Biochemical Analysis of Auxin-Binding Proteins Purified from Etiolated Mung Bean Hypocotyls

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Abbreviation

ABP	: auxin-binding protein
ADH	: alcohol dehydrogenase
BSA	: bovine serum albumin
2,4-D	: 2,4-dichlorophenoxyacetic acid
DEAE	: diethylaminoethyl
cDNA	: complementary DNA
EDTA	: ethylenediaminetetraacetate
ELISA	: enzyme-linked immunosorbent assay
ER	: endoplasmic reticulum
FDH	: formaldehyde dehydrogenase
GSH	: glutathione
GST	: glutathione S-transferase
HPLC	: high-performance liquid chromatography
IAA	: indole-3-acetic acid
IAAld	: indole-3-acetaldehyde
IEt	: indole-3-ethanol
KLH	: keyhole limpet hemocyanin
NAA	: naphthalene-1-acetic acid
NADH, NAD^+	: nicotinamide adenine dinucleotide, reduced and oxidized

NADPH, $NADP^+$: nicotinamide adenine dinucleotide phosphate, reduced and oxidized

NBCS : new-born calf serum

NPA : naphtylphthalamic acid

PBS : phosphate-buffered saline

PCIB : *p*-chlorophenoxyisobutylic acid

PMSF : phenylmethylsulfonyl fluoride

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TLC : thin-layer chromatography

Tris : N-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid

Abstract

Soluble auxin-binding proteins were isolated from an extract of etiolated mung bean hypocotyls by affinity chromatography on 2,4-dichlorophenoxyacetic acid (2,4-D)-linked Sepharose 4B. A 40-kDa polypeptide and a 39-kDa polypeptide could be detected in the fraction not only eluted with the solution containing 2,4-D, but also in that eluted with the solution containing indole-3-acetic acid (IAA). An auxin-binding protein containing 40-kDa polypeptide and an auxin-binding protein containing 39-kDa were purified by column chromatography. I designated the former protein ABP40 and the latter protein ABP39. The apparent molecular masses of ABP40 and ABP39 were estimated to be 80 kDa and 77 kDa by gel-filtration, respectively. These proteins should be dimers.

The dissociation constant of purified ABP40 for [^{14}C]-2,4-D was calculated to be 1 x 10^{-5} M. The binding of [^{14}C]-2,4-D was completely inhibited by *p*-chlorophenoxyisobutyric acid (PCIB) and weakly inhibited by IAA and naphthalene-1-acetic acid (NAA). Benzoic acid and tryptophan had no effect on the binding. The partial amino acid sequences of ABP40, obtained after chemical cleavage by CNBr, revealed a high homology to glutathione-dependent formaldehyde dehydrogenase (FDH; EC 1. 2. 1. 1.). The ABP40 had an enzyme activity of FDH.

The partial amino acid sequence of ABP39, obtained after cleavage by CNBr, revealed a high homology to the amino acid sequences of alcohol dehydrogenase (ADH; EC 1. 2. 1. 1.). While ABP39 was not capable of oxidizing ethanol, it catalyzed the reduction of indole-3-acetaldehyde (IAAld) to indole-3-ethanol (IEt). The IAAld reductase (EC 1. 2. 3. 1.) is specific for NADPH as a cofactor. The ABP39 also catalyzed the reduction of other aldehydes such as acetaldehyde, benzaldehyde, phenylacetaldehyde and propionealdehyde. Indole-3-aldehyde was a poor substrate. The FDH activity of ABP40 was inhibited by 2,4-D, and the IAAld reductase activity of ABP39 was inhibited by both IAA and 2,4-D. Immunological analysis revealed that both ABP40 and ABP39 existed mainly in the hypocotyls of etiolated mung bean seedlings and increased during the

elongation of the hypocotyls.

In conclusion, the present thesis demonstrates that auxins affect the enzyme activities of two ABPs directly and proposes that such modulations of enzyme activities of the ABPs cause physiological changes in plants.

Introduction

Plant hormones play a vital role in the control of growth and development within higher plants. It is now known that there are at least five major classes of plant hormones: auxins, gibberellins, cytokinins, abscisic acid and ethylene. Auxins were the first phytohormones discovered and are known to be of fundamental importance in the physiology of growth and differentiation. They affect various processes throughout the plant life cycle, such as cell division and elongation, root formation and development, leaf development, apical dominance, tropisms, flowering and fertility (Evans 1974).

The basis of our knowldge of auxins lies in Charles Darwin's (1896), published in *The Power of Movement in Plants.* Darwin investigated the phenomenon of phototropism and concluded that some influence is transmitted from the upper to the lower part, causing the latter to bend. Boysen-Jensen (1910) and Paal (1914) demonstrated that the growth-promoting influence was of a purely chemical nature. Paal suggested that this chemical acts as a correlative growth promoter. F.W. Went (1926) first separated the growth-promoting properties from a plant. The growth hormone was diffused from an excised oat (*Avena*) coleoptile tip to an agar block. The name "auxin" was given to the growth hormone produced by the tip of the coleoptile. Haagen-Smit *et al.* (1946) isolated indole-3-acetic acid (IAA) from immature corn kernels, the first isolation of crystalline auxin from a plant. Since the initial discovery of IAA as an auxin, it has been found that this substance occurs in most plant species, and it is thought to be the chief natural auxin. Since then, other compounds having auxin activity have been synthesyzed in many labolatories. These compounds, such as α-(indole-3)-propionic acid, 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA), are called synthetic auxins. A large number of physiological studies have been made to clarify the effects of auxins on the growth and differentiation of plants.

Various studies of the mechanism of auxin action at the molecular level have been made. Auxin-induced growth of soybean hypocotyl segments and RNA synthesis in

the segments were similarly inhibited by various concentrations of actinomycin D (Key *et al.* 1964). Pope and Black (1972) and Penny and Galston (1966) reported that auxin can cause a sizable elongation in antibiotic-treated tissues in which protein synthesis has been essentially eliminated. They suggested that the primary action of auxin is not at the level of protein synthesis. Cleland (1965) determined the effects of various treatments on the extensibility of *Avena* coleoptile cell walls, and noted that the effect of auxin on extensibility persists even after auxin-induced elongation has been completely blocked by actinomycin D. He concluded that RNA synthesis is not necessary for auxin-induced wall loosening, but is necessary for some other processes required in the elongation.

From the results of these experiments, at least two kinds of mechanisms of auxin action are thought to exist: 1) auxins affect the synthesis of mRNA and protein to cause physiological changes in plants and 2) auxins directly affect the pre-existing constituents. The former mechanism may involve receptor-like proteins that mediate the signals of auxin and resemble the receptors of animal hormones, while the latter may involve various pre-existing proteins such as enzymes, the proteins associated with the cell wall and plasma membrane. Auxin-induced gene expression is likely mediated by such receptor-like proteins. It is also likely that the physiological changes previously reported, such as enzyme activities, permeability of the plasma membrane and extensibility of the cell wall, were caused by the interaction of auxins and pre-existing proteins. Considering the effects of auxins, both the receptor-like proteins and the pre-existing proteins that interact with auxins recognize auxins specifically. The existence of these proteins that recognize auxins specifically has also been predicted by some physicochemical approaches described below.

The relationship between molecular structure and auxin activity has been investigated by comparing the structure and auxin activities of natural and synthetic auxins to clarify the structural requirments for auxin activity. Went and Thimann (1937) drew up a list of general structural requirements of a molecule for auxin activity. Hansch *et al.* (1963) proposed the two-point attachment theory. On the assumption that a carboxyl-terminated

side chain and a free *ortho* position on the ring system are essential for activity, they proposed that the basic reaction of an auxin within the cell involves two parts of the molecule, i.e., the carboxyl group of the side chain and an *ortho* position of the ring system. They proposed that there is a covalent bond formation at these two points between the auxin molecule and some constituent, possibly a protein, of the cell. Porter and Thimann (1965) pointed out that the molecules which are active as auxins contain a strong negative charge (arising from the separation of the carboxy group) which is separated from a weaker positive charge on the ring by a distance of about 5.5 Å (0.55 nm). These studies revealed that auxin activity is determined by structural and stereospecific properties of the molecules. It is predicted that there exist proteins that are able to recognize subtle differences between active and inactive analogues of auxin because proteins have the capacity to recognize the appropriate molecular structure of auxins.

To characterize these proteins, various auxin-binding proteins have been isolated (Klämbt 1990, Sakai 1992, Jones 1994). Hertel (1972) and his colleagues prepared a membrane fraction of maize coleoptiles with auxin-binding activity. They demonstrated that auxin-binding sites are located on the endoplasmic reticulum (ER), the tonoplast and the plasmalemma (Dormann *et al.* 1978). Löbler and Klämbt (1985 a,b) purified a 40-kDa ABP (ABP1) composed of two subunits of 22-kDa from the ER fraction of maize coleoptiles. The cDNA of ABP1 was cloned and the primary structure of the protein was elucidated (Tillmann *et al.* 1989, Hesse *et al.* 1989, Inohara *et al.* 1989). Several approaches such as immunological studies have been made to investigate the function of this protein. Other membrane associated ABPs in various plant species have been identified by photoaffinity labelling techniques. In tomato and zucchini, a 40- and 42-kDa auxin-binding doublet has been identified (Hicks *et al.* 1989, 1993). The 23-kDa ABP from the plasma membrane fraction of maize (pm23) was labelled by azide-NPA (NPA: naphthylphthalamic acid, an inhibitor of polar auxin transport) and thought to be a possible auxin efflux carrier (Zettl *et al.* 1992).

