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Effect of antidenaturant drugs on lysozyme deposit formation on soft contact lenses by liquid chromatography–electrochemical detection

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The effect of some antidenaturant drugs on the formation of lysozyme deposits on soft contact lenses was investigated by *in vitro* experiments. Results were obtained which encourage the use of this strategy to reduce lens spoilage. At the same time emphasis is given to the possibility that some functionalities (i.e. lysine groups) present in drug molecules could promote protein adsorption. In order to evaluate at a quantitative level the effectiveness of the drugs used to minimize the formation of deposits on soft contact lenses, a selective method for the determination of adsorbed lysozyme was developed. It is based on the hydrolysis of the 'lens/adsorbed lysozyme' system, followed by high performance liquid chromatography coupled to electrochemical detection of the released tryptophan residues. The lysozyme calibration curve was linear up to 5 mg ml^{-1} and the detection limit was 5 ng injected.

Keywords: Contact lenses, protein adsorption, high performance liquid chromatography–electrochemical detection

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Among biomaterials, soft contact lenses (SCLs) have become increasingly popular as they are capable of correcting a wide range of refractive disorders with good biotolerability, mainly due to high oxygen permeability. On the other hand, it is well known that, with daily use, polymers constituting SCLs can deteriorate and, more importantly, deposits (usually proteins) can accumulate on the SCL surface[1–3] provoking changes in the optical quality, oxygen permeability and eye tolerability. All this can originate symptoms[4,5] varying from slight eye irritation to giant papillary conjunctivitis.

Despite the difficulties of studying the interaction of hydrogel materials with natural tears due to the complex composition of these fluids, which also show individual-related differences in the quantity and the quality of their components, a large body of knowledge on protein adsorption onto SCLs has been developed[6–13]. Sack *et al.*[11] found that the chemical composition of hydrogels strongly influences the nature of the lens-bound protein layer. While in the case of poly(hydroxyethyl methacrylate) (PHEMA) based lenses the deposit consists of a thin, highly denaturated layer of proteins, a thick, loosely bound layer forms on lenses containing methacrylic acids. Moreover, investigations performed on both *ex vivo* and *in vitro* SCLs indicated that lysozyme (LSZ) is the main component of the protein deposit[1–3,14–16].

Different strategies can be developed in order to eliminate/minimize the protein adsorption phenomenon. In our laboratory, lens surface modification by the physical adsorption of selected molecules was attempted. Substances were chosen (i.e. pirfenoxone and bendazac salts) which were known to adsorb on PHEMA and to possess antidenaturant properties[17,18] on the basis of the following considerations:

(i) a preferential competitive adsorption of drugs could minimize protein layer formation;
(ii) proteins eventually co-adsorbed in the native (non-denatured) state can be quite easily removed from the lens surfaces by usual daily washing, by commercial oxidation or enzyme solution (reversible adsorption);

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(iii) proteins eventually co-adsorbed in the non-denatured state do not induce a heavy immune response in the conjunctiva.

In order to check the effectiveness of the proposed surface treatment, analytical methods which allow the early detection of LSZ adsorbed on SCLs should be available. Different methods have recently been developed[19,20] in our laboratory. Good sensitivity is shown by methods employing basic hydrolysis of protein deposits and spectrophotometric determination of free amino acids after derivatization by ninhydrin reagent. This method, however, could not be applied in the present investigation due to the formation of interfering products during the hydrolysis of drugs used to modify SCL surfaces.

In this paper a more selective approach was developed based on the alkaline hydrolysis of the LSZ layer adsorbed *in vitro* on SCLs and subsequent separation of free amino acids by high performance liquid chromatography and electrochemical detection (HPLC–ECD) of tryptophan (Trp) residues (five to eight residues per LSZ molecule can be found, depending on the protein source). The method has been successfully applied to the investigation of the effect of the two mentioned antidenaturant drugs on LSZ adsorption onto SCLs.

MATERIALS AND METHODS

Materials

LSZ (from chicken egg-white) and L-Trp were obtained from Sigma (St. Louis, MO, USA) and used as-received. Other chemicals were of analytical reagent grade. All solvents were HPLC grade (J.T. Baker, Deventer, Holland). Stock LSZ and L-Trp solutions were prepared in tridistilled water or mobile phase and stored in the dark at 4 C. More dilute solutions were prepared just before their use. SCLs (spin cast type, 61.4% PHEMA, 38.6% water) immersed in a phosphate-buffered saline solution were obtained from Bausch & Lomb (Rochester, NY, USA) and used as-received. Two drugs, commercially available in the form of eye-drops, were used in this work. The first one (called drug A in this work) is based on the bendazac {[(1-benzyl-1H-indazol-3-yl)oxy]acetic acid} lysine salt (0.5% w/w); drug A also contains boric acid, L-lysine, hydroxypropyl methyl cellulose and potassium chloride. The other eye-drop (drug B) is based on pirfenoxone (1-hydroxy-5-oxo-5H-pyrido [3,2-a]phenoxazine-3-carboxylic acid) sodium salt (0.5% w/w); drug B also contains 2-aminoethanesulphonic acid. Both eye-drops were used as-received.

