 3{\cdot}9 \times 10^{-3}$ units/ml ($n = 15$), which was double the value observed in bovine milk ($29{\cdot}1 \pm 1{\cdot}5 \times 10^{-3}$ units/ml, $n = 15$).

Purification of lysozyme

Purification was by cation exchange chromatography followed by size exclusion chromatography. Fig. 1 shows the profile of protein and lysozyme activity eluted from the CM-cellulose column. Binding of lysozyme to CM-cellulose was 60% of the activity present in milk and 99% of the bound activity was recovered when eluted

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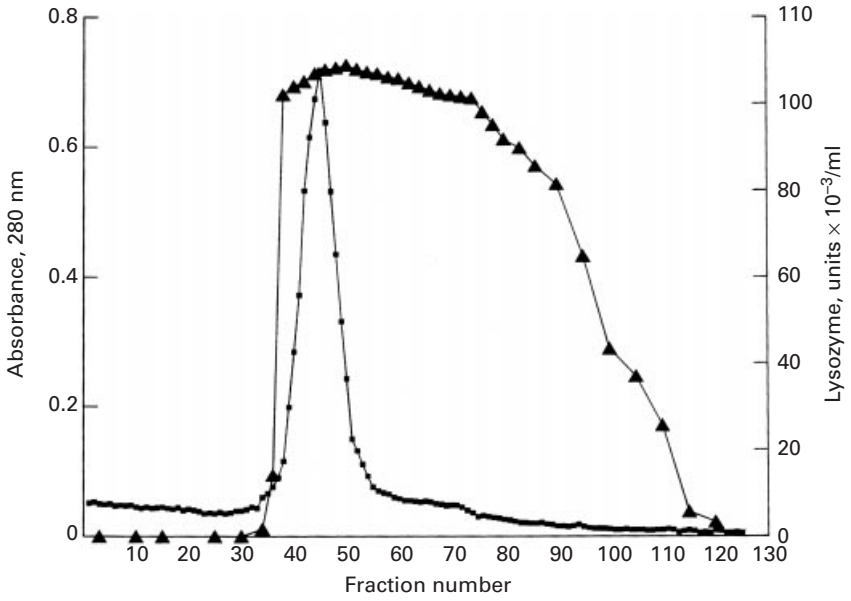

Fig. 1. Profile of lysozyme eluted from CM-cellulose. Lysozyme from buffalo skim milk was adsorbed onto activated CM-cellulose (100 g), packed into a glass column (5·25 × 60 cm) and eluted (14-ml fractions) with 1 M-NaCl in 0·05 M-sodium phosphate buffer (pH 6·5). Protein (■) was estimated at 280 nm and lysozyme activity (▲) determined as described in the text.

