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A STANDARD METHOD FOR DETERMINATION OF LYSOZYME ACTIVITY VIA CONTROLLING THE KINETICS OF SUBSTRATE - ENZYME INTERACTIONS

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Abstract

The role displayed by different criteria, to furnish the optimal experimental conditions, for controlling the rate of lysis of M. lysodeikticus substrate suspensions with lysozyme, has been well defined. According to the constructed calibration curve obtained, lysozyme concentrations, as low as, 0.3 and up to 50 ug/ml could be accurately determined with a high, analytical sensitivity and statistical reliability. Statistical analysis of different sets of experiments, carried out at various enzyme concentration levels, in terms of, the relative standard deviation and limit of error at P = 0.99, were found to be 2.55 percent and ± 0.032, respectively.

Evidence on the stability of M. lysodeikticus suspensions further revealed that, substrate dispersions stored in a refrigerator show neither any appreciable loss in their, initial absorbance, nor entire original lytic susceptibility towards lysozyme, within 10 days storage period.

INTRODUCTION

The susceptibility of microorganisms to lysozyme is mainly restricted to the ability of the enzyme to lyse the wall structure of bacterial

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cells. Lysozyme is a glycosidase which hydrolyses the peptidoglycan component of bacterial cell walls, specifically at the β (1 - 4) glycosidic linkages between the N-acetylmuramic acid and N-acetylglucosamine moieties (1-4).

The dissolution of bacterial cells, is the essential phenomenon, whereby the presence of lysozyme could be detected. Quantitatively, the assay method favored by most investigators entails, a spectrophotometric measurement of the rate at which an enzyme sample could clear up turbid suspensions of specified, microorganisms or substrates. The wavelengths at which measurements were monitored, the way of relating the empirical data to enzymic activity (5-15), the addition of stabilizers (16) and/or sensitizers (17,18), represent the main different features among these reported methods.

The clearing phenomenon is a complex process. Under appropriate conditions, the dissolution of the cell wall proceeds to or near completion. In other cases, the substrate - enzyme interactions do occur, but without disruption of the cells (19) and/or a substantial amount of debris may remain (20), where no or little absorbance change could be detected. The latter cases, may explain the divergence and lack of reproducibility among the optical data taken for a given sample of materials, in spite of, the fact that, all measurements were carried out under one and the same set of circumstances.

In a course dealing with drug - lysozyme interactions (21-25) and its consequences on the lytic activity of the enzyme (26), it was deemed of prime importance to establish first an accurate, simple and sensitive assay method for standardization of the enzyme, Such an assay must be devoid from additives which may show any tendency to interact with the main reactant components, namely, drug, enzyme and/or substrate. The addition of EDTA as a stabilizer (16), or bovine serum

albumin (BSA), as a sensitizer (17,18), by some workers, cannot be adopted herein. The chelating agent EDTA, not only potentiates the enzyme activity (27), but also may be considered as a lytic agent per se, towards certain bacterial strains (28-31). Similarly, the inclusion of BSA in the reaction medium, particularly, at a relatively higher molar ratios compared with that of lysozyme (17,18), is again not recommended in the present investigation. This may be attributed to the fact that, in analogy to other plasma proteins, BSA shows a binding tendency towards less smaller molecules or ligands(drugs and/or substrates).

The present investigation is therefore, a contribution to furnish the optimal experimental conditions for detection and micro-quantitation of substrate - lysozyme complexes, specifically, at the step where the reaction kinetics proceed to or near completion.

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. Micrococcus lysodeikticus, dried cells (M-o128), lot No. 98C-0266 , Sigma Chemical Co., St. Louis, MO. Other chemicals are of analytical grade. Triple distilled water from all glass apparatus was used throughout. Buffer solutions used for the preparation of all test samples were also previously sterilized by autoclaving at 15 lb. for 20 minutes.

