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INFLUENCE OF pH AND IONIC STRENGTH ON THE LYSIS OF *MICROCOCCUS LYSODEIKTICUS* CELLS BY SIX HUMAN AND FOUR AVIAN LYSOZYMES*

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SUMMARY

1. The activities of six human and four avian lysozymes (mucopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17) on cell suspensions of *Micrococcus lysodeikticus* have been examined as a function of ionic strength and pH: three-dimensional models were built.

2. Alkaline pH improves the rate of lysis at low ionic strength for all the lysozymes, with the exception of the goose enzyme.

3. The affinity of the various lysozymes for the substrate is independent of the ionic strength. The variations of $pK_{a,app}$ as a function of pH suggest an analogy of the chemical groups involved in the catalytic mechanism.

4. The percentage of inhibition of all the enzymes by *N*-acetylglucosamine is a function of pH, but not of ionic strength; it is particularly marked at alkaline pH where the rate of lysis is the highest.

5. Goose egg white lysozyme has a particular behaviour; the maximum activity occurs at acidic pHs (3.8 and 5.25) and at a higher ionic strength than for other lysozymes; the inhibition by *N*-acetylglucosamine remains very low.

INTRODUCTION

A study of the influence of pH and ionic strength on the enzymic activity of hen egg white lysozyme (mucopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17) was recently published by DAVIES *et al.*[1]. Our investigations, devoted to chromatographically pure lysozymes from various sources, led us to examine the importance of these two factors for the lysis of *M. lysodeikticus* cells by (a) three lysozymes isolated from human blood (plasma, normal leucocytes, leucocytes of patients suffering from chronic myelogenous leukemia, CML)[2], (b) three further human lysozymes (milk[3],

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two enzymes from the urine of patients suffering from acute myeloblastic leukemia, AML[4]) and (c) four bird egg white lysozymes (guinea-hen, duck II (ref. 5), duck III (ref. 5), goose[6]).

MATERIALS AND METHODS

All the lysozymes were chromatographically pure samples prepared in our laboratory[2–6] with the exception of hen egg white lysozyme which was a commercial sample (Worthington).

The initial velocity of the lysis of *M. lysodeikticus* cells ([*S*] = 150 mg/l) at various pH's, ionic strengths and at 25° was measured in the presence of 4–6 μg/ml of lysozyme. The results are expressed as percentage of the velocity (v%) obtained with the same concentrations of reagents ([*S*], lysozyme) under well-defined conditions of pH (6.2) and ionic strength (I = 0.107) (0.066 M phosphate buffer + 0.09% NaCl). The results are reported with the help of three-dimensional models (v%, pH, I) as a two-dimensional representation gives only a partial view of the results.

Two types of buffers were used: (a) buffers with a constant Na^+ concentration (0.08 M): 0.02 M citric acid–0.04 M disodium phosphate, following McILVAINE[7] (pH 3–8) or 0.1 M glycine–0.1 M NaOH, according to SØRENSEN[8] (pH 8.5–9.5); (b) buffers with constant ionic strength: 0.142 M veronal–0.142 M sodium acetate–0.1 M HCl (I = 0.118), according to MICHAELIS[9] (pH 3–9.4); buffers of MILLER AND GOLDER[10] containing glycine–HCl–NaCl (pH < 3.5), sodium acetate–acetic acid (pH 4–5.5), mono- and disodium phosphate (pH 6–7.5), HCl–sodium veronal (pH 8–8.5), glycine–NaOH (pH $\geqslant$ 9), stock solutions (I = 0.200).

The pH was measured in the buffers containing substrate, but before addition of lysozyme.

The apparent affinity constants $K_{a,app}$ and the inhibition of lysozymes by *N*-acetylglucosamine were determined according to LOCQUET *et al.*[11] and SAINT-BLANCARD *et al.*[12].

RESULTS

Influence of pH and ionic strength on the velocity of lysis of M. lysodeikticus cells

Case of the various lysozymes with the exception of the goose enzyme. Alkaline pH increased the velocity of lysis, particularly at low ionic strengths. At lower pH, the optimum velocity was situated in the area of higher ionic strengths.

For human lysozymes, the pH optimum was 8–8.2 for I = 0.025–0.0375. Human milk lysozyme however had two maxima, a first one at pH 5.5–7, I = 0.1 and a second one at pH 8.2, I = 0.0375–0.05 (Fig. 1a).

Under our conditions, the velocity of lysis of *M. lysodeikticus* cells by hen egg white lysozyme had its highest value at pH 8.6 and I = 0.025; DAVIES *et al.*[1] have mentioned a pH of 9.2. The conditions for optimum velocity of guinea-hen, duck II and duck III lysozymes were pH 8.5, I = 0.0250–0.0375; pH 8.5, I = 0.050 and pH 8–8.5, I = 0.050, respectively.

