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Barley Malt α-Glucosidase

IV. Studies on the Kinetics and the Active Groups

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The time course of the hydrolysis of maltose by an α-glucosidase from barley malt has been investigated to high degrees of reaction. It was found that the time course of the reaction for different initial concentrations of maltose, glucose, and enzyme could be described by a chronometric integral consisting of a sum of a first-order and a zero-order term. A reaction mechanism based on the chronometric integral found is suggested.

The influence of pH on the Michaelis constant K_m and on the maximum velocity V suggests that two ionizable groups with p$K_a \sim$ 4.0 and p$K_a \sim$ 5.8 are involved in the mechanism of the enzymatic hydrolysis.

The influence of sulfhydryl reagents, mercuric chloride, phenylmercuric acetate, iodoacetamide, and N-ethylmaleimide, has been examined; only mercuric chloride and phenylmercuric acetate showed inhibition.

In a previous paper initial rate studies of the hydrolysis of several sugars by an α-glucosidase from barley malt have been reported. [1] In the present investigation the hydrolysis of maltose has been studied further and the time course of the reaction has been followed to high degrees of reaction to examine the kinetics of the enzyme reaction. To obtain some information of the groups involved in the reaction, the variation with pH of the Michaelis constant K_m and the maximum velocity V has been studied and interpreted according to the theories of Dixon and Laidler.[2,3] With the same purpose the photo-oxidation of the enzyme and the inactivation by sulfhydryl reagents were examined.

MATERIALS AND METHODS

Sugars. Maltose and D-glucose were purified by charcoal adsorption.[1,4] ^{14}C-labelled D-glucose was obtained from The Radiochemical Centre, England.

Enzyme. The enzyme was extracted from high-diastase malt and purified as described previously.[5] The enzyme solution was stored at 0°C with 1 % of toluene and the time

course experiments were performed within a few days, in order to secure constant enzyme activity.

Time course studies

Glucose determinations. The hydrolysis of maltose was followed colorimetrically by determination of the glucose released with a tris-glucose-oxidase reagent as described by Dahlqvist,[6] although slightly modified (375 mg glucose-oxidase, 6 mg peroxidase, 1.5 ml 1 % *o*-dianisidine in 100 ml 0.5 M tris buffer pH 7.0). One ml of the reagent was added to 2.5 ml of the test solution. In all determinations duplicates of five standards containing 0, 5, 20, 40, and 50 μg of glucose per 2.5 ml were run simultaneously.

The relative standard deviation of the glucose determination was 1.5 – 2 %.

Procedure. Maltose solution (800 μl) and 0.2 M acetate buffer pH 4.6 (800 μl) were mixed and preheated in a water thermostat at 37°C; the reaction was started by adding 400 μl of preheated enzyme. The moment of half emptied pipette was taken as zero time. Aliquots (50 – 200 μl) of the enzyme reaction mixture were withdrawn at suitable intervals and pipetted into (2.45 – 2.30 ml) 0.5 M tris buffer (2-amino-2-hydroxymethylpropane-1,3-diol/HCl) pH 7.0 whereby the enzyme reaction is stopped. The moment of half emptied pipette was taken as the time of the withdrawal. Reaction mixtures with enzyme but without maltose were run simultaneously and samples from this reaction mixture were used as enzyme blanks. The glucose concentration was determined in all samples as described above.

Initial velocity studies

In the initial velocity studies the hydrolysis of the maltose did not exceed 5 %, below this limit the degree of hydrolysis is apparently proportional to the time and the enzyme concentration. The hydrolysis of maltose was determined by measuring the glucose formed.[5]

Enzyme assay procedure. The determination of the α-glucosidase activity was performed as described previously but with maltose (2 mM) as substrate, instead of isomaltose.[5]

Enzyme unit.[5] The enzyme unit, U, is defined using isomaltose (2 mM) as substrate. 1 U = 1000 mU. If maltose (2 mM) is used instead of isomaltose, the U-value is arrived at by dividing by 16, as maltose under these conditions is hydrolysed about 16 times faster than isomaltose.

