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STUDIES ON THE KINETICS OF ENZYMIC REACTIONS, III

Thermo-analytical Studies of the Degradation of Amylose by Action of Bacterial α-Amylase

By Jiro Osugi and Keitaro Hiromi

The previous reports[1] treated upon the mechanism and kinetics of the degradation of amylose by action of bacterial α-amylase. The present report concerns to the thermal nature of the enzymic reaction which is studied by the method of the thermal analysis[2].

Experimentals

A amylose solution was prepared from soluble starch or potato starch according to the hot water extraction proposed by K. H. Meyer[3].

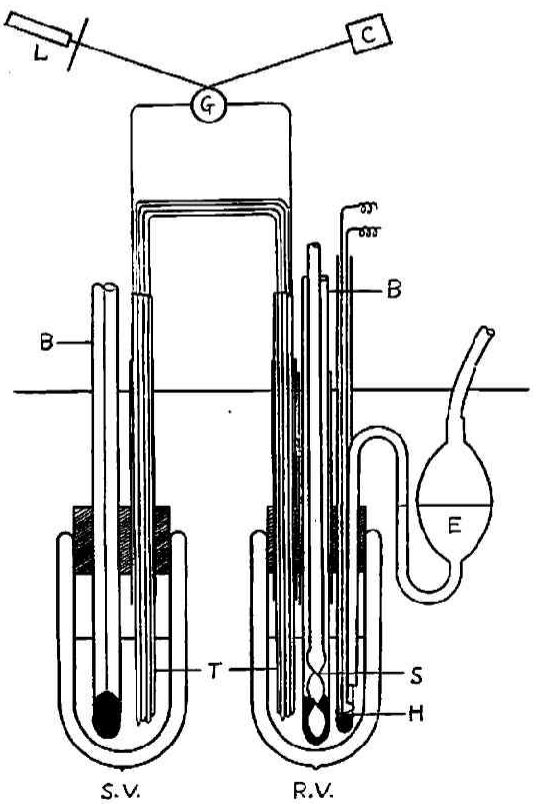

Fig. 1

S. V.: Standard vessel containing water
R. V.: Reaction vessel containing 50 ml of substrate solution
E : 10 ml of diluted enzyme solution
S : Stirrer
T : Five copper-constantan thermo-couples connected in series
B : Beckmann thermometers
H : Heater
G : Galvanometer
L : Light source
C : Camera

1) J. Osugi, *Proc. Japan Acad.*, 27, 241, 245 (1951)
2) S. Horiba, *This Journal*, 11, 439 (1937)
3) K. H. Meyer et al., *Helv. Chim. Acta.*, 23, 860 (1940)

A stock enzyme solution, crystalline bac. α-amylase in 0.01 M calcium acetate solution, was kindly given by Dr. J. Hukumoto.

20 ml of a diluted enzyme solution and 100 ml of a substrate solution were both divided into two equal amounts and for each, the reaction was carried out under the same condition. The reactions separately were measured by two different methods, thermal and analytical.

In order to make the thermal measurement easier, the reaction was performed at considerably rapid rate. The reproducibility of the experiments was sufficient.

(a) Thermal measurement

Very small amount of the heat liberated in the reaction (the temperature rise was usually 0.005°~0.01°C) made the measurement by means of a Beckmann thermometer inappropriate owing to its time-lag and inaccuracy in this small temperature change, so that thermocouples and galvanometer were employed. The apparatus is shown in Fig. 1.

The standard and the reaction vessels (Dewar's vessels) were dipped in a thermostat (40°±0.005°C) and the reaction was commenced by pouring an enzyme solution into the vessel. Temperature rise due to the reaction heat was recorded automatically with thermocouples and galvanometer on the film. The temperature equivalent of the galvanometer deflection was determined by raising the temperature of the solution by about 0.1°C and comparing the reading of a Beckmann thermometer with the deflection. 5 mm of the deflection corresponds to about 0.01°C of temperature. Accuracy of the temperature measurement was 1/10,000°C. In Fig. 2 (a) is shown the temperature change with time thus obtained. Sudden descend of temperature at zero minute is caused by the fact that the temperature of the reaction vessel is usually somewhat higher than that of the thermostat on account of stirring the solution. From this curve the rate of temperature rise dT/dt is obtained. And the rate of heat production dQ/dt can be calculated as follows:

