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The effect of phosphate buffer in the range of pH 5.80–8.07 on jack bean urease activity

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Abstract

The effect of phosphate buffer on the activity of jack bean urease was studied in the range of pH 5.80–8.07. The inhibition constants of phosphate buffer were determined by measuring initial reaction rates at each pH for a series of buffer concentrations at a series of urea concentrations. It was shown that: (1) at pH 5.80–7.49 the buffer is a competitive inhibitor of the enzyme with $K_{i,buffer}$ increasing from 0.54 mM for pH 5.80 to 362 mM for pH 7.49, (2) the values of $pK_{i,buffer}$ are pH-dependent exhibiting a slope of -1 at pH 5.80–6.5 and a slope of -2 at pH 6.5–7.49, (3) from pH 7.62 as the pH is further raised the competitive inhibition of urease by the buffer was not observed, (4) the true competitive inhibitor of urease is $H_2PO_4^-$ ion, and (5) pH 6.5 and 7.6 correspond to the ionization constants of the active site groups of urease responsible for the inhibitory strength of $H_2PO_4^-$ ion.

Keywords: Urease; Phosphate buffer; Inhibition

1. Introduction

Urease (EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and carbon dioxide via the intermediate formation of carbamate [1]:

$$(NH_2)_2CO \underset{H_2O}{\rightarrow} NH_3 + H_2NCOOH \underset{H_2O}{\rightarrow} 2NH_3 + CO_2 \quad (1)$$

In aqueous solution ammonia and carbon dioxide generate a net increase in pH. The reaction has been studied either in buffer-free systems [2–4] or, which is a more frequent case, in buffers. The former eliminates the interference of a buffer with the reaction, the latter the change in pH. A variety of buffers have been used, e.g., phosphate [2,5–13], citrate [5,6,9,11], maleate [5,6], malonate [5], borate [2], Tris [6,7,13,14], HEPES [13,15], MES [13,15]. Buffers interfere with the reaction and determine its scheme and parameters. On the nature of the buffer depend among others: (1) which products are formed in the reaction, e.g., NH_3 + carbamate in citrate and Tris, $NH_3 + CO_2$ in phosphate and maleate [6], (2) urease activity, the Michaelis constant and activation energy resulting from the inhibitory action of the buffer, e.g., phosphate buffer competitive at pH 7.0 [5], citrate buffer uncompetitive at pH 7.0 [11], Tris, HEPES and MES noninhibitory [13], (3) susceptibility of urease to inhibition by substrate, e.g.,

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$K_S = 420$ mM in phosphate, $K_S = 6.25$ M in citrate [11], (4) the type of inhibition by product, e.g., noncompetitive with $K_P = 14$ mM in phosphate, competitive with $K_P = 10$ mM in citrate [9], (4) the optimum pH, e.g., 6.7–7.6 in phosphate, 6.5–6.7 in citrate, 8.0 in Tris [6].

Phosphate buffer has been widely applied in kinetic studies of urease. It was observed as early as 1934 by Howell and Sumner [16] that phosphate buffer inhibits urease competitively at pH 7.0. This observation has been later confirmed by several authors [4,5,8,12,15], although it was also reported that this inhibition is of partially mixed type [11]. Kistiakowsky et al. [5] reported that at pH 7.48 phosphate buffer has no detectable inhibitory action on urease and that this inhibition increases rapidly with decreasing pH. It was concluded from this behaviour of the system that the true inhibitors in phosphate buffer are some less ionized species than HPO_4^{2-}. Some authors have suggested that responsible for urease inhibition in phosphate buffer is $H_2PO_4^-$ ion [8,15], some have suggested H_3PO_4 [17]. Phosphate buffer at neutral pH is a relatively weak inhibitor of jack bean urease ($K_i = 10$ mM at pH 7.0 [11], 21 mM at pH 6.96 [18], $K_{i,H_2PO_4^-} \approx 17$ mM at pH 7.07 [8]). Todd and Hausinger [15] in their study of the inhibition of bacterial urease *Klebsiella aerogenes* by phosphate buffer over the pH range 5.0–7.5 concluded that: (1) between pH 5.0 and 7.0 the inhibition is competitive ($K_{i,buffer} \approx 30$ mM at pH 7.0), and the values of $-\log K_{i,buffer}$ are pH-dependent exhibiting a slope of -1 from pH 5.0 to 6.3 and a slope of -2 from pH 6.3 to 7.0, thus revealing that the protonation of two groups of the system of $pK = 6.3$ and < 5.0 is necessary for the process, (2) at pH > 7.0 the inhibition is not purely competitive, and (3) at pH < 5.0 urease is labile. The authors did not define precisely which component of the system is characterized by $pK = 6.3$.