As auxins are considered to be synthesized in all plant cells and move easily through the cytoplasmic membrane, it is believed that auxin binding sites are also found in the cytosol and nucleus, not only in the membrane fractions. In fact, several auxin-binding proteins have been identified in both the cytosol and the nucleus. Soluble factors in the nuclear preparation of cultured tobacco cells were enriched sufficiently to detect high auxin binding activity (Libbenga *et al.* 1986). The addition of this preparation to isolated tobacco nuclei stimulates RNA polymerase II activity more than the addition of samples without auxin-binding activity (Libbenga *et al.* 1986). Jacobsen *et al.* (1987) demonstrated that two auxin-binding proteins (sABP1 and sABP2) isolated from pea epicotyls stimulated transcription in isolated nuclei in the presence of 10^{-8} M IAA. Sakai *et al.* (1983, 1984, 1985) has purified two proteins (ABP-I and ABP-II) from mung bean hypocotyls that have auxin-binding activity and may function in the nucleus. The addition of these ABPs to mung bean nuclei during *in vitro* run-on experiments causes an increase in [^{3}H]UMP incorporation into RNA. It has been suggested that the stimulation of RNA synthesis by both ABP-I and ABP-II was due to the activation of RNA polymerase II. The addition of IAA with ABP-I or -II causes quantitative changes among newly-synthesized transcripts (Kikuchi *et al.* 1989). A soluble 65-kDa ABP located in the nucleus has been identified by anti-idiotypic antibodies in different plant species. The localization of the ABP in the nuclei suggests that this ABP may be involved in controlling auxin-regulated gene expression (Prasad and Jones 1991).

The auxin-binding proteins that were identified as enzymes have been reported by several groups. Pat King *et al.* used photoaffinity labeling with 5-azido-[7^{3}H]IAA to detect ABPs in the soluble fraction of *Hyoscyamus muticus* cell cultures. 31-kDa and 25-kDa ABPs were identified as the basic form of β1,3-glucanase (Macdonald *et al.* 1991) and glutathione S-transferase (GST) (Bilang *et al.* 1993), respectively. A 24-kDa ABP isolated from *Arabidopsis* also has an activity of glutathione S-transferase (Zettl *et al.* 1994). Palme's group identified a 60-kDa soluble ABP (Zm-p60) from maize seedlings as a β-

glucosidase that specifically hydrolyzes cytokinin-glucoside conjugates, releasing an active cytokinin (Campos *et al.* 1992).

These ABPs are classified into two types accordings to their possible roles in auxin actions. One type of ABP may act as receptors of auxins in the signal transduction pathway, while the other type of ABP has an enzyme activity that may be modulated by auxins. However, there is still no evidence that indicates the function of these ABPs as receptors. It remains unclear whether auxin binding induces changes in the enzyme activities of these ABPs.

Recently, photoaffinity labeling has been used for the isolation of ABPs from plant materials. It has been well known that unspecific binding may occur. Careful consideration will be necessary to isolate the binding proteins by this method. Affinity chromatography is a powerful tool for the isolation of ABPs. Several ABPs have been isolated by affinity chromatography on IAA-linked or 2,4-D-linked Sepharose (Roy and Biswas 1977, Sakai 1983, 1985). 2-OH-3,5-diindobenzoic acid-linked Sepharose 4B (Löbler and Klämbt 1985) and naphthalene-1-acetic acid (NAA) -linked AH Sepharose 4B (Shimomura *et al.* 1986) have been widely used for the isolation of auxin-binding proteins from some plant tissues. In the present study, I isolated ABPs by affinity chromatography using 2,4-D-linked Sepharose 4B, which was used to isolate of ABP-I and ABP-II that are putative receptors of auxins (Sakai 1983, 1985).

Current knowledge of ABPs has not been sufficient to elucidate the mechanisms of auxin actions. As these actions are so diverse, there should be multiple ABPs in plant cells. A large number of ABPs should be involved in the physiological responses of plants to auxins. So, it is necessary to purify more ABPs and to characterize the proteins to clarify the mechanisms of auxin actions. In this thesis work, novel ABPs are isolated and purified from etiolated mung bean hypocotyls by affinity chromatography, and the properties of the proteins are characterized. The interaction of the ABPs to auxins is demonstrated and the significance of the binding of auxins to the ABPs in plants is discussed.

Materials and Methods

Plant materials

Mung bean (*Vigna radiata* L. Wilczek) seeds were soaked in water for 16 h and then grown on an agar-gel bed for 48 h at 28°C in darkness. Maize (*Zea mays* L.) seeds were soaked in water for 16 h and grown on moist paper towels for 5 days at 28°C in darkness.

Chemicals

IAAld was prepared from IAAld-sodium bisulfate (Sigma) according to the method of Brown and Purves (1976).

Preparation of ABP fractions

The following procedure was carried out at 0-4°C. 2,4-D-linked Sepharose 4B was prepared as described previously (Sakai and Hanagata 1983). Hypocotyls were excised from mung bean seedlings and homogenized in a Polytron (KINEMATICA, Swizerland) with extraction buffer [100 mM Tris-HCl buffer (pH 7.6), 0.2 M NaCl, 2 mM EDTA, 20 mM 2-mercaptoethanol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and filtered through two layers of Miracloth (CALBIOCHEM, U. S. A.). The filtrate was centrifuged at 200,000 x *g* for 30 min and the supernatant was applied to a column of Sephadex G-25 that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.6) that contained 0.1 M NaCl. Protein fractions were collected and applied to a column of 2,4-D-linked Sepharose 4B that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.6) that contained 0.1 M NaCl. The column was washed first with starting buffer and then washed with 50 mM Tris-HCl buffer (pH 7.6) that contained 1 M NaCl. The column was then eluted with 50 mM Tris-HCl buffer (pH7.6) that contained 0.1 M NaCl and 40 mM IAA or 50 mM Tris-HCl buffer (pH7.6) that contained 0.1 M NaCl and 40 mM 2,4-D. The eluted fractions were designated the ABP fractions and resolved by SDS-PAGE.

Purification of ABP40 and ABP39

The following procedure was carried out at 0-4°C. 2-Mercaptoethanol was added to each buffer used in the purificaton of ABPs to give a final concentration of 20 mM. The presence of ABP40 and ABP39 in each step of the purification process was examined by SDS-PAGE. Hypocotyls of etiolated mung bean seedlings were homogenized in a Polytron with an equal volume of 2x TSE buffer [100 mM Tris-HCl (pH 7.6), 0.2 M NaCl, 5 mM EDTA and 0.2 mM PMSF]. The homogenate was filtered through two layers of Miracloth and centrifuged at 200,000 x *g* for 30 min. Solid ammonium sulfate was added to the supernatant to 60% saturation. After stirring for 1 h, the precipitate was collected by centrifugation at 10,000 x *g* for 30 min, then dissolved in 50 mM Tris-HCl the buffer (pH 7.6) and dialyzed against the same buffer (pH 7.6) for 12 h with several changes of buffer. The dialyzate was centrifuged at 200,000 x *g* for 30 min and the supernatant was applied to a column of DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.6). The column was washed with the same buffer and then eluted with 50 mM Tris-HCl buffer (pH 7.6) that contained 0.1 M NaCl. The eluted fractions were applied to a column of 2,4-D-linked Sepharose 4B that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.6) that contained 0.1 M NaCl and the column was then washed successively with the same buffer and with 50 mM Tris-HCl buffer (pH 7.6) that contained 1 M NaCl. The fractions containing ABP40 and ABP39 were eluted with 50 mM Tris-HCl buffer (pH 7.6) that contained 40 mM 2,4-D and 0.1 M NaCl. The eluted fractions were combined, dialyzed against 50 mM Tris-HCl buffer (pH 7.6) and applied to a column of hydroxyapatite that had been equilibrated with the same buffer. The fractions containing ABP40 and ABP39 were eluted with 50 mM potassium-phosphate buffer (pH 7.6) and the eluate was dialyzed against 50 mM Tris-HCl buffer (pH 7.6). The dialyzate was applied to a column of Mono Q HR 5/5 (Pharmacia) that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.6) and was then eluted with a linear gradient of 0 to 0.3 M NaCl in 50 mM Tris-HCl buffer (pH 7.6) at a flow rate of 0.5 ml/min with a HPLC system. The fractions containing ABP40 and those containing ABP39 were pooled separately. The buffer of the fraction containing ABP40 was replaced by 0.025 M

imidazole-HCl buffer (pH 7.4) by gel filtration using NAP5 (Pharmacia) and concentrated with Ms. BTAURY-KN (ATTO, Japan). The concentrated solution was subjected to chromatofocusing on a column of Mono P (Pharmacia) that had been equilibrated with 0.025 M imidazole-HCl (pH 7.4) with a HPLC system. The fractions containing purified ABP40 were eluted with Polybuffer (pH 4.0; Pharmacia) at a flow rate of 1 ml/min. The fractions containing ABP39 obtained by the ion-exchange chromatography on Mono Q were applied to a column of Mono Q HR 5/5 in the same condition as the previous column chromatography. The fractions containing ABP39 were pooled and concentrated with Ms. BTAURY-KN (ATTO, Japan). The concentrated solution was subjected to gel-filtration on a column of Superose12 that had been equilibrated with 50 mM Tris-HCl (pH 7.6) that contained 0.1 M NaCl with a HPLC system. The fractions containing purified ABP39 obtained by this gel-filtration was used for the experiments.

