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STUDIES ON PEROXIDASE ISOLATED FROM ETIOLATED ALASKA PEA SEEDLINGS

II. EFFECT OF QUERCETIN ON THE OXIDATION OF INDOLE-3-ACETIC ACID

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SUMMARY

1. The effect of quercetin on the kinetic behaviour of Alaska pea peroxidase (H_2O_2 oxidoreductase, EC 1.11.1.7) at the time of oxidation of indole-3-acetic acid has been examined.

2. Plots of reaction rates against indole-3-acetic acid concentration gave the usual hyperbolic and sigmoidal curves in the absence and presence of quercetin, respectively at pH 5.5. Apparent K_m values and n values evaluated by Hill's plot were $4 \cdot 10^{-4}$ M and 1.9 in the presence of $1 \cdot 10^{-5}$ M quercetin, and $1.6 \cdot 10^{-2}$ M and 1.0 in its absence, respectively.

3. Plots of reaction rates against quercetin concentration showed activation and inhibition patterns. The apparent Michaelis constant of activation (K_a) and n value were independent of indole-3-acetic acid concentration, giving values of $1 \cdot 10^{-4}$ M and 0.75, at pH 5.5, respectively.

4. The effect of quercetin on the reaction rate was pH-dependent. Plots of the reaction rate against the indole-3-acetic acid concentration in the presence of $1 \cdot 10^{-5}$ M quercetin was hyperbolic at pH values lower than 4.5 with an n value of 1.0, whereas it was sigmoidal at a pH value higher than 4.5 with an n value greater than 1.0.

5. Quercetin was degraded by peroxidase treatment with a maximal activity at pH 5.5, to a purple compound with an absorption maximum at 530 mμ. Indole-3-acetic acid inhibited the reaction non-competitively with an apparent inhibition constant (K_i) of $1.5 \cdot 10^{-2}$ M at pH 5.5, which agreed well with the apparent K_m for the indole-3-acetic acid oxidation in the absence of quercetin.

6. KCN inhibited the reaction non-competitively with an apparent K_i of $1 \cdot 10^{-3}$ M in the absence and presence of quercetin.

7. The results obtained imply that: (a) each binding site of quercetin and indole-3-acetic acid is not that of heme; (b) the binding site of quercetin differs from that of indole-3-acetic acid. On the basis of kinetic analysis, two binding sites for indole-3-acetic acid were assumed, a catalytic site with a dissociation constant of

$3 \cdot 10^{-4}$ M and an activating site with a dissociation constant of $2 \cdot 10^{-1}$ M in the presence of $1 \cdot 10^{-5}$ M quercetin at pH 5.5. This assumption can explain the experimental data well.

INTRODUCTION

Indole-3-acetic acid is a well-known plant growth-regulating substance, which can be found in various plant tissues. The enzyme system which destroys this substance was first found in etiolated Alaska pea seedlings by TANG AND BONNER[1], and later it was identified as peroxidase. Peroxidase catalyzes the oxidation of indole-3-acetic acid in the presence of O_2 as well as the peroxidation of various phenolic compounds in the presence of H_2O_2 (ref. 2).

The oxidation of indole-3-acetic acid by peroxidase *in vitro* was reported to be inhibited or activated in the presence of poly- and monophenols, respectively[3]. Among these phenols, flavonoids raised much interest, because they are readily found as glucosides in various plant tissues[4,5]. FURUYA *et al.*[5] have found quercetin glucoside in etiolated Alaska pea seedlings, and showed that it strongly inhibited the indole-3-acetic acid oxidation by horseradish peroxidase.

One of the peroxidases of the b-group has been partially purified from etiolated Alaska pea seedlings, and named Alaska pea peroxidase b', which oxidizes indole-3-acetic acid with an optimal activity at pH around 3.0 (ref. 6).

The kinetic properties of the indole-3-acetic acid oxidation by this enzyme in the presence of quercetin were studied. Some results of interest are presented in this paper that quercetin can modify the kinetic behaviour of peroxidase and that the effect is pH-dependent.

MATERIALS AND METHODS

Peroxidase

Alaska pea peroxidase b' was partially purified according to the method described previously[6]. 4 kg of material was homogenized in 0.01 M phosphate buffer (pH 7.2). Coarse residues were excluded with filtration and solid $(NH_4)_2SO_4$ was added to give a 90% saturated solution. The precipitate was gathered and resuspended in the buffer. The solution dialyzed against the buffer was put onto a resin column of Amberlite CG-50. The reddish band adsorbed on the top of column was eluted with 0.05 M buffer (pH 7.2) and fractionated repeatedly with $(NH_4)_2SO_4$. The R.Z. value of the finally obtained solution was between 0.6 and 1.3. Cytochrome *c* was not contained. The enzyme concentration was calculated on the basis of a molar extinction coefficient of γ-band, $\varepsilon_{mM} = 100$.

