Kinetics of Hydrolytic Reaction Catalyzed by Crystalline Bacterial α-Amylase. I. The Influence of pH

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It is well known that enzyme reactions consist of two elementary processes, namely formation of an intermediate enzyme-substrate complex and the subsequent breakdown of the complex into reaction products and free enzyme. These processes are represented kinetically by two parameters, i. e., the Michaelis constant and the rate constant for the breakdown of the complex, respectively. Systematic investigation of the influence of various extrinsic factors such as temperature, pH, ionic strength, dielectric constant of the medium, pressure, inhibitors, etc., upon these parameters, provides a means for elucidating the mechanisms of the enzyme reactions. Recently certain enzyme reactions have been studied along these lines and important information on the mechanisms has been obtained[1-4].

Bacterial amyloclastic α-amylase of *Bac. amyloliquefaciens* Fukumoto has long been studied by Fukumoto and his co-workers, and was crystallized by Hagihara[5]. Although various investigations have been made about this crystalline enzyme[6,7], few kinetic studies have been reported on it[8,9]. In our laboratory the influence of various factors on the rate of reactions catalyzed by this crystalline enzyme has been studied from the kinetic point of view, using substrates as simple and pure as possible. In the present report, the influence of pH on the reaction rate was investigated with the hope of obtaining a certain clue for elucidating the mechanism of the enzyme reaction involved.

Experimental

Materials. — 1) *Substrate.* — Potato amylose (degree of polymerization ca. 900) manufactured by Nagase Sangyô Co. Ltd. was used. 2) *Enzyme.* —Bacterial amyloclastic α-amylase (six-times recrystallized; molecular weight 45,000) was kindly provided by Professor J. Fukumoto of Osaka City University. The enzyme was dissolved in four-times redistilled water, and the solution was stored in a refrigerator as the stock solution. The enzyme concentration in this solution was determined from the optical density at 280 mμ to be 3.88×10^{-6} M. The enzyme activity in this solution was constant throughout the whole experiment. 3) *Buffer solution.*—A solution of 0.15 M veronal sodium and sodium acetate was mixed with 0.1 N hydrochloric acid in various ratios. This buffer solution was confirmed to have no specific effect on the reaction rate and on the determination of products.

Methods. — A mixture of 10 ml. of amylose solution in 0.5 N sodium hydroxide and 13 ml. of the buffer was neutralized with 5 ml. of 1 N hydrochloric acid in a reaction vessel immersed in a thermostat adjusted to 25°±0.05°C, unless otherwise stated. After equilibrium temperature was reached, 2 ml. of the enzyme solution (containing an appropriate amount of the stock solution in 1/300 M calcium acetate) was added quickly to make the total volume 30 ml. The reaction mixture was stirred mechanically. Two ml. of the reaction mixture was pipetted into 1 ml. of 0.5 N sodium hydroxide to stop the reaction at intervals of one minute during the first four minutes of the reaction.

The reducing end-groups produced as the result of the reaction were determined photometrically with 3,5-dinitrosalicylate[10]. In lower concentrations of the products (below 1.4×10^{-4} mole of reducing end group per liter), the atomspheric oxygen made the determination impossible because of its oxidizing action. Therefore, the nitrogen was bubbled through the reaction mixture and the reagents used in the analytical procedure before and during the experiment.

The pH of the reaction mixture was measured by means of a quinhydrone electrode at the same temperature as that of the reaction. The error of the measurements was within ±0.01 pH. The concentrations of substrate and products were

