A Novel Electrophoretic Variant of Human Tear Lysozyme with Altered Kinetic and Thermodynamic Properties

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ABSTRACT

We report for the first time on an electrophoretic human tear lysozyme variant. This lysozyme displayed a shift in optimum pH towards the basic region. It was more susceptible to inhibition by the substrate analogues (NAG and (NAG)$_4$ and had a lower activity on the Micrococcus luteus natural substrate indicating altered kinetic and thermodynamic properties. The eyes of the individual from whose tears the variant lysozyme came looked apparently normal.

Keywords: Lysozymes, Lacrimal gland, Tissue specificity, Gene duplication, Gene expression, Digestive enzymes, Innate immunity, Antibacterial activity.

1. INTRODUCTION

Lysozyme has been one of the most widely used model systems for biochemical studies (Jollès and Jollès, 1984; Prager and Jollès, 1996; Sekiyama et al., 2008; Kou et al., 2005). These include molecular evolution (Jollès et al., 1989), regulation of protein synthesis and secretion (Saleh and Ibrahimi, 1995), clinical and pathological studies (Pepys et al., 1993), protein and enzyme function (Dobson et al., 1984) and immunological studies (Sen and Sarin, 1982).

This extensive use of lysozyme is mainly due to its ubiquitous distribution in various fluids and tissues of different organisms, its stability and small size (Jollès and Jollès, 1984). Up to now, the exact physiological role of lysozyme is not clear. Nonetheless, many activities have been attributed to lysozyme including, antibacterial and anti-HIV activity as well as a nutritional digestive role (Sen and Sarin, 1982; Lee-Huang et al., 1999; Dobson et al., 1989; Irwin et al., 1992).

Lysozyme is secreted from the lacrimal glands into the tear fluid (Caffery et al., 2008; Murphy et al., 2007). Its protective role in the eye as an antibacterial agent is not well-understood since some mammalian species have no detectable levels of lysozyme activity in their tears under normal conditions (Saleh et al., 1995). It has been shown that lysozyme production is induced by infection (Sen and Sarin, 1982), and variation of lysozyme activity has been observed under stimulation of the eye by different environmental stresses (Saleh et al., 1995; Ibrahimi et al., 2000).

We have been looking at the distribution and polymorphism of mammalian tear lysozyme (Jollès et al., 1990; Saleh and Ibrahimi, 1995; Saleh et al., 1995; Ibrahimi et al., 2000). In this investigation, we report on the discovery of an electrophoretic variant of human tear lysozyme. The eyes of the individual from whom the tear sample was obtained looked apparently normal. This is the first report of a human tear lysozyme electrophoretic variant that we know of.

2. MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma Chemical Company, Aldrich Chemical Company or BDH Chemicals Ltd, unless otherwise indicated. Chitotetraose (NAG)$_4$ and its monomer N-acetyl-glucose amine (NAG) were a gift from Dr. E. Prager (University of California, Berkeley, CAL).

Tear Samples

Tears were collected from 53 individuals residing in Al-Ain city. Tears were collected as drops on a piece of a parafilm from which they were recovered with the aid of an automatic micropipette and delivered into the bottom
of a small eppendorf tube and stored in the freezer (-20°C). one sample was collected from each individual.

Lysozyme Assay

Lysozyme activity in tears was measured at the optimum pH 6.2 and ionic strength of 0.125 using freeze-dried Micrococcus luteus powder at a concentration of 0.25 mg/ml and recording the decrease in turbidity at 540 nm every 0.5 minutes for a total of 4 minutes in a 1 ml assay at room temperature as previously described (Dobson et al., 1984; Hammer et al., 1987; Ito et al., 1990; Saleh et al., 1995). A unit of activity was defined as a 0.1 decrease in absorbance per minute as estimated from the initial slope of the line which relates turbidity to time. Chicken lysozyme was used as a standard to correct for assay variations. The cell suspension was always prepared fresh.

