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An in Vitro System of Indole-3-Acetic Acid Formation from Tryptophan in Maize (*Zea mays*) Coleoptile Extracts

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The formation of a product from tryptophan that had the same retention time as that of authentic indole-3-acetic acid (IAA) on high performance liquid chromatography was detected in crude extracts of maize (*Zea mays*) coleoptiles. The product was identified as IAA by mass spectrometry. The IAA-forming activity was co-purified with an indole-3-acetaldehyde (IAAld) oxidase activity by chromatography on hydrophobic and gel filtration (GPC-100) columns. During purification, the IAA-forming activity, rather than that of IAAld oxidase, decreased; but when hemoprotein obtained from the same tissue was added, activity recovered to the same level as that of IAAld oxidase. The promotive activity of the hemoprotein was confirmed by the result that the activity coincided with amounts of the hemoprotein after GPC-100 column chromatography. The hemoprotein was characterized and identified as a cytosolic ascorbate peroxidase (T. Koshiba [1993] Plant Cell Physiol [in press]). The reaction of the IAA-forming activity was apparently one step from tryptophan. The activity was inhibited by 2-mercaptoethanol. The optimum temperature for the IAA-forming system as well as for the IAAld oxidase was 50 to 60°C, and the activity at 30°C was one-third to one-half of that at 60°C. The system did not discriminate the L- and D-enantiomers of tryptophan.

IAA plays important roles in many physiological processes in plants, such as cell elongation, cell division, phototropism, and gravitropism. Many previous efforts to establish the pathway of IAA biosynthesis in plants have not yielded conclusive evidence. From the similarity of molecular structure between Trp and IAA, Trp was considered to be a precursor of IAA in plants, and two pathways from Trp that go through IPyA or TNH_2 have been generally accepted (Sembdner et al., 1981). These pathways were proposed mainly based on in vivo tracer experiments in which incorporation of radioactive Trp into the putative intermediates was investigated after a long period of feeding (6–72 h) (Khalifah, 1967; Sherwin and Purves, 1969; Gibson et al., 1972a, 1972b). However, after a short period of 1 to 3 h, incorporation of the label to the putative intermediates was not observed in maize tissues (Iino, 1982) or barley protoplasts (Sandberg et al., 1982).

Many in vitro experiments at the enzyme level have been done to confirm the presence of the hypothetical pathways. For example, the possible involvement in IAA biosynthesis of Trp decarboxylase (Sherwin, 1970; Elkinawy and Raa, 1974; Fernandez et al., 1989; Songstad et al., 1990), Trp aminotransferase (Truelsen, 1972; McQueen-Mason and Hamilton, 1989; Koshiba et al., 1993), TNH_2 oxidase (Phelps and Sequeira, 1967; Percival and Purves, 1974), and IAAld oxidase (Rajagopal, 1971; Purves and Brown, 1978; Miyata et al., 1981) has been extensively studied in plants. However, many of them showed wide substrate specificities, and no conclusive in vitro enzyme system that produces IAA from Trp has been obtained from any plant source. In addition, the pathways via IAOX (Helmlinger et al., 1987, Ludwig-Müller and Hilgenberg, 1988) and IAM (Kawaguchi et al., 1991) were recently reported. Thus, the IAA synthetic pathway from Trp in plants should be reinvestigated from different angles.

Recently, Cohen and co-workers suggested that Trp was not a precursor of IAA in Trp synthesis mutants of *Lemna* (Baldi et al., 1991) and maize (Wright et al., 1991). However, they reported more recently that IAA was synthesized from Trp in bean seedlings (Bialek et al., 1992). Another group demonstrated that a specific Trp pool that exhibited rapid turnover was involved in IAA biosynthesis in tomato shoots (Cooney and Nonhebel, 1991). Iino (1982) showed that IAA was synthesized from Trp in maize coleoptile tips and that the synthetic activity was inhibited by red-light irradiations. We confirmed the results with 2- to 3-mm tip sections of maize coleoptiles and found that an IAA-like product was produced from Trp in crude extracts of maize coleoptiles. The activity was retained after chromatography of the crude extract on hydrophobic and gel-filtration columns and was co-eluted with the IAAld oxidase, which had a relatively large mol wt of around 300,000. A similar in vitro system was reported only in extracts of oat (Lantican and Muir, 1967). Here, we report a novel IAA-forming system from maize coleoptiles.