Stock solutions of lysozyme (1×10^{-4}M), were prepared in 0.1M phosphate buffer adjusted at pH 7.4 with a pH-meter model 7020 (Electronic - Instruments Ltd. - England). The molarity of the enzyme was calculated on the basis of 14,400 (32, 33). Enzyme solutions were then filtered through 0.45 um pore size Millipore filter (Millipore-Bedford,MA) (34).

Substrate suspensions were made up by suspending 5 mg of M. lysodeikticus, dried cells, in 5 ml of the same buffer system, triturate gently for 2 minutes, but avoid shaking, then mix with an appropriate buffer to give a final dilution of ca. 0.25 mg/ml.

3 ml volume of substrate suspension was pipetted into a dry square silica cell, 1 cm path length. Lysozyme solutions were injected by an Eppendorf micropipette of 100 ul volume (Eppendorf - Hamburg, W. Germany), then mixed quickly with the substrate. Simultaneously, a Cecil Double Beem Digital U. V. Spectrophotometer, model CE 595, fitted with CE 500 Control - Record Module (Cecil Instruments - England), was arranged at the specified wavelength. In the meantime, the time-drive attachment was adjusted to give a rate of one chart unit/minute. In all cases, runs were recorded at 21 ± 1 ^{o}C, for 10 minutes, unless otherwise stated.

Lytic activities of lysozyme were expressed as percentages of the relative inhibition. This parameter was calculated according to the following equation:

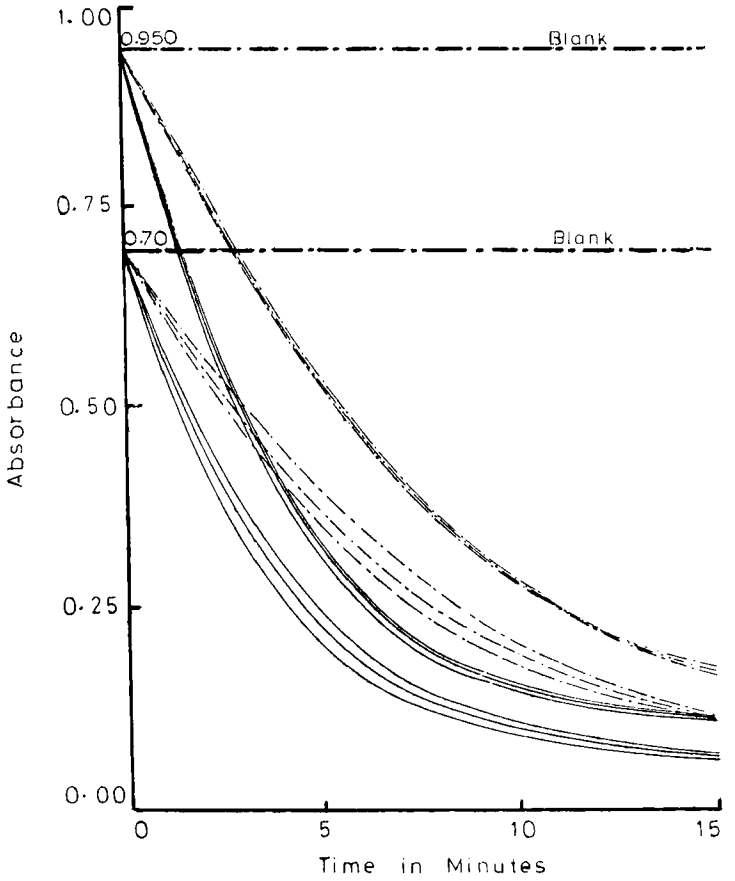
$$\frac{A_o - A_3}{A_o} \times 100$$

Where $A_o - A_3$, is the reduction in the optical density of substrate suspensions, induced after 3 minutes incubation time with the enzyme sample.

RESULTS AND DISCUSSION

Figure 1 illustrates the various absorbance - time profiles,for substrate - lysozyme interactions, compared at two enzyme concentration levels, viz., 2.5 and 5 x 10^{-5}M. To assess the reliability of the different runs, measurements were checked at 450 and 570 nm, the wavelengths arround which most reported assay methods have been conducted.