Goose lysozyme. The variations of the activity of goose lysozyme as a function of pH and ionic strength were quite different from those observed with all the other lysozymes. The highest activity was observed at pH 3.8 as well as at pH 5.25; I =

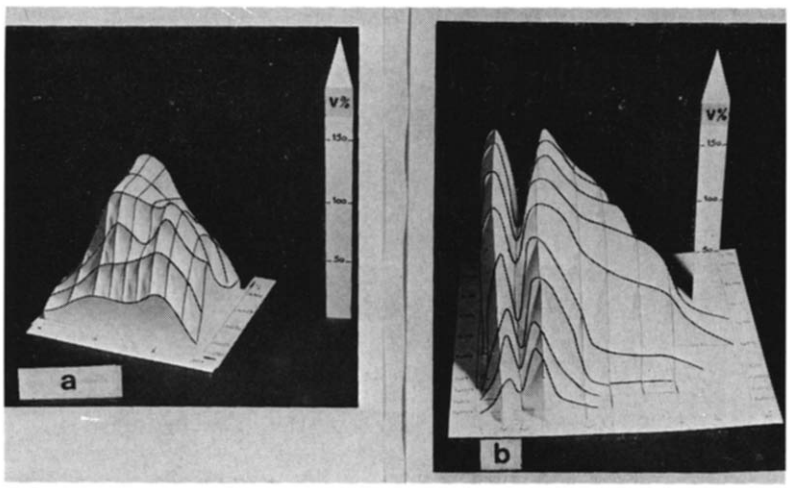

Fig. 1. Lytic activity of human milk (a) and goose egg white (b) lysozymes as a function of pH and ionic strength. Activity is recorded as a percentage of the velocity at pH 6.2, $I = 0.107$. pH 4–9 and I 0.0125–0.100 for a; pH 3–11 and I 0.0125–0.190 for b.

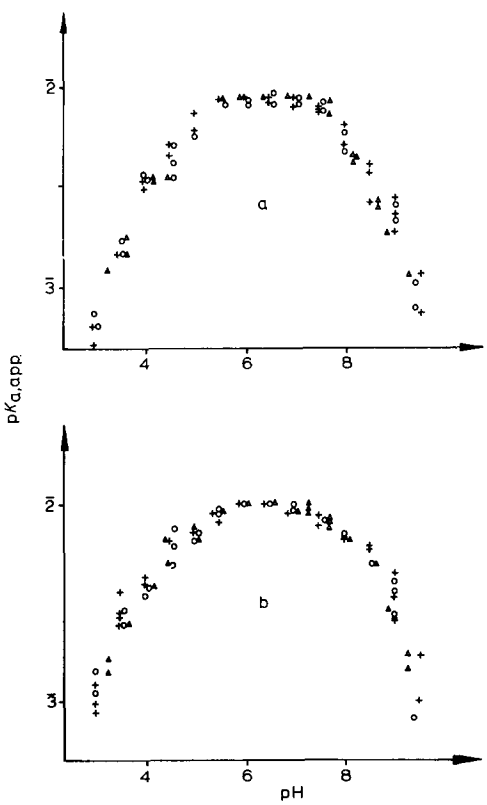

Fig. 2. Variation of $pK_{a,app}$ as a function of pH for hen egg white (a) and normal human leucocyte (b) lysozymes.

0.125–0.250. A minimum rate of lysis was observed at pH 4–4.5. Here again the behaviour of goose lysozyme was different from that of other lysozymes[13] (Fig. 1b).

Influence of pH and ionic strength on the apparent affinity constants ($K_{a,app}$)

At constant pH, the apparent affinity constant of a given lysozyme for the bacterial substrate remained the same, whatever the ionic strength of the buffer (I between 0.0125 and 0.2).

When the $-\log K_{a,app}$ ($pK_{a,app}$) of the various lysozymes (goose lysozyme included) are expressed as a function of pH, the profiles of the curves are very similar (Fig. 2). They all bend first between pH 3.5 and 4 and again between pH 8 and 9. As the observed variations are only due to pH and not to ionic strength (Table I),