Binding assay

The binding assay was performed by the method of ammonium sulfate precipitation, as described previously (Sakai and Hanagata, 1983). The protein preparation and 1.11 kBq of 2,4-dichlorophenoxy [2-^{14}C] acetic acid (2,183 GBq / mmol, American Radiolabeled Chemicals, U. S. A.) in 100 µl of 50 mM Tris-HCl buffer (pH 7.6) were incubated at 25°C for 20 min. After the incubation period, 400 µl of 50 mM Tris-HCl buffer (pH 7.6), saturated with solid ammonium sulfate, was added to the reaction mixture, which was left standing for 30 min on ice and then centrifuged at 15,000 x *g* for 30 min. The pellet was solubilized in 120 µl of 50 mM Tris-HCl buffer (pH 7.6) and 100 µl of the resultant solution was added to 5 ml of scintillator (ACSII, Amersham, UK). The radioactivity was measured in a liquid scintillation counter.

Assay of FDH activity

FDH activity was determined by following the generation of NADH as described by Wehner *et al.* (1993). The reaction mixture (3 ml) consisted of 100 mM sodium phosphate buffer (pH 8.0), 2 mM glutathione (GSH), 1 mM nicotinamide adenine dinucleotide (NAD^+), 1 mM formaldehyde and the protein fraction. Assay of ADH activity

toward ethanol and octanol was performed in a reaction mixture consisting of 100 mM glycine buffer (pH 10.0), 1 mM NAD^+ and the protein fraction. The enzymatic reaction was monitored at 340 nm in a 3-ml quartz cuvette (light path, 1 cm) at 25°C. The activity was calculated from the extinction coefficient of NADH, $\varepsilon = 6220\ cm^{-1}M^{-1}$. One unit of activity was defined as the amount of protein necessary to catalyze the conversion of 1 μmol of NAD^+ to NADH in 1 min under the conditions of the reaction.

Assay of IAAld reductase activity

A. HPLC analysis: The reaction product was determined by chromatography with a HPLC system. A reaction mixture (100 μl) consisting of 0.2 mM IAAld, 0.1 mM NAD(P)H and a protein fraction in 100 mM potassium phosphate buffer (pH7.6) was incubated for 10 min at 37°C. After the reaction, 100 mM sodium bisulfate (12 μl) and 0.1 N HCl (8 μl) were added to the reaction mixture to stop the reaction. Sodium bisulfate was added to separate the retention time of the peak of IAAld from that of IEt by conversion of IAAld to IAAld bisulfate. The reaction mixture was applied on a column of ODS 80 TM (Tosoh, Japan) equilibrated with a solvent of 10% (v/v) methanol containing 1% acetic acid. The column was eluted with a linear gradient of 10 to 70% (v/v) methanol containing 1% acetic acid at a flow rate of 1 ml/min.

B. Thin layer chromatography: A reaction mixture consisting of 0.2 mM IAAld, 0.1 mM NADPH and a protein fraction in 100 mM potassium phosphate buffer (pH 7.6) was incubated for 20 min at 37°C. After the reaction, each mixture was applied to a silica gel thin layer chromatographic plate (Kieselgel 60, MERCK). The plate was developed in an ethyl acetate/hexane (1/1, v/v) solvent system. The resulting chromatogram was sprayed with Ehrlich's reagent (Dawson *et al.* 1969).

C.Spectrophotometric assay: IAAld reductase activity was assayed by measurement of the oxidation of NADPH or NADH. The reaction mixture (0.4 ml) consisted of 100 mM potassium phosphate buffer (pH 7.6), 0.1 mM NADbPH, 0.2 mM IAAld and the protein

fraction. The enzymatic reaction was monitored at 340 nm in a 0.5-ml quartz cuvette (light path, 0.5 cm) at 25°C. The activity was calculated from the extinction coefficient of NADPH, $\varepsilon = 6220\ cm^{-1}M^{-1}$. One unit of the activity was defined as the amount of protein necessary to catalyze the conversion of 1 μmol of NADPH to $NADP^+$ in 1 min under the conditions of the reaction. The reductase activities using other aldehydes were also measured by the oxidation of NADPH.

Preparation of antisera against ABP40 and ABP39

Purified ABP40 and ABP39 were separately resolved by SDS-PAGE (Laemmli 1970). Then, each gel strip containing the purified protein was crushed in Freund's complete adjuvant and injected into rats at two-week intervals until the antibody titers against the proteins were significantly elevated.

Preparation of plant extracts for immunoblot analysis

The 2-day old etiolated mung bean seedlings were sectioned into eight regions (see Fig. 15A-a and 16A-a). Cotyledons, plumules, hook portions of hypocotyls, de-hooked hypocotyls and roots were homogenized with equal volumes of x2 TSE buffer by mortar and pistle, separately . After the centrifugation of the homogenate at 10,000 x*g* for 30 min, the supernatants were resolved by SDS-PAGE.

The crude extracts for the determination of quantitative changes of the ABPs were prepared from etiolated mung bean seedlings. After sawing on an agar-gel bed, the hypocotyls were collected at 12, 24, 36, 48, 60 and 72 h, respectively, and each hypocotyl was homogenized with equal volumes of x2 TSE buffer by mortor and pistle, separately . After the centrifugation of the homogenate at 10,000 x*g* for 30 min, the supernatants were resolved by SDS-PAGE.

Immunoblot analysis with antisera against ABP40 and ABP39

SDS-PAGE was performed by the method of Laemmli (1970) using a 12.5% polyacrylamide gel. Immunoblotting was done for 1.5 h at 40 V onto a nitrocellulose filter. After blotting, the filter was incubated with phosphate-buffered saline (PBS) containing 10% (v/v) new-born calf serum (NBCS) at room temperature for 30 min and then with anti-ABP40 or ABP39 sera in 10% NBCS-PBS for 2h. After the washing with PBS containing 0.1% tween 20, the filter was incubated with peroxidase-conjugated anti-rat IgG at room temperature for 2 h. The peroxidase activity was detected by incubation with PBS containing 0.03% 3-3' diaminobenzidine and 0.003% H_2O_2.

Preparation of a serum against a synthetic oligopeptide

A conjugate of the synthetic oligopeptide (Ile-His-Arg-His-Ser-Cys-Glu) and keyhole limpet hemocyanin (KLH) was obtained from TaKaRa Co. Ltd. (Kyoto, Japan). Anti- conjugate serum was raised in female New Zealand White rabbits. The rabbits were injected with the conjugate (200 μg) in Freund's complete adjuvant at two-week intervals until the antibody titer against the peptide was significantly elevated. Titration of the antibody was performed by an enzyme-linked immunosorbent assay (ELISA).

Preparation of a membrane fraction from etiolated maize coleoptiles

A membrane fraction was prepared by the method of Löbler and Klämbt (1985) with minor modifications. All procedures were carried out at 0-4°C. Etiolated maize coleoptiles were collected from 5-day-old seedlings, ground with a pestle and mortar in an equal volume of homogenization medium [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM $MgCl_2$ and 250 mM sucrose] and filtered through two layers of Miracloth. The filtrate was centrifuged at 5,000 x *g* for 30 min. The supernatant was centrifuged at 200,000 x *g* for 30 min and the pellet was collected as a membrane fraction.

Immunoblot analysis with a serum against a synthetic olidopeptide

SDS-PAGE was performed by the method of Laemmli (1970) using a 12.5% polyacrylamide gel. Immunoblotting was done for 1.5 h at 40 V onto a nitrocellulose filter. After blotting, the filter was incubated with phosphate-buffered saline (PBS) containing 10% (v/v) new-born calf serum (NBCS) at room temperature for 30 min and then with anti-synthetic oligopeptide sera in 10% NBCS-PBS overnight. To eliminate the reaction of antibodies to KLH, the antiserum was incubated with an excess of KLH [0.5% (w/v)] at 25°C overnight before immunoreaction. After the washing with PBS containing 0.1% tween 20, the filter was incubated with peroxidase-conjugated anti-rabbit IgG at room temperature for 2 h. The peroxidase activity was detected by incubation with PBS containing 0.03% 3-3' diaminobenzidine and 0.003% H_2O_2.

Results

Detection and purification of ABP40 and ABP39

Detection of 40-kDa and 39-kDa polypeptides in the ABP fractions

To obtain the ABPs retained on the affinity column, the column was eluted with the solutions containing natural auxin IAA, or the solution containing synthetic auxin 2,4-D. SDS-PAGE analysis showed that several proteins were eluted by both the solutions. A 40-kDa and a 39-kDa polypeptides could be eluted by both the solution containing 2, 4-D and the solution containing IAA (Fig. 1). We designated the protein consisting of the 40-kDa polypeptide and the protein consisting of the 39-kDa polypeptide as ABP40 and ABP39, respectively.