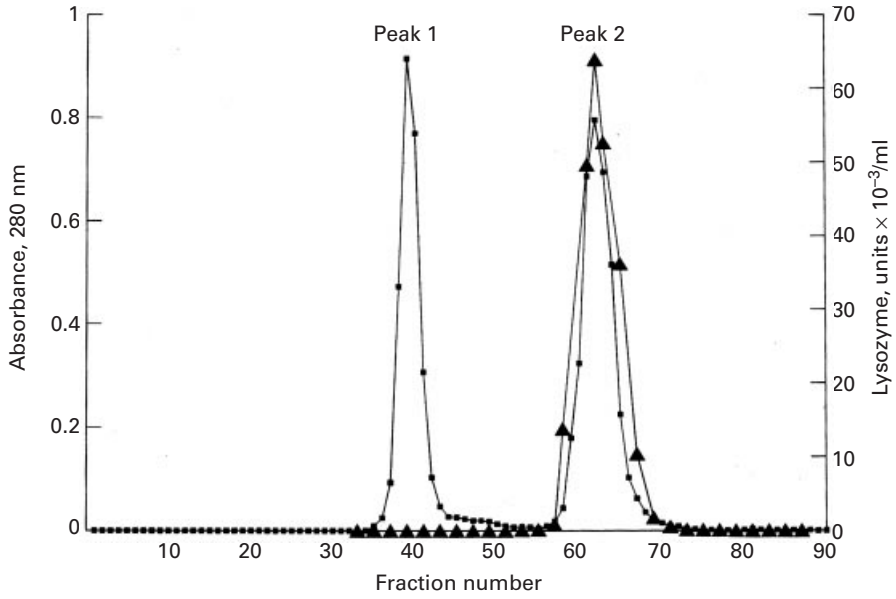

Fig. 2. Profile of buffalo-milk lysozyme eluted from Sephadex G-50. Concentrated eluate (11·6 ml containing 54·5 mg protein) from a CM-cellulose column was loaded onto a Sephadex G-50 column (3·18 × 93 cm) and eluted (6-ml fractions) with 0·2 M-NaCl in 0·05 M-sodium phosphate buffer (pH 7·0). Protein (■) was estimated at 280 nm and lysozyme activity (▲) determined as described in the text.

with 1 M-NaCl in 0·05 M-sodium phosphate buffer. This single step ion-exchange chromatography led to more than 3,000-fold purification of lysozyme. After concentrating the lysozyme-active fraction, it was resolved into two protein peaks by size exclusion chromatography (Fig. 2). Lysozyme activity was recovered in peak 2.

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Table 1. *An outline of the procedure used to purify buffalo-milk lysozyme*

Purification step	Volume, ml	Total activity, units†	Specific activity, units/mg protein	Yield, %
Skim milk	9000	8100	0·025	–
CM-cellulose chromatography	1091	4800	79·7	59·3
Ultrafiltration	66·3	4092	75·1	50·5
Vacuum concentration	11·6	4041	74·6	49·9
Gel filtration	90·0	3181	215	39·3

† One unit of lysozyme activity is defined as the change in unit absorbance per minute per millilitre/reaction mixture at 450 nm.

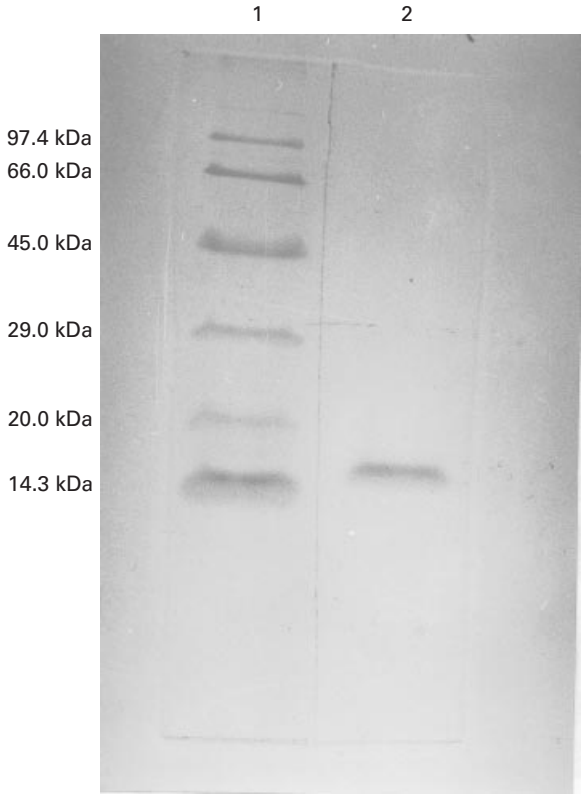

Fig. 3. SDS-PAGE profile of buffalo-milk lysozyme (10 μg) separated on 15% SDS-polyacrylamide gel and stained with coommassie brilliant blue. Lane 1, standard molecular weight markers (30 μg): phosphorylase b, 97·4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 20 kDa and egg-white lysozyme, 14·3 kDa. Lane 2, buffalo-milk lysozyme (peak 2 from Sephadex G-50 Column).

Recovery of lysozyme from Sephadex G-50 column was around 80% and the overall yield was 39·3% (Table 1). Specific activity of purified buffalo-milk lysozyme was 225 units/mg protein, which was more than three-times that of standard egg-white lysozyme.