Methods

The chromatographic system consisted of a Perkin–Elmer (Norwalk, CT, USA) model 3B pump module equipped with a Rheodyne (Cotati, CA, USA) model 7125 injection valve and a Bischoff (Leonberg, Germany) 125 × 4.6 mm i.d. reversed-phase ODS2 column, 5 μm packing. A Bischoff reversed-phase ODS2 pre-column (5 μm packing, 26 × 4.6 mm i.d.) fitted into a Bischoff holder was used to protect the analytical column. An EG&G (Princeton, NJ, USA) model 400 electrochemical detector including a thin-layer electrochemical cell with a glassy carbon working electrode and an Ag/AgCl, NaCl (3 M) reference electrode was used. The glassy carbon working electrode was cleaned by an alumina (0.05 μm particles) polishing procedure, extensive washing and sonication in tridistilled water. Signals were recorded on a Linseis (Linseis GmbH, Germany) model LS4 analogue recorder.

A method already developed[21] for the analysis of some electroactive amino acids in biological samples has been adapted for the chromatographic separation and detection of Trp in LSZ hydrolysates. The mobile phase was a phosphate–citrate buffer, pH 4, 20% v/v methanol. The flow rate was 1 ml min^{-1}, the injection volume 20 μl and the temperature ambient. The detection potential was +1.1 V versus Ag/AgCl.

LSZ adsorption on lenses was performed *in vitro* by soaking lenses in a phosphate-buffered saline solution (110 mM sodium chloride, 19 mM disodium hydrogen phosphate, 4.6 mM sodium dihydrogen phosphate, pH 7.3) containing 0.05% w/w LSZ (series I) or 0.05% w/w LSZ and 0.5% w/w drug A (series II) or 0.05% w/w LSZ and 0.5% w/w drug B (series III). 'Blank' samples were obtained by soaking lenses in a buffered saline solution or in a buffered saline solution containing the desired eye-drop. LSZ adsorption was performed at 37 C for 4 wk.

After the desired period of adsorption time, lenses were thoroughly rinsed with a buffered saline solution to remove any weakly adsorbed compound, cut into small pieces and hydrolysed with 500 μl of 2.5 M NaOH at 100 C in an oven for 48 h. To avoid any loss of protein by adsorption to the walls of the containing vessel, LSZ hydrolysis was performed in Teflon FEP tubes (Nalge Company, Rochester, NY, USA). The hydrolysate (150 μl) was then neutralized with 50 μl of glacial acetic acid, diluted with 400 μl of mobile phase and 20 μl injected.

Since the method of internal standard cannot be used in the present case, LSZ adsorbed on SCLs was quantified by the external standard method. The relevant calibration curve was constructed just before SCL analysis by using at least five different LSZ concentrations.

RESULTS AND DISCUSSION

Figure 1 compares typical chromatograms recorded on an alkaline hydrolysate of LSZ (*Figure 1a*) and on a Trp standard solution (*Figure 1b*). As can be seen, besides other chromatographic peaks due to electroactive compounds released because of LSZ hydrolysis, a well-resolved peak can be observed at the retention time expected for Trp. The identity of this peak was confirmed by both its chemical and electrochemical behaviour. Trp is mostly destroyed during the usual HCl protein hydrolysis conditions[22]. As a matter of fact, chromatograms relevant to LSZ standard hydrolysed by HCl did not show any peak eluting at the Trp retention time. Electrochemical confirmation

Figure 1 Chromatograms relative to a lysozyme standard solution (1 mg ml^{-1}) after alkaline hydrolysis for 48 h (**a**) and a tryptophan standard solution (100 ng injected; **b**). Other conditions as in Materials and Methods section.

was based on comparison of hydrodynamic voltammograms obtained from LSZ alkaline hydrolysate and Trp standard solutions. To this purpose, known amounts of Trp or LSZ alkaline hydrolysate were injected in the chromatographic system and detection was performed at several electrode potentials. The relevant chromatographic peak heights, after normalization, are plotted in *Figure 2* as a function of the detection potential employed. As can be seen, the two curves are practically coincident, confirming the identity of the chromatographic peak.

