DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 11(2&3), 473-430 (1985)

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LYTIC ACTIVITY OF LYSOZYME RELEVANT TO PRESERVATIVES - ENZYME INTERACTIONS

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A b s t r a c t

At physiological hydrogen-ion concentration, p-hydroxybenzoate esters have been found to exert a synergistic effect on the lytic activity of lysozyme; the effect being increased with increasing the size of the aliphatic chain. Although the investigated parabens were proved to bind in significant quantities with lysozyme, however, such additives fail to mask the formation of substrate - lysozyme complexes. The observation was further extended to some organic mercurials with essentially similar results.

At different enzyme concentration levels, phenylmercuric acetate was proved to be the most superior potentiator, whereas, methyl paraben displayed a negligible role.

in the light of the findings obtained, the synergistic effect was interpreted on the basis of the preferential orientation of the substrate on the enzyme binding sites. Such an orientation seems to be more accessible in presence of preservatives.

INTRODUCTION

Lysozyme is a low molecular weight cationic protein which plays a major role in the hygiene of the eye via endowing the lacrimal fluids

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0363-9045/85/1102-0473$3.50/0

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with a natural immunological system capable for destroying many potentially harmful microorganisms. Since its discovery by Fleming (1), lysozyme has been the subject of vast investigations (2). Only in recent years, evidences on drug - lysozyme interactions, particularly, those of ophthalmic interest, began to emerge (3-7). At physiological hydrogen-ion concentration, El-Nimr et al (3) showed that, parabens can bind in significant quantities to lysozyme, whereas, organic mercurials do not. Furthermore, the binding affinities of the different p-hydroxybenzoate esters were found to be independent on the size of the ester grouping.

Akin to other tear proteins, the binding of drugs to lysozyme could be expected to influence the pharmacokinetics of those drugs used for ophthalmic purposes. However, it is still not well established, whether, the preservatives - lysozyme interactions could also be reflected on the natural bacteriolytic activity of the enzyme ? and if this is the case, to what extent would the paraben ester branching participate ?

The objective of the present contribution is to assist in answering the raising questions.

EXPERIMENTAL

Materials and Methods

Hen egg-white lysozyme, 2-times crystallized, lot No. E2-3431 (specific activity 14,170 units/mg), Schwarz/Mann, Orangeburg, N. Y. Dried cells of Micrococcus lysodeikticus (M-0128), lot No. 98C-0266 ; methyl, ethyl and propyl parabens, all obtained from Sigma Chemical Co., St. Louis, MO. Phenylmercuric acetate and nitrate, Eastman Kodak Co., Rochester, N. Y. Other reagents are of analytical grade.

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In all cases, buffer solutions used throughout, were prepared by using triple distilled water from all glass apparatus, and then sterilized by autoclaving at 15 lb. for 20 minutes.

Stock solutions containing 1 x 10^{-4}M lysozyme were made up in 0.1M phosphate buffer, the pH being adjusted at 7.4 with a pH-meter model 7020 (Electronic Instruments Ltd. - England). The molecular weight of the enzyme was taken at 14,400 (8,9). When first prepared, lysozyme solutions were filtered through 0.45 um pore size Millipore filter (Millipore, Bedford, MA)(10). These solutions when stored in a refrigerator showed no appreciable loss of activity within one month (11).

Dried cell suspensions of M. lysodeikticus ($\simeq$ 5 mg/20 ml), were also prepared in the same buffer system, without and with the corresponding preservatives, at concentration levels as those popularly used in ophthalmic formulations. When stored in a refrigerator, substrate suspensions devoid of additives, have been found to retain all of their original absorbance, as well as, entire susceptibility towards lysozyme within 10 days (12).

Substrate samples, each 3 ml volume, were pipetted in a dry spectrophotometer silica cell, 1 cm light path. Lysozyme solution was injected by an Eppendorf micropipette of 100 ul volume (Eppendorf, Hamburg, W. Germany), then mixed quickly. Starting from a fixed absorbance value of 0.950, the lytic course of the enzyme, on the different substrate samples was simultaneously followed turbidimetrically for 10 minutes, at 450 nm and 21 $\pm$ 1^{o}C. Lysozyme assays were carried out on a Cecil Double Beam Digital U. V. Spectrophotometer, model CE 595, fitted with CE 500 Control - Record Module (Cecil Instruments - England), arranged to give a rate of one chart unit/minute.