TABLE I

$K_{a,app}$ OF VARIOUS LYSOZYMES FOR BACTERIAL SUBSTRATE AT DIFFERENT pH'S AND I'S

Lysozyme	*pH*	*I*	$K_{a,app}$ *(ref. 11) (mg/l)*
Human origin			
Normal plasma	7.5	0.025	107–103–105
	7.5	0.100	110–100
Normal leucocytes	7.5	0.025	108–105–111
	7.3	0.100	105–110–100
CML leucocytes, Peak I	8.5	0.025	250–243–227
	8.6	0.118	200–250
AML urine			
Peak α	8.5	0.025	250–234
	8.5	0.100	250
Peak β	8.5	0.0375	303–307
	8.5	0.100	300
Milk	6.2	0.075	120–112
	6.0	0.118	110
Avian egg whites			
Hen	8.6	0.025	330–400
Guinea-hen	7.9	0.075	312–310–300
	8.0	0.118	310–320
	8.6	0.0375	420–342–400
	8.5	0.100	400–357–416
Duck			
Lysozyme II	8.6	0.0375	328–360–333
	8.6	0.118	330–360–250
Lysozyme III	8.0	0.0375	320–350–280
	8.0	0.118	285–300
Goose	3.5	0.050	900
	3.8	0.125	1100
	5.2	0.125	300–370
	5.0	0.100	400–310

they do not represent the initial electrostatic linkage between lysozyme and cell walls. However it is possible that they reflect the ionisation of the enzyme–substrate complex, ES, or of the enzyme or of the substrate, as one is not able to distinguish between these different ionisations.

However the study of the effect of pH on the rate of lysis at very low substrate concentrations ($[S] = 40$ mg/l) by human leucocyte or hen egg white lysozymes

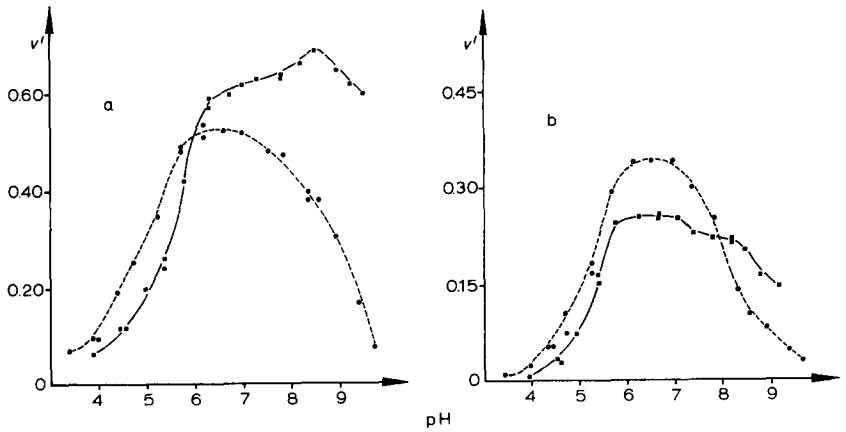

Fig. 3. Variation of the rate of lysis of a diluted suspension of *M. lysodeikticus* cells ([*S*] = 40 mg/l) as a function of pH by hen egg white (a) and normal human leucocyte (b) lysozymes. ■—■, $I = 0.025$; ●— — —●, $I = 0.100$.

suggests that the bends are primarily due to the ionisation of the free enzyme or of the substrate (Fig. 3).

The ionisation of the substrate is unknown. As the ionic strength has no influence on $K_{a,app}$ and by comparison of our results with those obtained by DAHLQUIST *et al.*[14] concerning the complexes formed between hen egg white lysozyme and chitotriose—a soluble and neutral low-molecular-weight substrate—it is possible to assume that the pH of the first bend corresponds to the pK of ionisation of the carboxylic groups implicated in the catalytic mechanism. As a change of one unit in the slope is observed and the curvature at the bends is such that the graphs miss the intersection point of neighbouring straight parts by a vertical distance of 0.3 unit (Fig. 2a), it can be concluded according to DIXON AND WEBB[15] that only one ionisable group is detected. It probably belongs to the free carboxylic group of glutamic acid No. 35 of hen lysozyme; such a residue was found in identical or very similar sequences in duck II (ref. 16) and duck III egg white lysozymes and in human milk lysozyme (ref. 17). In bovine α-lactalbumin which has a closely related structure[18] to hen lysozyme but possesses no lytic activity, the above-mentioned glutamic acid residue is replaced by a histidine residue.

As far as our results can be compared to those of DAHLQUIST *et al.*[14], they constitute a theoretical basis in favour of the analogy of the sites for, at least, one of the chemical functions implicated in the lytic activity.

The variation in value of $K_{a,pp}$ has also been studied as a function of the variation in the maximum velocities. The results obtained with hen and normal human leucocyte lysozymes, in the presence of substrate concentrations 3 times higher than that defined by the $K_{a,app}$, are indicated in Fig. 4. The increase of the lytic activity at $I = 0.025$ is particularly important at pH 8.6 for hen lysozyme and at pH 8.2 for the human lysozyme (Fig. 4).