Chemicals

Indole-3-acetic acid and quercetin (5,7,4',3'-tetraoxyflavonol) were purchased from Sigma Chemical Co. and Wako Pure Chemical Industries, respectively. Each was dissolved in ethanol to give a solution of 0.4 M indole-3-acetic acid and 0.01 M quercetin.

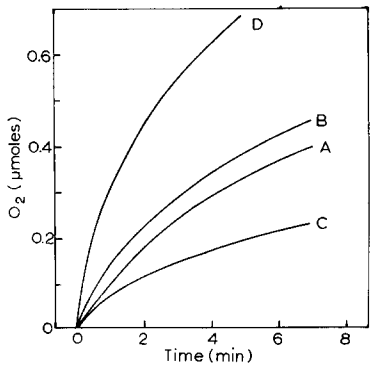

Fig. 1. Time-courses of O_2 consumption of the indole-3-acetic acid oxidation by Alaska pea peroxidase b'. Reacting mixture: 0.16 μM peroxidase, 0.05 M acetate; pH 5.5, in the absence of quercetin (A); pH 5.5, in the presence of $1 \cdot 10^{-5}$ M quercetin (B); pH 3.5, in the absence of quercetin (C); pH 3.5, in the presence of $1 \cdot 10^{-5}$ M quercetin (D). Final concentration of indole-3-acetic acid was $1 \cdot 10^{-2}$ M and $1 \cdot 10^{-3}$ M at pH 5.5 and pH 3.5, respectively. Temp., 25.5°.

Measurement of enzymatic activity

Indole-3-acetic acid is oxidized enzymatically by almost equivalent moles of O_2 (ref. 1), and this was also the case with Alaska pea peroxidase b'. The O_2 consumption was measured polarographically with a Clark O_2 electrode equipped with a Toa electronic polyrecorder, Model EPR 2T. An example is shown in Fig. 1. The initial rate was expressed in O_2 consumed per sec per 4 ml of reacting mixture. Each experimental condition was given in each figure.

The chemical change of quercetin was measured spectrophotometrically with a Hitachi recording spectrophotometer, EPS 3, equipped with a thermoregulated cell holder specified for the purpose of experiment. A 3-ml portion of reaction mixture contained 0.05 M buffer, various concentrations of quercetin and indole-3-acetic acid. At time zero, 50 μl of enzyme solution was added with a glass rod specified for the purpose. The reaction rate was expressed by the increase in absorbance per 2 sec per cm. An example is shown in Fig. 6a.

RESULTS

Effect of indole-3-acetic acid in the presence and absence of quercetin

Indole-3-acetic acid was oxidized aerobically and the reaction was activated at both the pHs 3.5 and 5.5, in the presence of $1 \cdot 10^{-5}$ M quercetin, at suitable concentrations of substrate (Fig. 1).

When the reaction rate was demonstrated as a function of indole-3-acetic acid concentration at pH 3.5 the usual hyperbolic curves were obtained in the absence and presence of $1 \cdot 10^{-5}$ M quercetin, whereas a sigmoidal curve was obtained in the presence of $1 \cdot 10^{-4}$ M quercetin. The O_2 consumption was essentially zero until the indole-3-acetic acid concentration was raised to $3 \cdot 10^{-4}$ M (Fig. 2a). The double-reciprocal plots gave straight lines, and apparent K_m values calculated were $1 \cdot 10^{-3}$ and $3.7 \cdot 10^{-4}$ M in the absence and presence of $1 \cdot 10^{-5}$ M quercetin, respectively (Fig. 2b).

At pH 5.5, similar plots gave hyperbolic curves only in the absence of quercetin,

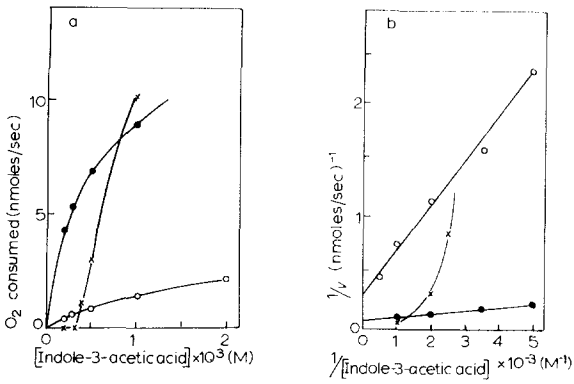

Fig. 2. (a) The rate of indole-3-acetic acid oxidation as a function of indole-3-acetic acid concentrations in the absence (○—○) and presence of $1 \cdot 10^{-5}$ M (●—●) and $1 \cdot 10^{-4}$ M (×—×) quercetin. Reaction mixture: 0.16 μM peroxidase, various concentrations of indole-3-acetic acid and 0.05 M acetate, pH 3.5. Temp., 25.5°. (b) Double reciprocal plots of data in (a).