Variation of K_m and V with pH. K_m and V were determined at different pH values by Lineweaver and Burk's method,[7] plotting reciprocals of initial velocities *versus* reciprocals of substrate concentrations. The pH was varied between pH 3.0 – 6.5 by means of citrate buffers (0.04 M), and at each pH-value the substrate concentrations 1.0, 2.0, 3.0, and 4.0 mM maltose were examined. Reaction temperature 37°C. Enzyme concentration 0.05 mU per 0.5 ml reaction mixture. The experiment was repeated with phosphate-acetate buffers.

Photo-oxidation of the enzyme. Test tubes with enzyme (17 mU/ml) in 0.02 M phosphate buffer pH 8.0 with and without methylene blue (50 μg/ml) were placed in the dark and in light at 37°C (see Table 11). In some of the test tubes the air was replaced by nitrogen. The exposure to light was performed by placing the test tubes in a water bath at 37°C with glass walls. The light source, a 250 W electric bulb cooled by a fan, was placed about 15 cm from the test tubes. After the exposure (25 min and 50 min) the enzyme solutions were diluted 75 times and the remaining enzyme activity was determined as under "enzyme assay procedure". Under these conditions the methylene blue is without effect on the tris-glucose-oxidase reagent.

Inhibition by sulfhydryl inhibitors. Enzyme (0.3 mU/ml) in 0.05 M acetate buffer pH 4.6 was incubated for 30 min with 1.6×10^{-4} M phenylmercuric acetate and 0.33×10^{-4} M mercuric chloride, respectively, at 37°C, and the remaining enzyme activity was determined. The experiment was repeated, but 5 min after the addition of the inhibitor, cysteine was added to a concentration of 0.5×10^{-4} M, and after a period of 25 min at 37°C the remaining enzyme activity was determined. Similar experiments were performed, in which the enzyme activity determinations were performed instantly after addition of the inhibitors to the enzyme.

Table 1.

a = 0.5 mM maltose
E = 1

t min	$x/2$ mM	t_{calc} min
3	0.0715	2.5
8	0.194	8.1
13	0.268	13.0
20	0.342	19.9
25	0.381	25.1
30	0.394	27.3

Table 2.

a = 1.0 mM maltose
E = 1

t min	$x/2$ mM	t_{calc} min
3	0.136	2.5
8	0.354	7.9
13	0.497	13.0
20	0.638	20.0
25	0.713	25.3
30	0.763	29.7
40	0.859	41.8
50	0.894	48.7
60	0.928	58.3

Table 3.

a = 2.0 mM maltose
E = 1

t min	$x/2$ mM	t_{calc} min
3	0.228	2.7
6	0.427	5.7
10.1	0.666	10.1
20	1.07	20.6
31.2	1.36	32.2
40	1.49	39.8
50	1.61	49.1
60	1.72	60.6
80	1.84	80.5

Table 4.

a = 3.0 mM maltose
E = 1

t min	$x/2$ mM	t_{calc} min
3	0.244	2.4
8	0.674	7.8
13	0.963	12.4
20	1.37	20.4
30	1.75	30.8
40	1.99	40.1
50	2.19	49.5
64	2.44	66.2
89	2.60	82.0

In another series enzyme (0.3 mU/ml) in 0.05 M acetate buffer pH 4.6 was incubated for 24 h with 2.5×10^{-4} M iodoacetamide, and 2.5×10^{-4} M N-ethylmaleimide, respectively, at 25°C. The remaining enzyme activity was determined.

In all the experiments appropriate blank determinations were run simultaneously.

At the conditions given the added reagents are without effect on the tris-glucose-oxidase reagent.

