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Biochemical characterization of buffalo (*Bubalus bubalis*) milk lysozyme

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Lysozyme, a low-molecular weight basic protein, is an important component of the antibacterial system in milk. Lysozyme activity is higher in buffalo milk ($60 \pm 3{\cdot}9 \times 10^{-3}$ units/ml) than in bovine milk ($29{\cdot}1 \pm 1{\cdot}5 \times 10^{-3}$ units/ml). Buffalo colostrum contains five-times more lysozyme activity than mature milk (Priyadarshini & Kansal, 2002a). Lysozyme activity in buffalo milk is not influenced by the parity of animal or stage of lactation, but it increases during extreme weather (winter and summer). Lysozyme in buffalo milk is more stable than in cow milk during storage and heat treatment. A sharp increase in milk lysozyme has been observed in buffaloes with sub-clinical mastitis (Priyadarshini & Kansal, 2002a).

The molecular weight of buffalo milk lysozyme is 16 kDa as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The sequence of 23 amino acid residues at the N-terminal end shows 56·5% homology with bovine milk lysozyme and 30·4% with equine milk lysozyme. The specific activity of buffalo milk lysozyme is 10-times that of bovine milk lysozyme. Buffalo milk lysozyme is active over a wide range of pH and its activity is strongly influenced by the molarity of the medium (Priyadarshini & Kansal, 2002b). The anti-bacterial activity of buffalo milk lysozyme against *Micrococcus luteus*, *Bacillus subtilis*, *Enterococcus faecalis* and *Lactococcus lactis* ssp. *lactis* has been demonstrated (Priyadarshini & Kansal, 2002b). In this paper we report further biochemical characterization of buffalo milk lysozyme.

Materials and Methods

Egg white lysozyme, lyophilized cells of *Micrococcus luteus*, CM-cellulose, Sephadex G-50 and bicinchonic acid were from Sigma (St. Louis, MO 63178, USA). Other chemicals were of analytical grade. Assay of lysozyme was as described by Selested & Martinez (1980) with some modifications (Priyadarshini & Kansal, 2002b). 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 lysozyme and 0·05 M-potassium phosphate buffer (pH 7·4) to a final volume of 3 ml. Absorbance was read at 450 nm before and after incubation at 37 °C for 6 h. The method was sensitive over the range 10–50 ng egg-white lysozyme. The unit of enzyme activity was defined as the change in unit absorbance per minute per milliliter reaction mixture.

Buffer molarity influenced the activity of buffalo-milk lysozyme. Optimum concentration at pH 7·4 was 0·05 M for potassium phosphate buffer and 0·075 M for Tris–HCl buffer. Lysozyme activity in 0·05 M-phosphate buffer was similar to that in 0·075 M-Tris–HCl buffer (Priyadarshini & Kansal, 2002b). Tris–HCl buffer was used in the study of the effect of heavy metal ions, since organic buffers are free from any traces of heavy metal ions. Moreover, with phosphate buffer, there is a possibility of precipitation of metal phosphates.

Lysozyme from buffalo milk was purified to homogeneity. The procedure comprised ion-exchange chromatography using CM-cellulose and size-exclusion chromatography using Sephadex G-50 (Priyadarshini & Kansal, 2002b). Purity of lysozyme was tested by SDS-PAGE (Laemmili, 1970) and immuno-electrophoresis (Bog-Hansen, 1990).

Results and Discussion

Milk is a complex mixture of various salts, which determines the ultimate activity of lysozyme in milk. The activity of buffalo milk lysozyme in 50 mM-phosphate buffer was three-times that observed in 10 mM-phosphate buffer, and in 75 mM-Tris–HCl buffer it was more than five-times that in 25 mM Tris–HCl buffer (Priyadarshini & Kansal, 2002b).