The findings obtained demonstrate that, measurements monitored at 570 nm, are nevertheless unsuitable for accurate evaluation of the



F I G U R E 1

Comparison of the sensitivity, reproducibility and initial linearity of the different runs at 21 ± 1 °C, as a function of monitored wavelengths and lysozyme concentration (M x 10^{-5}).

-·-·-·- 2.5 and ——— 5.0 x 10^{-5}M

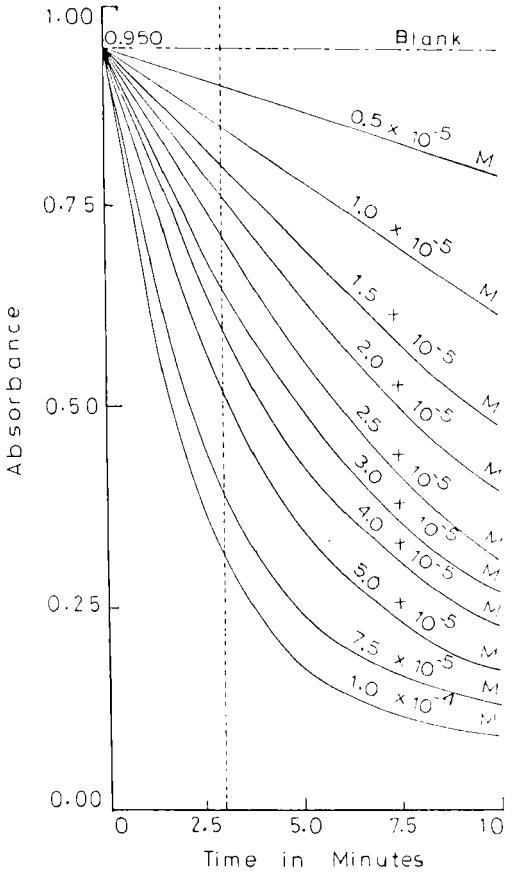
time of onset and completion of lysis reactions. The lack of reproducibility, limit of sensitivity (A_{570} ca. 0.7) and the curvilinear characteristics of the different runs performed at this wavelength, cannot be improved by the addition of either EDTA (16) or BSA (17,18), as previously declared.

On the other hand, runs carried out at 450 nm wavelength, under the same set of circumstances, were found to be more advantageous, in terms of, its high analytical sensitivity (A_{450} ca. 0.950), reproducibility and tracing of reaction completion, as clearly reflected by the appearance of an initial rectilinear part in conjunction with each curve.

Starting from a fixed absorbance value A_{450} = 0.950, the linear characteristics of the different runs were analyzed in relation to the enzyme concentration (5×10^{-6} - 1×10^{-4}M). It would appear from Figure 2 that, in all cases, a sensible initial straight line was constructed as a part of each curve. The linear sigments which deviated from linearity after varying time intervals, were found to increase with decreasing in lysozyme concentration.

Due to the complexity of the clearing phenomenon (16,19,20), it may be considered plausible to generate a calibration curve on the basis of the linear sigments obtained, in relation to the various enzyme concentration levels, where the kinetics of substrate -lysozyme interactions seems to proceed regularly with respect to time. Figure 2 illustrates again that, the intersection for the different curves after 3 minutes incubation time, would pass entirely through the linear sigments corresponding to enzyme concentrations, covering the range between 0.5 and 2.5×10^{-5}M . Plotting percentages of the relative inhibition versus lysozyme concentration (0.5 - 2.5×10^{-5}M), would result in a fairly straight line (Figure 3).