Purification of ABP40 and ABP39

Purification of ABP40

Detection of ABP40 was performed by SDS-PAGE. As a result of sequential column chromatography on DEAE Sepharose CL-6B, 2,4-D-linked Sepharose 4B, hydroxyapatite, Mono Q and Mono P, ABP40 was purified as a protein that gave a single stained band after the SDS-PAGE (Fig. 2).

The molecular mass of the polypeptide, as estimated by SDS-PAGE, was 40 kDa. The molecular mass of ABP40 was estimated to be about 80 kDa by gel-filtration on Superose 6, indicating that the native protein is probably a dimer (Fig. 3).

Purification of ABP39

Detection of ABP39 was performed by SDS-PAGE. As a result of sequential column chromatography on DEAE Sepharose CL-6B, 2,4-D-linked Sepharose 4B, hydroxyapatite, Mono Q and Superose 12, ABP39 was purified as a protein that gave a single stained band after the SDS-PAGE (Fig. 4).

The molecular mass of ABP39 was estimated to be about 77 kDa by gel-filtration on Superose 6, indicating that the native protein is probably a dimer (Fig. 5).

Characterization of ABP40

Kinetics of auxin binding

The binding properties of ABP40 were examined by the method of ammonium sulfate precipitation. Scatchard analysis (1949) was carried out to characterize the binding activity of ABP40 for 2,4-D. The apparent dissociation constant (Kd) of ABP40 for 2,4-D was 1 x 10^{-5} M. The analysis indicated that the binding of 2,4-D at saturation corresponded to 2 mol of 2,4-D bound per mol of ABP40 (Fig.6). The binding of [^{14}C]-2-4-D to ABP40 was strongly inhibited by PCIB and weakly inhibited by IAA and NAA . However, the binding was not affected by tryptophan or benzoic acid (Table 1).

Analysis of partial amino acid sequences of ABP40

Since the N-terminal amino acid of ABP40 was blocked, the chemical digestion of the purified ABP40 was carried out with CNBr (Deutscher 1990). Among the several peptides obtained by the digestion with CNBr, the 15-kDa and the 30-kDa peptides were subjected to amino acid sequencings. About 20 amino acid sequences of each fragment were obtained and compared to sequences in the Swiss-Prot data bank. Significant homologies were found to sequences in human FDH (Fig. 7). A similar degree of homology was found in comparisons with the amino acid sequences of FDH from other species such as horse, rat and yeast (Fig. 7).

Determination of FDH activity

The FDH activity of purified ABP40 was measured after chromatofocusing, which

was the last step in the purification of ABP40. SDS-PAGE analysis showed that ABP40 was purified as a protein that gave a single stained band (Fig. 8-B). The elution profile of the chromatofocusing and the FDH activity of the fractions indicated that an FDH had been successfully purified (Fig. 8-A). The specific activity of FDH was increased by each column-chromatographic step during the purification of ABP40. The specific activity of the purified ABP40 was about 2 units/mg protein (Table 2). Other fractions that did not contain ABP40 had no activity of FDH.

Since it has been reported that FDH also has ADH activity that catalyzes the oxidation of long-chain primary alcohols, the ADH activity of ABP40 was measured using octanol as a substrate. ABP40 catalyzed the oxidation of octanol (Table 3).

Characterization of ABP39

Analysis of partial amino acid sequences

The chemical digestion of the purified ABP39 was carried out with CNBr to determine the partial amino acid sequence (Deutscher 1990). The 18-kDa peptide, one of several peptides obtained by the digestion with CNBr, was subjected to N-terminal sequencing. Amino acid sequences of the fragment obtained were compared to sequences in the Swiss-Prot data bank. Significant homologies were found to the sequences of ADHs from several species such as *Saccaromyces cerevisiae* and *Aspergillus nidulans* (Fig.9).

Determination of IAAld reductase activity of ABP39

The ADH activity of the purified ABP39 was measured. No activity could be detected when ethanol was used as a substrate. The ADHs of animals have been classified into three classes (class I, class II and class III) which have been differentiated on the bases of their substrate specificities, structure and tissue distribution (Kaiser *et al.* 1989). The

partial amino acid sequence of ABP39 had a significant homology to the class III ADH. The class III ADH has a low affinity for ethanol but it has a high affinity for long-chain primary alcohols and alcohols that have aromatic rings. It has been reported that the ADHIII of the human liver has an activity that catalyzes the dehydration of indole-3-ethanol (Kaiser *et al.* 1988). So, I investigated the ADH reaction using indole-3-ethanol and indole-3-acetaldehyde as substrates. As the ADH activity that catalyzes the dehydration of indole-3-ethanol could not be detected in ABP39, the reverse reaction that catalyzes the reduction of indole-3-acetaldehyde was investigated. The HPLC profile of the reaction mixture consisting of IAAld, ABP39 and NADPH revealed the formation of indole-3-ethanol (Fig. 10). Little activity could be detected when NADH was used as a cofactor.

The results of thin layer chromatograms of the reaction mixtures showed a formation of indole-3-ethanol when the ABP39 was incubated with IAAld and NADPH (Fig. 11). The color and Rf of the deposit revealed that indole-3-ethanol was produced from IAAld by the enzymatic reaction of ABP39. The results also showed that the enzymatic reaction is a NADPH-specific reaction. Elution profiles of ion-exchange chromatography and gel-filtration and IAAld reductase activity of each fraction indicated that ABP39 was identical to an IAAld reductase (Fig. 12). The specific activity of IAAld reductase was increased by each column-chromatographic step during the purification of ABP39 and the specific activity of the purified ABP39 was about 36 units/mg protein (Table 4).

IAAld reductase activity was measured for each fraction obtained after gel-filtration, which was the last step in the purification of ABP39. SDS-PAGE analysis showed that ABP39 was purified as a protein that gave a single stained band (Fig. 13). The elution profile of the gel-filtration and the IAAld reductase activity of the fractions indicated that ABP39 had an IAAld reductase activity. ABP40 had no activity of IAAld reductase.

Substrate specificity

Substrate specificity of the reductase was measured with other aldehydes. As shown in Table 5, the ABP39-associated aldehyde reductase activity was not specific for IAAld as a substrate. Phenylacetaldehyde, propionealdehyde, benzaldehyde and acetaldehyde were good substrates for the reductase, while indole-aldehyde was a poor substrate.

Immunological properties of ABP40 and ABP39

Crossreactivity of antisera to ABP40 and ABP39

The reactivity of the two antisera raised against ABP40 and ABP39, respectively, to both ABPs was determined by immunoblotting. The fraction obtained after the chromatography on Mono Q during the purification of ABP39 (see Fig. 4, lane 5) was resolved by SDS-PAGE. From the results of immunoreaction, the antiserum raised against ABP40 strongly reacted to ABP40, but did not react to ABP39 (Fig. 14, lanes 3 and 4). The antiserum raised against ABP39 reacted to ABP39, specifically (Fig. 14, lanes 5 and 6).

Organ-specificity of ABP40 and quantitive changes of ABP40 during the development

In order to investigate roles of ABP40, the localization of ABP40 in etiolated mung bean seedlings was investigated by immunoblot analysis using the anti-ABP40 serum. ABP40 was mainly detected in the hypocotyls, and slightly in cotyledons, plumules and roots (Fig. 15-A).

The changes in the quantity of ABP40 in hypocotyls during the development of mung bean seedlings were investigated at different growth stages. Immunoblotting showed that ABP40 gradually increased during the elongation of the hypocotyls (Fig. 15-B).

Organ-specificity of ABP39 and quantitative changes of ABP39 during the development

The localization of ABP39 in etiolated mung bean seedlings was investigated by immunoblot analysis using the anti-ABP39 serum. The results of immunoblot analysis showed that ABP39 was mainly detected in the hypocotyls, but not in cotyledons, plumules and roots (Fig. 16-A).

The changes in the quantity of ABP39 in hypocotyls during the development of mung bean seedlings were investigated. The results of the immunoblotting using the anti-ABP39 serum showed that ABP39 increased during the elongation of the hypocotyls (Fig. 16-B).

Immunoblot analysis with the antiserum against the oligopeptide

The putative auxin-binding site of 22-kDa auxin-binding protein (ABP1) from maize coleoptiles was suggested by Venis *et al.* (1992). The serum raised against the conjugate of the synthetic oligopeptide and KLH was used for the immunoreaction. Immunoblotting of the ABP fraction that was retained on 2,4-D-linked Sepharose 4B revealed that the antiserum strongly recognized ABP40 in this fraction (Fig. 17-B). Both KLH and a 22-kDa polypeptide in a membrane fraction from maize were also recognized by the antiserum (Fig. 17-B). After incubation with an excess of KLH, the antiserum no longer reacted with KLH (Fig. 17-C). In contrast, the antiserum clearly reacted with the 40-kDa polypeptide in the ABP fraction and with the 22-kDa polypeptide in the membrane fraction from maize (Fig. 17-C) after the antiserum had been incubated with KLH. The antiserum did not react to ABP39 well, although the amount of the protein applied on SDS-PAGE was equal to the amount of ABP40. From these results, ABP39 differed from ABP40 immunologically and was not a derivative nor modified protein of ABP40.