Effect of pH on Lysozyme Activity

This was done according to the previously described procedure (Miller and Golder, 1950; Dobson et al., 1984; Saleh et al., 1995). The lysis of M. luteus was measured turbidimetrically at 540 nm at the different pH values (from 3 to 12) at a constant ionic strength of 0.125.

Lysozyme Thermostability

The procedure described before was used (Hammer et al., 1987; Saleh et al., 1995). The equivalent of one unit of lysozyme activity in 20 ml of 0.05 M sodium phosphate buffer, pH 7.5 and 1 mg/ml bovine serum albumin was placed at 64°C in a water bath. The tubes were removed at time intervals over a period of one hour. This was followed by centrifugation at 10,000 rpm for 20 minutes and the enzyme activity was measured using the turbidimetric assay described above.

Inhibition of Lysozyme Activity by (NAG) and (NAG)₄

This was done by modifying the procedure previously described (Jollès et al., 1968; Saleh et al., 1995). The inhibitor (NAG) at a concentration of (5-40)x10⁻³ M or (NAG)₄ at concentration of (5-60)x10⁻⁴ M in 0.20 ml water was added to 0.75 ml of a 0.25 mg/ml suspension of M. luteus in 0.055 M phosphate buffer (pH 6.2) containing 0.04 M NaCl. Fifty microliters of the proper dilution of lysozyme solution was added (Hammer et al., 1987; Jollès et al., 1995). Sample buffer was added to give 0.03% (w/v) methyl green and 15% (v/v) glycerol in the final sample. To minimize smearing, poly-l-lysine (5 mg/ml) was also added (Hammer et al., 1987; Jollès et al., 1990).

Electrophoresis

Polyacrylamide gel electrophoresis under denaturing conditions using (SDS) was done according to the method of Laemmli (Laemmli, 1970). It was generally through 12% gels at pH 8.3 as described before (Heussen and Dowdle, 1980). The samples were prepared for electrophoresis with or without precipitation with trichloroacetic acid followed by solubilization and reduction with DL-dithiothreitol (DTT) at 75°C for 5 minutes and alkylation with iodoacetamide at 37°C for 1 hr. Electrophoresis was carried out at a constant current of 20 mA for 5-7 hours. The gels were then stained with Coomassie Brilliant Blue G-250 and destained with a mixture of 35% methanol, 20% acetic acid in distilled water.

Non-denaturing gel electrophoresis was done as previously described (Reisfield et al., 1962; Saleh et al., 1995). It was performed at 4°C in vertical polyacrylamide gel slabs, 0.1 cm thick, 15 cm long and 13 cm wide, using the pH 4.3, β-alanine-acetate buffer system without a staking gel as described previously (Saleh and Ibrahim, 1995). Sample buffer was added to give 0.03% (w/v) methyl green and 15% (v/v) glycerol in the final sample. To minimize smearing, poly-l-lysine (5 mg/ml) was also added (Hammer et al., 1987; Jollès et al., 1990).

3. RESULTS

Screening for Lysozyme in Tears and Classification of Screened Individuals

Tears were collected from 53 individuals and screened for lysozyme by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The distribution of the individuals according to age, sex and nationality is shown in table 1. The banding pattern was identical in all cases.