Determination of purity and molecular weight of lysozyme

Peak 2 protein from the Sephadex G-50 column when subjected to SDS-PAGE, migrated as a single band with mobility slightly lower than that of standard egg-white lysozyme (Fig. 3), confirming the purity of the lysozyme preparation. Standard

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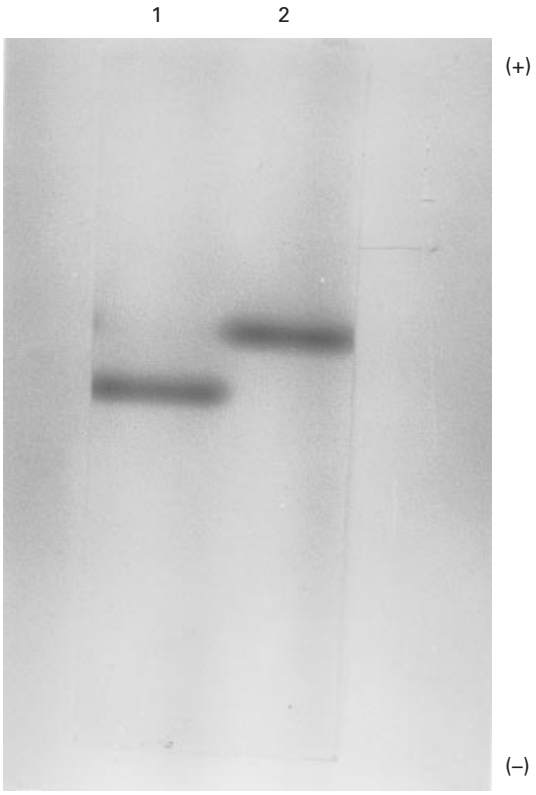

Fig. 4. Native-PAGE profile of buffalo-milk lysozyme (10 μg) and standard egg-white lysozyme (10 μg) separated on 12·5% polyacrylamide gel and stained with coommassie brilliant blue. Lane 1, buffalo-milk lysozyme (peak 2 from Sephadex G-50 Column). Lane 2, standard egg-white lysozyme.

molecular weight markers were subjected to SDS-PAGE in the lane adjacent to buffalo-milk lysozyme. From the plot of log molecular weight *v.* relative mobility (not shown), the molecular weight of buffalo milk lysozyme was estimated as 16 kDa. Furthermore, the peak 2 protein on native PAGE also migrated as a single band towards the cathode and in this case slightly faster than egg-white lysozyme (Fig. 4), suggesting the presence of greater positive charge on buffalo milk lysozyme than on egg-white lysozyme.

Immunological homogeneity was confirmed by immuno electrophoresis. Antibodies against buffalo-milk lysozyme raised in mouse showed a single precipitin line with the antigen and no cross reactivity with egg-white lysozyme (Fig. 5).

Amino acid composition and sequencing of lysozyme

Amino acid composition of lysozyme determined by reverse phase HPLC is shown in Table 2. Standard egg-white lysozyme was used as a control and its composition was compared with the reported values from its sequence analysis (Jolles & Jolles, 1972). Observed amino acid composition of egg-white lysozyme was close to reported values. Absence of tryptophan and poor recovery of cysteine were due to their destruction during acid hydrolysis. The reason for the higher value for proline is not clear. The single histidine residue present in egg-white lysozyme could not be detected. Buffalo-milk lysozyme contained 115 amino acid residues (excluding tryphophan and cysteine). Buffalo-milk lysozyme has ten lysine residues compared

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Table 2. *Amino acid residues in buffalo-milk and egg-white lysozyme*

Amino acid	Buffalo-milk lysozyme Observed†	Egg-white lysozyme Observed ‡	Egg-white lysozyme Theoretical§
Lysine	10	6	6
Isoleucine	4	6	6
Leucine	7	8	8
Phenylalanine	3	3	3
Cysteine	1	3	8
Methionine	2	1	2
Valine	7	5	6
Tyrosine	4	3	3
Alanine	9	12	12
Proline	4	5	2
Arginine	11	11	11
Threonine	9	8	7
Glycine	9	11	12
Serine	7	9	10
Histidine	ND	ND	1
Aspartic acid + asparagine	22	23	21
Glutamic acid + glutamine	6	5	5
Tryptophan	ND	ND	6

† Calculated by taking molecular weight as 16 kDa; values rounded to the nearest integer.
‡ Calculated by taking molecular weight as 14 kDa; values rounded to the nearest integer.
§ From sequence analysis (Jolles & Jolles, 1972).
ND, not detected.

with six in egg-white lysozyme, while the number of arginine residues is the same in both. The difference in lysine residues, therefore, accounts for the greater positive charge on buffalo milk lysozyme observed in native PAGE (Fig. 4).