and sigmoidal curves were obtained in the presence of the quercetin concentrations examined ($5 \cdot 10^{-6} - 2 \cdot 10^{-5}$ M) (Fig. 3a). Double-reciprocal plots showed a straight line in the absence of quercetin while a downward convex curve was observed in its presence (Fig. 3b). If the reciprocals of the reaction rate were plotted against the square of the reciprocal of the indole-3-acetic acid concentration, a straight line was obtained (Fig. 3b). Data in Fig. 3a were rearranged according to the description by LINEWEAVER AND BURK[7], and the resulting plots are shown in Fig. 3c. (They are identical with Hill's plot.) Slopes were almost 2 in the presence of quercetin and 1 in its absence. Apparent K_m values were evaluated from intercepts at $[S] = 1$ which agreed well with those calculated from double-reciprocal plots. The kinetic parameters obtained are summarized in Fig. 3d.

Effect of quercetin

When the reaction rate was determined as a function of the quercetin concentration at various indole-3-acetic acid levels, patterns of activation and inhibition were obtained (Fig. 4a). Double-reciprocal plots of the activating part gave straight lines as shown in Fig. 4b. The Michaelis constant of activation (K_a) was estimated to be about $1 \cdot 10^{-4}$ M from this plot. When the reaction rate as a function of the quercetin concentration was arranged for Hill's plot, slopes were 0.75 for all indole-3-acetic acid concentrations (Fig. 4c).

If K_a is assumed to be a parameter for the affinity of quercetin for the enzyme in the presence of indole-3-acetic acid, then its affinity for the enzyme is barely affected by indole-3-acetic acid. This implies that the binding site of quercetin differs from that of indole-3-acetic acid.

Effect of pH

As mentioned before, the effect of $1 \cdot 10^{-5}$ M quercetin changes considerably with pH values. When the reaction rate was demonstrated as a function of the indole-3-acetic acid concentration in the presence of $1 \cdot 10^{-5}$ M quercetin, the usual hyperbolic curves were obtained at a pH between 3.5 and 4.5, whereas sigmoidal ones were

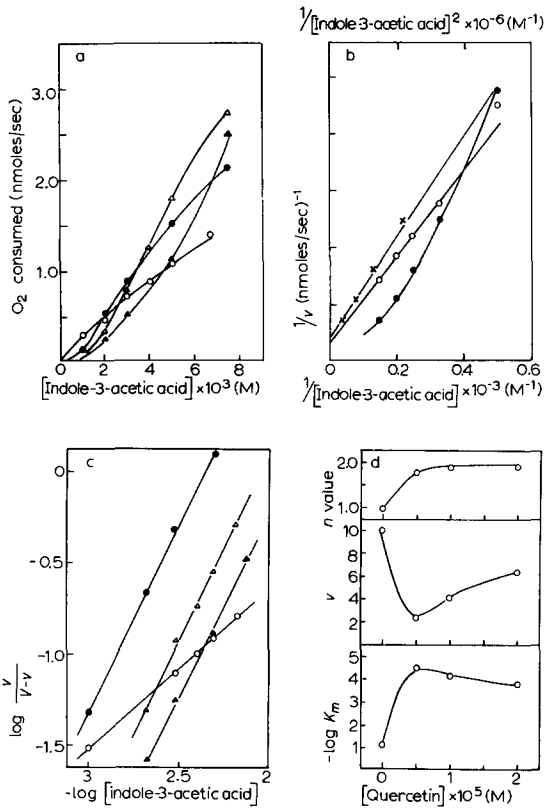

Fig. 3. The rate of indole-3-acetic acid oxidation as a function of indole-3-acetic acid concentration in the absence (○—○) and presence of $5 \cdot 10^{-6}$ M (●—●), $1 \cdot 10^{-5}$ M (△—△) and $2 \cdot 10^{-5}$ M quercetin (▲—▲). Reaction mixture: 0.16 μM peroxidase, various concentrations of indole-3-acetic acid and 0.05 M acetate, pH 5.5. Temp., 25.5°. (a) The reaction rate plotted against indole-3-acetic acid concentrations. (b) Double-reciprocal plots of data in (a). ○—○, control; ●—●, in the presence of $1 \cdot 10^{-5}$ M quercetin. The same data plotted against the square of reciprocal of indole-3-acetic acid concentration in the presence of $1 \cdot 10^{-5}$ M quercetin is also shown (×—×). (c) Hill plots of data in (a). (d) Kinetic parameters which were obtained by plots of (b) and (c) demonstrated as a function of quercetin concentrations. The V values are expressed in nmoles O_2 consumed per sec per 0.1 μM peroxidase.