Effects of auxins on the enzyme activities of ABP40 and ABP39

Effects of auxins on FDH activity of ABP40

The FDH activity of ABP40 was measured with and without 1mM auxins at several concentrations of NAD^+. The addition of 2,4-D inhibited FDH activity. Fig. 18 shows a Lineweaver-Berk plot from which the Km and Ki values were calculated and the type of inhibition was determined. The inhibition was caused in a competitive manner. The Km value for NAD^+ calculated from the plot was about 10 μM and the Ki value for 2,4-D was about 2.7 mM. The addition of IAA did not inhibit FDH activity.

Effect of auxin on IAAld reductase activity of ABP39

The IAAld reductase activity was measured with and without 1mM auxins at several concentrations of IAAld. IAAld reductase activity was inhibited by both IAA and 2,4-D. Fig. 19 shows two Lineweaver-Burk plots from which the Km and Ki values were calculated. The Km value for IAAld was 8 μM. These inhibitions were caused in a competitive manner. The apparent Ki value for IAA was about 1.1 mM (Fig. 19-A). A similar result was obtained when 2,4-D was added to the reaction. The apparent Ki value for 2,4-D was about 1.3 mM (Fig. 19-B).

Discussion

Auxin, a plant hormone, plays a crucial role in the growth and development of plants. The search for the primary sites of auxin actions has led to the identification of various auxin-binding proteins in plant cells. Although several ABPs were isolated and characterized, the molecular mechanisms of auxin action remain unknown. More information concerning auxin-binding proteins is required.

In this thesis, I purified two novel ABPs that have not been reported and investigated their properties. I found that a 40-kDa polypeptide and a 39-kDa polypeptide in a soluble fraction from etiolated mung bean hypocotyls were retained on 2,4-D-linked Sepharose 4B and eluted with both a solution containing 2,4-D and a solution containing IAA. I successfully purified a protein (ABP40) composed of two 40-kDa polypeptides and a protein (ABP39) composed of two 39-kDa polypeptides. From the results of amino acid sequences and enzyme activities of ABP40 and ABP39, I identified ABP40 and ABP39 as a glutathione-dependent formaldehyde dehydrogenase (FDH) and an indole-3-acetaldehyde (IAAld) reductase, respectively. It demonstrated that auxins inhibited these enzyme activities by binding.

The characteristics of these ABPs such as apparent molecular mass, kinetics of the bindings, partial amino acid sequences and enzyme activities indicated that these proteins were novel ABPs from the soluble fraction of plants. From the results of partial amino acid sequences, both ABP40 and ABP39 were highly homologous to ADH. However, ABP40 had no activity of IAAld reductase and ABP39 had no activity of glutathione-dependent formaldehyde dehydrogenase. The reactivities of the antisera against ABP40 and ABP39 to the ABPs (Fig. 14) and crude extracts of mung beans were different to each other (Fig. 15, Fig. 16). Immunoblott analysis using an anti-synthetic peptide serum revealed the differences of the reactability (Fig.17). The differences of the enzymatic properties and immunological properties between ABP40 and ABP39 indicated that ABP39 was not a derivative or a modified form of ABP40.

The binding assay revealed the auxin-binding activity of ABP40 and supported the

existence of a conserved auxin-binding site. From the results of inhibition assays, I found that ABP40 bound auxins and PCIB specifically. It was apparent that the specificity of the binding increased in the order 2,4-D, NAA, IAA. Further investigations are necessary to clarify the structure of the auxin-binding site of ABP40.

Glutathione-dependent formaldehyde dehydrogenase activity is widespread in plants (Uotila and Koivusalo 1979), animals (Uotila and Koivusalo 1974), bacteria (Schutte *et al.* 1976, Gutheil *et al.* 1992), and yeast (Wehner *et al.* 1993). However, the precise roles of this enzyme have not yet been demonstrated. Giese *et al.* (1994) studied the metabolism of formaldehyde in cultured cells of the spider plant (*Chlorophytum comosum* L.) and isolated a GSH-dependent FDH from these cells and from other plants. They suggested that FDH might have a crucial role in the detoxification of formaldehyde. The results of immunological analysis suggested that ABP40 would function in the hypocotyls. In addition, it has been reported that FDH also has an alcohol dehydrogenase (ADH) activity. Since the structure of FDH is homologous to that of ADH from animals (Uotila and Koivusalo 1974) and *E. coli.* (Gutheil *et al.* 1992), FDH was identified as a unique type of ADH that is ineffective with ethanol as substrate, but effective with long-chain primary alcohols as subatrate and is also capable of oxidizing formaldehyde in a glutathione-dependent reaction. ABP40 also has ADH activity that is effective with octanol as substrate (Table 3). These results indicated that ABP40 has the characteristics resembling those of FDHs in animals and bacteria. Further investigations such as the cloning of the cDNA of ABP40 would reveal the function of FDH in plants.

IAAld has been thought to be a key intermediate in IAA biosynthesis (Ludwig-Müller and Hilgenberg 1989). IAAld can either be oxidized to IAA or reduced to IEt. IAAld reductase is an enzyme that converts IAAld to IEt and seems to be important for IAA homeostasis in plants (Brown and Purves 1976). IAAld reductase activities were identified in Chinese cabbage, *Cucumis sativus* and *Phycomyces blakesleeanus* (Brown and Purves 1976, 1980, Ludwig-Müller *et al.* 1990 a,b). The molecular mass of the IAAld reductase of *P. blakesleeanus* was estimated by gel-filtration as 38 kDa (Ludwig-Müller *et al.* 1990 a). The molecular mass of the ABP39 estimated by SDS-PAGE was similar to that of *P.*

blakesleeanus. However, the molecular mass of the ABP39 estimated by gel-filtration was 77 kDa. The ABP39 would be different from other IAAld reductase reported before. Although previous reports suggested the distribution of IAAld reductase activity on gel-filtration and the characteristics of the enzyme using the partial purified fractions (Brown *et al.* 1976, 1980, Ludwig-Müller *et al.* 1990 a,b), the primarry structure of the enzyme has not been clarified. The results of this report suggest that the enzyme has the primary structure of alcohol dehydrogenase in the amino acid sequence and support the idea that the IAAld reductase might be a type of ADH.

The reductase activity associated with ABP39 was not specific for IAAld (Table 2). However, I think that the ABP39 may catalyze the reduction of IAAld *in vivo* for several reasons. First, the Km value for IAAld of the ABP39 (8 μM) was lower than other IAAld reductases reported before. The Km values of the reductases identified in the Chinese cabbage, *Cucumis sativus* and *P. blakesleeanus* were 125μM, 73μM, 130 μM and 24 μM respectively (Brown and Purves1976, 1980, Ludwig-Müller *et al.* 1990 a,b). Second, the reductases reported in Chinese cabbage and *P.blakesleeanus* were also not specific for IAAld (Ludwig-Müller *et al.* 1990 a,b). Third, during the purification of ABP39, no activity that can reduce IAAld could be detected in other fractions of ion-exchange chromatography and gel-filtration. Fourth, the results of affinity chromatography and the inhibitory effects of auxins on enzyme activity indicated that ABP39 has an affinity to IAA. From these results, the ABP39 likely acts as an IAAld reductase in mung beans. This study would represent the first extensive purification of IAAld reductase. Further investigations of ABP39 will be important to elucidate the regulation of the biosynthesis of IAA.

To clarify the molecular mechanisms of auxin actions, investigations of rapid responses to auxins of plants have been made (Davies 1973, Evans 1974). The rapid responses to auxin include the stimulation of RNA synthesis, synthesis of enzymes, changes of enzyme activities, increased permeability of plasma membrane, increased wall extensibility, stimulation of protoplasmic streaming and stimulation of hyperpolarization (Evans 1974). A number of studies on the relation of auxins to these physiological responses have been carried out (Berger and Avery 1943, Masuda and Wada 1966,

Vanderhoef and Stahl 1975). The following studies revealed that some of these responses are regulated at the transcriptional level (Key 1964) while others are not (Penny and Galston 1966, Pope and Black 1972). Considering such regulatory systems and the possible functions of ABPs that were previously reported for the auxin actions, ABPs can be classified into at least 2 types of proteins, i. e., receptor-like proteins and enzymes whose activities are modulated by auxins. ABP40 and ABP39 should be the latter type of ABP. It was strongly suggested that the inhibitory effects on the enzyme activities of ABP40 and ABP39 were caused by the binding of auxins to each enzyme (Fig. 18 and 19). As the precise roles of FDH have not been clarified, it is unclear what will happen when the FDH activity is modulated by auxins. Further investigations are necessary to elucidate the action of auxins on FDH in plants. Since IAAld has been thought to be the precursor of IAA, the effects of auxins on IAAld reductase activity may affect the biosynthesis of IAA. There should be a mechanisn that allows auxins to regulate the activity of IAAld reductase for the regulation of IAA contents, when plants require high levels of auxins. More knowledge of the regulation of IAA-biosynthesis should reveal the precise function of ABP39. Recently, several ABPs that have enzyme activities have been found. However, the effects of auxins on these enzyme activities were not clarified. So, this is the first report of ABPs that have enzyme activities, and the modulation of these activities by the direct-bindings of auxin.