The sequence of 23 amino acid residues at the N-terminal end of buffalo milk lysozyme was elucidated and compared with egg-white lysozyme and milk lysozymes of other species (Table 3). The N-terminal sequence of 23 amino acid residues of buffalo-milk lysozyme shows highest homology with bovine milk lysozyme (56·5 %) followed by human milk lysozyme (47·8 %), egg-white lysozyme (34·7 %) and equine milk lysozyme (30·4 %). Lys at position 1, Cys at 6, Ala at 9, Asp at 18 and Gly at positions 16 and 22 are conserved in all five lysozymes. Positions 3 and 20 have aromatic amino acids (Phe or Tyr) and positions 1 and 5 have basic amino acids (Lys or Arg) in all five lysozymes. Important variations in buffalo-milk lysozyme are Arg at position 4, Ile at 13, Asn at 7 and Ala at positions 8, 17 and 19, which differ from the other four lysozymes.

Characteristics of buffalo milk lysozyme

Effect of pH determined in 0·05 M-potassium phosphate and tris-HCl buffers showed that buffalo milk lysozyme was active over a wide range of pH with maximum activity at pH 7·4 (Fig. 6). Even at pH 6·5 and 8·0, the enzyme retained >90 % of its maximum activity. Buffer molarity also influenced the activity of buffalo-milk lysozyme. Optimum molar concentration at pH 7·4 was 0·05 M for potassium phosphate buffer and 0·075 M for tris-HCl buffer (Fig. 7). Optimum temperature for the enzyme was 37 °C (Fig. 8).

Antibacterial spectrum of buffalo milk lysozyme

Using standard egg-white lysozyme as a positive control, the antibacterial activity of buffalo-milk lysozyme was determined against 11 species of bacteria. Results were quantified by measuring the diameter of the zone of inhibition around

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Table 3. *N-terminal sequence of buffalo-milk lysozyme and comparison with lysozyme from other species*

Buffalo milk	Lys	Ile	Tyr	Arg	Arg	Cys	Asn	Ala	Ala	Arg	Thr	Leu	Ile	Lys	Ile	Gly	Ala	Asp	Ala	Tyr	Gly	Gly	Val
Bovine milk†	Lys	Lys	Phe	Gln	Arg	Cys	Glu	Leu	Ala	Arg	Thr	Leu	Lys	Lys	Leu	Gly	Leu	Asp	Gly	Tyr	Arg	Gly	Val
Human milk‡	Lys	Val	Phe	Glu	Arg	Cys	Glu	Leu	Ala	Arg	Thr	Leu	Lys	Arg	Leu	Gly	Met	Asp	Gly	Tyr	Arg	Gly	Ile
Equine milk§	Lys	Val	Phe	Ser	Lys	Cys	Glu	Leu	Ala	His	Lys	Leu	Lys	Ala	Gln	Glu	Met	Asp	Gly	Phe	Gly	Gly	Tyr
Egg-white‡	Lys	Val	Phe	Gly	Arg	Cys	Glu	Leu	Ala	Ala	Ala	Met	Lys	Arg	His	Gly	Leu	Asp	Asn	Tyr	Arg	Gly	Tyr
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23

† From White *et al.* (1988)
‡ From Jolles & Jolles (1972)
§ From McKenzie & Shaw (1985)

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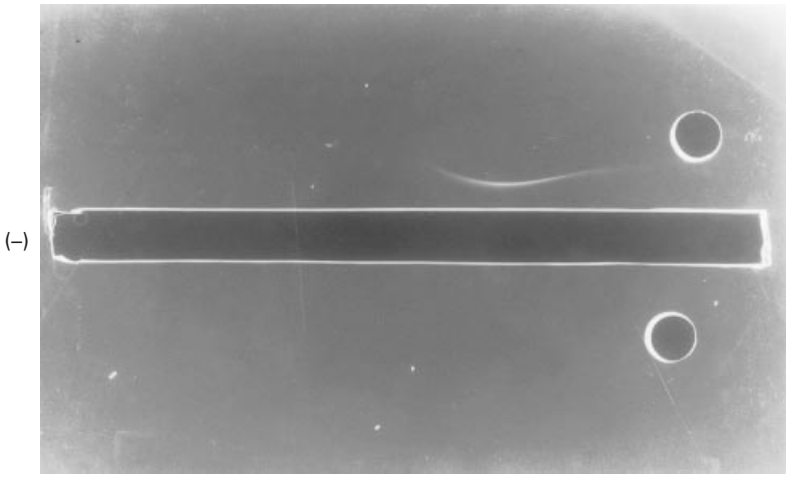