obtained at a pH higher than 5.0 (Fig. 5a). Double-reciprocal plots gave straight lines when the reaction rate was plotted against the nth power of the reciprocal of the indole-3-acetic acid concentration. The n values were 1.0 at a pH value lower than 4.5, 1.5 at pH 5.0 and 2.0 at pH 5.5 (Fig. 5b). These values agree well with those obtained by Hill's plots (Fig. 5c). The kinetic parameters obtained by these treatments are summarized in Fig. 5d. In the absence of quercetin, both the apparent K_m value and the maximal velocity increase as the pH value increases, while the n value is fixed at 1.0. In the presence of $1 \cdot 10^{-5}$ M quercetin, these parameters are essentially the same as those obtained in the absence of quercetin at a pH lower than 4.5. However, the apparent K_m decreases when n value increases near 2.0 at a pH higher than 5.0. This suggests that some kind of substrate co-operation takes place.

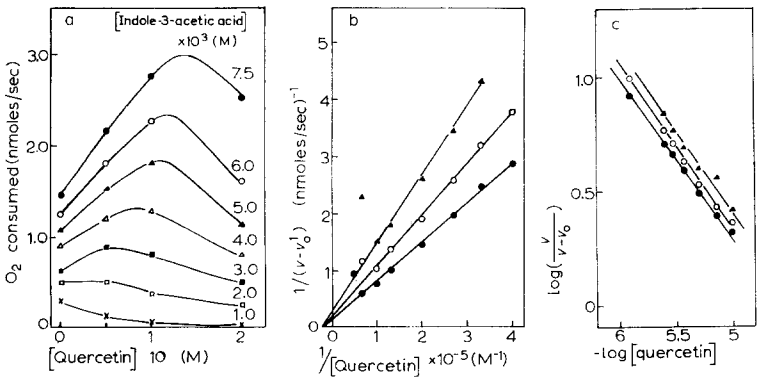

Fig. 4. Effect of quercetin concentration on the reaction rate at various concentrations of indole-3-acetic acid. The data of Fig. 3a are plotted. (a) The reaction rate plotted against quercetin concentrations at various concentrations of indole-3-acetic acid which are indicated in figure. (b) Double-reciprocal plots of data in (a). (c) Hill plots of data in (a). The slope is 0.75 at each straight line. Indole-3-acetic acid concentrations in (b) and (c), $5 \cdot 10^{-3}$ M (▲—▲), $6 \cdot 10^{-3}$ M (○—○) and $7.5 \cdot 10^{-3}$ M (●—●).