1) R. Lumry, E. L. Smith and R. R. Glantz, *J. Am. Chem. Soc.*, **73**, 4330 (1951).
2) R. Lumry and E. L. Smith, *Discussions Faraday Soc.*, No. **20**, 105 (1955).
3) K. J. Laidler, ibid., No. **20**, 83 (1955).
4) A. Stockell and E. L. Smith, *J. Biol. Chem.*, **227**, 1 (1957).
5) B. Hagihara, *Proc. Japan Acad.*, **27**, 346 (1951).
6) T. Yamamoto, *Bull. Agr. Chem. Soc. Japan*, **19**, 121 (1955).
7) T. Yamamoto, ibid., **20**, 188 (1956).
8) J. Osugi, *Rev. Phys. Chem. Japan*, **22**, 66, 71 (1952).
9) J. Osugi and K. Hiromi, ibid., **22**, 76 (1952).
10) G. N. Smith and C. Stocker, *Arch. Biochem.*, **21**, 95 (1949).

expressed in mole per liter of the bond hydrolyzable and hydrolyzed, respectively. The initial reaction rate was estimated from the tangent of the reaction-time curve at zero time. The error in this procedure was minimized by choosing appropriate concentrations of enzyme so that the curves would not show any marked curvature.

Results

The initial rate v was in good proportionality with the enzyme concentration [E], as shown in Fig. 1. The plots of the reciprocals of the initial rate v versus substrate concentration [S] were found to fulfil the linear relationship required by the Lineweaver-Burk equation[11],

$$\frac{1}{v} = \frac{\bar{K}_m}{\bar{k}_2[E]} \cdot \frac{1}{[S]} + \frac{1}{\bar{k}_2[E]} \qquad (1)$$

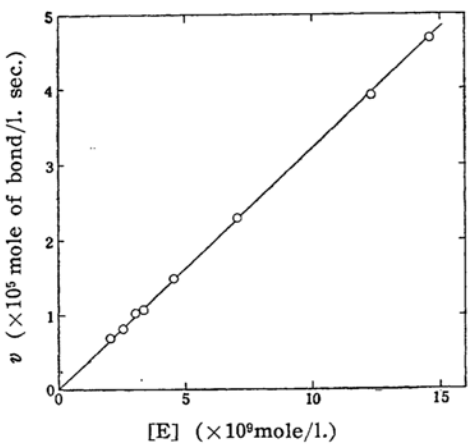


Fig. 1. Plot of initial rate versus enzyme concentration (substrate concentration, 0.0154 mole of bond/l.; 25°C; pH 5.41).

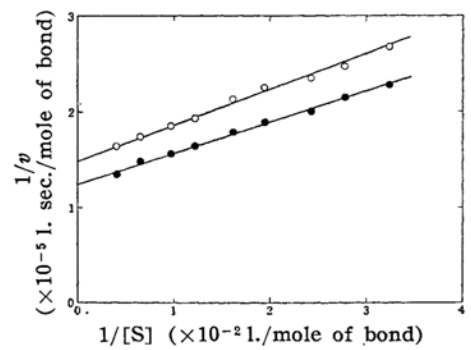


Fig. 2. Plot of $1/v$ versus $1/[S]$ at 25°C. ○ pH 7.51, ● pH 4.84

This relationship holds for any pH studied (see Fig. 2). The concentration of the enzyme [E] being known, the apparent Michaelis constant $\bar{K}_m$, and the apparent rate constant $\bar{k}_2$ for the breakdown of the enzyme-substrate complex, were obtained from the formula. The values of $\bar{K}_m$ and $\bar{k}_2$ at various pH's are summarized in Table I. In Fig. 3, $\log 1/\bar{K}_m$ and $\log \bar{k}_2$ are plotted versus pH. $\bar{K}_m$ is constant over the pH range examined (pH 3.6~8.4), while $\bar{k}_2$ decreases on both the acid and the alkaline side.