Paper chromatographic analysis. Whatman No. 1 filter paper was used for descending chromatography. The solvent system was ethyl acetate-pyridine-water (10:4:3, by vol.).[8] Location reagent: silver nitrate-sodium hydroxide.[9]

RESULTS AND DISCUSSION

The stability of the enzyme at optimum conditions (37°C, pH 4.6)[5] was examined and it was found that within a period of 2 h the inactivation of the enzyme was less than 1 %.

The influence of varying substrate concentration on the time course of the reaction. Experiments were performed at four different substrate concentrations (0.5, 1.0, 2.0, and 3.0 mM maltose) but with the same enzyme concentration (1 E), Tables 1—4. It was found that the time course of the reaction could not be described by simple zero-, first-, or second-order rate equations, but the

progress of the reaction could be described fairly well by an equation consisting of a sum of a first-order and a zero-order term:

$$t = A \ln \frac{a}{a-(x/2)} + B \frac{x}{2} \tag{1}$$

where t is time (min), a initial maltose concentration (mM), x concentration of glucose released (mM) at time t, and A and B are constants.

A straight line is obtained when $t/(x/2)$ is plotted against $\ln \frac{a}{a-(x/2)} \Big/ (x/2)$. The slope of this line is A and the intercept with the ordinate is B.

Tables 1—4 give the experimental values of x and t, and further t values (t_{calc}) calculated from eqn. (1) using the A and B values of Table 9.

The influence of varying enzyme concentration on the time course of the reaction. In two experiments the enzyme concentration was varied (0.75 E and 1.5 E), the maltose concentration was 1.0 mM; Tables 5 and 6. The experimental results again fit eqn. (1) with the A and B values given in Table 9.

The influence of added glucose on the time course of the reaction. From initial rate studies it was found that glucose inhibits the enzyme catalysed hydrolysis

Table 5.

a = 1.0 mM maltose
E = 0.75

t min	$x/2$ mM	t_{calc} min
3	0.125	3.0
8	0.298	8.2
13	0.423	13.1
20	0.561	20.5
30	0.694	30.7
40	0.772	39.4
50	0.834	49.1
60	0.870	56.7
100	0.964	97.9

Table 6.

a = 1.0 mM maltose
E = 1.5

t min	$x/2$ mM	t_{calc} min
3	0.204	2.8
8	0.458	7.8
13	0.629	13.2
18	0.731	18.0
23	0.805	23.0
28	0.849	27.0
33	0.895	32.7
40	0.939	41.6
50	0.957	47.3

Table 7.

a = 2.0 mM maltose
g = 0.5 mM glucose
E = 1

t min	$x/2$ mM	t_{calc} min
3	0.261	3.8
8	0.480	7.8
13	0.708	12.8
20	0.891	17.8
30.5	1.25	31.2
40	1.40	39.8
50	1.55	50.2
60.1	1.65	59.6
90	1.80	81.0

Table 8.

a = 2.0 mM maltose
g = 1.0 mM glucose
E = 1

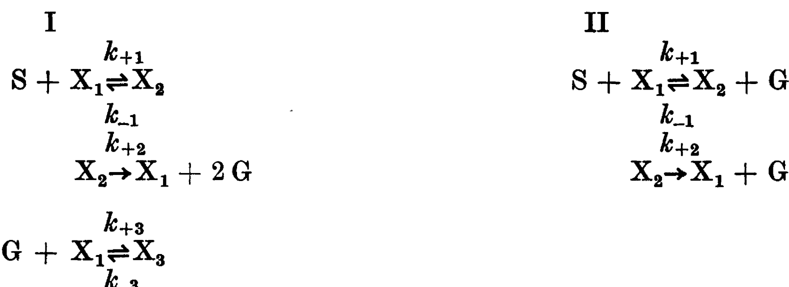
t min	$x/2$ mM	t_{calc} min
3	0.197	3.2
8	0.430	7.8
13	0.669	13.5
20	0.899	20.5
30	1.16	30.9
40	1.37	42.3
55	1.54	55.6
75	1.71	75.4
100	1.83	99.0

of maltose. Two experiments were performed with the same initial concentration of maltose (2 mM) and with the same enzyme concentration (1 E) but with two different initial concentrations of glucose (g) 0.5 mM and 1.0 mM, respectively. The results are given in Tables 7 and 8, the A and B values are given in Table 9.