Effects of salts

NaCl concentration up to 50 mM increased the activity of lysozyme with 12 and 18% activation observed at 25 and 50 mM, respectively (Fig. 1). KCl at 25 mM showed no

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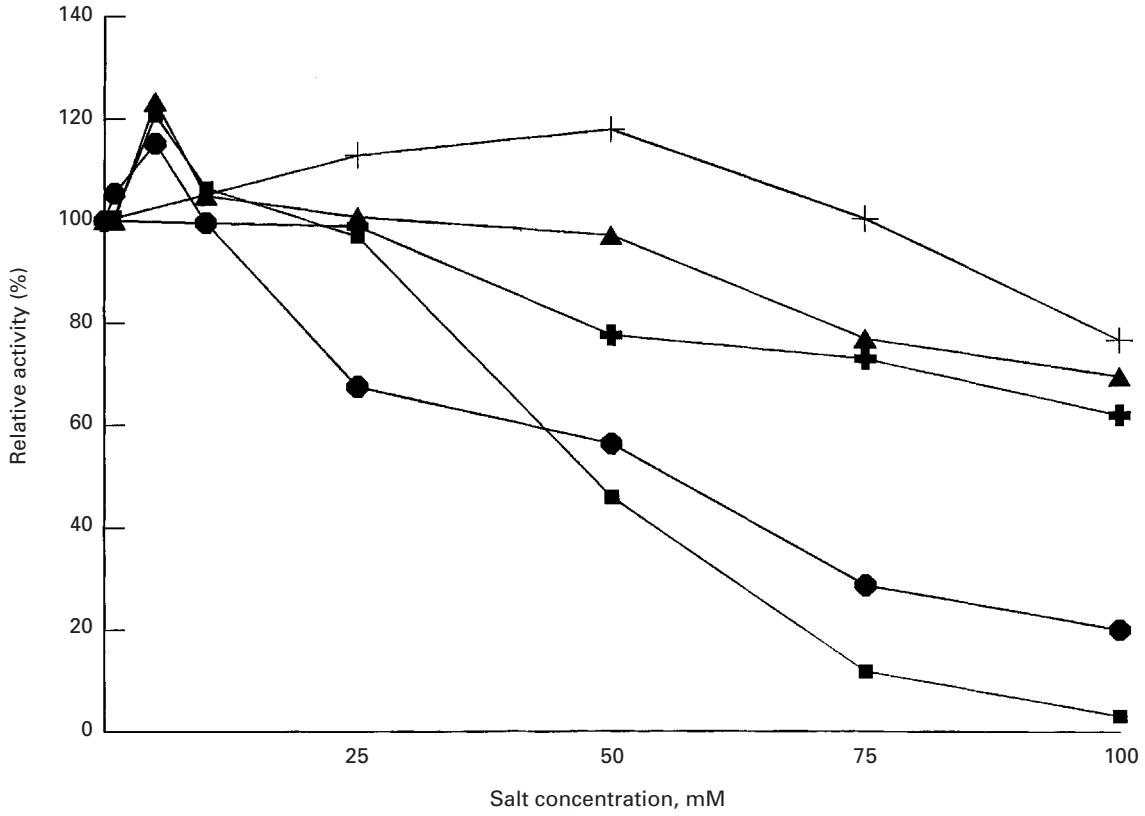

Fig. 1. Effects of NaCl (+), KCl (✚), sodium citrate (■), NH_4Cl (▲) and K_2SO_4 (●) on the activity of purified buffalo-milk lysozyme (3 milliunits in 3 ml reaction mixture). Values are means of 3 determinations expressed as percent activity.

effect on lysozyme activity, but 20% inhibition was observed at 50 mM and the inhibition increased further at higher KCl concentration. Sodium citrate, NH_4Cl and K_2SO_4 at low concentrations increased the activity of buffalo milk lysozyme. At 5 mM, the activation of lysozyme by citrate, NH_4Cl and K_2SO_4 was 20, 22·5 and 17%, respectively. At 25 mM, NH_4Cl and sodium citrate showed no effect on lysozyme activity, while K_2SO_4 caused 25% inhibition. Lysozyme activity declined by 31, 80 and 97% at 100 mM concentrations of NH_4Cl, K_2SO_4 and sodium citrate, respectively, which shows that high ionic strength is inhibitory to buffalo milk lysozyme.