MATERIALS AND METHODS

Plant Material

Seeds of maize (*Zea mays* L. cv Golden Cross Bantam 70) were soaked in running tap water overnight. The seeds were germinated on layers of wet paper at 24°C under red light (0.8 W/m^2) for 2 d and in the dark for another 2 d. Coleoptile

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Abbreviations: APX, ascorbate peroxidase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAOX, indole-3-acetaldoxime; IPyA, indole-3-pyruvic acid; TNH_2, tryptamine.

tips (8 mm long) were harvested, frozen with liquid N_2, and stored at −30°C.

Enzyme Extraction and Partial Purification

Extraction and purification of the enzyme were carried out at 0 to 4°C and the buffers used contained 1 mM $MgCl_2$. The coleoptile tips (1 cm long, 9.17 g) were homogenized in 18 mL of 0.1 M potassium phosphate buffer, pH 7.4, and centrifuged at 25,000*g* for 20 min. The supernatant (crude extract) was fractionated with ammonium sulfate. Precipitate obtained between 0.3 and 0.8 saturation was dialyzed against the phosphate buffer containing 20% (w/v) ammonium sulfate for 30 min. The enzyme solution was centrifuged at 25,000*g* for 15 min, and the supernatant was applied to a hydrophobic column, Phenyl-5PW (Tosoh, 8 × 75 mm), which had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, containing 20% (w/v) ammonium sulfate. The column was connected to an HPLC system (Tosoh, multipump CCPM) with a UV detector. After the column had been washed with 10 mL of the salt buffer, the adsorbed proteins were eluted with a linear gradient of ammonium sulfate from 20 to 0% (w/v) in the phosphate buffer at a flow rate of 0.667 mL/min. The elution of proteins was monitored at 280 nm, and 2-mL fractions were collected. The amount of hemoprotein was monitored at 405 nm, and IAA-forming and IAAld oxidase activities were assayed as described below. Fractions of hemoprotein and IAA-forming activity that had co-eluted with IAAld oxidase were separately pooled (hatched area in Fig. 1) and concentrated by ultrafiltration with Centrisart I (Sartorius, CS-10). Each sample was then applied to a gel-filtration column (GPC-100, SynchroPak, 7.8 × 300 mm) equilibrated with 50 mM phosphate buffer, pH 6.8, containing 0.15 M KCl. The column was developed with the same buffer at a flow rate of 0.3 mL/min.

Enzyme Assay

The reaction mixture (100 μL) for the IAA-forming activity contained 5 to 50 μL of an enzyme solution, 20 mM L-Trp, 0.1 M phosphate buffer, and 1 mM $MgCl_2$, pH 7.4, in the absence or presence of hemoprotein (5 μL, A_{403} = 0.1). The hemoprotein had been partially purified from maize coleoptiles by ammonium sulfate fractionation (0.5–0.8 saturation), Phenyl-5PW, and GPC-100 column chromatography in a manner similar to that described above. After incubation at 60°C for 40 min, 8 μL of 1 N HCl, 5 μL of 2 M $NaHSO_3$, and 50 μL of 100% methanol were added successively to the mixture. The mixture was centrifuged and 100 μL of the supernatant was injected onto the HPLC system (Tosoh, CCPM). HPLC was performed with an ODS C-18 column (Tosoh, 0.5 × 15 cm, 5-μm particle size) at a flow rate of 1 mL/min. The solvent system was a 10 to 50% methanol gradient containing 0.2% acetic acid. The indole compounds were monitored at 280 nm and the amount of IAA was determined from its peak area. One unit of the activity was defined as the activity required to produce 1 nmol of IAA under the assay conditions.

IAAld oxidase activity was assayed in 100 μL of reaction mixture that contained an enzyme solution, 0.15 mM free IAAld (prepared from IAAld bisulfite as described by Bower et al. [1978]), 0.1 M phosphate buffer, 1 mM $MgCl_2$, pH 7.4. Other procedures were the same as those described for the IAA-forming activity assay.

MS

A large-scale reaction was carried out to identify the product by MS. The reaction mixture (2 mL) consisted of 300 μL of an enzyme solution obtained after Phenyl-5PW column chromatography, 100 μL of partially purified hemoprotein (A_{403} = 1.0), 20 mM L-Trp, 0.1 M phosphate buffer, and 1 mM $MgCl_2$, pH 7.4. After incubation at 60°C for 40 min, 160 μL of 1 N HCl was added to the mixture. The acidic and neutral materials were partitioned to ether phase (4 mL × 2). After the ether was removed at 40°C, the residue was dissolved in 20 μL of methanol. Samples of 5 μL were analyzed with a direct capillary inlet to a JMS-DX300 mass spectrometer at 70 electron volts.

