Studies on Branching Habits in Crop Plants

VI. On some characteristics of indoleacetic acid oxidase in stem tissue of corn plants

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INTRODUCTION

It is well known that auxin has an important role in apical dominance in higher plants, that is, auxin produced in the apical part of the plant inhibits the growth of the axillary buds^{1,2,10,20,21,24}). Auxin in plant tissue is destroyed by an enzyme system "indoleacetic acid (IAA) oxidase" which contains peroxidase and possibly flavoprotein.¹⁶) Some physiological phenomena such as growth and abscission which are known to be controlled by auxin have been reported to be related with IAA oxidase.^{7,8,16,17,20}) In addition, there are many naturally occurring and synthetic inhibitors and promoters of this enzyme system.^{5,11,12,15,16,19}) For instance, kaempferol-3-triglucoside (KG), its *p*-coumaric acid ester (KGC) and various monophenols act as promoters, while quercetin-3-triglucoside (QG) and its *p*-coumaric acid ester (QGC) and various *o*- and *p*-diphenols act as inhibitors of IAA oxidation.

In order to estimate the activity of this enzyme, a procedure to eliminate the inhibitors and promoters from the crude enzyme preparation must be carried out. The purification of the enzyme can be achieved by means of a) dialysis^{6,12,14}) or b) precipitating the enzyme with an organic solvent such as acetone⁶) or c) passing through a column of Sephadex.¹⁷)

The author conducted some experiments on the characteristics and method of purification of IAA oxidase from stem tissue of young light-grown corn plants in order to get informations needed about the enzyme, before dealing with the relationship between branching character and auxin metabolism in corn plants.

MATERIALS AND METHODS

Corn (*Zea mays* L.) cultivars, White Dent Corn and Golden Cross Bantam, obtained from Yamato Seeds and Implements Co. Ltd., Tokyo, were used as experimental materials. Seedlings were grown in the field at Komaba, Tokyo, several times in spring and summer in 1965. Young stem tissue above the 3rd node was collected from the seedlings which had been harvested at leaf age* 5 or 6, then frozen with dry ice immediately. After freezing, the stem tissue was lyophilized and kept at -20°C in a deep freezer until use.

The lyophilized stem tissue was ground in 1/50 M phosphate buffer, 2 ml for each mg of tissue, with a Potter-Elvehjem homogenizer in ice bath, extracted at 0°C for 30 min and centrifuged at $3,000 \times g$ for 10 min. The supernatant liquid was used as crude enzyme preparation. In order to remove the inhibitors and promoters from the crude enzyme solution, the cold acetone technique by Galston and Baker⁶) was used. Assay of IAA oxidase activity was carried out by the method in which the destruction of IAA by the enzyme in reaction mixture was estimated colorimetrically. The standard reaction mixture was composed of 2 ml of 10^{-3}M IAA, 5 ml of 1/15 M phosphate buffer pH 5.6, 1 ml of 10^{-3}M 2,4-dichlorophenol (DCP), 1 ml of 10^{-3}M manganese chloride (MnCl_2) and 2 ml of enzyme preparation. The reaction mixture in a test tube was kept in water bath at 26°C . One ml of the reaction mixture was taken out at definite interval and was added into 2 ml of Salkowski reagent. Then, after the color had developed at 30°C for

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* The age when leaf blade of the 5th leaf has just emerged out of the leaf sheath of the 4th leaf is referred to as leaf age 5.

30 min, the optical density of the mixture was measured at 530 $m\mu$.

Peroxidase activity was estimated with pyrogallol according to the method of Sumner and Gjessing.¹³⁾

RESULTS AND DISCUSSION

1. Destruction of IAA by crude enzyme

In the preliminary experiment which was carried out under diffusing light with a crude enzyme solution prepared as mentioned above, lag period as long as about 3.5 or 11.5 hr were observed as shown in fig 1. In some kinds of plant tissue, lag periods which are due to the presence of inhibitors have been reported.^{7,12)} Some experiments, therefore, were carried out to find out the way to remove the inhibitors.