A kinetic study of LSZ hydrolysis was necessary both to check the chromatographic resolution at the different hydrolysis time and to optimize full Trp release. In fact, although protein hydrolysis usually requires 18–24 h, in some cases a longer time (e.g. 120 h) may be necessary[22]. LSZ samples at known concentrations were hydrolysed as described in the Materials and Methods section and sampled at different hydrolysis times for HPLC–ECD analysis. The results are shown in *Figure 3*, where the chromatographic peak heights relevant to the released Trp, normalized with respect to the maximum value, are plotted as a function of the time of alkaline hydrolysis. As expected, Trp release increased on increasing the time of hydrolysis until reaching a maximum at time values of about 48 h. The signal decrease recorded at longer hydrolysis time could be ascribed to the alkaline attack of Trp itself. Furthermore, no significant changes in the chromatographic pattern (e.g. chromatographic interference) were observed whatever the hydrolysis time. Similar results were observed at several LSZ concentrations, suggesting that the kinetics of Trp release are practically independent of LSZ concentration. As a consequence, 48 h was chosen as the optimum hydrolysis time. Lower hydrolysis times can obviously be used when sensitivity is not important.

A comparison between the Trp found after 48 h and a Trp standard permitted an estimate of the number of Trp molecules released per molecule of LSZ after its alkaline hydrolysis. On considering[23] the molecular weight of egg-white LSZ (14 400 ± 100) and its Trp content (about six Trp residues per LSZ molecule), a mean recovery of about 47% was calculated, i.e. only one-half of the 'electrochemical labels' can be generated by alkaline hydrolysis per LSZ molecule.

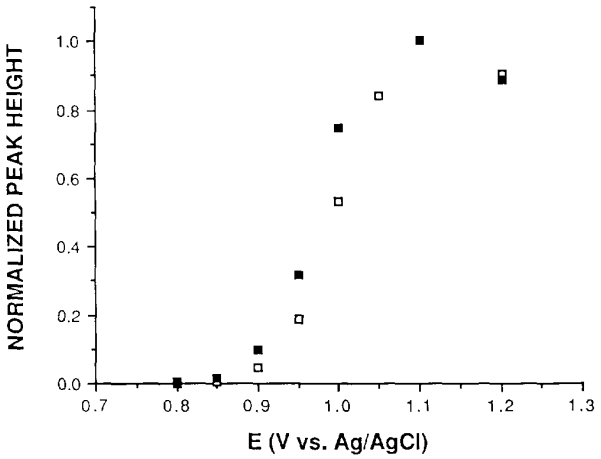

Figure 2 Hydrodynamic voltammograms deriving from: ■, a lysozyme alkaline hydrolysate (1 mg ml^{-1}, hydrolysis time 48 h); □, a tryptophan standard solution (100 ng injected). Other conditions as in Materials and Methods section.

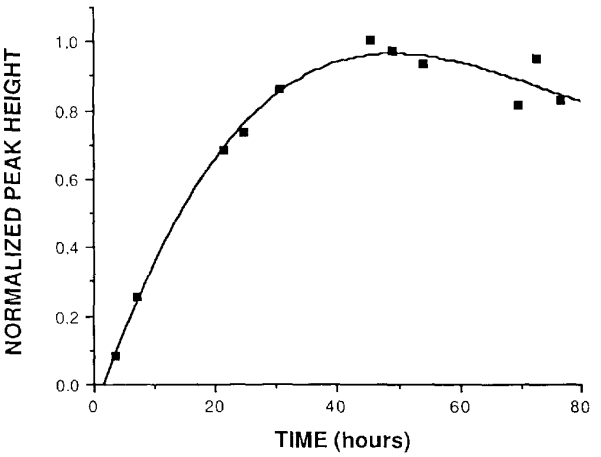

Figure 3 Normalized peak height as a function of time of alkaline hydrolysis of a lysozyme solution (1 mg ml^{-1}). Other conditions as in Materials and Methods section.

Methods recently developed[24] for Trp determination in LSZ and myoglobin showed recoveries ranging from 42 to 61%.

The LSZ calibration curve was linear up to 5 mg ml^{-1}, with a typical correlation coefficient of 0.9998 and intercept not significantly different from zero at the 95% confidence level. Repeatability evaluated at the 100 ng injected level was 1.2% ($n = 8$), very similar to that observed for Trp (0.92% at the 100 ng level; $n = 10$). This excluded any electrode fouling effect sometimes observed in the case of protein electrochemistry. *Figure 4* shows a chromatogram relevant to a hydrolysate of LSZ at a concentration near to its detection limit. This latter parameter, evaluated at a signal-to-noise ratio of 3 (noise calculated peak to peak in a blank chromatogram at the elution time of interest), was found to be 5 ng LSZ injected, corresponding to a relative amount of 0.2 ng of Trp. Such a detection limit compared well with that already reported[21].