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Lytic activity of the enzyme were expressed as percentages of inhibition relative to the original activity of lysozyme in absence of the preservatives (blank), induced after 3 minutes incubation time with substrate. A more detailed description of the experimental methodology adopted, as well as, computation of the data obtained, were entailed in a previous article (12).

RESULTS AND DISCUSSION

Figure 1 demonstrates the different potentiation patterns exerted by the various preservatives, on the lytic activity of lysozyme. It should emphasized that, no lytic activity was noted in conjunction with any of the preservatives so far investigated. As regards the p-hydroxybenzoate esters, it is apparent that, a mixture of parabens exert a well marked synergism on the lytic activity of lysozyme, whereas, no appreciable change could be detected in presence of methyl paraben alone. The other members show an intermediate effect. It is also interesting to note that, the synergistic effect of the individual parabens is strongly dependent upon the size of the aliphatic chain ; being increased with increasing the chain branching.

The data obtained in relation to phenylmercuric nitrate recall a close similarity to those observed in presence of parabens (Figure 1). At various lysozyme concentration levels, the catalytic action of phenylmercuric nitrate was found to increase in proportion to the preservative concentration.

Comparing the catalytic role displayed by the different preservatives at various enzyme concentrations, it is quite clear that, of all investigated preservatives, phenylmercuric acetate has been proved to be the most superior. Furthermore, Table 1 shows that, the potentiating action of the different preservatives is generally less pronounced at

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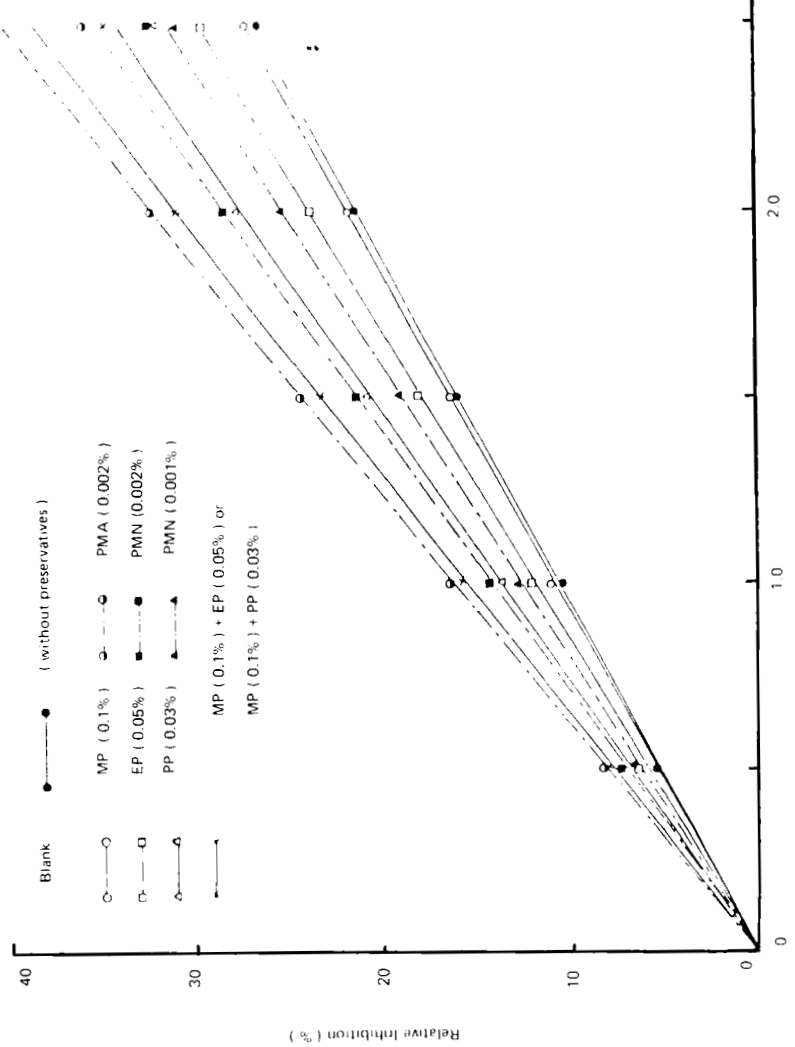

Figure 1. Synergistic patterns of different preservatives on the lytic activity of lysozyme towards M. lysodeiktic substrate suspensions, at various enzyme concentration levels and 21+ 1 °C.