RESULTS

When a crude extract from maize coleoptiles was incubated with L-Trp, a product that had the same retention time as that of authentic IAA was detected on HPLC. As described below, the product was identified as IAA by MS. The IAA-forming activity was partially purified by ammonium sulfate fractionation and chromatography on Phenyl-5PW and GPC-100 columns (Table I). The activity was co-purified with

Table I. *Partial purification of IAA-forming system from maize coleoptiles*

Crude extract was prepared from maize coleoptile tips and IAA-forming system was purified by $(NH_4)_2SO_4$ fractionation and Phenyl-5PW and GPC-100 column chromatography. IAA-forming activity in the absence or presence of hemoprotein and IAAld oxidase were assayed as described in the text. One unit of the activity was defined as the activity required to produce 1 nmol of IAA under the assay conditions.

Purification Step	IAA-Forming Activity		IAA-Forming Activity + Hemoprotein		IAAld Oxidase Activity	
	units	%	*units*	%	*units*	%
Crude extract	219	100	213	100	268	100
30–80% $(NH_4)_2SO_4$	160	73	126	59	192	72
Phenyl-5PW	20	9	64	30	55	21
GPC-100	6	3	36	17	42	16

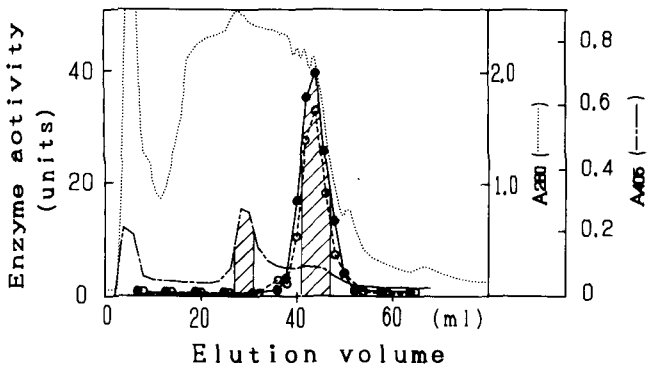

Figure 1. Hydrophobic (Phenyl-5PW) column chromatography of IAA-forming system. Enzyme extraction and procedures of the chromatography are described in the text. IAA-forming activity in the presence of hemoprotein (—●—) and IAAld oxidase activity (- - -O- - -) were assayed as described in the text. Proteins (·····) and hemoproteins (— - —) were monitored by A_{280} and A_{405}, respectively.

IAAld oxidase activity on chromatography (Figs. 1 and 2), but the ratio of IAA-forming activity to IAAld oxidase activity decreased during purification (Table I). However, the IAA-forming activity was restored to the same level as IAAld oxidase activity by addition of a hemoprotein that was obtained from the same extract. The promotive activity of the hemoprotein was also confirmed after chromatography on a GPC-100 column (Fig. 3). The promotive activity coincided with amounts of the hemoprotein, which had a mol wt of about 28,000. The hemoprotein was characterized and identified as cytosolic APX (Koshiba, 1993), and the concentration

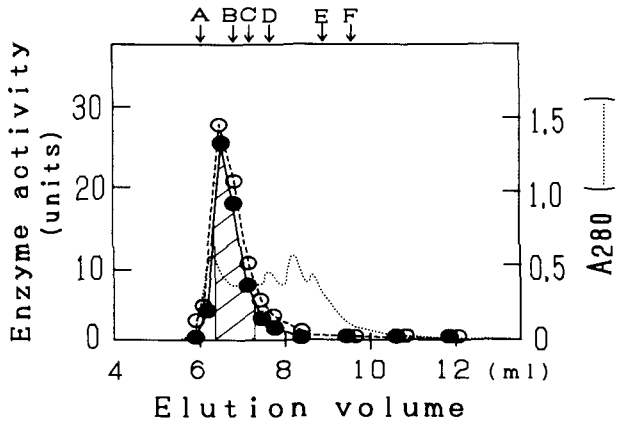

Figure 2. Gel filtration (GPC-100) column chromatography of the IAA-forming system. Fractions of the IAA-forming system co-eluted with IAAld oxidase on Phenyl-5PW column chromatography (hatched area in Fig. 1) were subjected to GPC-100 column chromatography. IAA-forming activity in the presence of hemoprotein (—●—) and IAAld oxidase activity (- - -O- - -) were assayed as described in the text. Proteins (·····) were monitored by A_{280}. Arrows indicate positions of molecular markers: A, appoferitin (440,000); B, alcohol dehydrogenase (150,000); C, BSA (67,000); D, ovalbumin (45,000); E, carbonic anhydrase (29,000); F, Cyt c (12,500).