Fig. 1. Time course of IAA destruction by crude enzyme preparation from stem tissue of light-grown corn plants. O.D. on ordinate describes residual IAA, measured colorimetrically.

2. Purification of IAA oxidase with acetone.

Galston and Baker removed inhibitors from crude enzyme preparation of pea plant by precipitating the enzyme with 4 ml cold acetone per 10 ml of crude enzyme.⁶⁾ But, in case of corn stem tissue, very little enzyme activity was precipitated with this concentration of acetone. Therefore, varying amounts of acetone were added. Five, 10, 12.5 or 15 ml of acetone which had been fully chilled with dry ice were added into 5 ml of crude enzyme solution extracted in 1/50 M phosphate buffer pH 5.6, which had been found optimal in another experiment (see below). After standing at 0°C in ice water for 10 min, the mixture was centrifuged at 3,000×g for 3 min. The supernatant was discarded, and the precipitate was redissolved in 1/50 M buffer to original volume, kept

Fig. 2. IAA oxidase activity of enzyme preparation purified through precipitation with various amount of acetone added to 5 ml of crude enzyme preparation.

at 2 to 3°C overnight, and centrifuged at 3,000×g for 5 min. The supernatant fluid was used as a purified enzyme preparation, whose estimated activity is shown in fig 2. High activity of enzyme was obtained with 10, 12.5 or 15 ml acetone, while very low activity was detected with 5 ml of acetone. As an unrefrigerated centrifuge was used (though the centrifuge tube and container had been fully chilled with dry ice before use), long centrifugation should be avoided in order to prevent decay of enzyme activity. Thus in next experiment, the crude enzyme solution was centrifuged for 3 min or 5 min after being

Fig. 3. Lag period and activity of IAA oxidase preparation purified through centrifugation at 3,000×g for 3 or 5 minutes. Various amounts of acetone added to 5 ml of crude enzyme preparation prior to centrifugation.

Table 1. Lag period, IAA oxidase and peroxidase activity of enzyme preparations after different centrifugation regime. Various amounts of acetone added to enzyme during purification.

Centrifugation	Amount of Acetone*	IAA	Oxidase	Peroxidase Activity***
		Lag Period	Activity**	
20,000 ×g 10 min	2	6'	0.18	4.52
	5	1'51"	2.28	8.22
	10	1'28"	3.40	11.86
3,000×g 3 min	10	41"	3.90	12.00
Crude Enzyme Prep.		—	—	14.72
L.S.D. 0.05		29"	0.34	0.91

* Expressed with ml acetone added to 5 ml of crude enzyme preparation.

** μg IAA oxidized/mg fresh wt/10 min

*** μg purpurogallin formed/mg fresh wt/15 min

added to the acetone. Both enzyme activity and length of the lag period, the latter due to small amount of inhibitor which were not removed by a single acetone precipitation, were estimated. The results are shown in fig 3. After centrifugation for 3 min, the tendency was almost the same as that in fig 2. But, after 5 min of centrifugation, the enzyme to which much acetone had been added had lower activity than that with less acetone. This appears to be due to the rise of temperature during the centrifugation. As for the lag period, it was longer after 5 min centrifugation than after 3 min, and longer with much acetone than with less acetone.

One question remained to be resolved, however, was there any possibility that the necessity for the addition of much acetone in corn stem IAA oxidase was due to failure of enzyme precipitation by such a short centrifugation as 3 or 5 min at low speed? To solve this problem, another experiment was carried out. Five ml of crude enzyme solution was centrifuged at 20,000×g for 10 min after being mixed with 2, 5 or 10 ml of cold acetone, or at 3,000×g for 3 min with 10 ml of cold acetone. In this experiment, a refrigerated centrifuge was used, preventing enzyme denaturation due to temperature rise. Table 1 shows the result, from which the following facts are clear. Five or 3 ml acetone for 5 ml crude enzyme solution is still

insufficient to precipitate all the activity even though rapid and long centrifugation was used. Centrifugation at 3,000×g for 3 min is enough for complete precipitation, and is better than at 20,000×g for 10 min, if the lag period is taken into account. The reason for the longer lag period with stronger centrifugation is not clearly known, but it might be due to precipitation of some inhibitor with protein. Estimation of peroxidase activity also shows that 3,000×g for 3 min is enough. About 80% of the original activity of peroxidase remained after a single purification procedure.