Differentiation of equation (1) gives:

$\Delta t = (A/(a-(x/2)) + B)\ (\Delta x/2)$, substituting the values of A, B, a, x, and $\Delta x \sim 0.02\ x$ into this equation we may arrive at an approximate estimation of the allowed deviation of t_{calc}. The values given in Tables 1–8 show a reasonable agreement with Δt values calculated from the above equation.

Reaction mechanism. Reaction schemes of one or two steps if necessary with an additional product inhibition reaction have been examined. Several of these schemes lead to a rate equation similar to the one found. The following two schemes are subjected to further examination:[10,11]



(where X_{1-3} are different enzyme forms, S maltose, and G glucose). The reaction with one mole of water which is necessary for the hydrolysis is omitted as this reaction cannot be detected by the method used.

Scheme I represents Michaelis-Henry scheme with competitive glucose inhibition.

Using the steady state method as described by Christiansen[12] we obtain the following chronometric integrals.[10]

Scheme I

$$t = \frac{k_{+2}+k_{-1}}{Ek_{+1}k_{+2}}\left[1+\frac{k_{+3}}{k_{-3}}(g+2a)\right]\ln\frac{a}{a-(x/2)} + \frac{k_{+2}+k_{-1}}{Ek_{+1}k_{+2}}\left[\frac{k_{+1}}{k_{+2}+k_{-1}} - \frac{2k_{+3}}{k_{-3}}\right]\frac{x}{2} \qquad \text{(I)}$$

Scheme II

$$t = \frac{1}{Ek_{+1}}\left[1+\frac{k_{-1}}{k_{+2}}(g+2a)\right]\ln\frac{a}{a-(x/2)} + \frac{1}{Ek_{+2}}\left[1-\frac{2k_{-1}}{k_{+1}}\right]\frac{x}{2} \qquad \text{(II)}$$

(where E is the total enzyme concentration, a the initial maltose concentration (mM), g the initial glucose concentration (mM), x the glucose (mM) released at the time t (min), and k_i reaction constants).

Comparison of these equations with that found empirically shows that a straight-line relationship between A and $(g + 2a)$ should be expected and that B should be independent of a and g. This seems to be fulfilled; see Fig. 1

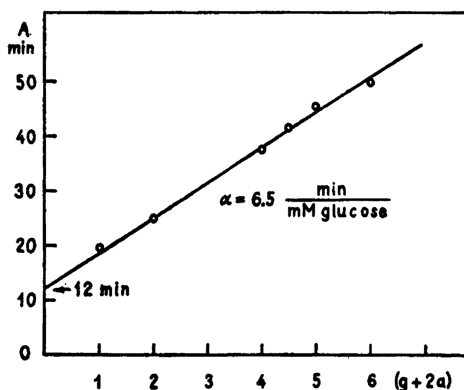


Fig. 1. Values of A as a function of $(g + 2a)$, g initial glucose concentration (mM), a initial maltose concentration (mM).

and Table 9. Also the expected inverse proportionality between A and E, and between B and E is fulfilled (Table 9).

It is not possible on the basis of the kinetic experiments mentioned to distinguish between reaction schemes I and II.

When eqn. (II) is compared with the straight line in Fig. 1 it is found that the slope of the line 6.5 min/mM glucose is equal to $k_{-1}/k_{+1}k_{+2}E$ and the intercept with the ordinate 12.0 min is equal to $1/k_{+1}E$. From these figures we find $k_{-1}/k_{+2} = 0.54$ mM glucose^{-1}.

From reaction scheme (II) it may be deduced that the ratio of the velocity (v_{-1}) of the reaction $X_2 + G \rightarrow X_1 + S$ to the velocity (v_{+2}) of the reaction $X_2 \rightarrow X_1 + G$, $v_{-1}/v_{+2} = k_{-1}(x + g)/k_{+2} = 0.54(x + g)$.

