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APPARENT AFFINITY CONSTANTS OF LYSOZYMES FROM DIFFERENT ORIGINS FOR *MICROCOCCUS LYSODEIKTICUS* CELLS*

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SUMMARY

Lysozymes (mucopeptide *N*-acetylmuramylhydrolases, EC 3.2.1.17) from different origins have qualitatively the same biological activity, but possess different chemical structures and different reaction velocities. In order to express these latter differences, affinity constants of 7 different lysozymes for the bacterial substrate were determined. The various lysozymes can be ranged into two groups: a group including the human lysozymes which have very similar affinity constants, and a group including the bird egg-white lysozymes where important differences between the constants are noted from one enzyme to another, even between the lysozymes isolated from the duck egg-white. However hen egg-white lysozyme appears to have a similar affinity constant as the human lysozymes.

INTRODUCTION

Lysozymes (mucopeptide *N*-acetylmuramylhydrolases, EC 3.2.1.17) from different animal species[1] and sometimes also from different organs or secretions of the same animal[2] are chemically different, but have qualitatively the same biological activity: they provoke the lysis of a suspension of dead cells of *Micrococcus lysodeikticus*. Quantitatively the reaction velocity is different from one enzyme to another. Preliminary experiments by JOLLÈS *et al.*[3,4] have already indicated that human lysozymes reacted more slowly with different substrates than the enzymes from avian sources (hen, duck, goose egg-whites). Goose egg-white lysozyme has the highest initial velocity, but its action is very short[5,6].

In the course of our comparative studies devoted to lysozymes of different origins[1], we wanted to express the differences mentioned above by the determination, for each enzyme, of an affinity constant for the bacterial substrate. Its insolubility constituted a difficulty for the resolution of this problem. However, the type of

* 62nd communication on lysozymes. 61st communication, P. JOLLÈS AND J. JOLLÈS, *Helv. Chim. Acta*, 51 (1968) 980.

kinetics which was choosen seemed to follow the classical mechanism of the enzymatic kinetics. The determination of an apparent affinity constant (K_a app.) became possible by expressing in units of substrate and not by the usual molarity system.

MATERIALS AND METHODS

Lysozymes

The lysozymes from normal leucocytes and plasma were prepared following a procedure analogous to that described by CHARLEMAGNE AND JOLLÈS[7]; lysozyme from human milk was obtained following the procedure of JOLLÈS AND JOLLÈS[8]. Hen egg-white lysozyme was obtained from Worthington, Freehold, N.J. (lot 642). Duck egg-white lysozymes II and III and goose egg-white lysozyme were prepared following the procedures of JOLLÈS, SPOTORNO AND JOLLÈS[9] and DIANOUX AND JOLLÈS[5], respectively. All the lysozymes were chromatographically pure samples, except hen egg-white lysozyme which was a commercial sample.

Determination of the lytic activity

The lytic activity was determined by observing spectrophotometrically the increase of transmittance which occured during the lysis of a suspension of dead cells of *M. lysodeikticus*[10] (Reagent Vial, from Worthington or cells from Miles; 0.066 equiv/l phosphate buffer (pH 6.2); 0.09% NaCl; 25°; 650 mμ, spectrophotometer Lérès Spila, DMS; initial transmittance of 30% in a cuvette of 1 cm path when distillated water was set at 100% transmittance). In these conditions with hen egg-white lysozyme used as a standard, a linear increase of the transmittance is observed over 3–4 min; the time of linear increase is shorter with lysozymes of other origins. The slope of the line is proportional to the concentration of the enzyme. By this technique, 1–6 μg lysozymes per ml (± 0.2 μg/ml) can be determined.

Determination of the apparent affinity constant

The apparent affinity constant K_a app. was evaluated graphically according to LINEWEAVER AND BURK[11]. After measurement of the initial velocity for different substrate concentrations, $1/v$ (v = initial velocity) was plotted *versus* $1/[S]$ ($[S]$ = substrate concentration). The intersection of this line with the $1/[S]$ axis occured at a point corresponding to $-1/K_a$ app.

For the cell concentrations used in these studies (60–500 mg/l) Beer-Lambert's law was valid. The absorbance (A) is proportional to the substrate concentration $[S]$ ($\text{mg}\cdot\text{l}^{-1}$). The slope of the experimental line, $dA/d[S]$ is in our conditions $2.5\cdot10^{-3}$ ($\text{l}\cdot\text{mg}^{-1}$).

If $[S]$ is the instantaneous concentration of the substrate which is not lysed, the velocity v at the same moment can be expressed as follows:

$$v = -\frac{d[S]}{dt}\left(\text{in fact} - \frac{d(ES)}{dt}\right) \tag{1}$$

(ES = enzyme–substrate complex).