In the analysis of LSZ adsorbed on SCLs, alkaline hydrolysis had the twofold aim of stripping away the protein from the lens and generating the electrochemical labels (i.e. Trp) for HPLC–ECD determination. In order to test the absence of interfering species eventually produced by the alkaline attack of lenses, 'blank' SCLs were hydrolysed for 48 h and the relevant hydrolysate analysed by HPLC–ECD. No chromatographic interference was observed. Electrode fouling effects were tested by repeatedly alternating injections of blank SCL hydrolysates with Trp standard injections. Differences in the chromatographic behaviour and in the electrode response were never observed.

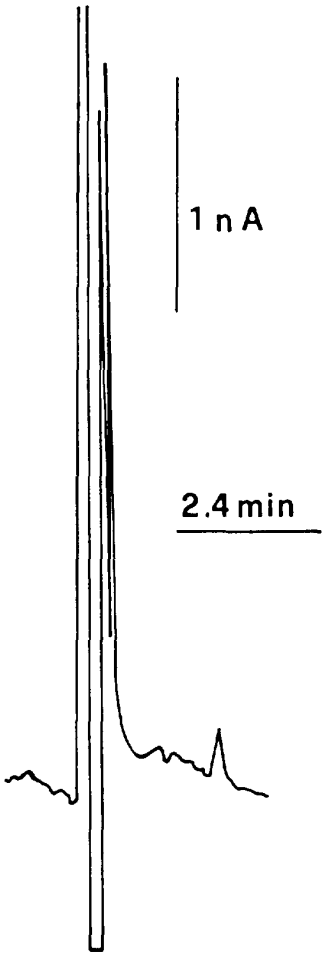

Figure 4 Chromatogram relative to a hydrolysate of lysozyme at a concentration (250 ng ml^{-1}) proximal to its detection limits. Other conditions as in Materials and Methods section.

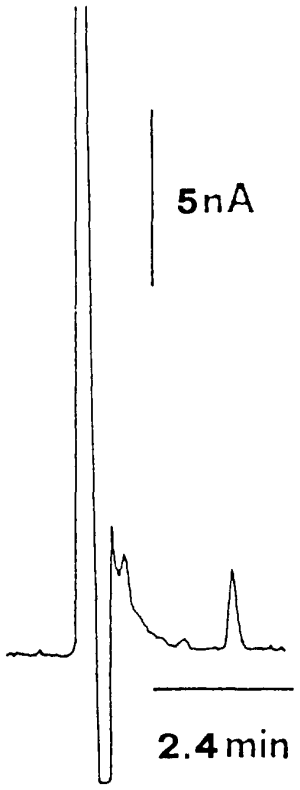

Figure 5 Typical chromatogram recorded on a 48 h alkaline hydrolysate of a soft contact lens of series I (see text).

Figure 5 shows the chromatogram relevant to a hydrolysate of a typical sample of series I. As can be seen, the presence of a peak eluting at Trp retention time suggested a significant LSZ adsorption on the SCL. The identity of this peak was confirmed as described previously.

Since it is well known that large molecules can diffuse deeply inside the polymers[7], protein desorption, even under very drastic conditions such as alkaline hydrolysis, could not have been complete. The efficiency and completeness of the hydrolysis step was evaluated in two ways: the same sample was submitted to the hydrolysis procedure on two occasions, and the amount of LSZ found in the hydrolysate was compared with the difference found in the LSZ content of the solution employed for protein adsorption before and after the adsorption stage. The former experiment showed no signal due to Trp after the second successive hydrolysis of the same sample;

the latter experiment indicated a good agreement between the two values.

The analytical procedure described here was successfully employed to study the effect of the mentioned commercial eye-drops on LSZ adsorption on SCLs. Since these drugs may interfere in LSZ analysis, a preliminary investigation of their chromatographic and electrochemical behaviour was performed. Eye-drop standards, hydrolysates of eye-drop standards and of SCLs soaked in solution containing only the eye-drop of interest were analysed by HPLC–ECD. No chromatographic interference and fouling of electrode surface were observed whatever the eye-drop used, thus confirming the selectivity of the developed method.