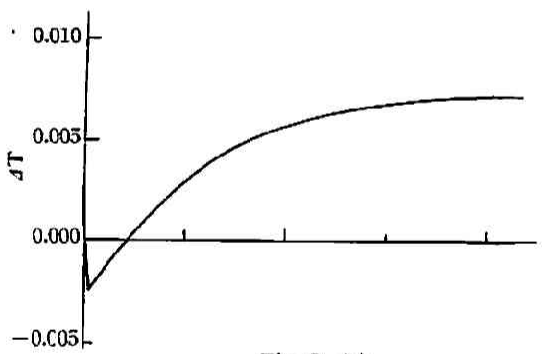

Fig. 2 (a)

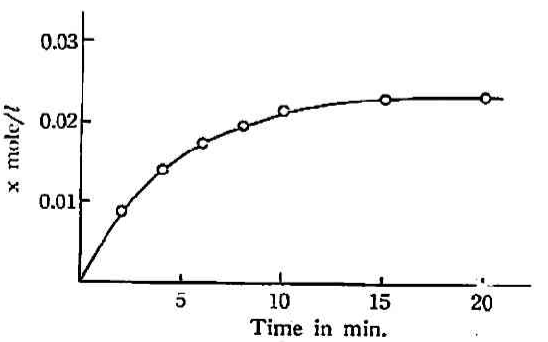

Fig. 2 (b)

$$\frac{dQ}{dt} = W\left\{\frac{dT}{dt} + k(T - T_0)\right\}, \quad (1)$$

where W is the water equivalent of

the reaction system, and the term $k(T-T_0)$ is a correction for cooling. The values of W, k, and T_0 are experimentally determined.

The influence of stirring was examined and it was found that one revolution per minute caused 4×10^{-6}°C per minute of temperature rise for 60ml of a reaction mixture. The error is quite negligible when attention is paid, so that the change of revolution might not exceed ten revolutions per minute. The revolution was kept at 240±6 r.p.m. throughout the experiment.

The effect of the heat of dilution of amylose, enzyme and salt solutions was carefully examined, but at the concentrations used no effect was perceived.

(b) Analytical measurement

1 ml of the reaction product was sucked up and the reaction was stopped within ±5 second by pouring the product into 0.4 N NaOH solution. The increase of the reaction product, the reducing end, was determined photometrically with 3,5-dinitrosalycilic acid[4]. Fig. 2 (b) shows the curve of reducing end increased x against time. From the curve the rate of increase of product dx/dt can be calculated.

Results and Discussions

From the results of the two different measurements, the heat of reaction Q_α is calculated according to the following equation:

$$\frac{dQ}{dt}=Q_\alpha\frac{dx}{dt}. \qquad (2)$$

Q_α obtained should be constant, but it is not always the case in the present experiment. In some cases, Q_α becomes large with time.

Two typical cases are shown in Fig. 3 and Table 1. When the ratio of enzyme to substrate is not so high, Q_α remains constant at 0.37 kcal* (case A). But when the ratio is high enough, Q_α gradually increases as the reaction proceeds, until it reaches a constant value of 0.83 kcal (case B).

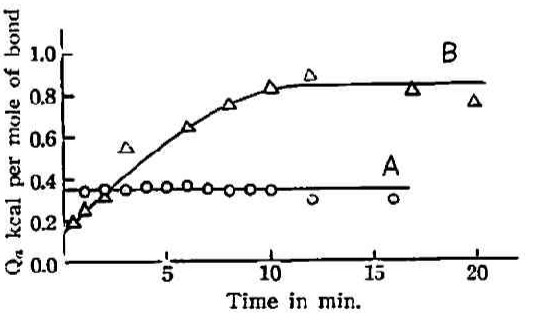

Fig. 3
A: Substrate 2.01%, stock enzyme solution used 0.11 ml
B: Substrate 2.03%, stock enzyme solution used 0.17 ml

These phenomena cannot be understood without assuming the presence of some endothermic change in the reaction processes besides the exothermic one. Thus, it is interpreted that, in case A, since the rate of exothermic

* The unit is kcal/mole of 1,4-glucoside linkage. The number of moles of the bond is obtained from: gr maltose/l of increased reducing end/molecular weight of maltose. This means the heat (kcal) when one mole of the bond splits.