By introducing the absorbance, it is possible to write:

$$v = -\frac{d[S]}{dA}\cdot\frac{dA}{dt} \tag{2}$$

$d[S]/dA$ is a constant of our system and can be determined from the standard curve. Its value is $10^3/2.5 = 400$. dA/dt is the slope of the tangent to the curve which represents the variation of the absorbance as a function of time. This instantaneous value can be measured on the recorder. The reaction velocity ($mg \cdot l^{-1} \cdot sec^{-1}$) can be determined at every moment, especially at the beginning of the reaction (t_0).

The reaction is followed at 25 ± 0.1°, at 650 mμ and is recorded on a paper graduated in linear absorbance (1 unit of absorbance for 20 cm). The velocity of the paper on the recorder is 3 cm/min. If the reaction velocity is followed during 200 sec (10 cm = 200 sec), it is possible to write $dA/dt = x/dt$. In our system,

$$v = -400 \cdot \frac{x}{200 \times 20} = -\frac{x}{10}$$

where x is expressed in cm.

The values of $1/v$ as a function of $1/[S]$ which are in correlation are used for the statistical determination of K_a app. by the method of least squares which allows calculation of the line of regression ($y = ax + b$) and its parameters.

TABLE I

APPARENT AFFINITY CONSTANTS OF LYSOZYMES FROM DIFFERENT ORIGINS FOR *Micrococcus lysodeikticus* CELLS

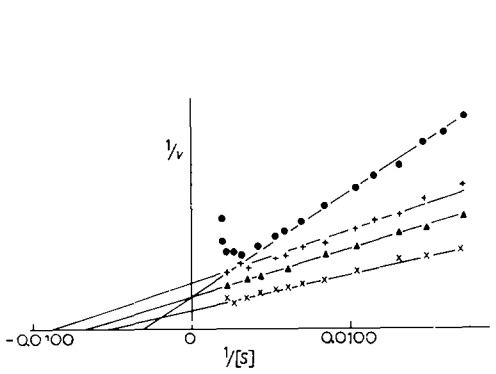
Lysozyme	*Number of assays*	K_a *app.* *(mg/l)*
Human		
Normal leucocytes	16	90 ± 10
Normal plasma	8	100 ± 5
Milk	13	110 ± 10
Avian		
Hen egg-white	10	115 ± 10
Duck egg-white II	8	150 ± 10
Duck egg-white III	17	200 ± 20
Goose egg-white	9	400 ± 100

RESULTS

The experimental data are summarized in Table I. Fig. 1 indicates the determination of the apparent affinity constants of human leucocyte lysozyme, of the two duck egg-white lysozymes II and III (refs. 2, 12) and of goose egg-white lysozyme. An inhibition of the lysis by an excess of substrate was observed with goose lysozyme. If the reaction velocity of this latter is plotted *versus* the logarithm of the substrate concentration, a bell-shaped curve is obtained (Fig. 2).

CONCLUSION

From Table I, where the different apparent affinity constants are indicated, it can be concluded that the various lysozymes employed in this research can be ranged into two groups: a group including the human lysozymes and a group including the bird egg-white lysozymes, although hen lysozyme appears to have a



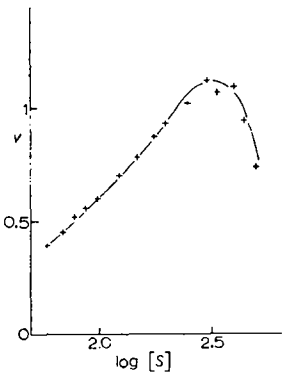


Fig. 1. Variation of $1/v$ *vs.* $1/[S]$ for different lysozymes. ●- -●, goose; +—— +, hen; ▲—▲, duck II; ×— ×, duck III.

Fig. 2. Variation of v *vs.* log $[S]$ of goose egg-white lysozyme.

similar constant as the human lysozymes. The differences (from 90 ± 10 to 110 ± 10 mg/l) noted for the K_a app. of the human lysozymes seem to be, at the time of these investigations, not significant; they can perhaps be related to slight differences in the primary structures (this point is under investigation) or in the secondary structures, as the purification procedures of all these enzymes are not entirely identical. Small differences in the specific activities could be observed. However, important differences between the apparent affinity constants of hen egg-white, two duck egg-white and goose egg-white lysozymes are noted. These differences can probably be related to the primary structures of these enzymes which are quite different[2,5,12]. In this relationship, it is noteworthy to point out that the rather slight structural differences between the two duck lysozymes II and III (refs. 2, 12) have, however, an effect on their chromatographic behaviour, on their behaviour in the presence of a competitive inhibitor like *N*-acetyl-glucosamine[13] and also on the value of their apparent affinity constants.

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