Inhibition of various lysozymes by N-acetylglucosamine at different pH and ionic strengths

At each pH, the changes of the ionic strength (between 0.0125 and 0.118) do

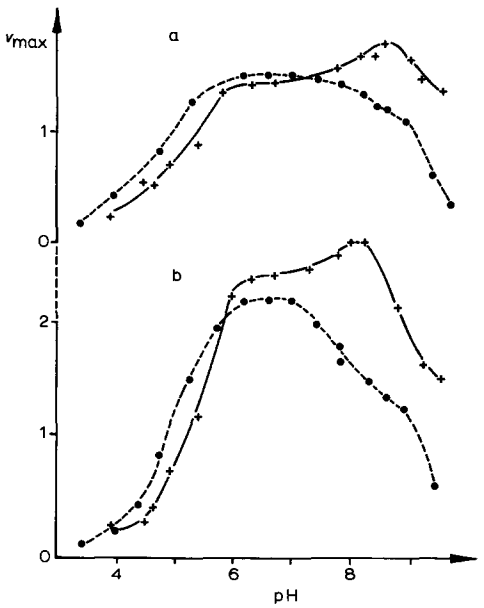

Fig. 4. v_{max} (mg·l^{-1}·sec^{-1}) as a function of pH for hen egg white (a) and normal human leucocyte (b) lysozymes. +—+, I = 0.025; ●— — —●, I = 0.100.

not modify the inhibition with *N*-acetylglucosamine of human plasma and leucocyte lysozymes as well as of hen, guinea-hen, duck II and III egg white lysozymes. These results are in accordance with the observations made by DAVIES *et al.*[1] for hen egg white lysozyme.

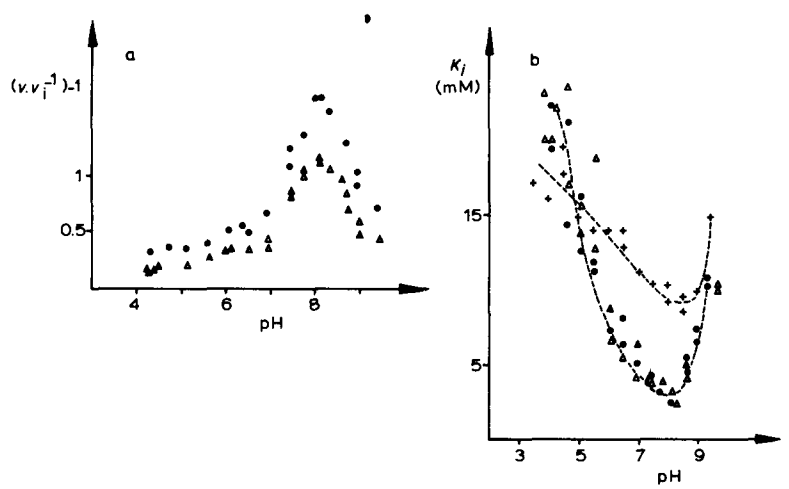

Fig. 5. a. Variation of $(v \cdot v_i^{-1}) - 1$ as a function of pH for human plasma lysozyme. *N*-Acetyl glucosamine concn.: △, 5 mM; ●, 10 mM. b. K_i as a function of pH for normal human plasma (●) and leucocyte (△) lysozymes and hen egg white lysozyme (+). v_i = initial velocity in the presence of *N*-acetylglucosamine (inhibitor); K_i = inhibition constant.

The inhibition with *N*-acetylglucosamine of the lytic action of the various lysozymes, with the exception of the goose enzyme, is particularly marked at those pHs where the activity of the lysozymes for *M. lysodeikticus* cells is the highest; the curves obtained with human plasma and normal leucocyte lysozymes are in accordance with this statement (Fig. 5a).

The effect of the pH on the value of the inhibition constant was also studied, especially in the cases of hen egg white lysozyme and human plasma and normal leucocyte lysozymes. The constant K_i decreases from acidic pHs until pH 8.2 (human enzymes) or pH 8.6 (hen enzyme) and then increases again. A bend between pH 3.5 and 5.5 suggests the involvement of a group possessing a dissociation constant situated in this zone of pK; an inverse bend is found in the basic area (Fig. 5b).

Goose egg white lysozyme was not inhibited at pHs 3.8, 5.25, or 6.2. The results expressed in the Dixon system gave rise to parallel lines and suggested an apparent and discrete uncompetitive inhibition at several pHs[12].

CONCLUSION

In the case of MEYER *et al.*[19] and many other authors, the curves of lytic activity of hen lysozyme *versus* pH exhibit a unique maximum situated between pH 5.8 and 7. Others do not agree with this result and indicate that the lysis of bacterial cell walls is also possible or even increased at alkaline pHs. These different interpretations can probably be attributed to the different experimental conditions which were used. The buffer ionic strength plays an outstanding role, as demonstrated by DAVIES *et al.*[1] in studies devoted to hen egg white lysozyme. Our results, obtained with six human and four avian lysozymes are in accordance with the interpretations of DAVIES *et al.*[1] and suggest a close similarity in the mode of action of the ten lysozymes employed in this research; however the special behaviour of the goose enzyme has once more been demonstrated.

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