TABLE I

VALUES OF $\bar{K}_m$ AND $\bar{k}_2$ AT VARIOUS pH'S AT 25°C

pH	[E]×10⁹ mole/l.	$\bar{K}_m$×10³ mole of bond/l.	$\bar{k}_2$×10⁻³ sec.⁻¹
3.59	7.05	2.3±0.4	0.37±0.03
3.73	9.94	2.3±0.3	0.61±0.02
4.16	3.38	2.3±0.2	1.91±0.05
4.84	2.56	2.6±0.2	3.20±0.04
5.60	2.56	2.6±0.2	3.94±0.15
5.95	2.06	1.8±0.2	3.91±0.11
6.25	1.54	2.1±0.2	3.80±0.10
6.92	2.06	2.6±0.1	3.90±0.05
7.51	3.08	2.4±0.2	2.18±0.04
8.23	4.58	2.1±0.3	0.75±0.02
8.40	14.6	2.6±0.2	0.47±0.02

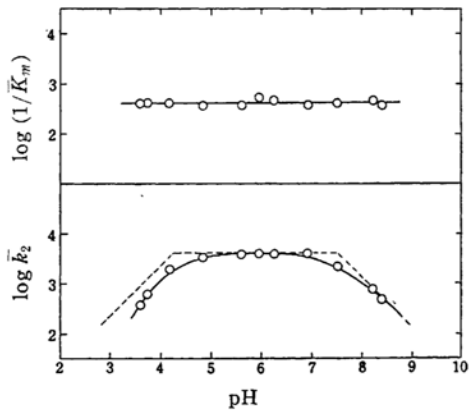


Fig. 3. Plots of $\log (1/\bar{K}_m)$ and $\log \bar{k}_2$ versus pH at 25°C.

The results of another series of experiments are shown in Fig. 4, in which the ratio of the initial rate v at a given pH, to that v_{max} at the optimum pH, measured at constant substrate and enzyme concentration, was plotted against pH. A nearly symmetrical bell-shaped curve was obtained. Neither the shape nor the position of the curve was affected by varying the substrate concentration. The solid line in Fig. 4 represents the theoretical curve which will be described below. The experimental points satis-

11) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

factorily fit the theoretical curve except in the narrow region below pH 4, where the occurrence of a rapid irreversible inactivation of the enzyme was observed. The influence of temperature on the v/v_{max}—pH curve was also investigated, and the results are presented in Table III, which will be discussed below.

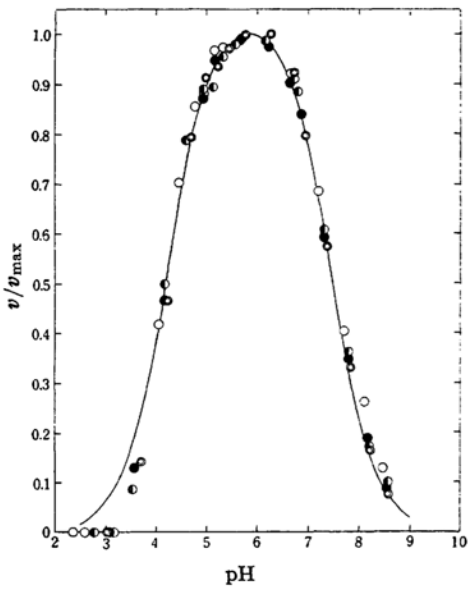
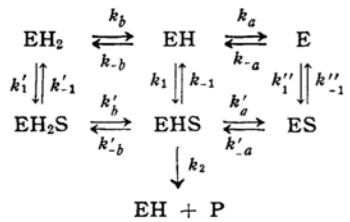


Fig. 4. Plot of v/v_{max} versus pH at various substrate concentrations at 25°C.
○ 0.0247, ◐ 0.0123, ● 0.00617,
◎ 0.00308 mole of bond/l.

Discussion

The results will be analyzed according to the following scheme which has been employed by various authors[12-15]:



where E represents the enzyme in a particular state of ionization, H a proton, S the substrate and P the products. Of the three forms of enzyme-substrate complexes, only the complex EHS is assumed to break down into the reaction products. The various rate constants k's are indicated above.