When the inhibitory effect of high concentrations of chloride salts of three monovalent cations (Na^+, K^+ and NH_4^+) were compared, inhibition was in the order $KCl < NH_4Cl < NaCl$. Thus, the inhibition of buffalo milk lysozyme by cations of equal valency at particular ionic strengths is not similar, suggesting the involvement of factors such as size and mobility of the cations. Although the greater inhibition by sodium citrate than by sodium chloride, and by K_2SO_4 than by KCl appears to be due to the higher ionic strength of sodium citrate and K_2SO_4 than NaCl and KCl, respectively, the possibility of the nature of anions influencing the activity of buffalo milk lysozyme is not ruled out.

The effect of $MgCl_2$ and $CaCl_2$ at concentrations up to 25 mM was studied and the results are shown in Fig. 2. $MgCl_2$ at 5 mM induced 20% activation of lysozyme activity, while at higher concentration it turned inhibitory with 38% inhibition occurring at 25 mM. Concentrations of $CaCl_2$ up to 5 mM did not significantly influence lysozyme activity, but at 25 mM $CaCl_2$ about half of the enzyme activity was lost. The ionic strength of $MgCl_2$ and $CaCl_2$ are similar, therefore the differential effects of two salts is due to the nature of the cations.

The exact mechanism by which ions influence lysozyme activity is not clear. The activation observed at low salt concentration may be due to interaction of salts with polar groups on the cell surface causing altered permeability. Electrostatic forces play an important role in binding a positively charged lysozyme onto the negatively charged cell surface. Favourable electrostatic forces are decreased at higher ionic concentration, making the substrate less susceptible to enzyme action.

The concentration at which Na^+ is present in buffalo milk is stimulatory to lysozyme activity (Table 3). K^+ and Ca^{2+} do not stimulate lysozyme, and their concentrations in buffalo milk are less than their inhibitory levels. Concentrations of Mg^{2+} and citrate in buffalo milk are within the ranges that are stimulatory to lysozyme activity. Concentrations of Cl^- and phosphate in buffalo milk are 21 mM and 11·6 mM, respectively, which are also not inhibitory to lysozyme. Buffalo milk lysozyme is active over a wide range of pH with maximum activity at pH 7·4

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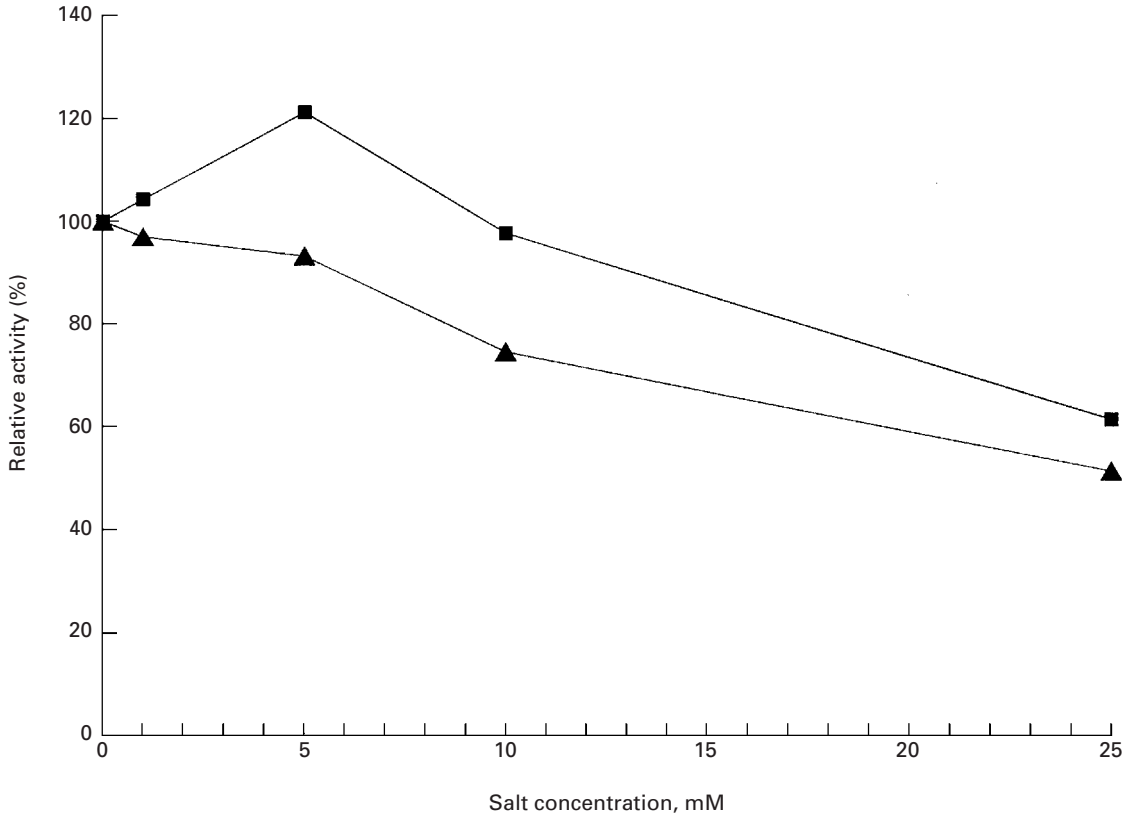