In conclusion, this thesis demonstrates the possibilities that auxins affect enzyme activities directly in plant cells, and should provide a clue to elucidating the mechanisms of auxin action in plants.

Conclusion

In this thesis work, novel auxin-binding proteins (ABP40 and ABP39) were purified from the soluble fraction of etiolated mung bean seedlings by affinity chromatography. ABP40 and ABP39 were identified as FDH and IAAld reductases, respectively. The enzyme activities of these ABPs were inhibited by auxins. From the results of the inhibition of their enzyme activities, it is suggested that auxins affect the activities of these enzymes directly. Fig. 20 summarizes the mechanisms of auxin actions that were put forward by the results of this thesis work and predicted by previous reports (Theologis 1986, Libbenga *et al.* 1986).

Recent investigations on the molecular mechanisms of auxin actions have been paying attention to auxin-induced gene expressions and the receptor-like proteins that mediate auxin signals through gene expressions. However, this thesis demonstrates the possibility that the direct effects on enxyme activities cause physiolosical changes and provides a clue to the elucidation of the mechanisms of auxin actions in plants.

Acknowledgements

I wish to express my sincere appreciation to Professor S. Sakai of the University of Tsukuba for his invaluable suggestions, advice and constructive criticism throughout the course of my research and the preparation of this thesis. I am also indebted to Professor H. Harada and Professor T. Fujii of the University of Tsukuba for their invaluable suggestions and constant encouragement throughout the course of my research.

My sincere thanks also go to Professor H. Kamada, Assistant Professor S. Satoh and Dr. M. Wada of the University of Tsukuba. Special thanks must also be given to all the members of the laboratory for their many useful discussions of my results.

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Figures and Tables

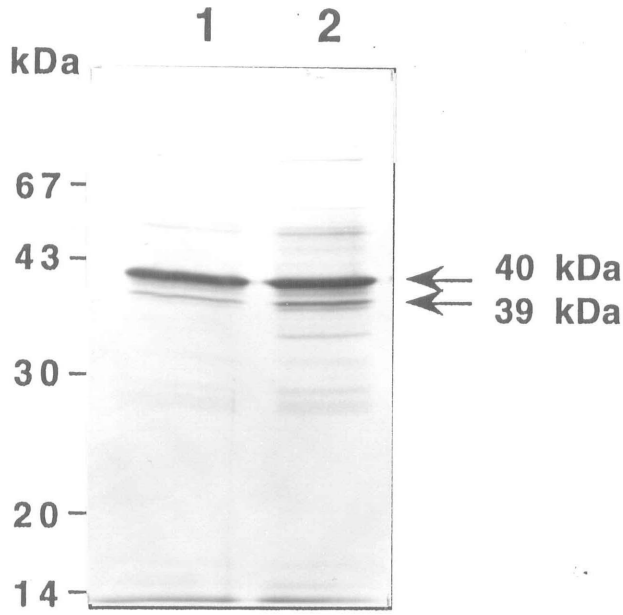

Fig. 1.

Analysis of the ABP fractions obtained by column chromatography on 2,4-D-linked Sepharose 4B. Proteins were detected with silver staining. Lane 1, proteins eluted with 50 mM of Tris-HCl buffer (pH7.6) containing 0.1 M of NaCl and 40 mM of IAA; lane 2, 40 mM of 2,4-D.

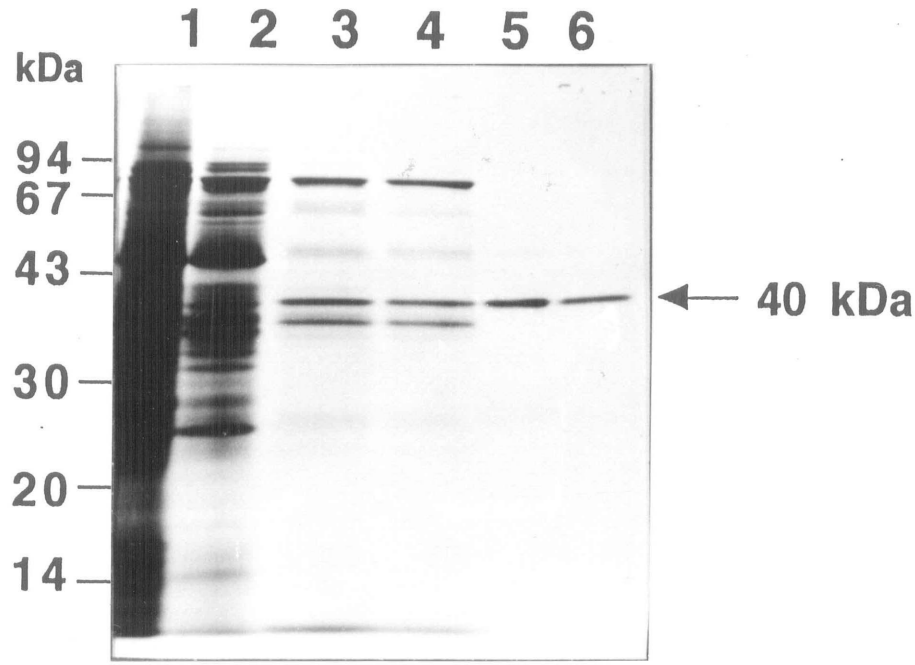

Fig. 2.

SDS-PAGE of fractions obtained during the purification of ABP40. Proteins were detected with silver staining. Lane 1, proteins obtained from ammonium sulfate precipitation (60% saturation); lane 2, after chromatography on DEAE-Sepharose CL-6B; lane 3, on 2,4-D-linked Sepharose 4B; lane 4, on hydroxyapatite; lane 5, on Mono Q; lane 6, on Mono P.

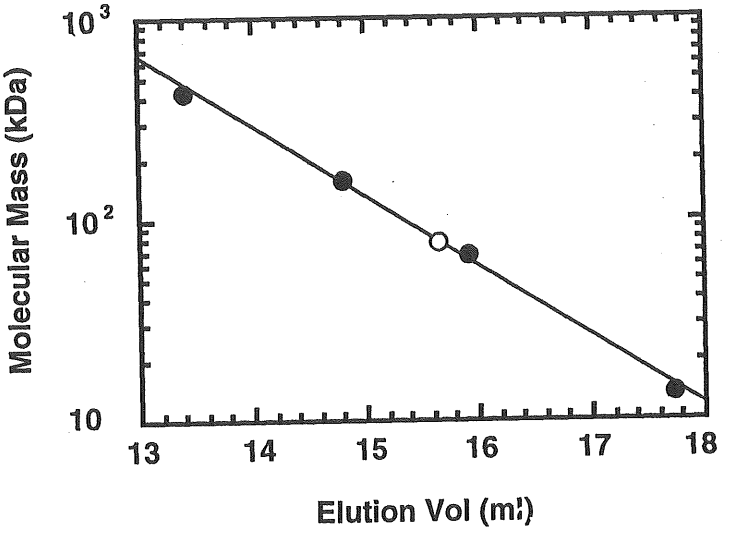

Fig. 3.

Estimation of the molecular mass of ABP40. The standards (●) were ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa). ○, ABP40.

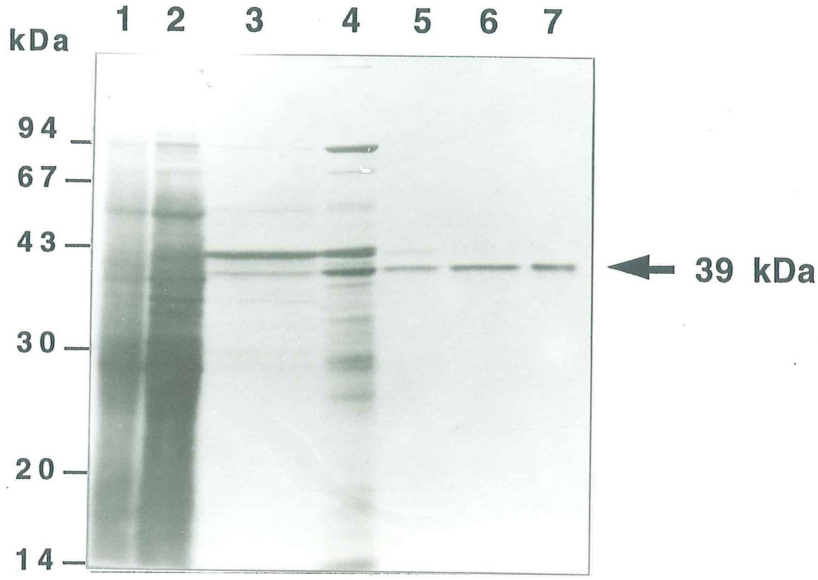

Fig. 4.

SDS-PAGE of fractions obtained during the purification of ABP39. Proteins were detected with silver staining. Lane 1, proteins obtained from ammonium sulfate precipitation (60% saturation); lane 2, after chromatography on DEAE-Sepharose CL-6B; lane 3, on 2,4-D-linked Sepharose 4B; lane 4, on hydroxyapatite; lane 5, on Mono Q; lane 6, after rechromatography on Mono Q; on Superose12.

