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pH DEPENDENCE OF THE KINETIC PARAMETERS OF NITROTYROSYL-ASPARAGINASE

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In a number of papers devoted to the study of the enzyme L-asparaginase the question is discussed of the possible localization of the catalytically important hydroxy group of the tyrosine residue in the active center of the enzyme [1-3]. The change in the nature of the pH dependence of the activity when the tyrosine residues are subjected to selective chemical modification leading to a change in the pK value of the phenolic hydroxyl shows its direct participation in the catalytic act [2]. In the present paper we consider the influence of the pH on the kinetic parameters of the hydrolysis of L-asperagine.

The values of the Michaelis constant, K_M, for the hydrolysis of L-asparagine catalyzed by nitrotyrosyl-asparaginase, obtained by using three buffer solutions in the range of pH values from 4.50 to 8.85 are given in Fig. 1. The observed threefold increase in K_M takes place at pH values of 5.0-5.5 at which nitrotyrosyl-asparaginase shows its optimum activity in the reaction under consideration [2].

It followed from a comparison of the kinetic parameters of the reaction obtained by using modified and native L-asparaginase (K_M = 0.008 mmole; $k_{cat} = 0.41 \cdot 10^3$ min^{-1}; pH 8.0) that the nitration of the tyrosyl residue leads to a marked increase in K_M (by more than an order of magnitude) and an insignificant change in k_{cat} (Fig. 2). It follows from Fig. 2 that the catalytic constant k_{cat} changes little with a change in the composition of the buffer in the given pH range.

Thus, since the modification of the tyrosine residue of the enzyme with tetranitromethane leads to a marked change in K_M, it may be assumed that the tyrosine residue participates in the formation of the enzyme—substrate complex.

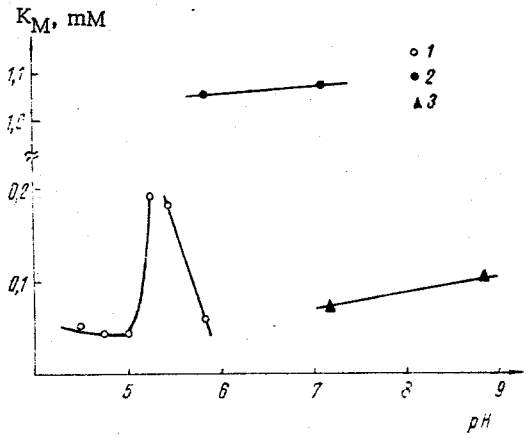

Fig. 1. Dependence of K_M on the pH: 1) sodium acetate buffer; 2) universal buffer; 3) potassium phosphate buffer.

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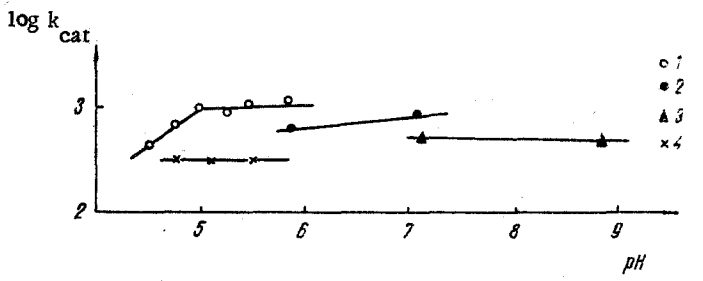

Fig. 2. Dependence of the logarithm of the catalytic constant on the pH: 1-3) nitrotyrosyl-asparaginase; 4) native asparaginase.

The inadequate sensitivity of the method of analysis used to NH_4^+ did not enable us to obtain the complete dependence of K_M on the pH in universal buffer. The values of K_M obtained in a narrow pH range differ sharply from those obtained in phosphate and acetate buffers.* The latter may be connected with the inhibiting action of the boric acid present in the universal buffer. The inactivating influence of a borate-containing buffer has been shown by Parrott and Shifrin [4] for trinitrophenylated L-asparaginase. According to the literature [5], no such specific influence of universal buffer on K_M is observed for the native enzyme.

We have determined the kinetic parameters of the hydrolysis of L-asparagine in the presence of boric acid [experimental conditions: pH 8.0; 37°C; 0.05 M phosphate buffer; $(E_o) = 1.4 \cdot 10^{-7}$ M]:

$[H_3BO_3] \cdot 10^3$, M	K_M, mmole	V_{max}, mmole/min
—	0,049*	0,076
8,58	0,048	0,078
36,14	0,012	0,060

At a concentration of H_3BO_3 close to that used in the universal buffer, decreases in V_{max}, in the initial rate of hydrolysis ($V_i/V_o = 0.86$), and in K_M are observed, and therefore the sharp rise in K_M in universal buffer is apparently connected with a complex effect of the whole buffer system.