In the kinetic studies of urease in phosphate buffers of different pHs attention should be paid to the fact that pH, concentrations and ionic strengths of the buffers do not vary independently. Such studies always ignore certain minor differences between the solutions. The partially mixed inhibition of urease by phosphate buffer pH 7.0 reported in Ref. [11] seems to originate from disregard for this fact.

In this study the effect of phosphate buffer in the range of pH 5.80–8.07 on jack bean urease was reinvestigated in an attempt to elucidate the mechanism of phosphate inhibition.

2. Materials and methods

The jack bean urease was Sigma type III of specific activity 33 units/mg protein. One unit of activity corresponds to the amount of the enzyme that liberates 1.0 μmol NH_3 from urea per minute at pH 7.0 and 25°C. Urea, sodium phosphates ($NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 12H_2O$) and EDTA, all of them of analar grade, were obtained from POCh, Gliwice, Poland. Phosphate buffers pH 5.80, 6.02, 6.22, 6.45, 6.72, 6.96, 7.14, 7.35, 7.49, 7.62, 7.93 and 8.07 were prepared by mixing the above phosphates. The above given pHs were measured in 22 mM buffer solutions with a pH-meter. Phosphate buffers of a given pH and of needed concentrations were prepared from the stock solution by diluting. Each buffer contained 1 mM EDTA added upon dilution.

The initial reaction rates of urease-catalyzed hydrolysis of urea were measured at each pH in the ranges of concentration of buffers and of urea selected accordingly to the activity of urease at given pHs, between 22–155 mM for the buffers and 2–300 mM for urea. The volume of the reaction mixtures was 25 cm^3, and the concentration of urease was 0.025 mg/cm^3. The reaction was initiated by addition of concentrated urease solution to the urea–phosphate buffer solutions. The initial reaction rates were determined by measuring the amount of ammonia by the phenol–hypochlorite method [19] in samples removed from the reaction mixtures at time intervals. The rates were expressed in mmol

NH_3/min mg protein. The measurements were performed at 25°C.

3. Results

The measured initial rates of urease-catalyzed hydrolysis of urea at each phosphate buffer concentration at each pH in the studied range 5.80–8.07 were fitted to the Michaelis–Menten equation by nonlinear regression. The obtained values of the Michaelis constant K_M and of the maximum reaction rate V_{max} demonstrated that the inhibitory action of the buffer decreases with an increase in pH, and that at pH between 5.80 and 7.49 the buffer is a competitive inhibitor of urease. From pH 7.62 as the pH is further raised the buffer exerts some very weak inhibition on the enzyme. The type of this inhibition (presumably no inhibition at all) is difficult to analyze, as at the limits of buffering capacity the buffer changes its pH upon dilution, e.g., at pH 7.93 by about 0.2 on dilution from 155 to 22 mM. The ratio K_M/V_{max} at each pH plotted against phosphate buffer concentration (Fig. 1) illustrate the above observations. From the plots in Fig. 1 for the competitive