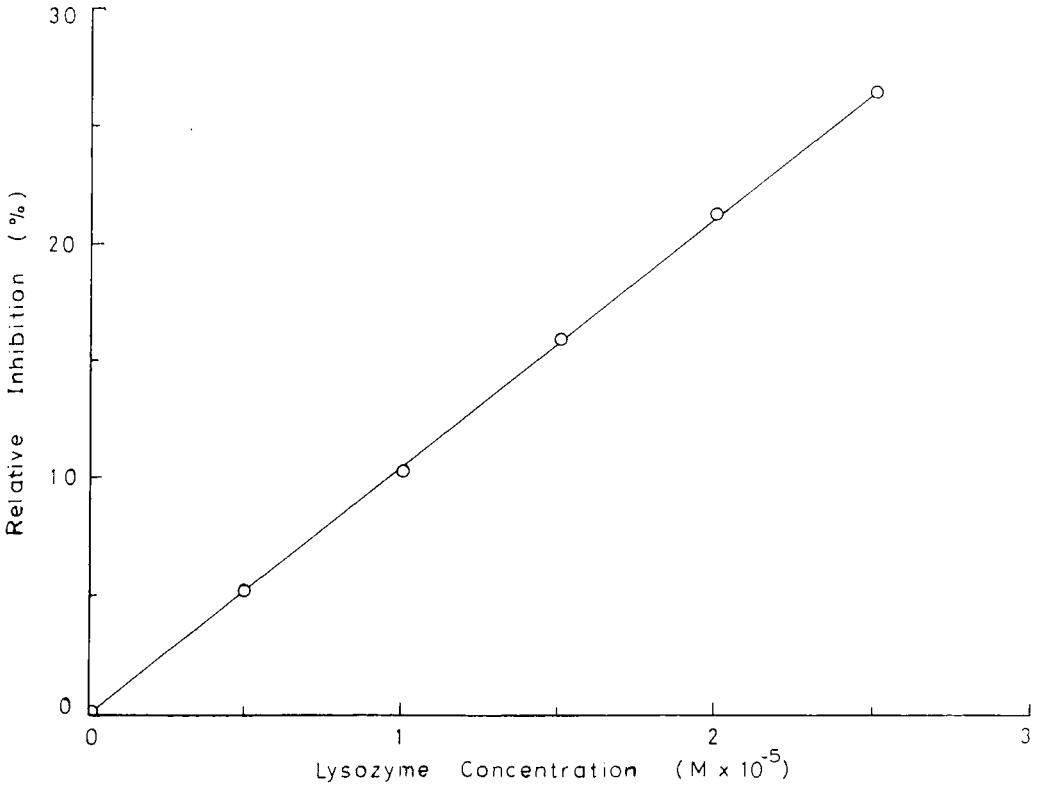
The reliability of the assay method was further checked at different enzyme concentrations, each concentration level was presented by 10 determinations. For each assay, an identical freshly prepared test samples were examined under one and the same experimental conditions previously described. Statistical analysis of the different sets of measurements, in terms of, the relative standard deviation (R.S.D.)



F I G U R E 2

Effect of lysozyme concentration, on the rate of lysis of M. lysodeikticus substrate suspensions at 21 ± 1 °C. Substrate dispersions were prepared in 0.1M phosphate buffer of pH 7.4 and standerdized at an initial A_{450} = 0.950

and limit of error at P = 0.99 (L.E. $_{0.99}$), are shown in Table 1. The average values for R.S.D. and L.E.$_{0.99}$, were found to be 2.55 percent and ± 0.032 , respectively. Compared with calculations based on , tangents drawn at zero time or after reaction times of maximum one minute (14,35-37), the magnified values of ΔA_{450} obtained after 3 minutes



F I G U R E 3

A calibration curve for lysozyme. Percentages of relative inhibition versus enzyme concentration at 450 wavelength and 21 ± 1 ^{o}C.

incubation period, have been proved also to be highly reproducible and ultrasensitive.

The present investigation considered also, one of the major shortcoming interrelated to almost all published assay methods, namely, restriction on the usage of only freshly prepared substrate suspensions. Under the experimental conditions adopted, dried cell suspensions of M. lysodeikticus, stored in a refrigerator were found to retain all their, initial A_{450} values, as well as, original susceptibility towards lysozyme, within 10 days storage period. A substrate sample withdrawn

TABLE 1

Lytic Activity of Lysozyme on M. lysodeikticus Substrate Suspensions, as a Function of Enzyme Concentration, in 0.1M Phosphate Buffer, at pH 7.4 and 21 ± 1°C.