Fig. 2. Effects of $MgCl_2$ (■) and $CaCl_2$ (▲) on the activity of purified buffalo milk lysozyme (3 milliunits in 3 ml reaction mixture). Values are means of 3 determinations expressed as percent activity.

Table 1. Effect of heavy metal ions on the activity of buffalo milk lysozyme. Values are % original activity; mean ± SEM for $n=3$

Metal ions (0·1 mM)†	Percent activity
Ni^{2+}	85·4 ± 0·4
Co^{2+}	83·2 ± 1·2
Mn^{2+}	73·5 ± 1·0
Zn^{2+}	73·0 ± 0·6
Fe^{2+}	68·7 ± 0·8
Cu^{2+}	65·3 ± 1·2
Hg^{2+}	54·5 ± 0·8
Fe^{3+}	25·2 ± 1·1

Lysozome activity was determined in a reaction mixture (3 ml) containing 30 milliunits of enzyme in 0·075 M-Tris–HCl buffer (pH 7·4)
† Metal chlorides were used

Table 2. Thermal stability of buffalo milk lysozyme†. Values are % original activity; mean ± SEM for $n=3$

	Temperature (°C)		
Time (min)	63	74	100
1	98·4 ± 0·2	98·8 ± 0·9	70·7 ± 1·3
15	98·0 ± 0·8	91·0 ± 1·1	24·5 ± 0·5
30	99·4 ± 1·1	66·0 ± 0·7	0·4 ± 0·4

† Enzyme (126 µg/ml) in 0·2 M-NaCl–0·05 M-sodium phosphate buffer (pH 7·4) was subjected to temperatures as shown in table and then its activity determined

(Priyadarshini and Kansal, 2002b). Even at the pH of buffalo milk (6·9), the enzyme retains 95% of its maximum activity.

Effects of heavy metal ions

Effects of heavy metal ions on buffalo milk lysozyme are shown in Table 1. All heavy metal ions tested at 0·1 mM were inhibitory; the inhibition ranged from 15% for Ni^{2+} to 75% for Fe^{3+}.

Thermal stability of buffalo milk lysozyme

Buffalo milk lysozyme, at 126 µg/ml in 0·2 M-NaCl–0·05 M-sodium phosphate buffer (pH 7·4), was subjected to temperatures of 63, 74 and 100 °C for 1, 15 and 30 min. Lysozyme retained full activity after 1 min at 74 °C or 30 min at 63 °C (Table 2). The enzyme lost only 10% of its activity after 15 min at 74 °C and 30% of its activity after 1 min at 100 °C. After 30 min at 74 °C about 34% of