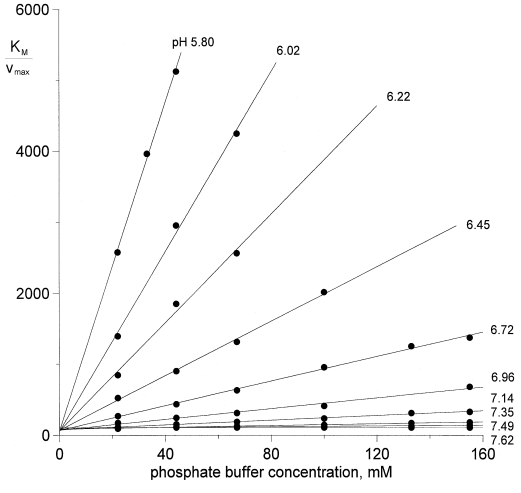

Fig. 1. The ratio of the Michaelis constant to the maximum reaction rate K_M / V_{max} plotted against phosphate buffer concentration for pHs between 5.80 and 7.62.

inhibition at pH between 5.80 and 7.49 the inhibition constants of the buffer $K_{i,buffer}$, and of the Michaelis constants extrapolated to buffer concentration 0 mM K_M^0, were calculated. K_M differs from K_M^0 in that its value reflects the inhibitory effect of both the concentration of the inhibitor and of pH, whereas K_M^0 depends only on pH. Thus, determined constants: K_M^{22} (obtained for 22 mM buffers), V_{max}, K_M^0 and $K_{i,buffer}$ are listed in Table 1.

The logarithmic plots of the above constants vs. pH allow to analyze the process of inhibition of urease by phosphate buffer. By applying the Dixon rules [20] concerning the effect of pH on enzymes, the ionization constants of the components of the process were obtained. Fig. 2a shows the plot of pK_M^{22} vs. pH whose straight lines, one with a slope of zero and the other one with a slope $+1$ intersect at pH 7.2. The plot corresponds to that of log $[H_2PO_4^-]$ vs. pH in 22 mM phosphate buffer (Fig. 2b), and the value 7.2 corresponds to $pK_{H_2PO_4^-} = 7.2$. This finding provides evidence that the true inhibitor in phosphate buffer is $H_2PO_4^-$ ion. The lower is its concentration in the buffer upon approaching pH 7.2, the weaker becomes the inhibitory action of the buffer (K_M^{22} decreases), and when the ion gets deprotonated at pH 7.2, the buffer gradually loses its power of inhibition. The same patterns with the intersection point near pH 7 ($pK_{H_2PO_4^-}$ is known to be affected by ionic strength and buffer composition) were obtained for each pH series at all the studied buffer concentrations (data not shown). The inhibition constants recalculated for $H_2PO_4^-$, $K_{i,H_2PO_4^-}$, are added to Table 1.

Fig. 3 presents the plots: $pK_{i,buffer}$ vs. pH (closed circles) and $pK_{i,H_2PO_4^-}$ vs. pH (open circles). The straight lines of the plot: $pK_{i,buffer}$ vs. pH of the slopes -1 and -2 intersect at pH 6.5. The plot is similar to that reported for *K. aerogenes* urease [15], revealing the existence of an ionizable group with a similar ionisation constant pK. The plot of pK_M^0 vs. pH in Fig. 4a shows that this value is the ionization constant of the enzyme, $pK_E = 6.5$. The plot $pK_{i,H_2PO_4^-}$

Table 1
Kinetic parameters of the studied urease–urea–phosphate buffer (pH 5.80–8.07) system

pH	K_M^{22} [mM] (22 mM buffer)	V_{max} [(mmol NH_3)/(min mg)] (22 mM buffer)	K_M^0 [mM] (0 mM buffer)	$K_{i,buffer}$ [mM]	$K_{i,H_2PO_4^-}$ [mM]
5.80	95	0.0367	2.3	0.54	0.53
6.02	51	0.0366	2.4	1.0	0.96
6.22	34	0.0407	2.5	1.6	1.5
6.45	23	0.0444	2.8	3.6	3.1
6.72	12	0.0443	3.0	7.7	5.9
6.96	8.0	0.0466	3.3	19	12
7.14	5.7	0.0490	3.8	47	24
7.35	4.5	0.0450	3.8	132	55
7.49	4.0	0.0392	3.8	362	123
7.62	3.8	0.0349	3.8[a]	–	–
7.93	3.3	0.0292	3.3[a]	–	–
8.07	2.8	0.0268	2.8[a]	–	–