4) K. H. Meyer et. al., *J. Phys. Coll. Chem.*, 53, 319 (1949)

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Table 1

Case A			Case B		
Time in min.	Percent of Hydrolysis	Q_α kcal per mole	Time in min.	Percent of Hydrolysis	Q_α kcal per mole
1	10.7	0.338	1	21.6	0.245
2	21.4	0.334	2	37.0	0.308
3	29.4	0.344	3	46.8	0.559
4	36.8	0.360	6	61.4	0.650
6	49.3	0.368	8	64.2	0.756
8	58.9	0.336	10	65.7	0.825
10	66.5	0.336	12	66.8	0.895
12	72.2	0.295	16	68.9	0.815
15	78.7	0.290	20	71.0	0.762

change and that of endothermic one are equal, Q_α appears constant, while in case B, the endothermic change is dominant in the initial stage of the reaction, gradually diminishes and finally disappears. Accordingly Q_α gradually increases and finally reaches a constant value.

In order to know what the endothermic change is and how the two different cases appear, the nature of the endothermic change will be examined.

When B curve in Fig. 3 reaches a constant value, the endothermic change must entirely disappear and there must exist only the exothermic one, which corresponds to the step of product formation. So the value of 0.83 kcal is considered to be the heat of reaction, the reaction which forms product. Let it be Q_0. Assume that the rate of heat absorption due to endothermic change in question is dq/dt, it can be represented as follows:

$$\frac{dq}{dt} = Q_0\frac{dx}{dt} - \frac{dQ}{dt}. \qquad (3)$$

In Fig. 4 (a) is shown how dq/dt changes with time, while in (b) dx/dt with time, and comparing these two, one can easily see that the resemblance is quite remarkable, and we can imagine that the endothermic change might be closely connected to the reaction which forms product. The relation between dq/dt and dx/dt is shown in

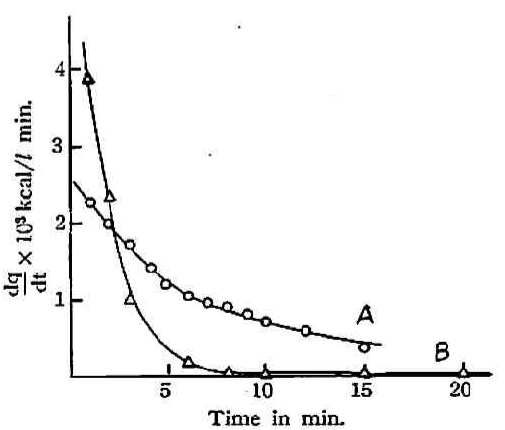

Fig. 4 (a)

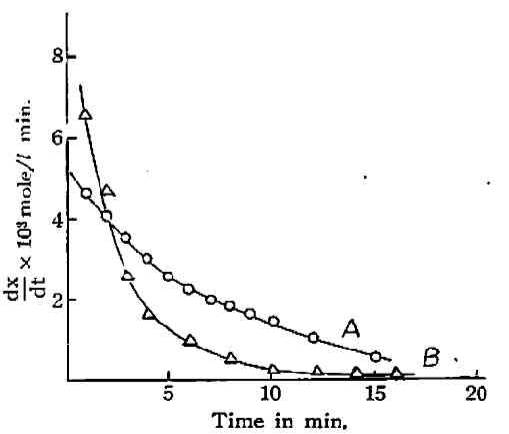

Fig. 4 (b)

Fig. 5. In case A, it makes a straight line passing origin.