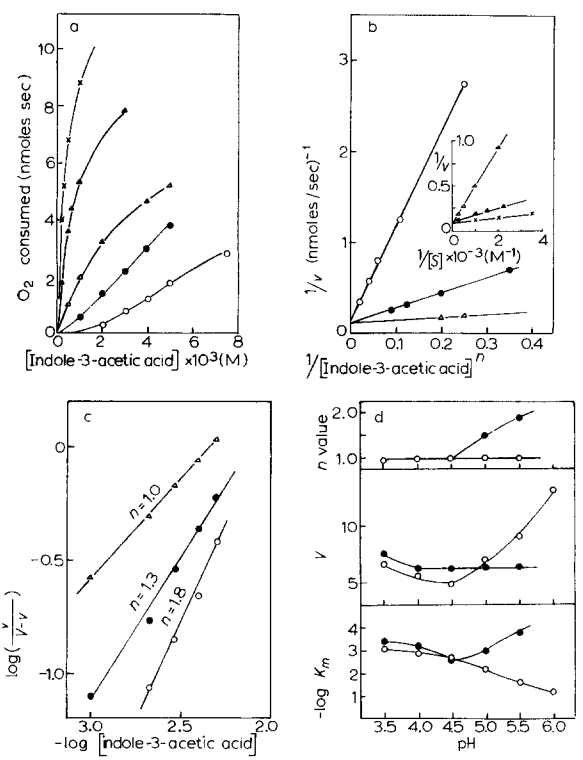

Fig. 5. (a) Effect of pH on the reaction rate as a function of indole-3-acetic acid concentrations in the presence of $1 \cdot 10^{-5}$ M quercetin. Reaction mixture: 0.16 μM peroxidase, various concentrations of indole-3-acetic acid and 0.05 M acetate, pH 3.5 (×—×); pH 4.0 (▲—▲); pH 4.5 (△—△); pH 5.0 (●—●); pH 5.5 (○—○). Temp., 25.5°. (b) Double-reciprocal plots of data in (a). The n values are 1.0 at pH values lower than 4.5, 1.5 at pH 5.0 and 2.0 at pH 5.5. Symbols which represent pH values are the same as those in (a). (c) Hill plots of data in (a). (d) Effect of pH on the kinetic parameters which were obtained from (b) and (c). The V values are expressed in nmoles O_2 consumed per sec per 0.1 μM peroxidase. ○—○, control (in the absence of quercetin); ●—●, in the presence of $1 \cdot 10^{-5}$ M quercetin.

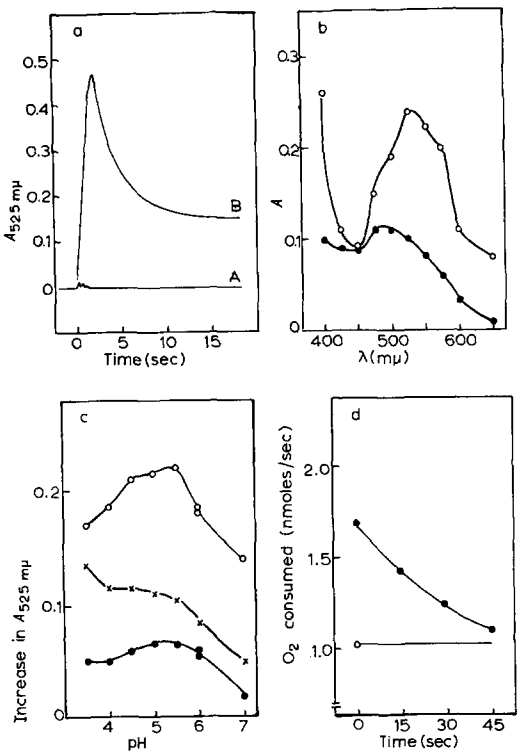

Fig. 6. (a) The time-course of quercetin degradation by peroxidase. The assay method is stated in the text. Reaction mixture: $6.7 \cdot 10^{-5}$ M quercetin, 0.07 μM peroxidase, 0.05 M acetate, pH 5.5. Temp., 25°. A, control (reaction mixture without enzyme); B, reaction mixture with enzyme. (b) Absorption spectra of the reaction products measured 2 sec (○—○) and 10 sec (●—●) after the start of reaction. (c) Effect of pH on the quercetin degradation by peroxidase. The reaction rate was measured at 2 sec (○—○), 10 sec (×—×) and 30 sec (●—●) after the start of reaction. (d) Decrease of effect of quercetin by a time lag between the addition of quercetin and indole-3-acetic acid. Reacting condition: $5 \cdot 10^{-3}$ M indole-3-acetic acid was added at various intervals after the addition of $1 \cdot 10^{-5}$ M quercetin to the reaction mixture containing 0.16 μM peroxidase and 0.05 M acetate, pH 5.5. Temp., 25.5°.

Chemical change of quercetin by peroxidase

When the enzyme solution was added to the reaction mixture containing quercetin, the color of the reaction mixture changed to purple for a few seconds in the absence and presence of indole-3-acetic acid. This color change monitored by a recording spectrophotometer revealed that quercetin changed *via* a short-lived intermediate (purple compound) to the final stable compound (Fig. 6a). The absorption spectra of the products are shown in Fig. 6b. The purple compound measured 2 sec after the start of the reaction had an absorption maximum at around 530 mμ, and the final product at 490 mμ. The reaction was maximal at pH 5.5 (Fig. 6c), which was identical with the case of guaiacol peroxidation by this enzyme[6]. It must be noted that no O_2 was consumed by this reaction and that H_2O_2 had no effect.