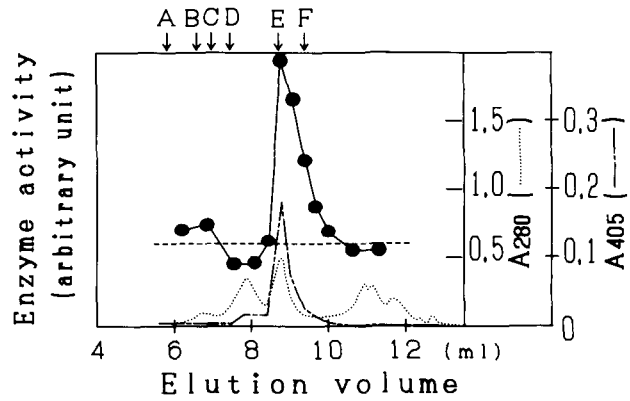

Figure 3. Promotive activity of hemoprotein for IAA-forming activity of IAAld oxidase fraction. The peak fractions of hemoprotein on Phenyl 5-PW column chromatography (hatched area in Fig. 1) were subjected to GPC-100 column chromatography and each fraction was tested for its promotive activity for IAA-forming activity of the IAAld oxidase fraction. The oxidase fraction was obtained from GPC-100 column chromatography (hatched area in Fig. 2). IAA-forming activity in the presence of 10 μL of each fraction was assayed as described in the text. The broken line represents the level of IAA-forming activity without addition of the fraction. Hemoprotein and protein were monitored for A_{405} (— - —) and A_{280} (·····), respectively. Arrows indicate positions of the same molecular markers as described in the legend for Figure 2.

needed for maximum activation of the IAA-forming activity was about 15 nM (the concentration was calculated from the extinction coefficient of α-band at 556 nm, 20 = 1 mM [1-cm light path], and from its mol wt of 28,000). Higher concentration of the protein caused the production of many unknown products other than IAA. Under these conditions, the IAA-forming activity was almost the same as that of IAAld oxidase on Phenyl-5PW and GPC-100 column chromatography (Figs. 1 and 2, Table I). The M_r of the IAA-forming system as well as IAAld oxidase estimated on gel filtration was about 300,000 (Fig. 2).

The reaction products of the IAA-forming system were analyzed by HPLC. A peak having the same retention time as authentic IAA was detected (Fig. 4a). No other product such as IPyA, TNH_2, or IAAld was observed. After the reaction of the hemoprotein (15 nM APX) and Trp, neither IAAld nor IAA was detected (Fig. 4b). A large-scale reaction (2 mL of the mixture) of the IAA-forming system was carried out, and the product was analyzed by MS. The mass spectra of authentic IAA showed major fragment ions of m/z 175 (M^+), 130 (base peak), 131, 103, and 77 (Fig. 5a). The same fragments were observed in the peaks of the reaction product, with small amounts of unknown contaminants, such as m/z 167, 149, 71, 56, and 53 (Fig. 5b). Thus, the product was identified as IAA.

Some properties of the IAA-forming system were characterized (Table II). The reaction did not proceed under anaerobic conditions and was inhibited by reductants such as 2-mercaptoethanol. The activity was completely lost when the system was boiled for 5 min. The system did not discriminate between the L- and D-enantiomers of Trp as its substrates.