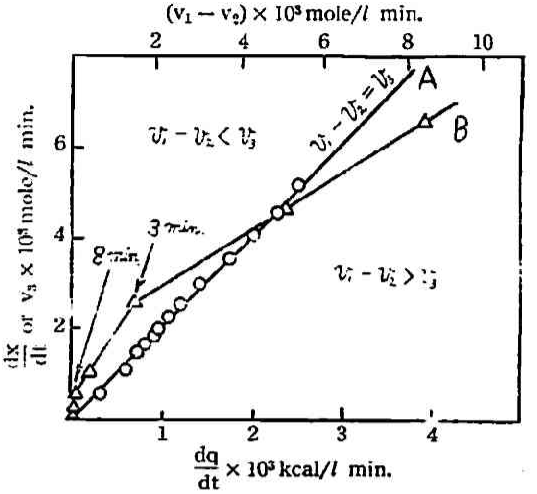

Fig. 5

Here we will assume that the endothermic change in question is identical with the intermediate process forming the complex between enzyme and substrate. And we are going to see whether this assumption can account for the variation of Q_a mentioned above.

Let the heat of formation of complex be $-q_0$, and the scheme of enzymic reaction can be written as follows:

$$E + S \xrightarrow{v_1} ES - q_0, \qquad (I)$$

$$ES \xrightarrow{v_2} E + S + q_0, \qquad (II)$$

$$ES \xrightarrow{v_3} E + P + Q_0, \qquad (III)$$

where E is enzyme, S substrate, P product, and v_1, v_2 and v_3 are the rates of the steps I, II and III respectively, so $v_3 = dx/dt$. Considering the reverse process II, the resultant rate of complex formation is, $v_1 - v_2$, and so the next relation holds:

$$dq/dt = q_0(v_1 - v_2). \qquad (4)$$

When the stationary concentration of ES is kept during the reaction, $v_1 - v_2$ is equal to v_3, and from the above equation (4),

$$dq/dt = q_0 dx/dt \qquad (5)$$

is obtained. Equation (5) means the proportionality of dq/dt to dx/dt, and the straight line in Fig. 5, (A) exactly corresponds to this relation.

The constancy of Q_a in case A is explained as follows: from Eqs. (2), (3) and (4), Q_a is written in the following relation.

$$Q_a = Q_0 - q_0\left(\frac{v_1 - v_2}{v_3}\right). \qquad (6)$$

Since the stationary state is kept in case A, $v_1 - v_2 = v_3$, so Q_a is equal to $Q_0 - q_0$ (constant) all over the reaction.

Thus the value of q_0 is calculated to be 0.46 kcal, Q_0 being 0.83 kcal and Q_a in case A 0.37 kcal. The value can be calculated in another way according to Eq. (5) from the slope of Fig. 5, which of course coincide with the above value.

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When the graduation of the abscissa of Fig. 5 is divided by q_0, the figure represents the relation between v_1-v_2 and v_3, and the straight line A corresponds to the stationary state, $v_1-v_2=v_3$. In the right side of the line the relation $v_1-v_2>v_3$ holds, and in left, $v_1-v_2<v_3$.

As for the curve B, therefore, $v_1-v_2>v_3$ in the earlier stage, $v_1-v_2<v_3$ in the later, and $v_1-v_2\fallingdotseq 0$ towards the end of the reaction.

According to the scheme of the enzymic reaction, the facts above mentioned may be interpreted as follows: under the condition in which the case B occurs, i.e., the ratio of enzyme to substrate is sufficiently large, the complex formation occurs in such a high rate, that the second and third steps in the reaction scheme cannot follow the first step, and temporarily v_1-v_2 exceeds v_3, thus the concentration of the complex becomes higher, and then on account of high concentration of ES and decreased concentrations of S and E, v_3, on the contrary, will exceed v_1-v_2, and finally v_1 and v_2 become comparable. And in this case, as shown in Eq. (6) and Fig. 3 (B), the value of Q_a increases as $(v_1-v_2)/v_3$ decreases, until at $v_1-v_2=0$, it becomes a constant of Q_0 in Fig. 3. These behaviors are naturally expected to occur under such a condition that the amount of enzyme is exceedingly large.