EXPERIMENTAL

In nitration we used a $1.9 \cdot 10^{-5}$ M solution of *E. coli* L-asparaginase of the Riga Medical Preparations Factory in 0.05 M phosphate buffer, pH 8.0. The modification was carried out with a 20-fold excess of tetranitromethane, using a 10% (by volume) solution of "Serva" tetranitromethane in 96% ethanol. The nitration was carried out at room temperature with constant stirring for 60 min. It was stopped by eliminating the excess of reagent by gel filtration through a column of Sephadex G-25 (1.5 × 35.0 cm).

The molar concentration of the 3-nitrotyrosine residues in the asparaginase solution was determined from the absorption at 428 nm ($\varepsilon^M_{428} = 4100$) [6].

The hydrolase activity of the 3-nitrotyrosyl-asparaginase was determined by the direct Nesslerization of the product of the enzymatic reaction — ammonia [7]. The concentration of protein in the solution was determined by Lowry's method [8]. The incubated mixture used in the experiments consisted of 0.8 ml of the solution of L-asparagine in buffer, 0.6 ml of buffer, and 0.2 ml of enzyme solution. The reaction was stopped by the addition of 0.4 ml of a 25% solution of trichloroacetic acid. All the solutions were prepared in double-distilled water. Reagents of "kh. ch." ["chemically pure"] and "ch.d.a." ["pure for analysis"] grades were used.

The initial rate V_o of the hydrolysis of L-asparagine was measured from the accumulation of the NH_4^+ ion, the concentration of which was determined by the phenol–hypochlorite method [9] on an SF-4 spectrophotometer; each value of V_0 is an average of 4-5 measurements. The consumption of substrate at the moment of stopping the reaction (reaction time 30 sec) did not exceed 20% of the initial concentration. The measurements were performed at a temperature of 37 ± 0.1°C.

*These results and those shown in Fig. 1 were obtained on different portions of nitrated enzyme.

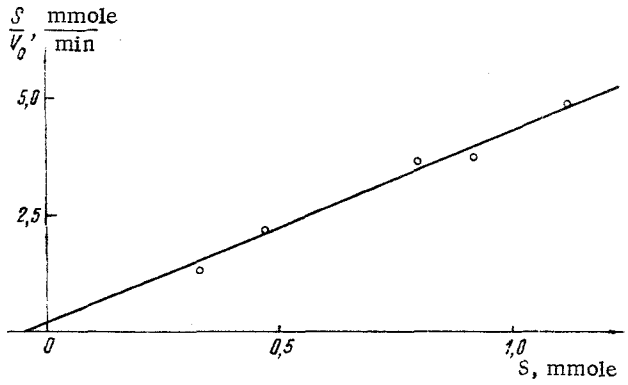

Fig. 3. Linearization of the dependence of the initial rate on the concentration of the substrate in plots of S/V_0 versus S. pH 5.85, 1 N sodium buffer, enzyme — nitrotyrosyl-asparagine.

The possibility of rectifying the curves in plots of S/V_0 versus S permitted the conclusion that in the range of concentrations of substrate studied the hydrolysis of L-asparagine follows the Michaelis–Menten equation (Fig. 3).

The most probable values of the kinetic parameters K_M and k_{cat} were determined by the method of least squares with expansion in a Taylor series of the sum of the squares of the absolute residue, i.e., the difference between the observed and the calculated magnitudes according to a program proposed by M. Sakoda and K. Hiromi using a WANG 2200 computer. The coefficient of linear correlation was not less than 0.95.

The following buffer solutions were used in the investigation: 1 N sodium acetate (pH 4.50-5.84), 0.04 M universal buffer (pH 5.84-7.15), and 0.05 M potassium phosphate buffer (pH 7.10-8.85).

SUMMARY

The kinetic parameters, K_M and k_{cat} of the hydrolysis of L-asparagine by nitrotyrosyl-asparaginase have been determined over a wide pH range of 4.50-8.85. It has been shown that the modification of the asparaginase leads to a marked increase in the Michaelis constant and to an insignificant change in k_{cat}. A hypothesis has been put forward concerning the possible participation of the hydroxy group of the tyrosine residue in the formation of the enzyme–substrate complex.

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