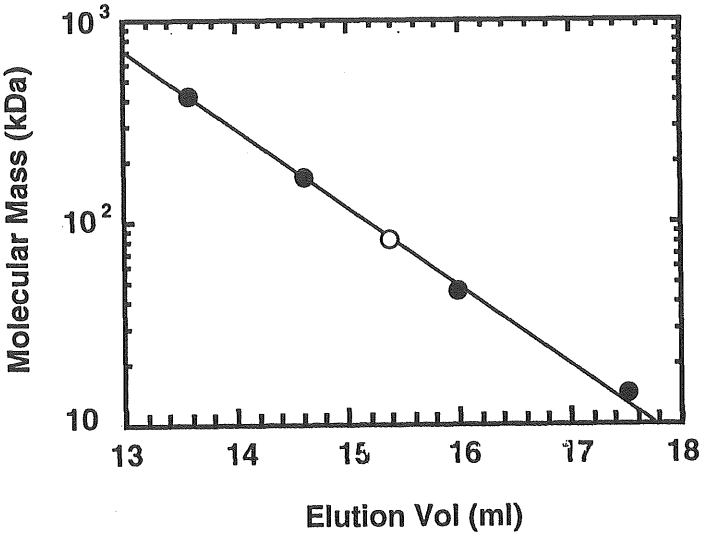

Fig. 5.

Estimation of the molecular mass of ABP39. The standards (●) were ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) and ribonuclease A (13.7 kDa).

○, ABP39.

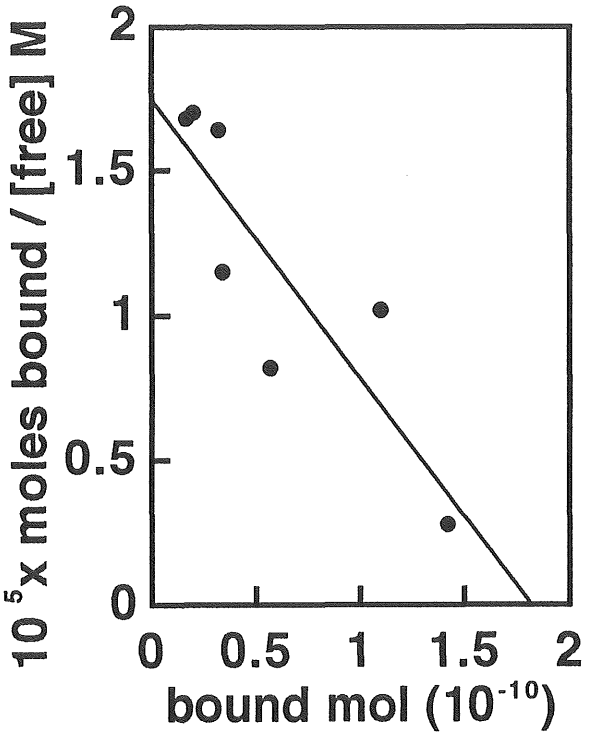

Fig. 6.

Scatchard analysis of 2,4-D-binding by ABP40. Purified ABP40 (8 μg) was used for each experiment. The apparent dissociation constant (Kd) of ABP40 for 2,4-D was calculated by the method of Scatchard (1949). Each point indicates the mean of results from 3 experiments.

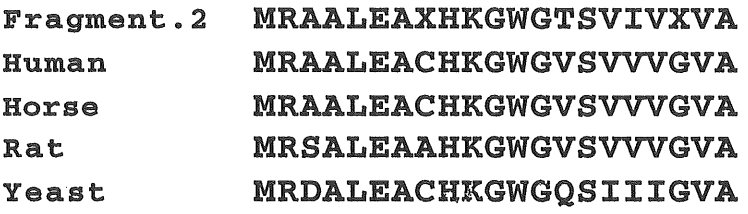

Fig. 7.

Comparison of partial amino acid sequences of ABP40 (upper sequences) with those of sequences of FDH (ADHIII) purified from a human liver (Kaiser *et al.*, 1988), horse (Kaiser *et al.* 1989), rat (Julia *et al.* 1988) and yeast (Wehner *et al.* 1993).

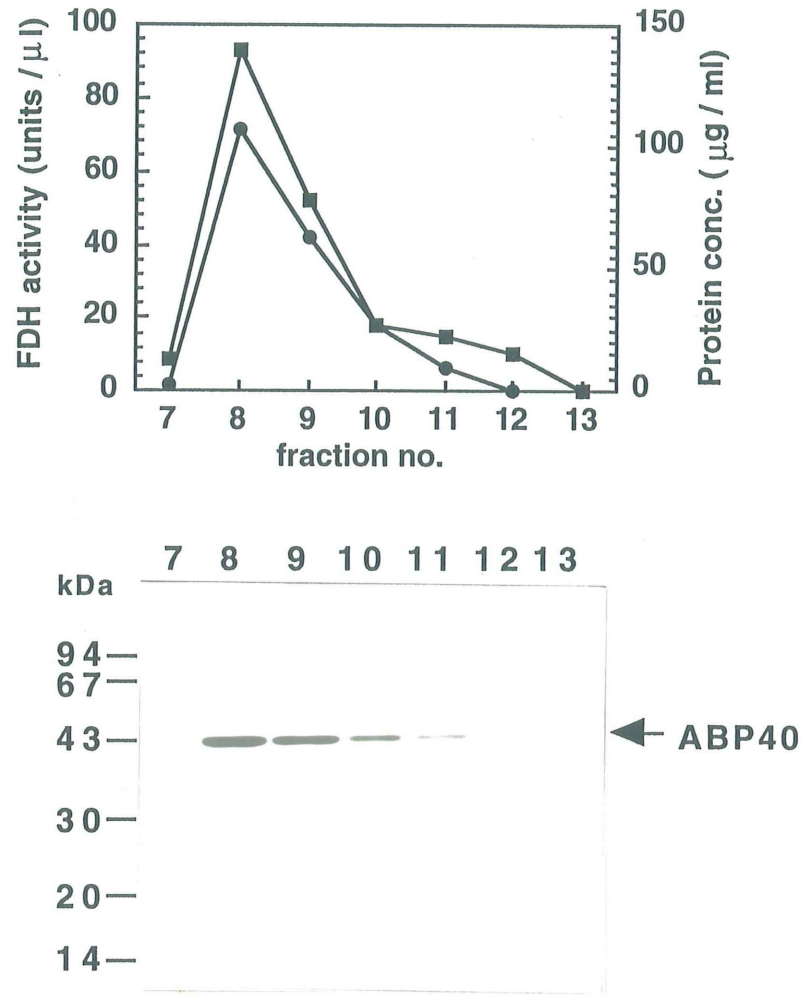

Fig. 8.

SDS-PAGE, elution profile and FDH activities of the fractions obtained by chromatofocusing. A, Elution profile and FDH activities; B, Silver staining. (■) Protein concentration. (●) FDH activity. Numbers indicate fraction numbers.

ABP39			APLLLAGIXV	
ADH5	*S.Cerevisiae*	153	APILCAGITV	162
ADH3	*A.Nidulans*	152	APILCAGITV	161
ADH2	*S.Cerevisiae*	149	APILCAGITV	158
ADH1	*A.Nidulans*	150	APVLCAGITV	159
ADH1	*S.Cerevisiae*	149	APVLCAGITV	158

Fig. 9.

Comparison of partial amino acid sequences of ABP39 (upper sequences) with those of ADHs in the data base.

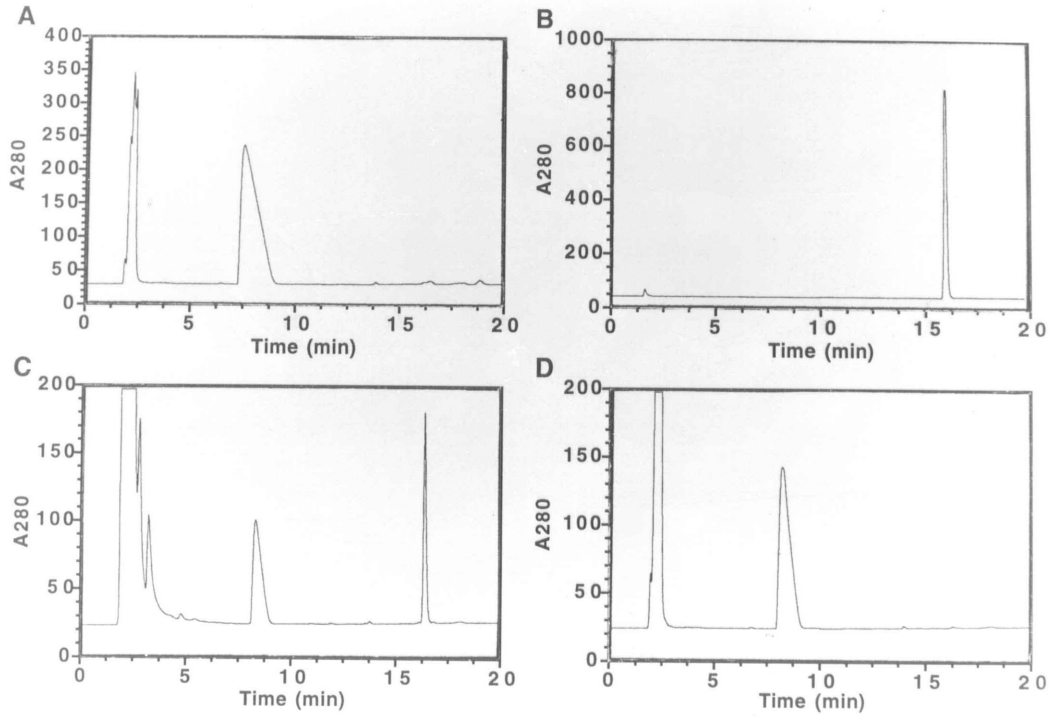

Fig. 10.