Experiment 8 was then repeated but with the addition of ^{14}C-labelled glucose representing 9×10^3 counts/min per 2 μl of the reaction mixture. When the maltose had been hydrolysed to an extent of 50 % by the enzyme, the reaction

Table 9.

Expt.	a mM maltose	g mM glucose	E	A min	B min/mM maltose	$A \times E$	$B \times E$
1	0.5		1	19.5	− 7.5		
2	1.0		1	25.2	− 8.7	25.2	−8.7
3	2.0		1	37.5	− 7.7		
4	3.0		1	49.8	− 7.2		
5	1.0		0.75	32.7	−11.5	24.5	−8.6
6	1.0		1.5	16.5	− 5.0	24.8	−7.5
7	2.0	0.5	1	41.5	− 7.5		
8	2.0	1.0	1	45.5	− 7.5		

was stopped by boiling. An aliquot (2 μl) of the reaction mixture was applied to a paper for chromatography. After the chromatographic separation of glucose and maltose the radioactivities of the glucose and maltose were measured directly on the paper chromatograms with a Geiger-Müller counting assembly. The glucose spot gave 9×10^3 counts/min and the maltose spot gave 2 counts/min. From the rate of the hydrolysis at about 50 % hydrolysis (Table 8) we find $v = v_{+2} \sim 0.053$ mM glucose per min and from $v_{-1}/v_{+2} = 0.54\ (2 + 1) = 1.62$, ($x = 2$ mM at 50 % hydrolysis and $g = 1.0$ mM), we obtain $v_{-1} = 1.62 \times 0.053 = 0.086$ mM glucose per min, or 2.9 % of the total amount of glucose present. This amount of glucose would have exchanged with the maltose in the last minute of the reaction, if reaction scheme II were valid. The maltose spot should have given considerably more than 260 counts/min (2.9 % of 9×10^3), as only the last minute of the reaction is taken into account. The maltose spot gave only 2 counts/min, therefore reaction scheme II is not valid.

Substituting the Michaelis constant $K_m = (k_{+2} + k_{-1})/k_{+1}$, the competitive inhibition constant $K_I = k_{-3}/k_{+3}$, and the maximum velocity $V = k_{+2}E$ into eqn. I we have:

$$t = \left[\frac{K_m}{V} + \frac{K_m}{VK_I}(g+2a)\right] \ln \frac{a}{a-(x/2)} + \left[\frac{1}{V} - \frac{2K_m}{VK_I}\right]\frac{x}{2} \quad \text{(III)}$$

The slope of the straight line in Fig. 1 is then equal to $K_m/VK_I = 6.5$ min/mM glucose and the intercept with the ordinate is equal to $K_m/V = 12.0$ min. The mean value of the B values -7.8 min/mM maltose is equal to $(1/V) - (2K_m/VK_I)$ in eqn. (III). From these figures we find $K_m = 2.3$ mM maltose, $K_I = 1.85$ mM glucose and $V = 0.192$ mM maltose/min $\times$ 1 E. The enzyme concentration chosen as unity (1 E) is equal to 5.9 mU/ml as defined previously. In this unit and in μmole glucose per hour the value of V is 3.9 μmole glucose/h $\times$ ml $\times$ mU. In initial rate studies [1] with the same enzyme and substrate the following values were found K_m 2.0 mM maltose, K_I about 1 mM glucose and V 3.9 μmole glucose/h $\times$ ml $\times$ mU in fairly good agreement with the values found here.