Polyacrylamide Gel Electrophoresis

The collected tear samples were subjected to native gel electrophoresis. The banding patterns were identical, except for one male case, an Indian who was 37 years old. In the tears of this individual, the expected band for lysozyme disappeared and a new band with slower Electrophoretic mobility appeared. Lysozyme from this sample and a normal tear sample were subjected to partial purification by binding to carboxymethyl cellulose on a small scale as previously described (Saleh and Ibrahim, 1995) and the resulting lysozyme samples were subjected to non-denaturing gel electrophoresis as described in
materials and methods. Five tear samples and purified chicken lysozyme were included for comparison. The results are shown in figure (1). Indeed, the concerned tear sample showed an altered Electrophoretic mobility of lysozyme relative to other tear samples and standard chicken lysozyme. The mutant tear sample together with other tear samples were subjected to sodium dodecyl sulfate denaturing gel electrophoresis to check for any alteration in molecular mass of the lysozyme. Figure (2) shows a representative sample electrophoretic run including the mutant case (Lane 4). In addition figure (3) shows the results of denaturing gel electrophoresis of tears from nine unrelated Jordanians together with standard chicken egg white lysozyme. Besides the variability in the intensity of the bands, all samples showed identical banding patterns which include lysozyme and other tear proteins. This reflects the fact that the level of lysozyme and possibly other tear proteins is very sensitive to the condition prevailing during tear collection and the activity of lysozyme in consecutive tear samples (Lal et al., 1991; Temel et al., 1991; Brightman et al., 1991; Ibrahim et al., 2000; Saleh et al., 1995).

The Effect of pH on the Activity of Human Mutant Tear Lysozyme

The activity of the human mutant lysozyme as measured by the rate of lysis of M. luteus cells, was compared to that of standard human and chicken lysozymes at pH 6.2 and an ionic strength of 0.125. The rate of change in percent transmittance during the linear phase was 1.03, 1.027 and 0.76 /min for chicken, human standard and mutant lysozymes, respectively.

The activity of these lysozymes was measured as a function of pH at constant ionic strength of 0.125. The results are shown in figure (4). The optimum activity ranges for chicken and human standard lysozymes are very similar, while that of the mutant lysozyme was different with a shift towards the basic region.

Lysozyme Thermostability

The effect of temperature on the activity of the mutant lysozyme was compared with those of standard human and chicken lysozymes. Figure (5) shows that the half-life of standard human and chicken lysozymes at 60°C are very similar, being 18-20 minutes respectively; while the mutant lysozyme was more thermostable with a half-life of 9.7 minutes as shown in figure (5). All three lysozymes were very stable when stored at -20°C with no significant loss of activity after one month. However, more than 50% of the activity was lost after 10 hours at room temperature.

Lysozyme Susceptibility to Inhibition

The neutral oligosaccharide Chitotetraose (NAG)₄ and its monomer N-acetylglucosamine (NAG) were used as inhibitors to compare the inhibition effect on the mutant lysozyme and the standard human and chicken lysozymes. The degree of inhibition was expressed in terms of the ratio Kₒ/K. Figure (6) shows the inhibition by (NAG)₄. The inhibition effect was least on chicken lysozyme followed by human standard lysozyme and mutant lysozyme on which the inhibition effect was most prominent. Similar results were obtained with NAG but at much higher concentrations. The concentration of (NAG)₄ necessary for 50% inhibition of chicken lysozyme was 3.7x10⁻⁴ M compared to 3.9x10⁻² for (NAG).

4. DISCUSSION

It has been almost 80 years since the discovery of human lysozyme in nasal secretions by Fleming (Fleming, 1927). Since then, lysozyme was found to be one of the most adaptive and ubiquitous enzymes (Dobson et al., 1984; Hammer et al., 1987; Stewart et al., 1987; Godovac-Zimmermann et al., 1988; Jollès et al., 1989; Irwin et al., 1992; Van-Seuningen et al., 1992; Ito et al., 1993; Irwin, 1995). We have been lately engaged in looking for lysozyme polymorphs and variants in tears and indeed we found that tear lysozyme is highly polymorphic (Saleh and Ibrahim, 1995; Saleh et al., 1995; Ibrahim et al., 2000). Further more, there is differential regulation of the synthesis of the various polymorphs among the different mammalian species and within the same species (Padgett and Hirsch, 1967; Hindenburg et al., 1974; Janssen and Van Bijsterveld, 1983a; Lie and Solbu, 1983; Prieur 1986; Hammer and Wilson, 1987; Camara et al., 1990; Brightman et al., 1991; Bron et al., 1992; Irwin, 1995).