Taking into account these results, 12.5 ml of cold acetone was added to

each 5 ml crude enzyme preparation, and the mixture was centrifuged at 3,000×g for 3 min in the succeeding experiments.

3. Effect of pH on IAA oxidase activity

To determine optimal pH for the IAA oxidase, the

Fig. 4. Effect of pH on the activity of purified IAA oxidase preparation from stem tissue of light-grown corn plant.

Fig. 5. Effect of concentrations of manganese chloride and 2,4-dichlorophenol on the activity of purified IAA oxidase preparation from stem tissue of light-grown corn plants.

enzyme was redissolved in different pH of phosphate buffers, 1/50 M, after precipitated by acetone, and assayed for the activity with the standard reaction mixture. The results presented in fig 4 showed that the optimal pH was around 5.6. Thus IAA oxidase from corn stem differs from other IAA oxidase as regard pH optimum.^{6,7,8,12,14,20,22}

4. Effects of manganese and 2, 4-dichlorophenol concentration.

The effects of concentration of MnCl₂ and DCP on

Fig. 6. Courses of destruction of IAA by different concentrations of purified IAA oxidase preparations from stem tissue of light-grown corn plants. IAA concentration is expressed as usual.

* mg dry wt of stem tissue/ml enzyme prep.

the activity of IAA oxidase purified as above were examined. The results are shown in fig 5. According to the result, both MnCl₂ and DCP are needed for the action of this enzyme. IAA destruction was highest when both MnCl₂ and DCP were present at the concentration of 10⁻⁴ M. This result coincides with those for other IAA oxidase.^{5,9,16,22}

5. Destruction of IAA by different concentration of the enzyme preparation.

IAA was destroyed by various concentration of enzyme preparation (2, 4, 6, 8 or 10 mg dry wt of tissue in 1 ml enzyme preparation) prepared by diluting

Fig. 7. Courses of destruction of IAA by different concentrations of purified IAA oxidase preparations from stem tissue of light-grown corn plants. IAA concentrations are expressed in log scale.

* see Fig. 6.

Fig. 8. Courses of destruction of IAA by purified enzyme preparation from stem tissue of light-grown corn plants at different conditions of pH. IAA concentrations are expressed in log scale.

the highest concentration. The reaction proceeded in the presence of 10^{-4} M MnCl_2 , 10^{-4} M DCP and at pH 5.6. The course of destruction of IAA is shown in fig 6. In this experiment, a lag period was observed which was longer in high than in low concentration of the enzyme.

In order to calculate the true velocity of the reaction excluding lag period, the lag period must be estimated with the least possible error. It was found that the reaction proceeds as a function of the log of IAA concentration (fig 7), indicating a first order reaction. If the pH results are expressed in the same way, it is clear that the reaction is first order near the pH optimum (fig 8). From these results, it is clear that the length of lag period, and hence the reaction velocity after the lag period, can be estimated much more precisely by expressing the IAA concentration in a log scale. In addition, if so, the enzyme activity can be represented by a first order reaction velocity constant calculated by the following formula:

$$k = \frac{2.303}{t} \log_{10} \frac{a_0}{a_t}$$

Here, a_0 and a_t are concentrations of IAA at time 0 and t respectively.

The rate of IAA destruction by different concentration of the enzyme was expressed in two ways, that is, as μg IAA destroyed in 10 min and as a k value (table 2). The k value seems to reflect the enzyme activity better than does the amount of IAA oxidized, although it is not exactly related to the enzyme concentration.

SUMMARY

In connection with studies on the relationship between branching character and auxin metabolism in corn plants, experiments were carried out on the purification and characterization of the indoleacetic acid (IAA) oxidase of the stem tissue.

In the oxidation of IAA by crude enzyme preparations from corn stem, a lag in the reaction appeared to be due to the presence of inhibitors, as reported in other plants. From the results of experiments, the

Table 2. IAA oxidase activity of various concentration of enzyme preparation expressed by amount of IAA oxidized or reaction velocity constant, k .