Fig. 5. Immuno-electrophoresis of buffalo-milk (well *a*) and egg-white (well *c*) lysozymes against mouse anti-buffalo milk lysozyme (well *b*).

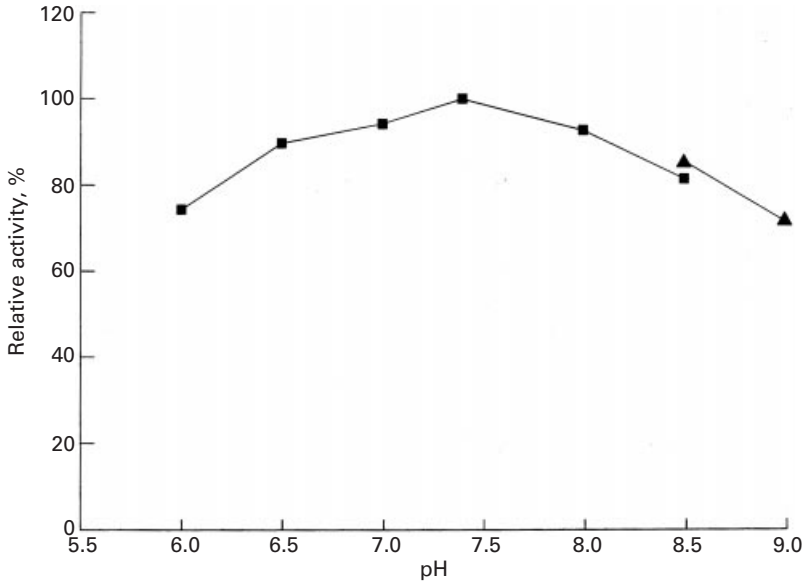

Fig. 6. Effect of pH on activity of buffalo-milk lysozyme (3 mU) at 37 °C in a reaction mixture containing 0·05 M-potassium phosphate buffer (■) or 0·075 M-tris-HCl buffer (▲). Values are means of three determinations expressed as percentage of maximum activity observed at pH 7·4. Differences between replicates and the mean are $< 2{\cdot}8\%$ of mean.

the well in which the lysozyme was layered (Table 4). *Mc. luteus*, *Bacillus subtilis* and *Lactobacillus lactis* ssp. *lactis* were inhibited by both buffalo-milk and egg-white lysozymes. *Enterococcus faecalis* was inhibited by buffalo-milk lysozyme, but not by egg-white lysozyme. *Staphylococus aureus* was inhibited by egg-white lysozyme, while buffalo-milk lysozyme had no effect. Two other Gram-positive bacteria (*B. cereus* and *Lc. delbrueckii* ssp. *bulgaricus*) and all the Gram-negative bacteria

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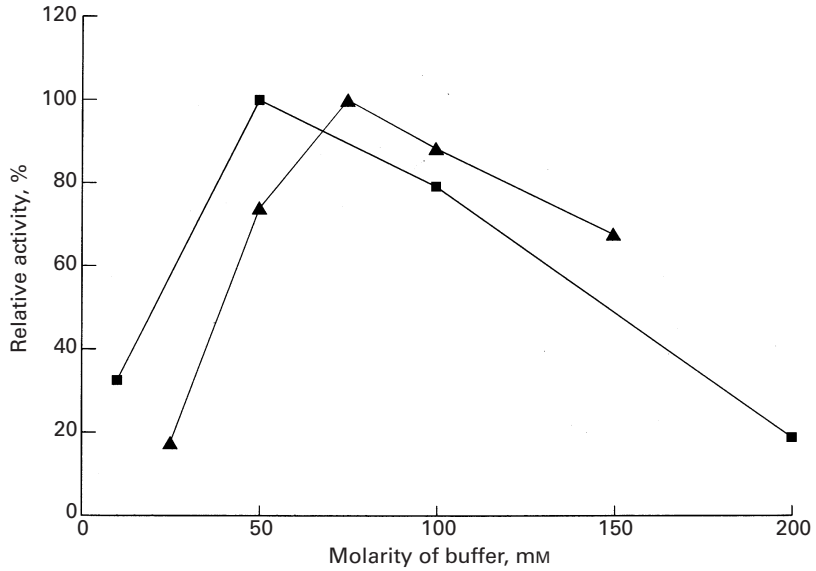