When indole-3-acetic acid was added a little after the addition of quercetin to the reaction mixture containing the enzyme, the activation effect of quercetin on the indole-3-acetic acid oxidation was reduced (Fig. 6d). This suggests that quercetin

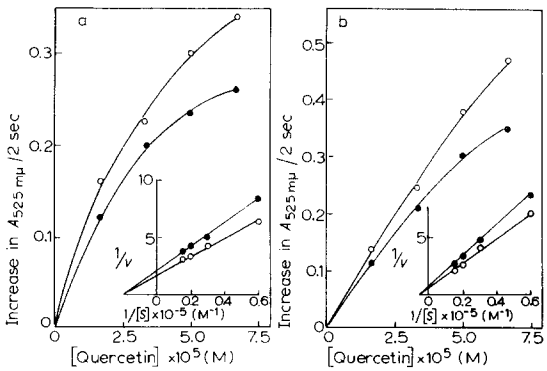

Fig. 7. The rate of quercetin degradation as a function of quercetin concentrations in the absence (○—○) and presence (●—●) of indole-3-acetic acid. Reaction mixture: 0.07 μM peroxidase, various concentrations of quercetin and 0.05 M acetate. (a) Measured at pH 3.5, indole-3-acetic acid concentration, $1 \cdot 10^{-3}$ M. (b) Measured at pH 5.5, indole-3-acetic acid concentration, $3.3 \cdot 10^{-3}$ M.

itself and/or the short-lived purple compound is responsible for the dynamic effect on the indole-3-acetic acid oxidation.

The effect of quercetin concentration upon the formation of the purple compound in the presence and absence of indole-3-acetic acid was examined, and curves of the Michaelis–Menten type were obtained (Fig. 7). The apparent K_m calculated from the double-reciprocal plots were $5 \cdot 10^{-5}$ and $2.5 \cdot 10^{-4}$ M at pH 3.5 and 5.5, respectively. Indole-3-acetic acid inhibited the reaction non-competitively at both pHs of 3.5 and 5.5. The apparent inhibition constant (K_i) was $3 \cdot 10^{-3}$ and $1.5 \cdot 10^{-2}$ M at pH 3.5 and 5.5, respectively.

Inhibition studies

KCN is well-known to inhibit the enzymatic reaction of peroxidase by binding

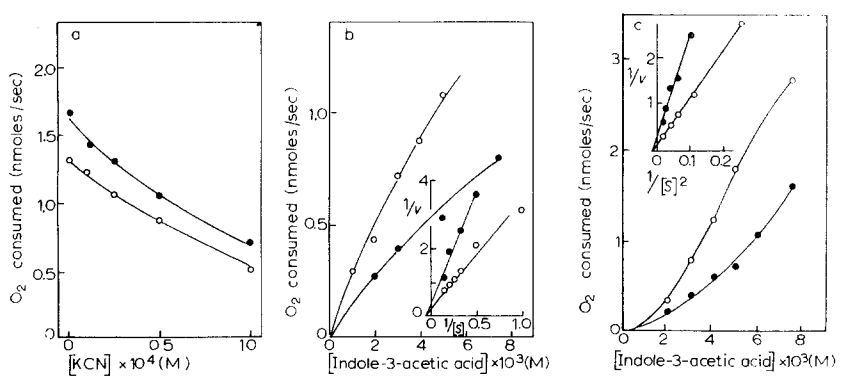

Fig. 8. (a) Effect of KCN concentrations on the indole-3-acetic acid oxidation in the absence (○—○) and presence (●—●) of $1 \cdot 10^{-5}$ M quercetin. Reaction mixture: 0.16 μM peroxidase, $5 \cdot 10^{-3}$ M indole-3-acetic acid and 0.05 M acetate, pH 5.5. Temp., 25.5°. (b) Effect of substrate concentrations on the indole-3-acetic acid oxidation in the absence (○—○) and presence (●—●) of $1 \cdot 10^{-4}$ M KCN. Reacting mixture: 0.16 μM peroxidase, various concentrations of indole-3-acetic acid and 0.05 M acetate, pH 5.5. Temp., 25.5°. (c) A similar experiment to (b) except that $1 \cdot 10^{-5}$ M quercetin was present.

to the heme. This was also the case with the indole-3-acetic acid oxidation by Alaska pea peroxidase. However, even in the presence of KCN, $1 \cdot 10^{-5}$ M quercetin promoted the reaction to the extent of 20% over the whole KCN concentration examined (Fig. 8a). When the reaction rate was determined as a function of indole-3-acetic acid concentration in the presence and absence of $1 \cdot 10^{-4}$ M KCN, hyperbolic and sigmoidal curves were obtained in the absence and presence of $1 \cdot 10^{-5}$ M quercetin, respectively. Double-reciprocal plots gave straight lines, and KCN inhibited the reaction non-competitively with the apparent K_i of $1 \cdot 10^{-3}$ M in the presence and absence of quercetin (Figs. 8b, 8c). These results suggest that heme does not bind quercetin nor indole-3-acetic acid.