MP: Methyl paraben EP: Ethyl paraben PP: Propyl paraben
PMA:Phenylmercuric acetate PMN: Phenylmercuric nitrate

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TABLE 1

Percentages of Potentiation Action Exerted by Different Preservatives on the Lytic Activity of Lysozyme, at Various Enzyme Concentration Levels and 21 ± 1°C.

	Preservatives	Lysozyme Concentration (M x 10^{-5})				
		0.5	1.0	1.5	2.0	2.5
	Blank (without preservative)	0.0	0.0	0.0	0.0	0.0
1-	Methyl paraben (o.1%)	0.6	4.4	3.1	2.5	2.5
2-	Ethyl paraben (0.05%)	19.6	13.9	13.3	11.6	12.0
3-	Propyl paraben (0.03%)	36.8	30.1	30.4	30.0	21.9
4-	Methyl paraben (0.1%) + Ethyl paraben (0.05%)	50.0	49.0	46.9	46.2	31.8
5-	Methyl paraben (0.1%) + Propyl paraben (0.03%)	50.0	49.0	46.9	46.2	31.8
6-	Phenylmercuric nitrate (0.001%)	19.6	19.6	18.6	18.6	17.9
7-	Phenylmercuric nitrate (0.002%)	40.5	39.6	36.7	34.8	21.9
8-	Phenylmercuric acetate (0.002%)	59.5	54.8	53.2	50.0	36.0

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a higher enzyme concentration level (2.5×10^{-5}M). Such an effect is reflected, in turn, on the deviation of the representative points from the linear trend of the corresponding runs (Figure 1). The deviation of these points which proceeds in proportion to the synergistic effect is mainly attributed to the fact that, the linear range permissible by the present assay is quite limited beyond 2.5×10^{-5}M lysozyme concentration. Under these circumstances, enzyme dilution is required.

In a preceding communication, it has been reported that, p-hydroxybenzoate esters can bind to lysozyme and that such interactions are independent on the aliphatic chain length, whereas, organic mercurials do not. Using a fluorescence quenching technique, the different binding parameters for the three parabens were also quantitated, under almost the same experimental conditions, followed in the present investigation. The stoichiometry of the reaction (n), binding constant (K) and free energy of formation (ΔG), were derivated and found to be 1 : 1 type of interaction, 15,000 $\pm$ 500 mol^{-1} and -5650 Cal mol^{-1}, respectively (3).

In the light of these findings, it was anticipated that, parabens - lysozyme interactions, could exert a some sort of inhibition on the formation of substrate - lysozyme complexes. In contrast, the experimental findings collected, revealed in all cases, a catalytic role of varying degree, rather than an inhibitory action ; regardless of whether, the preservative considered shows a binding tendency towards lysozyme (parabens) or not (organic mercurials). The incapability of the parabens to interfere with substrate - lysozyme interactions, in spite of, the presence of excess from both competitive ligands in the reaction medium, namely, drug and substrate, at a relatively higher molar ratios compared with that of the enzyme, could be attributed to the preferential orientation of the substrate on the enzyme active sites. On the other hand, although the preservatives so far investigated are

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not considered as bacteriolytic agents per se, however, in the presence of such additives, a synergistic action on the lytic activity of lysozyme revealed itself. Again, such observations could be interpreted on the basis that, the substrate - enzyme interactions become more accessible in the presence of the preservatives. This accessibility, in turn, was found to increase with increasing the hydrophobicity of the preservative. However, in the scope of the present study, it is still rather difficult to explain the mechanism of synergism between these antimicrobials and lysozyme, this needs a further investigation.

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