

PREPARATION AND PROPERTIES OF LYSOZYME-CONTAINING EYE DROPS FOR TEAR-SUBSTITUTIVE THERAPY

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Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 53, No. 8, pp. 41 – 44, August, 2019.

Original article submitted December 15, 2016.

Lysozyme incorporated in polymer solutions is shown to be stabilized during prolonged storage at low temperatures (0 – 4°C) for 12 months with 96% preservation of the hydrolytic activity. Variations of the pH and temperature activity profiles of the enzyme incorporated in polymer solutions promotes its activation under physiological conditions during acidosis associated with inflammations of various etiologies and upon development of dry eye syndrome. Investigation of the biochemical and physicochemical properties of a lysozyme-containing solution showed that the developed eye drops met requirements applicable to them (density, viscosity, osmolality, refractive index, sterility). Ophthalmic safety studies showed that the solution was non-allergenic.

Keywords: eye drops, lysozyme, ophthalmic safety, dry eye syndrome.

Dry eye syndrome (DES) is a complicated disease with 9 – 18% incidence [1]. The causes of the disease are uncontrolled use of antidepressants and antihistamines, contact lenses, low-quality cosmetics, prolonged habitation of air-conditioned rooms, work with computers, etc. [2 – 4]. Tear-substitutive therapy using a broad spectrum of medicines such as eye drops and gels [2, 5] with various viscosities and chemical compositions are some of the modalities for treating DES. It is noteworthy that these artificial tears do not contain the “natural antibiotic” lysozyme (EC 3.2.1.17), a bacteriolytic enzyme that also possesses immunostimulating, anti-inflammatory, regenerative, and analgesic activity [6]. The lysozyme content in tear fluid is 20 – 30% total protein [7]. Its level drops with ophthalmological diseases, including DES [8]. Therefore, development of a tear-replacement preparation containing lysozyme is crucial.

The aim of the research was to develop and study the biochemical, physicochemical, medical, and biological properties of “artificial tear” lysozyme-containing eye drops.

EXPERIMENTAL PART

Drops were prepared using hydroxypropyl methylcellulose (HPMC Benecel™, mol. mass 75,000, Ashland Inc., USA), dextran 60 (Biotika Bohemia s.r.o., Czech Rep.), and lysozyme (40,000 U/mg, Sigma-Aldrich, Canada) [9]. The solvent was normal saline. Solutions of HPMC (0.39%), lysozyme (0.1%), and dextran (0.1%) were mixed in a 2:1:1 ratio; sterilized by filtration (Millipore filter), dispensed into sterile glass vials, and stored at 0 – 4°C. Lysozyme activity was determined using a bacteriolytic method [10]. The enzyme unit activity was taken as that amount that reduced in 1 min the optical density of a suspension of *Micrococcus lysodeikticus* ATCC No. 4698 cells (Sigma-Aldrich, USA) by 0.001. Protein content was monitored by the Lowry – Hartree method [11]. Viscosity was measured using an Ostwald viscometer with capillary diameter 0.73 mm. The density of polymer-stabilized lysozyme solution was measured using an aerometer; the refractive index, a refractometer. The optimal pH for lysozyme activity in the drops was determined by adding *M. lysodeikticus* cell suspensions in buffers (0.1 M; citrate-phosphate, pH 3.0 – 7.0; phosphate, pH 8.0 – 9.0) to samples of the same activity. The optimal temperature for lysozyme hydrolytic activity was determined in the temperature range 20 – 50°C. Polymer solutions with

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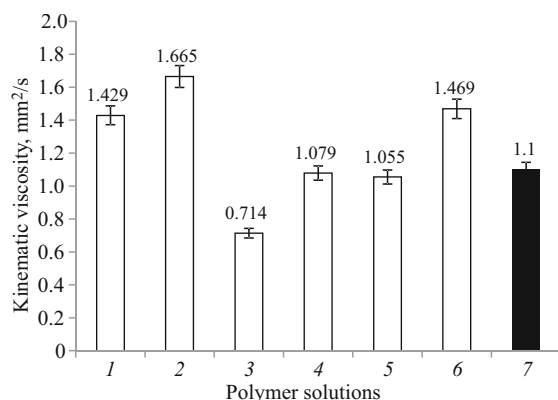


Fig. 1. Kinematic viscosity of various polymer solutions: HPMC (0.39%) (1); dextran (0.1%) (2); lysozyme (0.1%) (3); HPMC (0.39%) + lysozyme (0.1%) (4); dextran (0.1%) + lysozyme (0.1%) (5); HPMC (0.39%) + dextran (0.1%) (6); HPMC (0.39%) + dextran (0.1%) + lysozyme (0.1%) (7).

lysozyme were stored at 0 – 4°C. Osmolality was determined using a Tear Lab Osmolarity System. Results were processed statistically as before [12].