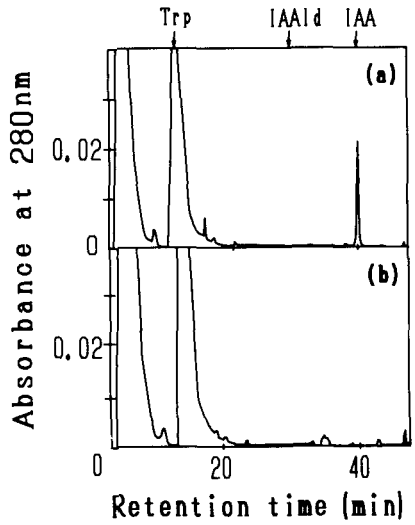

Figure 4. HPLC analysis of reaction products of the IAA-forming system. The IAAld oxidase fraction obtained from GPC-100 column chromatography (hatched area in Fig. 2) was used for the reactions. Elution profiles of the products of the IAA-forming system in the presence of homoprotein (a) and only hemoprotein with Trp (b) were recorded by monitoring the A_{280}. The positions of Trp, IAAld, and IAA are indicated with arrows.

Optimum concentration of Trp for the activity was more than 10 mM. The optimum temperature for the IAA-forming system was at 60 to 70°C, which was similar to that of IAAld oxidase (Fig. 6). At 30°C, the activity was about one-third of that at 60°C. The pH optima of the IAA-forming system and also IAAld oxidase were pH 6.5 to 7.5. Effects of various compounds, such as NAD(P), NAD(P)H, ascorbate, H_2O_2, Mn^{2+}, pyridoxal-5-phosphate, FAD, FMN, vitamin K_3, and pyrrolo-quinoline quinone, on the activity were tested in the presence or absence of the hemoprotein. Among these, FAD, FMN, and pyrrolo-quinoline quinone showed a significant promotive effect. However, FAD and FMN had a nonenzymic catalytic activity of conversion from Trp to IAAld (our unpublished observation), and pyrrolo-quinoline quinone was also reported to have such activity (Itoh et al., 1984).

DISCUSSION

In the present study, we found an IAA-forming activity from Trp in maize coleoptile tips. The activity was co-purified with IAAld oxidase and required a hemoprotein, extracted from the same tissue, for its full activity. The hemoprotein, which was identified as cytosolic APX (Koshiba, 1993), showed no activity for Trp as substrate at least at the optimum concentration (15 nM) for the IAA-forming activity. These results present two possibilities.

The first possibility is that IAAld oxidase itself has a catalytic activity to form IAA from Trp, and APX or some other factor(s) promotes the activity. Animal aldehyde oxidase, which contains two FAD, two Mo, eight Fe, and two

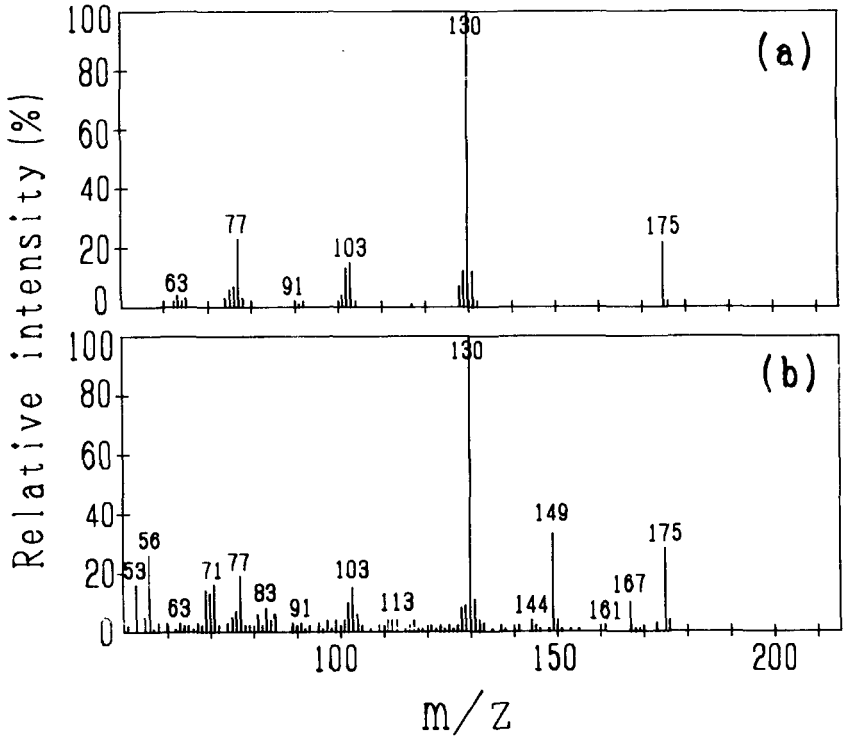

Figure 5. Mass spectra of authentic IAA and the product of the IAA-forming system. Procedure details are described in the text. a, Fragment patterns with MS of authentic IAA. b, The product obtained from large-scale reaction of the IAA-forming system. Authentic IAA (500 ng) and 300 ng of the product dissolved in methanol were subjected to MS.