The initial rate v can be expressed as follows:

$$v=\frac{k_2[E][S]}{K_m\left(1+\frac{K_a}{[H]}+\frac{[H]}{K_b}\right)+[S]\left(1+\frac{K_a'}{[H]}+\frac{[H]}{K_b'}\right)} \quad (2)^*$$

where

$$K_m=(k_{-1}+k_2)/k_1$$

and

$$K_a=k_a/k_{-a}, \quad K_b=k_b/k_{-b}$$
$$K_a'=k_a'/k_{-a}', \quad K_b'=k_b'/k_{-b}'$$

Thus K_a, K_b, K_a' and K_b' are the ionization constants of the two ionizable groups taken into account in the scheme, in the substrate-free enzyme and enzyme-substrate complex, respectively.

Comparing Eqs. 1 and 2, $\bar{K}_m$ and $\bar{k}_2$ are represented in the following formula;

$$\bar{K}_m=K_m\cdot\frac{1+\frac{K_a}{[H]}+\frac{[H]}{K_b}}{1+\frac{K_a'}{[H]}+\frac{[H]}{K_b'}} \quad (3)$$

$$\bar{k}_2=\frac{k_2}{1+\frac{K_a'}{[H]}+\frac{[H]}{K_b'}} \quad (4)$$

From our results which show that $\bar{K}_m$ is constant over a wide range of pH in which $\bar{k}_2$ varies (see Fig. 3), it is concluded that K_a equals to K_a' and K_b equals to K_b' in Eq. 3; that is to say, the ionization of the two groups considered is not affected by the substrate binding. If these ionizable groups were directly involved in the complex formation, their ionization constants would be changed considerably by the combination of the substrate with the enzyme[16]. Thus it may be most reasonable to conclude that these groups in the enzyme molecule are not directly responsible for the complex formation. The variation of $\bar{k}_2$ with pH is in accordance with the prediction of the theory (see Fig. 3 and Eq. 4) and it is evident that the breakdown of the complex occurs only when group A (whose ionization constant is K_a) is undissociated and group

12) S. G. Waley, *Biochim. et Biophys. Acta*, **10**, 27 (1953).
13) R. A. Alberty and V. Massey, ibid., **13**, 347 (1954).
14) K. J. Laidler, *Trans. Faraday Soc.*, **51**, 528 (1955).
15) A. Takamiya, "Kôso-kenkyûhô", edited by S. Akabori, Asakura Shoten, Tokyo (1955), p. 198.

* The exact steady-state treatment[14] leads to the expression which includes the perturbation term in the denominator of Eq. 2. But this term is reasonably neglected in the present case (see the foot-note** below).
16) K. J. Laidler, *Trans. Faraday Soc.*, **51**, 540 (1955).

B (whose ionization constant is K_b) is dissociated.

When K_a equals to K_a' and K_b equals to K_b', Eq. 2 reduces to a simpler form;

$$v=\frac{k_2[\mathrm{E}][\mathrm{S}]}{\left(1+\frac{K_a}{[\mathrm{H}]}+\frac{[\mathrm{H}]}{K_b}\right)(K_m+[\mathrm{S}])} \tag{5}$$

Let v_{max} denote the initial rate at the optimum hydrogen ion concentration $[\mathrm{H}]_{opt}$ with a given substrate concentration [S]. Then differentiation of Eq. 5 with respect to [H] leads to the following relationships;

$$[\mathrm{H}]_{opt}=\sqrt{K_a K_b} \tag{6}$$

$$v_{max}=\frac{k_2[\mathrm{E}][\mathrm{S}]}{(1+2\sqrt{K_a/K_b})(K_m+[\mathrm{S}])} \tag{7}$$

Consequently,

$$\frac{v}{v_{max}}=\frac{1+2\sqrt{K_a/K_b}}{1+\frac{K_a}{[\mathrm{H}]}+\frac{[\mathrm{H}]}{K_b}} \tag{8}$$

Thus v/v_{max} should be a function independent of the substrate concentration over the whole range of pH. The results shown in Fig. 4 obviously fulfil this requirement, again confirming the validity of the theory.