Ophthalmic safety of the eye drops was studied according to GLP requirements and methodical recommendations of the SPC, MH of Ukraine [13] using conjunctival samples from three guinea pigs (250 – 300 g) with clean non-hyperemic conjunctivae. The right eye of each guinea pig was treated with the tested eye drops; the left (control) eye, with normal saline. Possible reactions were monitored by visible observation 15 min and 1, 24, and 48 h after treatment.

RESULTS AND DISCUSSION

The amount of lysozyme included in the polymer solutions was the same as its normal content in human tears (0.81 ± 0.31)–(1.68 ± 0.66) mg/mL [7].

Polysaccharide polymers (hydroxypropyl methylcellulose, dextran) at concentrations 0.39 and 0.1% were used for the artificial-tear eye drops because they are nontoxic [14], are broadly used in pharmacy for ophthalmological formulations [14], and contain many hydroxyls capable of forming intermolecular H-bonds to the enzyme, thereby stabilizing it and mimicking the mucosal properties of the mucin layer of the corneal tear film [3]. The polymer concentrations were selected to correspond to those used in several artificial-tear preparations [15, 16].

The kinematic viscosity of the solution during the viscosity studies varied depending on the solution composition (Fig. 1). The viscosity observed after adding the enzyme to polymers dropped by 24.5% for HPMC (compare solutions 1 and 4), by 36.6%, for dextran (compare solutions 2 and 5), and by 25.1% for the mixed polymers (compare solutions 6 and 7), indicating that the polymers interacted with lysozyme and formed protein–polymer associates [17]. Furthermore, the kinematic viscosity of a complex preparation containing the final concentrations of lysozyme (0.1%), dextran (0.1%), and HPMC (0.39%) (solution 7) was 1.1 mm²/s, which agreed with normal human tears [16].

The quantitative protein content (0.1%) and complete retention of hydrolytic activity of lysozyme ($38,200 \pm 2,000$) U/mg after inclusion in the studied polymer solutions were also noteworthy.

The polymers stabilized the retention level over time of lysozyme activity. Storage for one month in an aqueous solution led to a 90% loss of enzyme activity; after 3 – 4 months, total loss. The lysozyme activity did not change over a year in the polymer solutions (Fig. 2).

A study of the hydrolytic activity of polymer-stabilized lysozyme as a function of incubation medium pH (Fig. 3) showed that the optimal pH shifted from 6.0 by 1 unit toward neutral (pH 7.0), which made the drops more efficacious because the pH of normal human tears lies in the range

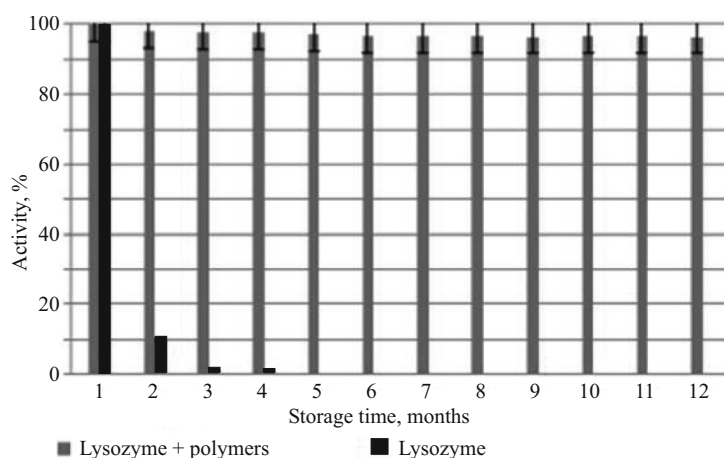


Fig. 2. Dependence of bacteriolytic activity of free and stabilized (by 0.1% dextran and 0.39% HPMC) lysozyme on storage time.