	Lysozyme Concentration ($M \times 10^{-5}$)									
	0.5		1.0		1.5		2.0		2.5	
	A_{450}	Concn.	A_{450}	Concn.	A_{450}	Concn.	A_{450}	Concn.	A_{450}	Concn.
Blank	0.950	---	0.950	---	0.950	---	0.950	---	0.950	---
1-	0.904	0.460	0.850	1.000	0.796	1.539	0.748	2.018	0.705	2.448
2-	0.900	0.499	0.847	1.029	0.803	1.469	0.750	1.998	0.698	2.519
3-	0.901	0.490	0.853	0.969	0.800	1.499	0.745	2.049	0.700	2.499
4-	0.903	0.470	0.852	0.980	0.795	1.549	0.748	2.018	0.695	2.548
5-	0.898	0.519	0.850	1.000	0.800	1.499	0.745	2.049	0.705	2.448
6-	0.900	0.499	0.855	0.949	0.802	1.479	0.752	1.978	0.696	2.539
7-	0.902	0.479	0.848	1.020	0.803	1.469	0.747	2.029	0.699	2.508
8-	0.897	0.530	0.852	0.980	0.795	1.549	0.753	1.969	0.695	2.548
9-	0.902	0.479	0.850	1.000	0.805	1.449	0.750	1.998	0.704	2.458
10-	0.903	0.470	0.855	0.949	0.798	1.519	0.752	1.978	0.698	2.519
* R. S. D. (%)		4.220		3.020		2.400		1.510		1.590
** L. E.$_{P = 0.99}$		± 0.022		± 0.031		± 0.037		± 0.031		± 0.041

* Percentages of relative standard deviation

** Limit of error at P = 0.99

daily during the storage time and analyzed, at various enzyme concentration levels, yield in all cases, identical calibration curves, without any appreciable change in the reliability of the assay method.

REFERENCES

1. M. R. J. Salton, Biochim. Biophys. Acta 10, 512 (1953).
2. C. Weibull, J. Bacteriol., 66, 688 (1953).
3. N. D. Zinder and W. F. Arndt, Proc. Natl. Acad. Sci., U. S. A., 42, 586 (1956).
4. M. R. J. Salton, in "The Bacterial Cell Wall", Elsevier, Amsterdam 1964.
5. E. H. Boasson, J. Immunol., 34, 281 (1938).
6. D. Shugar, Biochim. Biophys. Acta 8, 302 (1952).
7. A. N. Smolelis and S. E. Hartsell, J. Bacteriol., 58, 731 (1949).
8. G. Jollès and C. Fromageot, Biochim. Biophys. Acta 11, 95 (1953).
9. L. Colobert, Bull. Soc. Chim. Biol., 39, 1155 (1957).
10. P. Jollès, in "Methods in Enzymology", S. P. Colowick and N. O. Kaplan, Eds., Vol. V , p. 137. Academic Press, New York 1962.
11. S. R. Dickman and C. M. Proctor, Arch. Biochem. Biophys. 40, 364 (1952).
12. G. P. Kerby and G. S. Eadie, Proc. Soc. Exptl. Biol. Med. 83, 111 (1953).
13. E. L. Smith, J. R. Kimmel, D. M. Brown and E. O. P. Thompson, J. Biol. Chem., 215, 67 (1955).
14. A. L. N. Prasad and G. Litwack, Anal. Biochem., 6, 328 (1963).
15. J. Saint-Blancard, J. P. Locquet and P. Jollès, Protides Biol. Fluids 16, 191 (1969).
16. G. Gorin, S. F. Wang and L. Papapavlou, Anal. Biochem., 39, 113 (1971).
17. M. E. Selsted and R. J. Martinez, Infect. Immunity 20, 782 (1978).
18. M. E. Selsted and R. J. Martinez, Anal. Biochem., 109, 67 (1980).