The following reaction schemes: ($S + X_1 \rightarrow X_1 + 2G$; $X_1 + G \rightleftharpoons X_2$), ($S + X_1 \rightarrow X_2 + 2G$; $X_2 \rightleftharpoons X_1$; $X_1 + G \rightleftharpoons X_3$), ($S + X_1 \rightarrow X_2 + G$; $X_2 \rightleftharpoons X_1 + G$), ($X_1 \rightleftharpoons X_2$; $S + X_2 \rightarrow X_1 + 2G$; $X_2 + G \rightleftharpoons X_3$) give rise to chronometric integrals identical with the one found and it is not possible by this kinetic study to exclude these schemes. The reaction schemes I and II are more in line with schemes usually accepted in enzyme kinetics, and at the present the Michaelis-Henry scheme (I) seems to be the most probable.

Variation of K_m with V. Table 10 shows that for pH-values in the range 4.6—6.5 the change of the K_m-value is small compared with the change of the V-value. This suggests that the K_m-value $(k_{+2} + k_{-1})/k_{+1}$ equals the true equilibrium constant (substrate constant) $K_s = k_{-1}/k_{+1}$ of the reaction $S + X_1 \rightleftharpoons X_2$, because otherwise the variation in $V = k_{+2}E$ should be reflected in K_m.[13] Examination of the inhibition of the hydrolysis of maltose by isomaltose in "mixed substrate" experiments, where the Michaelis constant for the hydrolysis of isomaltose seems to be equal to the inhibitor constant, and the fact that K_m and V for the hydrolysis of several substrates by the enzyme change differently [1] likewise points toward the conclusion that $K_m \sim K_s$.[13]

Table 10. Hydrolysis of maltose by an α-glucosidase from barley malt. The effect of pH on the Michaelis constant K_m and the maximum velocity V.

pH	K_m M $\times$ 10^3	V $\frac{\mu \text{ mole glucose}}{\text{ml} \times \text{h} \times \text{mU}}$
3.1	25	3.3
3.5	5.6	3.9
4.1	2.0	3.9
4.6	2.0	3.9
5.1	2.0	3.3
5.6	2.0	2.4
6.1	2.0	1.4
6.5	1.9	0.8

Variation of K_m and V with pH. The hydrolysis of maltose was studied at 37°C by initial rate studies at pH 3.0—6.5. At each pH-value the substrate concentration was varied from 1 to 4 mM and the Michaelis constant K_m and the maximum velocity V were determined for each pH-value by the method of Lineweaver and Burk,[7] Table 10. The variation of pK_m ($-\log K_m$), $\log V$, and $\log (V/K_m)$ with pH has been studied according to Dixon and Laidler [2,3] (Fig. 2).

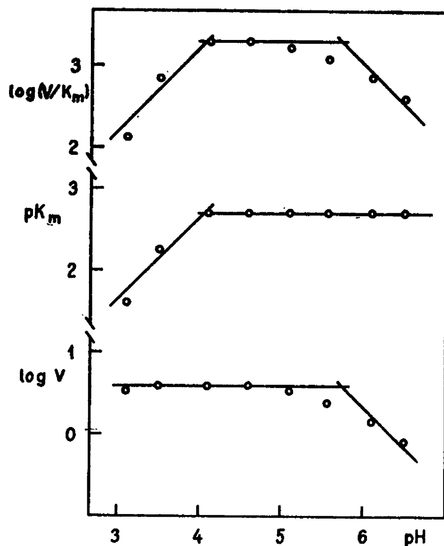


Fig. 2. Effect of pH on pK_m ($-\log K_m$), $\log V$, and $\log (V/K_m)$ for the hydrolysis of maltose by an α-glucosidase from barley malt. K_m Michaelis constant, V maximum velocity. The straight-line sections are drawn with the slopes + 1, 0, and -1.

The biphasic curve obtained when pK_m is plotted against pH suggests that an ionizable enzymatic group with a pK_a of about 4.0 is involved in the combination of the enzyme with the non-ionized substrate. This group may be represented by a carboxyl group (pK_a 3.0—4.7).[14] When $\log V$ is plotted against pH a biphasic curve is again obtained but with an inflection point at pH about

5.8 indicating an ionizable group with a pK_a about 5.8. This group which is not involved in the combination of the enzyme with the substrate, but in the subsequent cleavage of the enzyme-substrate complex, may be an imidazolyl group in a histidine residue (pK_a 5.6—7.0).[14] The plot of log (V/K_m) against pH reflects both the inflection points.