Moreover, several conclusions of considerable importance can be drawn from the foregoing results. (i) The apparent Michaelis constant $\bar{K}_m$ at any pH is equal to the true Michaelis constant K_m (see Eq. 3). (ii) The constancy of $\bar{K}_m$, as pointed out by Morales[17] and Laidler[16], leads to a reasonable conclusion that $k_2 \ll k_{-1}$**, i. e., there is equilibrium between enzyme, substrate and complex. The Michaelis constant may be regarded as the dissociation constant of the enzyme-substrate complex, i. e., $K_m=k_{-1}/k_1$. (iii) The apparent rate constant $\bar{k}_2$ at the optimum pH is equal to $k_2/(1+2\sqrt{K_a/K_b})$ (compare Eqs. 1 and 7), about 5 per cent less than the true rate constant k_2 at 25°C.

The ionization constants of the groups, K_a and K_b, were evaluated[13,15] according to Eq. 8, using the experimental values shown in Fig. 4. These values expressed in pK at various substrate concentrations at 25°C are presented in Table II. The mean values of these are 7.45 for pK_a and 4.22 for pK_b, from which the optimum pH is found from Eq. 6 to be 5.84, which is in good agreement with the experimental value 5.85. The solid line in Fig. 4 represents the theoretical curve drawn according to Eq. 8 using the mean pK values thus obtained. Except for the narrow region below pH 4, where the irreversible inactivation of the enzyme was detected, the coincidence between the theory and the experiment is remarkable.

TABLE II

THE pK VALUES AT VARIOUS SUBSTRATE CONCENTRATIONS AT 25°C

$[\mathrm{S}]\times10^2$ mole of bond/l.	pK_b	pK_a
2.47	4.18	7.49
1.23	4.20	7.45
0.617	4.24	7.45
0.308	4.27	7.41
Mean	4.22	7.45

TABLE III

TEMPERATURE DEPENDENCE OF pK'S

Temp. °C	pK_b	pK_a
6.0	4.44	7.60
12.0	4.29	7.56
18.0	4.38	7.46
25.0	4.24	7.45
32.0	4.28	7.37

Substrate concentration: 0.00617 mole of bond/l.

The pK values of the groups at various temperatures are summarized in Table III. The heats of ionization of these groups are calculated to be $\Delta H_a=4$ kcal. for group A, and $\Delta H_b=2$ kcal. for group B. From these values of pK and ΔH, one can infer the nature of the ionizing groups under consideration by comparing them with the reported values of various ionizing groups found in proteins[18]. Considering some deviations from these values which might be expected in the actual enzyme molecule, it may be reasonable to conclude that group B, having a pK of 4.22 and a ΔH of 2 kcal. is a carboxyl group, and that group A, having a pK of 7.45 and a ΔH of 4 kcal., may be either an imidazolium or an ammonium group (the ΔH value is in favor of the former possibility). As already stated, these groups are not considered to be involved in the complex formation, but to be essential in the breakdown of the complex into reaction products in the forms, respectively, of COO^- and $>NH^+$ (or $-NH_3^+$).

17) M. F. Morales, *J. Am. Chem. Soc.*, **77**, 4169 (1955).

** This is the condition under which the perturbation term which would appear in the denominator of Eq. 2, can be neglected (see ref. 14).

18) J. T. Edsall, "Proteins, Amino Acids and Peptides", Reinhold Pub. Corp., New York (1943), p. 444.

Yamamoto[6] examined the activity of this enzyme treated with several specific reagents, and concluded that the phenoxyl group of the tyrosine residue is essential. Since the pK value of this group lies outside the pH range in which kinetic studies are feasible (see Fig. 4), it is impossible to determine whether this group is involved in the formation or the breakdown of the complex. The above-stated constancy of $\bar{K}_m$ over the pH range examined (3.6~8.4) implies that no ionizable group having its pK in this range is involved in the complex formation. However, it does not exclude the possibility that some group having its pK outside this region may also participate in the formation and/or breakdown of the complex. His conclusion, therefore, is not incompatible with our findings.