Concentration of enzyme prep.*	Amount of IAA oxidized		Reaction velocity constant	
	μg IAA/10 min	Index	k value	Index
2	12.42	100	0.051	100
4	16.18	130	0.072	142
6	19.15	154	0.095	186
8	20.20	162	0.104	204
10	21.41	172	0.115	226

* concentration is expressed with mg dry weight of tissue per 1 ml final enzyme preparation.

following method was recommended for the acetone purification of the enzyme from stem tissue of corn: Add and mix 2 to 3 ml of fully chilled acetone into each ml of crude enzyme preparation, centrifuge at $3,000 \times g$ for about 3 min, redissolve the precipitate in the same volume of buffer solution as the original, keep it at 2 or 3°C overnight, and centrifuge again. Longer or more rapid centrifugation for precipitating the enzyme was rather harmful, because the lag period was prolonged without effect on the enzyme activity.

The following characteristics of this enzyme were observed:

1. The optimal pH for the reaction was around 5.6.
2. The reaction proceeded most rapidly when both manganese chloride (MnCl_2) and 2, 4-dichlorophenol (DCP) were present at the concentration of 10^{-4} M.
3. IAA oxidation by the enzyme proceeded as a first order reaction as a whole. Considering this fact, it is best a) to express the IAA concentration on a log scale for describing the course of reaction, in order to estimate the length of lag period with the least error, and b) to express the enzyme activity with the velocity constant, k , of the reaction instead of the amount of IAA destroyed.

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〔和 文 摘 要〕

作物の分枝性に関する研究

第6報 とうもろこし茎部のインドール酢酸酸化酵素の性質について

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とうもろこしの分枝性とインドール酢酸酸化酵素活性との関係を研究するに先立つて、同酵素の性質ならびに純化方法について、圃場で生育させたとうもろこし幼植物の茎部から抽出した酵素を用いて若干の研究を行なった。

従来、いくつかの植物について、インドール酢酸酸化酵素の阻害物質が存在することが知られているが、とうもろこし茎部からの粗酵素液についても阻害物質による著しい反応開始の遅れ (lag time) がみられた。そこで、アセトンを用いて阻害物質を除去する方法を検討した結果、この酵素では粗酵素液 1 ml に対して冷アセトン 2~3 ml を加えて攪拌し 0°C 下に約 10 分間おいた後、3,000×g で 3 分間程度遠心分離し、その沈殿をはじめの粗酵素液と同量の緩衝液に溶かす方法がよいことがわかった。この際もつと強い遠心分離を行なつても、ただ lag time が大きくなるのみで、酵素活性は増加しなかつた。

この方法で純化した酵素液を用い、いくつかの性質について実験を行なった結果、次のことが認められた。

1. 反応の最適 pH は 5.6 近辺であつた。
2. マンガン (MnCl_2) および 2, 4-dichlorophenol (DCP) がこの酵素の反応に必要なことが他の植物で知られているが、とうもろこしの酵素でも両者ともに 10^{-4}M 程度の濃度で存在する時、反応が最も速やかであつた。
3. 酵素濃度を変えて反応を行なわせた場合および上記 pH の影響に関する実験の際の反応の進み方を検討した結果、この酵素によるインドール酢酸 (IAA) の酸化反応が全体として 1 次反応に従つて進むことが認められた。また、最適 pH から離れた pH では、反応が 1 次反応として期待される速度よりも遅れてくることがみられた。1 次反応の場合の反応速度恒数 $k \left(= \frac{2.303}{t} \log_{10} \frac{a_0}{a_t} \right)$ で表わした場合の酵素活性の値と、一定時間内の IAA の酸化量で表わした場合の酵素活性を比較すると、前者の方が真の酵素濃度に近いことが認められた。また lag time を正確に知るためには、従来用いられている普通目盛によるグラフよりも、IAA 濃度を対数で示したグラフによる方が、反応が直線で示されるため誤差が少ないことが知られた。