[a] Values obtained in 22 mM buffer.

vs. pH on the other hand, forms two straight lines of the slopes -1 and -2 with the intersection point at pH 7 (corresponding to $pK_{H_2PO_4^-}$), which confirms the earlier conclusion (Fig. 2) that $H_2PO_4^-$ ion is the inhibitor in the system. Fig. 4a provides further information on urease: pK_E is little altered upon combination of the enzyme with the substrate (from 6.5 to 6.7), K_M^0 is only moderately affected by pH, i.e., a small decrease in K_M^0 is observed upon protonation of a group with $pK_E = 6.5$ (from 3.8 to 2.4 mM), and the inhibition of urease by H^+ and OH^- ions is noncompetitive only at pH between 7 and 8.

The ionization constants of the enzyme–substrate complex were determined by nonlinear

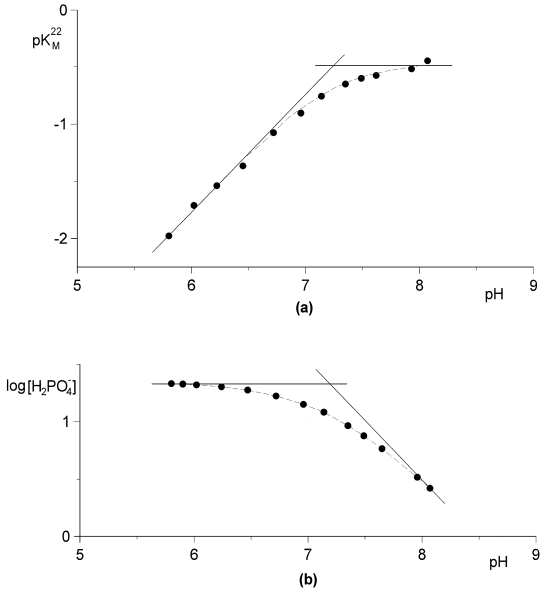

Fig. 2. Logarithmic plots of: (a) the Michaelis constant determined in 22 mM phosphate buffers K_M^{22} vs. pH, (b) concentration of $H_2PO_4^-$ ion calculated for 22 mM phosphate buffers vs. pH.

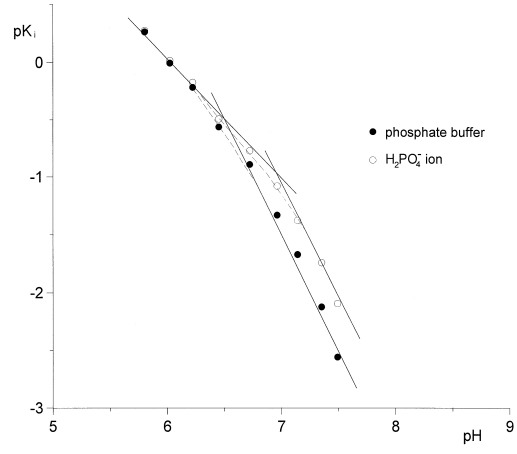

Fig. 3. Logarithmic plots of: the inhibition constant of phosphate buffer $K_{i,buffer}$ vs. pH (closed circles ●), and the inhibition constant of $H_2PO_4^-$ ion $K_{i,H_2PO_4^-}$ vs. pH (open circles ○).