Recently mechanisms of certain hydrolytic enzyme reactions have been considered in terms of bifunctional catalysis, or concerted mechanism[19-21]. It seems likely that the acidic (proton-donating) and basic (proton-accepting) groups of the enzyme function as an acid and a base simultaneously in the enzyme-catalyzed hydrolysis. From this point of view, a tentative scheme on the mechanism of this enzyme reaction is presented in Fig. 5.

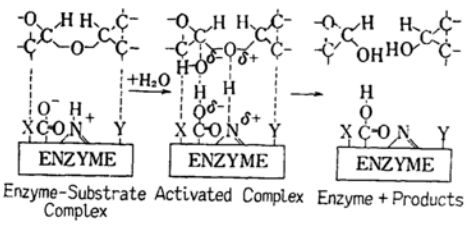


Fig. 5.

In this scheme it is supposed that the imidazolium cation, acting as an acid, attacks the etheric oxygen atom of the glucosidic bond, and simultaneously the carboxylate anion, acting as a base, pulls the proton from the water molecule which is situated between C_1 atom of the glucoside residue at the left and the carboxylate anion. X and Y represent the unknown groups of the enzyme which are responsible for binding the substrate in a geometrically favorable position so that the carboxylate and imidazolium groups will be able to form an activated complex with the substrate. The nature of groups X and Y cannot be determined from the present study. If they are ionizable groups, their pK's must lie either below 3.6 or above 8.4. Another possible mechanism is presented in the scheme[22] shown in Fig. 6.

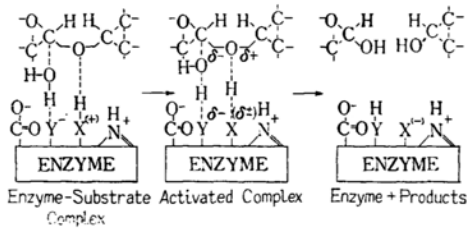


Fig. 6.

The possible charge separation in the activated complex, as depicted in each scheme, can be inferred from the study of the solvent effect upon this enzyme reaction, which will be reported in a later paper.

Summary

The influence of pH on the rate of the hydrolytic reactions of amylose catalyzed by the crystalline bacterial α-amylase of *Bac. amyloliquefaciens* Fukumoto has been examined at various substrate concentrations and temperatures.

The apparent Michaelis constant $\bar{K}_m$ is constant over the pH range examined (3.6~8.4), whereas the apparent rate constant $\bar{k}_2$ for the breakdown of the enzyme-substrate complex into the reaction products and free enzyme decreases on both the acid and the alkaline side of the optimum pH 5.85. The relative rate - pH curves are not affected by the substrate concentration. From these facts, it is concluded that at least two ionizable groups in the enzyme are involved in the breakdown of the complex, although these groups are not directly responsible for the formation of the enzyme-substrate complex.

The ionization constants and the heats of ionization of the two groups involved in the reaction have been determined. From these values, the ionizing groups are inferred to be the carboxyl and imidazole (or amino) groups. The ionized

19) C. G. Swain and J. F. Brown, *J. Am. Chem. Soc.*, **74**, 2534, 2538 (1952).
20) H. Lindley, *Advances in Enzymol.*, **15**, 271 (1954).
21) K. J. Laidler, "Introduction to the Chemistry of Enzymes", McGraw-Hill Book Co., New York (1954), p. 155.
22) S. Ono, K. Hiromi and Y. Yoshikawa, *Symposia on Enzyme Chem. Japan*, **12**, 1, 291 (1957).

form of the former and the positively charged form of the latter are considered to be essential for the breakdown process.

Tentative mechanisms of the reaction are proposed.

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