Fig. 7. Effect of buffer molarity on activity of buffalo-milk lysozyme (3 mU) at 37 °C in a reaction mixture (3 ml) containing potassium phosphate buffer (■) or tris-HCl buffer (▲), pH 7·4. Values are means of three determinations expressed as percentage of maximum activity. Differences between replicates and the mean are < 2·6% of mean.

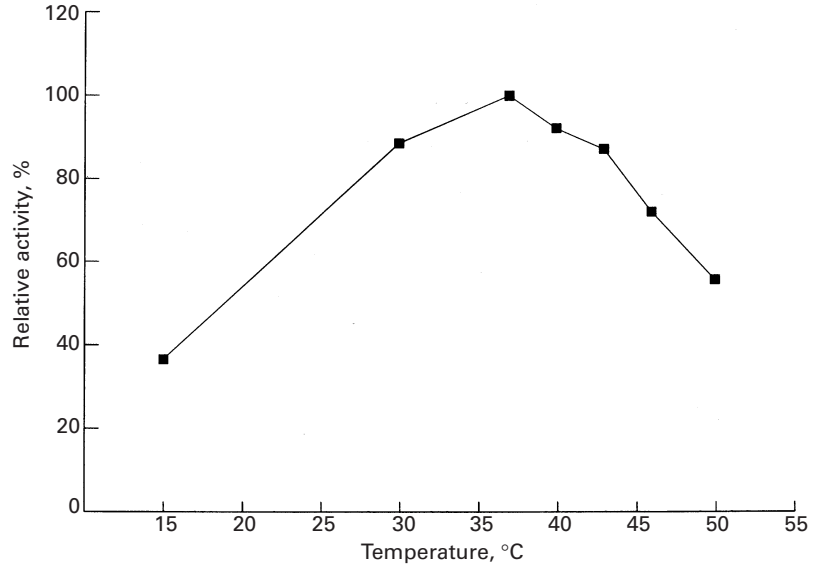

Fig. 8. Effect of temperature on activity of buffalo-milk lysozyme (3 mU) in a reaction mixture (3 ml) containing 0·05 M-potassium phosphate buffer (pH 7·4). Values are means of three determinations expressed as percentage of maximum activity. Differences between replicates and the mean < 2·9% of mean.

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Table 4. *Antibacterial activity of lysozyme from buffalo milk (BML) and egg-white (EWL)*†

(Values are means for $n = 2$)

Bacteria	Diameter of zone of inhibition, mm †‡	
	BML	EWL
Micrococcus luteus	16·0	19·5
Bacillus subtilis	13·5	15·0
Bacillus cereus	0	0
Lactococcus lactis ssp. *lactis*	13·5	15·5
Enterococcus faecalis	10·0	0
Lactobacillus delbrueckii ssp. *bulgaricus*	0	0
Staphylococcus aureus	0	12·5
Escherichia coli	0	0
Proteus vulgaris	0	0
Pseudomonas aeruginosa	0	0
Salmonella typhi	0	0

† Concentration of BML and EWL was 2·3 units in each well.
‡ See text for details.

(*Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Salmonella typhi*) were not inhibited by either of the lysozymes.

DISCUSSION

Buffalo-milk lysozyme was purified to homogeneity using cation-exchange and molecular exclusion chromatography. Earlier protocols for purification of lysozyme from cow, human and baboon milks used Amberlite IRC-50 as the cation-exchanger (Chandan *et al.* 1965; Dalaly *et al.* 1970; Buss, 1971). In the present study, Amberlite IRC-50 was found to be unsatisfactory because of poor recovery of buffalo-milk lysozyme during elution (data not shown). Recovery of bound buffalo milk lysozyme from CM-cellulose was 99%. Purification steps adopted in the present investigation conferred 39·3% recovery of lysozyme from buffalo milk. Recoveries recorded in earlier protocols were 15% from cow milk (White *et al.* 1988) and 24% from camel milk (Duhiman, 1988).