The enzyme therefore exists in the following forms, in the pH-range tested: $XH_2^+ \rightleftharpoons X^-H^+ + H^+ \rightleftharpoons X^- + 2H^+$, and X^-H^+ is the active form possibly containing a carboxylate and an imidazolium group.

In similar studies of β-fructosidase from yeast by Myrbäck and Björklund [15] and of calf-intestine β-galactosidase by Wallenfels and Fischer [16] similar ionizable groups were found.

Photo-oxidation of the enzyme. The plot of log V against pH gave some evidence for the existence of a histidine residue at the active site. As shown by Weil *et al.*[17] histidine and tryptophan residues are easily destroyed by light in the presence of methylene blue and oxygen. It was then to be expected that the enzyme would be inactivated by photo-oxidation.

Table 11. Inactivation of α-glucosidase activity from barley malt by photo-oxidation.

Concentration of methylene blue μg/ml		Time of exposure	Remaining enzyme activity, %
0	unexposed		100
0	exposed	25 min	100
50	unexposed		96
50	exposed	25 min	78
0	unexposed		100
0	exposed	50 min	100
50	unexposed		96
50	exposed	50 min	38
50	exposed	50 min	78*

* Air expelled by nitrogen.

Table 12. Inactivation of α-glucosidase activity from barley malt by sulfhydryl reagents.

Inhibitor	Inhibitor concentration M/l	Time of incubation with inhibitor	Cysteine concentration M/l	Remaining enzyme activity, %
None	0		0	100
Mercuric chloride	0.33×10^{-4}	0 min	0	51
—	0.33×10^{-4}	30 min	0	0
—	0.33×10^{-4}	5 min*	0.5×10^{-4}	41
Phenylmercuric acetate	1.6×10^{-4}	0 min	0	91
—	1.6×10^{-4}	30 min	0	66
—	1.6×10^{-4}	5 min*	0.5×10^{-4}	80
Iodoacetamide	2.5×10^{-4}	24 h	0	100
N-Ethylmaleimide	2.5×10^{-4}	24 h	0	100

* Time of incubation with inhibitor and cysteine: 25 min.

The result of the photo-oxidation of the enzyme is shown in Table 11. It is seen that the enzyme is inactivated by light in the presence of methylene blue and that the inactivation is considerably lower when the air has been expelled by nitrogen.

The experiment is in accordance with the hypothesis that the group in the enzyme with $pK_a \sim 5.8$ is a histidine residue.

Sulfhydryl inhibitors. As shown in Table 12 the enzyme activity is inhibited by mercuric chloride (0.33×10^{-4} M) and phenylmercuric acetate (1.6×10^{-4} M) and the inhibition by these reagents is partly reversed by cysteine (0.5×10^{-4} M). Iodoacetamide and N-ethylmaleimide (2.5×10^{-4} M) do not seem to be inhibitors even after prolonged reaction. The inhibitory effect of mercuric chloride and phenylmercuric acetate was dependent on the reaction time.

The inhibitory effect of mercuric compounds but not of iodoacetamide or N-ethylmaleimide makes it difficult to draw definite conclusions as to whether or not SH-groups are involved in the catalytic mechanism of the enzyme. Mercuric compounds are less selective sulfhydryl reagents than iodoacetamide and N-ethylmaleimide, but the inhibitory effect of the two last mentioned reagents seems to be greatly dependent on reaction conditions.[18]

As shown by initial rate studies [1] the hydrolysis of isomaltose catalysed by the same enzyme points toward a Michaelis-Henry reaction scheme. It is therefore to be expected that the hydrolysis of isomaltose follows the same reaction mechanism as that found here for the hydrolysis of maltose.

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