The exact physiological role which is played by lysozyme in tears is not very obvious (Schaumberg et al., 2003; Dogru et al., 2005). Various conditions have been linked to variations in the level of lysozyme in tears (Padgett and Hirsch, 1967; Atkins et al., 1971; Sen and Sarin, 1982; Janssen and Van Bijsterveld, 1983a; Janssen and Van Bijsterveld, 1983b; Jollès et al., 1990; Brightman, et al., 1991; Bron et al., 1992).

Up until 1993, only one type of human lysozyme has
been known in the various fluids, secretions, tissues and cells that have been looked at (Pepys et al., 1993). The first two variants of human lysozyme were reported in amyloid deposits around the viscera (Pepys et al., 1993). These were point mutations resulting in one amino acid substitution in each variant. This is the first case where lysozyme has been clearly associated with a disease condition. We have recently started looking for lysozyme variants in human tears were no such variants, to our knowledge, have been reported.

Extensive screening of individuals from the same ethnic background revealed no variants. In this report we show that an Indian male who was 37 years old showed a lysozyme polymorph. Native gels revealed that the lysozyme in the tears of this individual is indeed a lysozyme variant with reduced electrophoretic mobility at pH 4.3. This results in making the lysozyme less thermostable, with a shift in the optimum pH range to the basic region and increased sensitivity to inhibition. The reduced electrophoretic mobility suggests that the variant lysozyme has a reduced positive charge at pH 4.3. The change could probably involve substituting a neutral or basic amino acid by an acidic one, or a basic amino acid by a neutral one assuming that the variant results from a single amino acid substitution.

The eyes of the concerned individual was apparently normal. These results should open the way for identifying naturally occurring human lysozyme variants and associating them with precise physiological conditions, an approach which should yield better results than studying artificially induced mutants (Imoto et al., 1987; Kuroki et al., 1992a; Kuroki et al., 1992b; Kuroki et al., 1992c; Muraki et al., 1992; Omura et al., 1992; Taniyama et al., 1992; Wilson et al., 1992; Harata et al., 1993; Yamada et al., 1993; Shoichet et al., 1995).

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**Distribution of individuals screened for tear lysozyme.**

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Figure 1. Non-denaturing gel electrophoresis at pH 4.3 of normal, variant and chicken lysozyme. Electrophoresis, staining and destaining were done as described in the methods. Lane (1) contained partially purified variant human tear lysozyme, lane (2) contained partially purified normal human tear lysozyme, lanes (3-7) contained total normal human tears and lane (8) contained pure chicken egg white lysozyme. Arrows refer to the lysozyme bands.

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of tears from six individuals. Electrophoresis was done as described in the methods. The gel was stained with Coomassie brilliant blue and then destained. Lane (4) contained tears from the Indian individual with variant lysozyme. Lysozyme is pointed to with an arrow and it comigrated with chicken lysozyme.
Figure 3. SDS-PAGE of different individual tear samples. Lanes 1-9 contain tear samples from different individuals, lane 10 contains pure standard chicken egg white lysozyme. Arrow refers to the lysozyme band.

Effect of pH on lysozyme activity

Figure 4. The effect of pH on lysozyme activity. All measurements were made at an ionic strength 0.125 and with initial substrate concentration of 0.5 mg/ml. The activities were expressed relative to the value at the pH optimum. (■) chicken lysozyme, (●) and (▲) are normal and mutant human tear lysozymes, respectively.
Figure 5. The effect of temperature on lysozyme activity. Thermostability assays were done as described in methods. The relative activity of lysozyme at pH 6.2 and ionic strength of 0.125 was measured after incubation at 65°C for the indicated periods. Chicken lysozyme (▲), normal human tear lysozyme (■) and variant human tear lysozyme (●), are shown.

Figure 6. Inhibition of lysozyme activity by (NAG)₄. This was performed as indicated in the methods. Chicken lysozyme (●), normal human tear lysozyme (■) and variant human tear lysozyme (▲), are shown.
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