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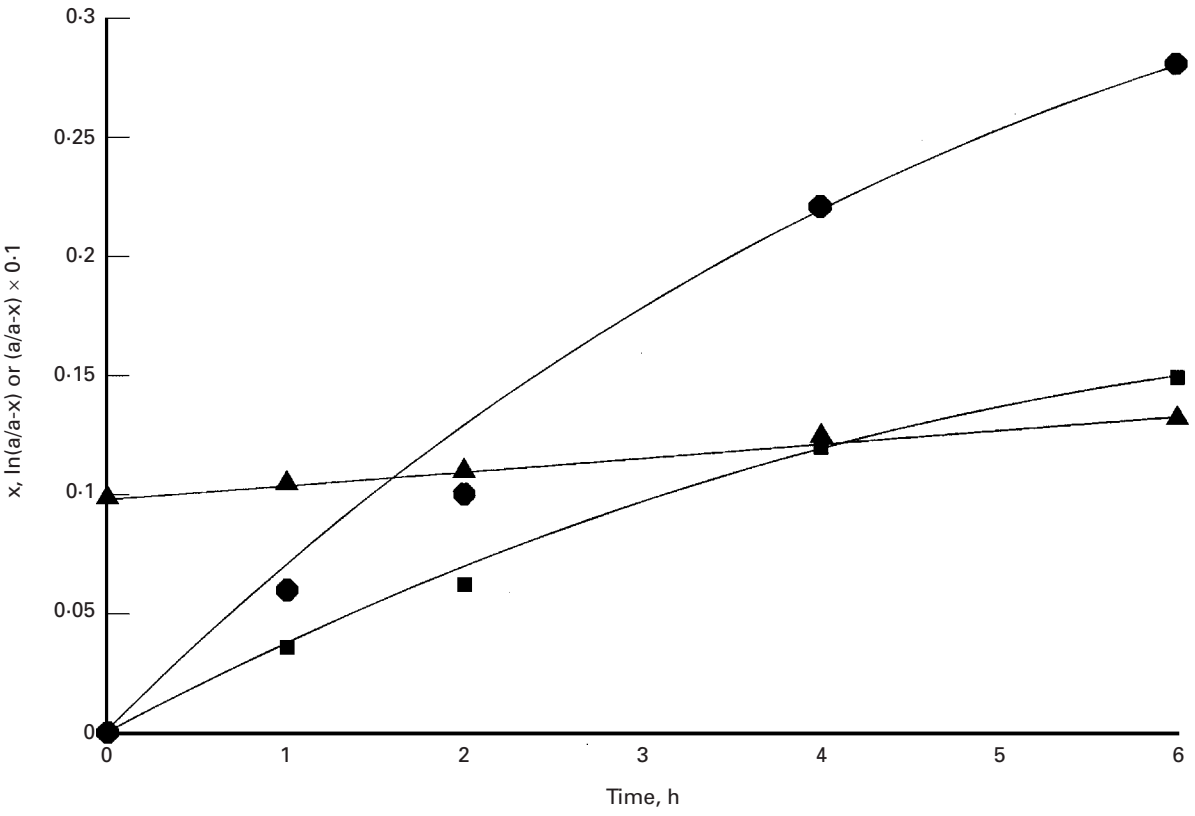

Fig. 3. Kinetics of buffalo milk lysozyme (3 milliunits in 3 ml reaction mixture). The change in absorbance was recorded at different time intervals. ■, x; ●, $\ln(a/a-x)$; ▲, $(a/a-x)$. These terms are equivalent to ΔA, $\ln(A^0/A^t)$ and (A^0/A^t), respectively.

Table 3. Ions in buffalo milk and their effective concentration for stimulation/inhibition of lysozyme

Ions	Concentration in milk (mM)	Effective concentration (mM) for	
		Activation	Inhibition
Na^+	18·6†	25–50	⩾100
K^+	26·8†	—	⩾50
Ca^{2+}	4·1‡	—	⩾5
Mg^{2+}	2·2‡	⩽5	⩾10
Citrate	6·4¶	⩽10	>25
Cl^-	21·0¶	—	>25
SO_4^{2+}	2·0¶	⩽5	>10
PO_4^{3+}	11·6¶	⩽50‖	>50‖

† Sindhu & Roy (1973)
‡ Sharma & Sindhu (1999)
¶ Sindhu & Roy (1976)
‖ Priyadarshini & Kansal (2002b)

activity was lost, and inactivation was complete after 30 min at 100 °C.