It is also anticipated that the relation between v_1-v_2 and v_3 may reflect upon the kinetic behavior of the reaction. According to the previous work[1], the following equation has been obtained on the degradation of amylose by bac. α-amylase.

$$\frac{dx}{dt}=\frac{k_3K_A(a-x)[E]}{1+K_A(a-x)+K_B(x)}, \tag{7}$$

where a: initial concentration of substrate,
x: concentration of product,
[E]: concentration of enzyme,
k_3, K_A, K_B: characteristic constants.

One can see whether the reaction follows the above equation or not by examining the linear relationship between k_a and v in the following equation. By the integration of Eq. (7),

$$mk_a=n+v \tag{8}$$

is obtained, where $k_a=1/t\ln a/(a-x)$ and $v=x/t$,
m, n: characteristic constants.

On deriving Eq. (7), it is tacitly assumed that the concentration of ES is always kept constant, i.e., the stationary state is established. If the stationary state is not kept during the reaction, the rate will not follow the kinetic equation (7) and the linear relation between k_a and v will no longer hold.

Fig. 6 shows $k_a\sim v$ diagram, where A makes a straight line, while B has some breaks, and further, comparing Fig. 6 with Fig. 5, the times where the breaks occur, are nearly the same in the two. This means the stationary condition does not hold

in case B, and the above discussions which are based on the endothermic complex formation is valid.

The discussions mentioned above confirm the reasonability of the assumption which we have made in the beginning. Strictly speaking, however, the endothermic change has only to be a step which precedes the step (III) which produces the product. But it seems most probable to consider endothermic change to be the step (I) which forms the intermediate complex.

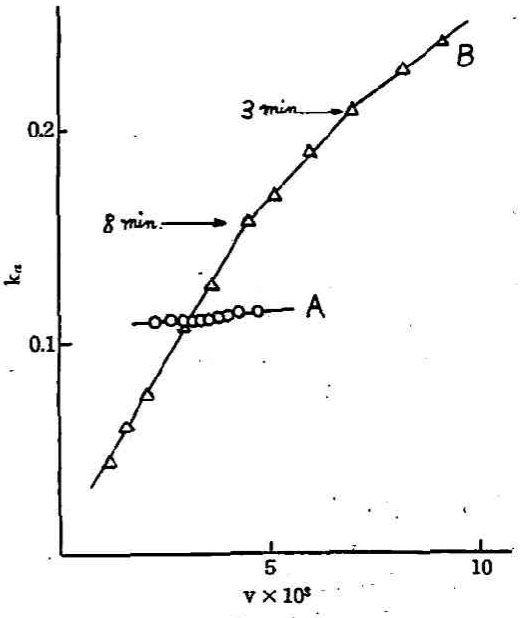

Fig. 6

Then, we find the heat of formation of the intermediate complex between substrate and enzyme to be -0.46 kcal per mole of 1,4-glucoside linkage, and the heat of the decomposition of the complex into product to be 0.83 kcal per mole, the net heat of reaction, therefore, to be 0.37 kcal per mole.

Recently, Laidler and his co-workers have calculated the enthalpy and entropy changes of enzyme-substrate complex formation for several enzymic reactions[5]. Their calculation is derived from the temperature coefficient of the equilibrium constant of $(E + S \rightleftarrows ES)$ which is found in the rate equation of the enzymic reactions. Their result is that the enthalpy change of complex formation is positive (endothermic) and the entropy change is also positive in the enzymic reactions studied. There is no report for α-amylase, but our conclusion of the endothermic complex formation which is directly measured is coincident with their calculated result, and seems to be an interesting fact on the mechanism of the enzymic reactions[6] [7].

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5) K. J. Laidler et al., *J. Am. Chem. Soc.*, **72**, 2159, 2489 (1950)
6) A. G. Evans and S. D. Hamman, *Trans. Farad. Soc.*, **47**, 25 (1951)
7) I. M. Klotz and J. M. Urquhart, *J. Am. Chem. Soc.*, **71**, 847 (1949)