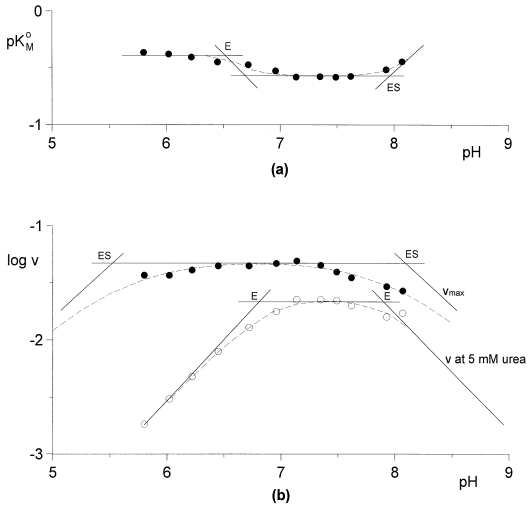

Fig. 4. Logarithmic plots of: (a) the Michaelis constant extrapolated to 0 mM phosphate buffers K_M^0 vs. pH, (b) the maximum reaction rate V_{max} vs. pH (closed circles ●), and the reaction rate measured at low substrate concentration (5 mM urea) V vs. pH (open circles ○).

regression applied to pH-dependence of V_{max} according to Eq. (2) [20]:

$$V_{max}(pH) = \frac{V_{max}(pH_{opt})}{1 + \frac{K_{ES1}}{[H^+]} + \frac{[H^+]}{K_{ES2}}} \quad (2)$$

Their values are: $pK_{ES1} = 8.1$ and $pK_{ES2} = 5.5$ of which the former confirms that in Fig. 4a. The results are shown graphically in Fig. 4b (closed circles). The optimum pH of the reaction equal to 7.14 is in agreement with the general observation that the optimum pH of urease is close in value to the pK of the applied buffer [6]. Fig. 4b also presents the logarithmic plot of the reaction rate measured at low substrate concentration (5 mM urea) vs. pH (open circles). This plot indicates that ionization constants of the enzyme pK_E are close to 6.7, which is comparable with that in Fig. 4a, and 7.9.

4. Discussion

In the studied range of pH 5.80–8.07 phosphate buffer was found to act on jack bean urease as a competitive inhibitor at pH between 5.80 and 7.49. pH 6.5 was found to correspond to the ionization constant of the enzyme, pK_E. In the solutions of further increased pH, starting from pH 7.62 the competitive inhibition was not observed. It was also shown that the inhibitory component of phosphate buffer is $H_2PO_4^-$ ion. The binding site it competes with urea for is the active site of the enzyme. The active site of urease contains two Ni(II) ions [21]. The X-ray crystal structure analysis of urease from *K. aerogenes* [22,23] demonstrated that the two Ni ions are 3.5 Å apart and bridged by carbamylated Liz-217 (through O-atoms). Ni-1 is further coordinated by His-246 and His-272 (through N-atoms), while Ni-2 is further coordinated by His-134 and His-136 (through N-atoms), Asp-360 (through O-atom) and by a water molecule. Other essential active site residues are His-219, His-320 and Cys-319, of which the last two belong to the mobile flap that covers the active site. In the mechanism of bacterial urease catalysis, based on the classic model proposed by Blakeley and Zerner [1] and Dixon et al. [24] for plant urease and extended for bacterial urease by Jabri et al. [22] and Mobley et al. [23], it is assumed that urea coordinates via its carbonyl oxygen to Ni-1 with stabilization provided by His-219. The Ni-2 coordinated water, either activated by a general base or as a Ni-bound hydroxide, binds to the urea carbonyl to form an intermediate from which, with the participation of a general acid protonating the urea nitrogen, ammonia and carbamate are released. Karplus et al. [25] in their newly proposed mechanism of urease catalysis based on a reverse protonation scheme, postulate that pK_E values: ~ 6.5 and ~ 9 [26] belong to His-320 acting as a general acid and to the Ni-bound water, respectively. This implies that His-320 must be protonated and the Ni-bound water deprotonated for the catalytic process.

By comparison with the above cited model of urease catalytic mechanism it might be postulated that the values of pH: 6.5 and 7.6 obtained in this study, characterizing the behaviour of the

enzyme in phosphate buffer over the pH range 5.80–8.07 are equivalents of the pK_E values of *K. aerogenes* urease, the former corresponding to the histidine acting as a catalytic acid, the latter to the Ni-bound hydrolytic water. Although the latter value is lower than that of *K. aerogenes* urease, it coincides with those of other urease-related metallo-enzymes referred to by Karplus et al. [25], i.e., Ni-substituted bacterial phosphotriesterase, $pK_E = 7.4$ [27] and yeast adenosine deaminase, $pK_E = 7.5$ (the other $pK_E = 6.5$) [28]. Also, the obtained value near 7.6 was confirmed in this study by the value 7.9 determined from the pH-dependence of the initial reaction rate at low substrate concentration. The constants characterizing urease–urea–phosphate buffer system obtained in this study are compared in Table 2 with those reported in the literature. The data in Table 2 are only partly confirmatory proving the complexity of the problem.