Kinetics of lysozyme reaction

Absorbance of the lysozyme reaction mixture at 450 nm was linearly related to substrate concentration. If a is the concentration of cells in the reaction mixture at zero time and A^0 the corresponding absorbance at 450 nm and x is the concentration of cells lysed at time t then the concentration of cells remaining is $(a-x)$ with absorbance A^t. If the kinetics of reactions are zero, first or second order, the plots of

x against t, or $\ln a/(a-x)$ against t, or $a/(a-x)$ against t

will be linear with intercepts of zero, zero or unity, respectively (McKenzie & White, 1986), and the variables x, $\ln(a/a-x)$ or $a/(a-x)$ are proportional to ΔA, $\ln(A^0/A^t)$ or A^0/A^t, respectively. The plot of reaction kinetics of buffalo milk lysozyme (Fig. 3) shows that the enzyme followed a second order reaction over a period of 6 h. McKenzie & White (1986) also observed second order kinetics for cell lysis by bovine milk and egg-white lysozyme.

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DR Frank Dodd – Obituary.

Dr Frank Dodd, formerly of the National Institute for Research in Dairying (NIRD) at Shinfield, Reading, has died aged 78. He had suffered ill health for the last few years but insisted on continuing his enthusiastic gardening and his voluntary work of chauffeuring the ''old folk'' to hospital and elsewhere.

Dr Dodd was internationally recognised as a leading research worker on mastitis. He joined the Dairy Husbandry Department of the NIRD in 1945 after graduating BSc at Reading University and remained there until his retirement in 1985, at which time he was Head of Department and Deputy Director. In his first studies, for which he was awarded his PhD, he investigated the properties of the milking machine in relation to the speed of milking.

The foundation of Frank Dodd's outstanding reputation was the series of Mastitis Field Experiments in the 1950s and 1960s. His team included Frank Neave and Roger Kingwill at the NIRD and, later, Douglas Wilson of the Ministry of Agriculture's Central Veterinary Laboratory at Weybridge. Dr Dodd's diplomacy and his clarity of vision enabled the large team to complete three field experiments which are still beacons in the world of mastitis research. The outcome was what became known as the ''Five point plan'' which was a simple but highly effective programme applied by farmers to reduce both clinical and, just as importantly, subclinical mastitis in their dairy cows. This mastitis control programme has been widely adopted in most dairying regions of the world. It was effective in reducing mastitis in the UK by two thirds over the 15 years from the end of the 1960s, improving the welfare of the dairy cow and saving the dairy industry many millions of pounds.

Dr Dodd was in great demand as a speaker in many countries. His relaxed but clear expression allowed him to project his ideas in a persuasive way to a wide range of people including farmers, veterinary surgeons and research scientists. His friendly reception of other scientists resulted in a host of international visitors to his department at the NIRD by whom he will be remembered with affection. He led the team at Reading which organised the highly successful first International Seminar on Mastitis Control in 1975. Subsequently he was chairman of the International Dairy Federation's Group of Experts on Mastitis for nine years. He was one of the earliest members of the British Cattle Veterinary Association, at a time when it had fewer than 100 members.

Frank was fortunate to have a close and loving family. In 1950 he married Mary Phillips who also worked at the NIRD and they had four daughters and now five grandchildren, to all of whom he was devoted. At home he was a keen gardener and, with Frank Neave, shared a common interest in bee-keeping.

A few of the tributes paid to Frank since his death bear testimony to the esteem in which he was held.

''I was saddened to hear about Frank Dodd. I believe that he and the Shinfield team made the most significant advance in the control of mastitis this century. A man who has left his mark.'' M Pott

''Frank was the most influential figure in our field and his work lives on. I am proud to say he was my friend.'' N Philpot

''I would like to extend my condolences to Frank's family and acknowledge a great mastitis research worker who became the cornerstone of academic thinking on mastitis. He pioneered concepts for managing the disease and was a great leader in the global directions undertaken to better understand the disease. From New Zealand we say farewell to Frank and remember his greatness.'' G Duirs

''I met Frank Dodd on several occasions in particular at the time of IDF mastitis group meetings. I had great esteem

for the scientist and for the person. I consider he was one of the most important contributors to the knowledge of mastitis and discussions with him were stimulating.'' B Poutrel

''Frank was certainly an icon in the mastitis world.'' R Harmon

James Booth, July 2003

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