DISCUSSION

Kinetics at pH 3.5

Both quercetin and indole-3-acetic acid are destroyed by peroxidase, and each modified the kinetic behaviour in the destruction of the other. Their kinetic parameters are summarized in Table I. The enzyme destroys quercetin with an apparent

TABLE I

RELATIONSHIPS BETWEEN KINETIC PARAMETERS FOR PEROXIDASE AT THE TIME OF REACTION WITH INDOLE-3-ACETIC ACID AND QUERCETIN AT pH 3.5 AND 5.5

E,S and X represents the enzyme, indole-3-acetic acid and quercetin, respectively.

Equations	*pH 3.5*	*pH 5.5*
$E + S \rightleftharpoons ES \rightharpoonup$	$K_s = 1 \cdot 10^{-3}$ M, $n = 1.0$	$K_s = 1.6 \cdot 10^{-2}$ M, $n = 1.0$
$E + X \rightleftharpoons EX \rightharpoonup$	$K_x = 5 \cdot 10^{-5}$ M	$K_x = 2.5 \cdot 10^{-4}$ M, $n = 0.75$
	K_i of $S = 3 \cdot 10^{-3}$ M	K_i of $S = 1.5 \cdot 10^{-2}$ M
$ES + X \rightleftharpoons ESX^{**} \rightharpoonup$	$K_{x'} = 5 \cdot 10^{-5}$ M	$K_{x'} = 2.5 \cdot 10^{-4}$ M, $n = 0.75$
		K_a of $X = 1 \cdot 10^{-4}$ M
$EX + S \rightleftharpoons EXS^{**} \rightharpoonup$	$K_{s'} = 3.7 \cdot 10^{-4}$ M*, $n = 1.0$	$K_{s'} = 4 \cdot 10^{-4}$ M*, $n = 1.9$

* In the presence of $1 \cdot 10^{-5}$ M quercetin.
** ESX is reduced to EX and S', and not to ES and X'.

K_m of $5 \cdot 10^{-5}$ M. Indole-3-acetic acid inhibited the quercetin destruction non-competitively, that is only the enzyme–quercetin complex is active and enzyme–indole-3-acetic acid–quercetin and enzyme–indole-3-acetic acid complexes are inactive for quercetin destruction. The apparent K_i of indole-3-acetic acid for this reaction is $3 \cdot 10^{-3}$ M, which must be same theoretically with the dissociation constant for the enzyme–indole-3-acetic acid complex. Apparent K_m for the indole-3-acetic acid oxidation is $1 \cdot 10^{-3}$ M, which agrees well with its K_i in the quercetin destruction. On the other hand the apparent K_m for the indole-3-acetic acid oxidation in the presence of $1 \cdot 10^{-5}$ M quercetin is $3.7 \cdot 10^{-4}$ M. If various Michaelis constants are assumed to be parameters for the affinity of substances to the enzyme, these results indicate that the affinity of quercetin to the enzyme is constant in the presence and absence of indole-3-acetic acid, though the reaction rate is inhibited by the enzyme–indole-3-acetic acid complex formation, and that once the enzyme–quercetin complex is formed, the affinity of indole-3-acetic acid to the enzyme increases much, resulting

in the activation of the reaction. When the quercetin concentration increases, the enzyme activity may change, giving a sigmoidal response to indole-3-acetic acid concentration, which will be mentioned later.

Kinetics at pH 5.5

Quercetin shows a dynamic effect on the indole-3-acetic acid oxidation, though quercetin itself shows a similar behaviour to the enzyme when compared with that at pH 3.5. Their kinetic parameters are summarized in Table I. The apparent K_m of the enzyme–quercetin complex is $2.5 \cdot 10^{-4}$ M, and indole-3-acetic acid inhibited the reaction non-competitively with an apparent K_i of $1.5 \cdot 10^{-2}$ M, which agrees well with the apparent K_m, $1.6 \cdot 10^{-2}$ M, of the indole-3-acetic acid oxidation in the absence of quercetin. The apparent K_m of quercetin for the enzyme-indole-3-acetic acid–quercetin complex is also $2.5 \cdot 10^{-4}$ M, which agrees with the apparent activation constant (K_a), $1 \cdot 10^{-4}$ M, of quercetin for the indole-3-acetic acid oxidation. In the presence of $1 \cdot 10^{-5}$ M quercetin, the apparent K_m of the indole-3-acetic acid oxidation increases to $4 \cdot 10^{-4}$ M. These results are the same as those obtained at pH 3.5, and activation should therefore take place. However, the indole-3-acetic acid oxidation is inhibited at low substrate concentration and promoted as the substrate concentration increases.