In view of the foregoing the inhibition of urease by $H_2PO_4^-$ ion can be explained as follows (Fig. 3): at pH > 6.5 the active site group of the enzyme (catalytic histidine) is deprotonated and repulses $H_2PO_4^-$ ion, which results in weak inhibition. At pH < 6.5 the group is protonated and consequently the ion has free access to the site and the inhibition attains its strongest stage. The inhibition constants recorded for this stage (~0.5 mM) classify phosphate buffer as an inhibitor of moderate strength, similar to β-mercaptoethanol (K_i = 0.72 mM) [8] and boric acid (K_i = 0.12 mM) [18], as compared to strong inhibitors like acetohydroxamic acid (K_i = 4 μM [34], 34 μM [35]) and fluoride ion (K_i = 30 μM [36]). β-mercaptoethanol and phosphate buffer pH 7.07 were shown to be mutually exclusive competitive inhibitors of urease [8], and β-mercaptoethanol independently was shown to inhibit the enzyme by binding to nickel ions in the active site

Table 2
Constants characterizing urease–urea–phosphate buffer system

K_M^0 [mM] pH 7.0	$K_{i,buffer}$ [mM] pH 7.0	$K_{i,H_2PO_4^-}$ [mM] pH 7.0	pK_{E1}	pK_{E2}	pK_{ES1}	pK_{ES2}	pH_{opt}	Ref.
Jack bean urease								
–	–	–	9.0	6.6	9.0	–	–	[29]
–	–	–	–	–	9.2	6.1	–	[30]
1.25	–	–	7.5	5.8	8.10	5.11	–	[31]
1.8	–	–	–	–	8.14	4.44	–	[3]
2.9	–	–	6.5	2.0	9.0	6.25, 3.0	7.25	[24]
–	–	17[a]	–	–	–	–	–	[8]
4.2	10	–	–	–	–	–	7.2	[11]
1.63	10.8	–	–	–	–	–	–	[12]
2.47	–	–	–	–	9.07	5.62	7.2	[4]
3.3[b]	21[b]	13[b]	–	–	–	–	–	[18]
3.6[c]	31[c]	14[c]	7.6–7.9	6.5	8.1	5.5	7.14	[d]
Bacterial ureases								
1.13	–	–	8.1	6.65,5.6	8.1	6.65	7.5	[32]
13	–	–	9.2–9.4	6.25	–	–	–	[33]
–	–	–	8.85	6.55	–	–	–	[26]
2.4	~30	–	–	–	–	–	–	[15]

[a] Value for pH 7.07.
[b] Values for pH 6.96.
[c] Values estimated for pH 7.0 from Figs. 3 and 4; N.B. $K_{i,H_2PO_4^-}$ estimated from Fig. 3 for pH 7.07 is 18 mM, confirming that determined by Dixon et al. [8].
[d] This paper.

[8,37]. It can therefore be assumed that $H_2PO_4^-$ ion occupies the same binding site, possibly by coordinating to one or both Ni ions: Ni–O^-–$PO(OH)_2$ or Ni–O^-–$P(OH)_2$–O–Ni. At pH near 7.6–7.9 the other active site group (Ni–water) gets deprotonated and by repulsing $H_2PO_4^-$ ion protects the enzyme from the phosphate inhibition.

The recorded behaviour of jack bean urease–urea–phosphate buffer system, consistent with that of *K. aerogenes* urease [15] confirms similarity of the active site and of the mechanism of action of ureases of different origins [25,37,38].

Acknowledgements

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