Specific activity of buffalo-milk lysozyme is ten-times that of bovine milk lysozyme (White *et al.* 1988), five-times that of camel-milk lysozyme (Duhiman, 1988), three-times that of baboon- (Buss, 1971) and horse- (Bell *et al.* 1981) milk and egg-white lysozymes and similar to that of human-milk lysozyme (Parry *et al.* 1969). There appears to be a relationship between charge on lysozyme and its specific activity. Bovine- (Chandan *et al.* 1965) and horse- (Bell *et al.* 1981) milk lysozymes, which possess less positive charge than egg-white lysozyme, also possess less lytic activity. Human-milk (Parry *et al.* 1969) and buffalo-milk (present study) lysozymes, which possess greater positive charge than egg-white lysozyme, are about three-times more active than the latter.

The molecular weight of buffalo-milk lysozyme was 16 kDa compared with 14·3 kDa for standard egg-white lysozyme. Molecular weights reported for other milk lysozymes are 15 kDa for human (Finkelstein & Finkelstein, 1982; Wang & Kloer, 1984), 14·4 kDa for camel- (Elagamy *et al.* 1996) and horse- (Bell *et al.* 1981), and 18 kDa for bovine- (Eitenmiller *et al* 1975) milk lysozymes. Milk lysozymes from different species are antigenically different. Egg-white lysozyme showed no cross-reactivity with anti-buffalo-milk lysozyme. Eitenmiller *et al.* (1974) also observed no

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immunological cross-reactivity amongst bovine-milk, human-milk and egg-white lysozymes. Similarly, anti-camel-milk lyzosyme did not cross-react with egg-white and bovine-milk lysozymes (Elagamy *et al.* 1996).

Buffalo-milk lysozyme showed maximum activity at pH 7·4, similar to that reported for bovine- (McKenzie & White, 1986), feline- and canine- (Halliday *et al.* 1993) milk lysozymes. Reported optimum pH for human-milk (Parry *et al.* 1969) and egg-white (Chandan *et al.* 1965) lysozymes are 6·5 and 6·6, respectively. Optimum temperature for buffalo-milk lysozyme was 37 °C, similar to that reported for egg-white (Smolelis & Hartsell, 1952) and bovine-milk (McKenzie & White, 1986) lysozymes. Further studies on biochemical characterization of buffalo-milk lysozyme are in progress.

Lysozyme from different milk sources is not always similarly effective against each microorganism. *Mc. Luteus* and *B. subtilis* are inhibited by both buffalo-milk and egg- white lysozymes. Bovine- and human-milk lysozymes are also effective against these microorganisms (Vakil *et al.* 1969). *Staph. aureus* is inhibited by egg-white (present study) and camel-milk (Elagamy *et al.* 1992) lysozymes, but not by buffalo- (present study), bovine- and human-milk lysozymes (Vakil *et al.* 1969). *Esch. coli* is inhibited by bovine and human milk lysozymes (Vakil *et al.* 1969) but not by buffalo milk lysozyme. *Ec. faecalis* was inhibited by buffalo-milk lysozyme, but not by egg-white lysozyme. All Gram-positive bacteria are insensitive to lysozyme. The susceptibility of bacteria to lysozyme depends on several factors including the ratio of glucosamine to muramic acid residues in the cell wall and the presence of substituent groups. Resistance of *B. cereus* to buffalo-milk lysozyme might be due to the absence of N-acetyl groups on the glucosamine residues (Amano *et al.* 1980). Gram-negative bacteria are insensitive to lysozymes because the peptidoglycan content in their cell walls is very much less (5–10 % of cell wall constituents) and is buried beneath an array of lipoproteins and lipopolysaccharides that prevent the lysozyme from reaching its substrate (Taylor, 1983). Although it is difficult to assess the actual contribution of lysozyme towards preventing the growth of Gram-positive bacteria in buffalo milk, the favourable ionic environment of buffalo milk (unpublished work), and the high specific activity of lysozyme might play an important role in preventing the growth of some Gram-positive bacteria.

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