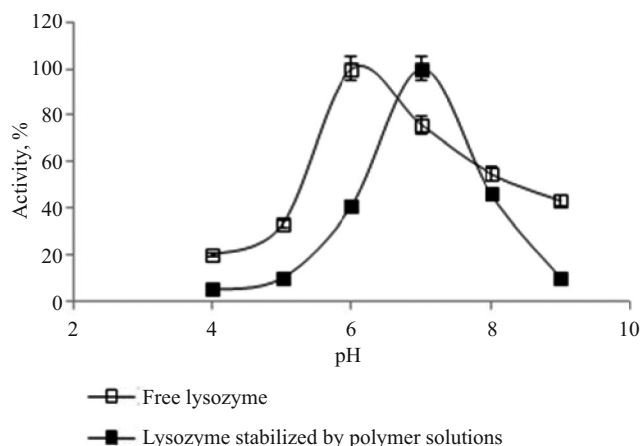


Fig. 3. Dependence of hydrolytic activity of free and stabilized (by dextran and HPMC) lysozyme on incubation medium pH.

7.11 ± 0.31 ; with DES, $6.75 - 7.75$. The pH of tears can change in different directions with inflammations [18].

Temperature effects on the activities of free and polymer-stabilized lysozyme increased significantly the activity of the latter in the range $30 - 40^\circ\text{C}$ (Fig. 4). Thus, the difference in activities at 37°C was 35%, which could be due to interaction of the enzyme and polymers and the appearance of more active lysozyme forms [10].

The density of the developed lysozyme-containing drops was 0.996 ± 0.003 g/mL and was close to that of human tears (1 g/mL). The osmolality of the preparation was 298 ± 2 mOsm/L, which was also normal for tears ($275 - 316$ mOsm/L). The refractive index was 1.3339.

Sterile filtering of the polymer solution had little effect on the preparation activity, i.e., 89.3% of the initial value. The solution was checked after filtration and found to be microbiologically pure [19].

An ophthalmic safety study of the lysozyme drops did not find reddening near the tear duct 15 min after treatment. Hyperemia of conjunctivae and reddening of the tear duct and sclera were not observed after 1 h in any of the guinea pigs. A conjunctival sample remained negative after 24 and 48 h, which indicated that the preparation was nonallergenic.

Thus, inclusion of lysozyme in polymer solutions stabilized the enzyme, enabling prolonged storage at $0 - 4^\circ\text{C}$ for 12 months in glass vials with a smooth neck, rubber stopper, and metal cap. The hydrolytic activity was 96% preserved. Variations of pH and temperature activity profiles of the enzyme incorporated in polymer solutions activated it under physiological conditions and with acidosis associated with inflammations of various etiologies due to development of DES. Studies of the biochemical and physicochemical characteristics of artificial tears showed that the developed drops met requirements applied to them (density, viscosity, osmolality, refractive index, and sterility). An ophthalmic safety study showed that the developed drops were nonallergenic.

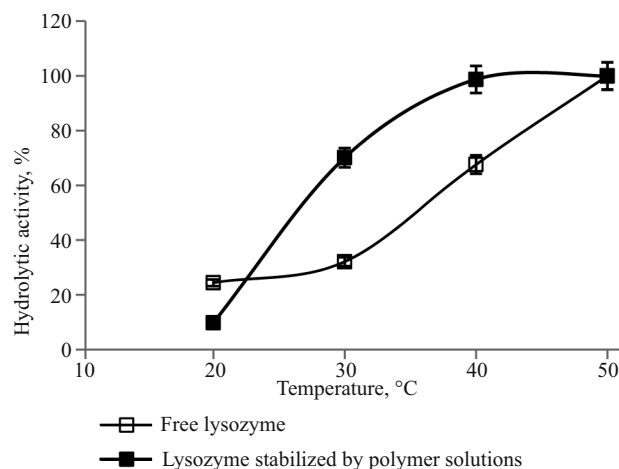


Fig. 4. Dependence of hydrolytic activity of free and stabilized (by dextran and HPMC) lysozyme on incubation medium temperature.

The developed lysozyme solution as drops had the following advantages over the lysozyme preparation manufactured by Bryntsalov-A PAO as a powder:

use of polymers adhering to mucous (HPMC, dextran) that prolonged the effect of artificial tears;

stabilization of lysozyme by polymer solutions enabled prolonged storage whereas the prepared aqueous solution of the enzyme lost most hydrolytic activity within one year;

lysozyme solution did not have to be prepared *ex tempore*, which made it convenient to use.

The developed eye drops with lysozyme stabilized by polymer solutions were promising for further studies in tear-substitutive therapy.

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