coenzyme Q_{10} as prosthetic groups, has broad substrate specificity (Rajagopalan and Handler, 1966; Felsted et al., 1973; Yoshihara and Tatsumi, 1985). The enzyme also has various catalytic activities other than oxidation of aldehydes, such as oxidation of nitrogen-containing heterocyclic compounds (Hall and Krenitsky, 1986), reduction of diphenyl sulfoxide (Yoshihara and Tatsumi, 1986), nitroreduction of aromatic heterocyclic compounds (Bauer and Howard, 1991), and oxime-metabolizing activity (Tatsumi and Ishigai, 1987). The enzyme sometimes exhibits strong activity against a specific substrate if some cofactor is present (K. Tatsumi, personal communication). The mol wt of 260,000 to 350,000 and the heat-stable property of the animal enzyme are similar to those of IAAld oxidase investigated here. Plant aldehyde oxidases have been reported in potato tubers (Rothe, 1974) and oat coleoptiles (Rajagopal, 1971) and IAAld oxidases in cucumber (Purves and Brown, 1978) and pea (Miyata et al., 1981) seedlings, but no detailed characterization with purified enzyme has been reported.

The second possible explanation for our results is that APX has a catalytic activity to form IAAld from Trp in the presence of IAAld oxidase, which catalyzes successively the reaction from IAAld to IAA. APXs, a kind of peroxidase, are known to have specificity for ascorbate as an electron donor. Chloroplastic APX is known to have a function in the scavenging of hydrogen peroxide in plant cells (Asada and Takahashi, 1987), but the physiological role of cytosolic APX is obscure. The maize APX in the present work was purified and characterized as a cytosolic APX (Koshiba, 1993). Multifunctional properties of plant peroxidases are well known, as are some oxidative activities in relation to phytohormone metabolism, such as the oxidation of IAA (Nakano et al., 1982), the production of IAM (Riddle and Mazelis, 1964, 1965) and IAOX (Ludwig-Müller et al., 1990) from Trp, and the ethylene formation from ACC (Boyer and De Jaegher, 1986; Acosta et al., 1991). APX from *Acetabulaia*, mung bean, and sugar beet was also reported to have the activity for converting ACC to ethylene (Kevers et al., 1992). The mechanisms of these peroxidase actions are unknown, but it is possible that Trp molecules are oxidized by APX (or peroxidase) to form IAAld under some conditions.

To reveal in more detail the mechanism of the IAA-forming reaction and also the possibility of its involvement on IAA production in vivo, we are now conducting further purification and characterization of IAAld (or aldehyde) oxidase and in vivo tracer experiments using [^{14}C]Trp in combination with the in vitro reaction as shown in the present study.

Table II. *Some properties of the IAA-forming system from maize coleoptiles*

Standard reaction mixture (100 μL) contained 20 mM L-Trp, 5 μL of hemoprotein (A_{403} = 1.0), 0.1 M potassium phosphate buffer, pH 7.4, 1 mM $MgCl_2$, and partially purified IAAld oxidase obtained from GPC-100 column chromatography

Treatment	Relative Activity
	%
Complete	100
+ 2-Mercaptoethanol (1 mM)	16
Anaerobic condition	13
Boiled for 5 min	0
− L-Trp, + D-Trp	97

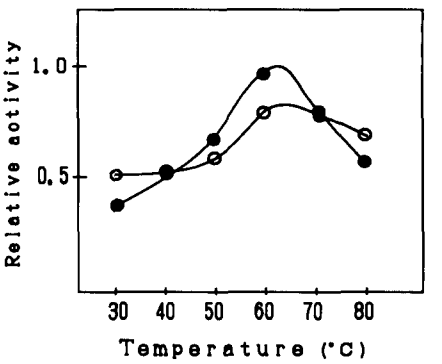

Figure 6. Effect of reaction temperature on IAA-forming and IAAld oxidase activities. The activities of the IAA-forming system in the presence of hemoprotein (—●—) and IAAld oxidase (—O—) were assayed as described in the text at the temperatures indicated.

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