The amount of LSZ adsorbed on SCLs in the presence of eye-drops was quite different depending on the nature of the investigated drug. Samples of series II and III, prepared under the same experimental conditions except for the nature of the drugs, gave the results shown in *Figure 6a* and *b*, respectively. *Figure 6a* shows a typical chromatogram relevant to a sample of series II. A comparison of this chromatogram with that reported in *Figure 5* shows that the amount of LSZ adsorbed is about 35 times higher than that found in the absence of eye-drops. The opposite behaviour was observed in the presence of drug B (series III samples). In this case, no appreciable LSZ adsorption was found (see chromatogram in *Figure 6b*). Replicates always gave the same results; the increase of the amount of adsorbed protein in the presence of drug A ranged between 20 and 40 times more than in the absence of drug.

The results clearly show that while the presence of drug B in some way prevents the adsorption of LSZ on SCLs, the presence of drug A seems to enhance protein deposit formation. The latter results present some discrepancy with respect to previously reported data[25], but it is to be underlined that the experimental conditions used in the present investigation, mainly concerning the adsorption stage, are completely different.

Parallel experiments were performed by soaking SCLs in solutions containing LSZ and the individual eccipients (boric acid, hydroxypropyl methyl cellulose, potassium chloride, 2-aminoethanesulphonic acid) present in the eye-drops used (see Materials and Methods section). No significant difference was found in the quantity of adsorbed LSZ with respect to samples of series I.

A tentative explanation of the results reported in this paper can be given on the basis of considerations about the surface charge of contact lenses, other than the antidenaturant properties of the drugs used. It has been shown that PHEMA surfaces exhibit a net negative charge[26]; under our conditions it can reasonably be hypothesized that while the film adsorbed on SCLs in the presence of drug B consists of a sodium ion layer followed by a layer of pirfenoxone anions, both of which have no specific affinity with LSZ, in the case of drug A the first adsorbed layer probably consists of lysine ions, which have a good affinity with LSZ and, in general, with proteins. It is well known, in fact, that lysine behaves as an efficient electrode

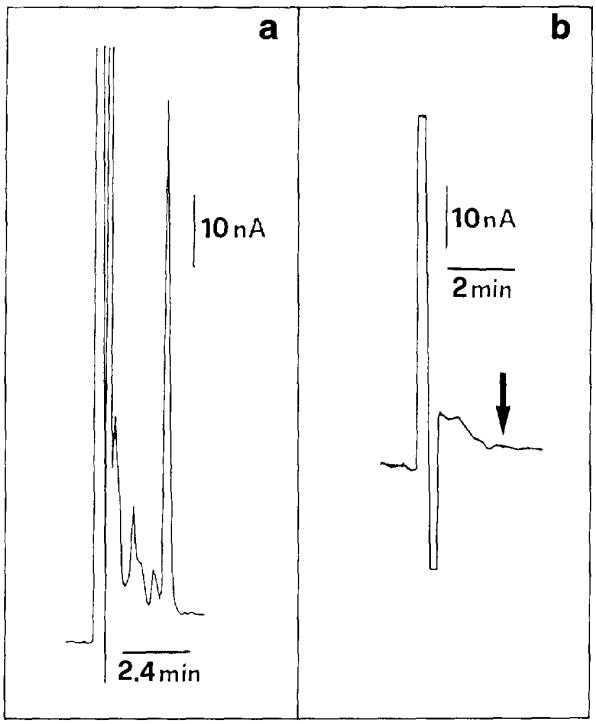

Figure 6 Comparison of chromatograms recorded on 48 h alkaline hydrolysates of soft contact lenses of series II (**a**) and series III (**b**).

modifier for the electron transfer process of proteins by promoting their adsorption on electrode surfaces. In other words, the lysine groups in our case could act as a link between the SCL surface and the LSZ. In order to support this hypothesis, a series of samples was prepared under the same conditions described for series II, but the bendazac lysine salt was substituted with lysine chloride salt. An enhancement (about 15–20 times) in the LSZ adsorption with respect to series I was observed in this case too, thus indicating that lysine, more than the bendazac species, is responsible for the observed phenomenon. Work is in progress to establish whether or not the adsorbed protein is in the denatured or in its native state. In conclusion, the use of pirfenoxone seems to be promising in the prevention of LSZ deposit formation on SCL surfaces. It could be of interest to test its effectiveness in the presence of other tear proteins charged at ocular pH, such as lactoferrins (positively charged) and albumin (negatively charged), together with LSZ. *Ex vivo* studies are required before any definite conclusions can be drawn.

The use of lysine, on the other hand, should be taken into account in those cases in which the biocompatibility of some interfaces relies on the formation of a suitable protein layer[27].

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