To explain this sigmoidicity, the assumption is made that indole-3-acetic acid can be bound to two distinct sites of the enzyme, and that the second site is opened when the enzyme binds quercetin. Because the binding site of quercetin is independent from those of indole-3-acetic acid, the enzyme–quercetin complex may be treated as a whole like an ordinary allosteric enzyme, and the following equation can be derived.

$$\begin{array}{ll} S_1 \text{ site } K_1 & E' + S \rightleftharpoons E'S_1 \\ S_2 \text{ site } K_2 & E'S_1 + S \rightleftharpoons E'S_1S_2 \end{array}$$

where E' represents the enzyme–quercetin complex; $E'S_1$, the enzyme with the first site occupied by indole-3-acetic acid; $E'S_1S_2$, the enzyme with two sites occupied by indole-3-acetic acid. By assuming equilibrium conditions, the reaction velocity, v, can be expressed as follows:

$$v = \frac{V[S]^2}{K_1K_2 + K_2[S] + [S]^2}$$

which may be rearranged as

$$\left[\frac{V}{v} - 1\right][S] = K_2 + \frac{K_1K_2}{[S]}$$

If $((V/v) - 1)[S]$ is plotted against $1/[S]$, a straight line will be obtained. The ordinate intercept is K_2, and the slope of the straight line is K_1K_2. As shown in Fig. 9, K_1 and K_2 were evaluated to be $2 \cdot 10^{-1}$ and $3 \cdot 10^{-4}$ M, respectively. The reaction may be characterized by K_2 which will be equal to the actual K_m. As the apparent K_m for the indole-3-acetic acid oxidation in the presence of $1 \cdot 10^{-5}$ M quercetin was calculated to be $4 \cdot 10^{-4}$ M, the theoretical assumption agrees with the experimental data.

When the enzyme binds quercetin, some conformational alteration of the enzyme may occur, and the second binding site for indole-3-acetic acid will exposed with a dissociation constant of $3 \cdot 10^{-4}$ M. At the same time, however, this confor-

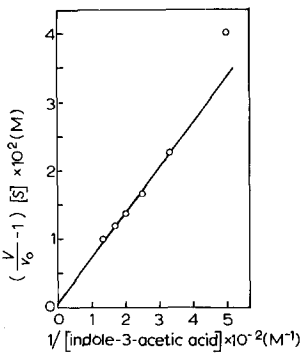

Fig. 9. The rate of indole-3-acetic acid oxidation as a function of indole-3-acetic acid concentrations in the presence of $1 \cdot 10^{-5}$ M quercetin at pH 5.5. Data in Fig. 3a are plotted according to a theoretical assumption stated in the text.

mational change makes the affinity of the first binding site for indole-3-acetic acid lower from $1.6 \cdot 10^{-2}$ to $2 \cdot 10^{-1}$ M. The two sites may interact with each other; the substrate at the second site is not oxidized unless the first site is occupied by the substrate. In this case, the first site may be the activation site and the other, the catalytically active site. The assumption can explain the inhibition effect at low substrate condition and the activating effect with increasing substrate concentration in the presence of quercetin. It is also supported by kinetic parameters such as the n value and various Michaelis constants.

The reason why the modified kinetic behaviour is pH-dependent is not clear. But the basic pattern seems to be unchanged, that is, the sigmoidal response to increasing the indole-3-acetic acid concentration can take place at any pH examined. Whether it occurs or not, depends upon the quercetin concentration, K_m for the enzyme–indole-3-acetic acid complex, state of the enzyme, *etc.*

Further studies are necessary to certify that peroxidase acts like an allosteric enzyme in the presence of modifier, and especially direct evidences which show the conformational alteration and the presence of two binding sites must be experimented.

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REFERENCES

1 Y. W. Tang and J. Bonner, *Arch. Biochem.*, 13 (1947) 11.
2 I. Yamazaki and L. H. Piette, *Biochim. Biophys. Acta*, 77 (1963) 47.
3 P. L. Goldacre, A. W. Galston and R. L. Weintraub, *Arch. Biochem. Biophys.*, 43 (1953) 358.
4 F. E. Mumford, D. H. Smith and J. E. Castle, *Plant Physiol.*, 36 (1961) 752.
5 M. Furuya, A. W. Galston and B. B. Stowe, *Nature*, 193 (1962) 456.
6 H. Sano, *Plant Cell Physiol.*, 11 (1970) 747.
7 H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658.