HPLC profiles of the product of the enzyme reaction. A, Authentic standard of IAAld bisulfate; B, Authentic standard of IEt; C, Reaction mixture of IAAld with ABP39 and NADPH; D, Reaction mixture of IAAld and ABP39 (-NADPH or NADH).

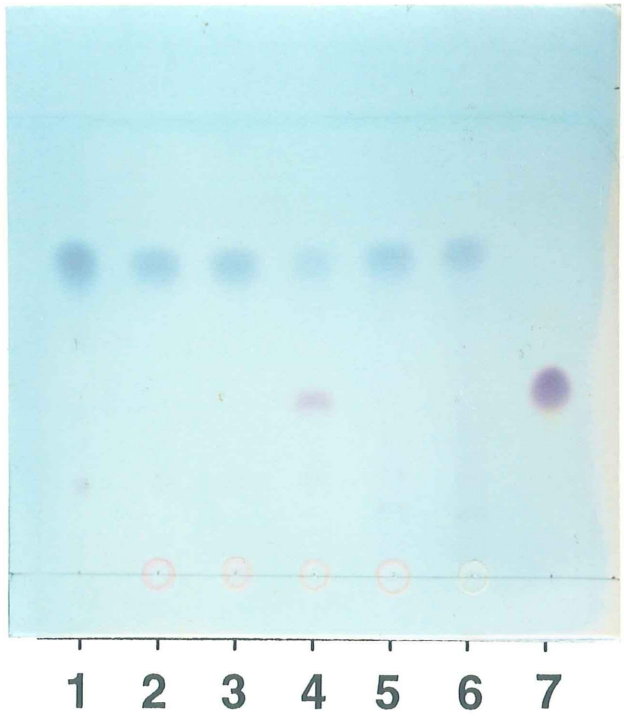

Fig. 11.

Thin layer chromatography of the product of the enzyme reaction. Lane 1, authentic standard of IAAld; lane 2, - ABP39; lane 3, 0 time; lane 4, +NADPH; lane 5, + NADH; lane 6, -NAD(P)H; lane 7, authentic standard of IEt.

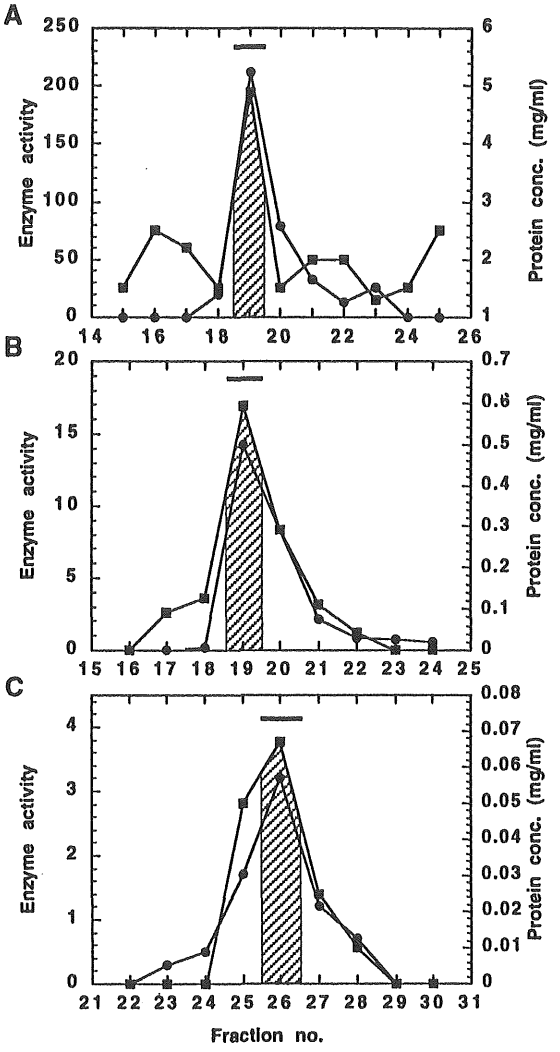

Fig. 12.

Elution profiles and IAAld reductase activities in the fractions obtained by chromatography on Mono Q, rechromatography on Mono Q and on Superose12. A , Mono Q; B, Rechromatography on Mono Q; C, Superose 12.(■); Protein concentration (mg/ml). (●) IAAld reductase activity (units/ml). Numbers indicate fraction numbers and bars indicate the fraction containing ABP39.

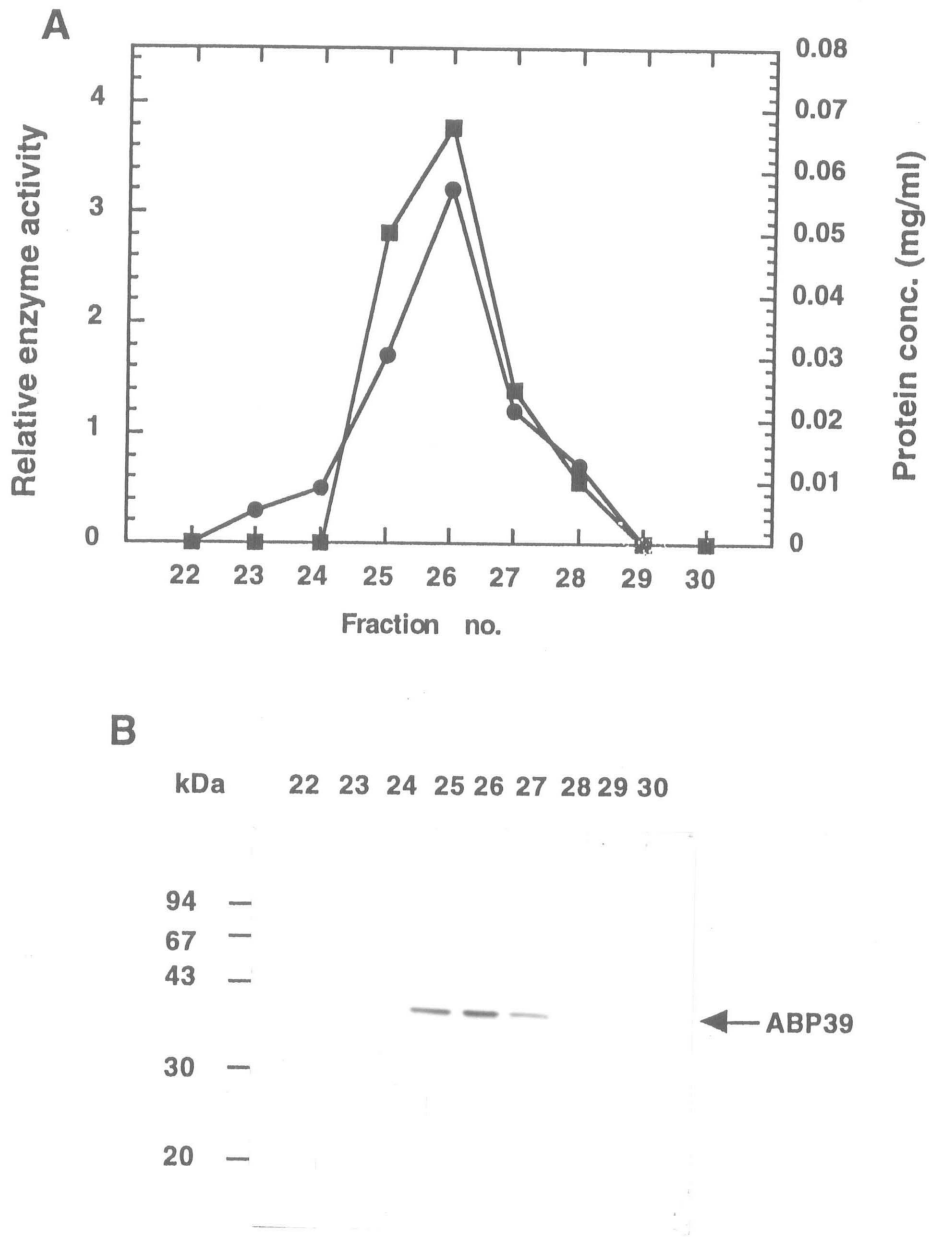

Fig. 13.

SDS-PAGE, elution profile and IAAld reductase activities of the fractions obtained by chromatography on Superose 12. A, Elution profile and IAAld reductase activities; B,Silver staining. Protein concentration (■) and IAAld reductase activity (●). Numbers indicate fraction numbers.

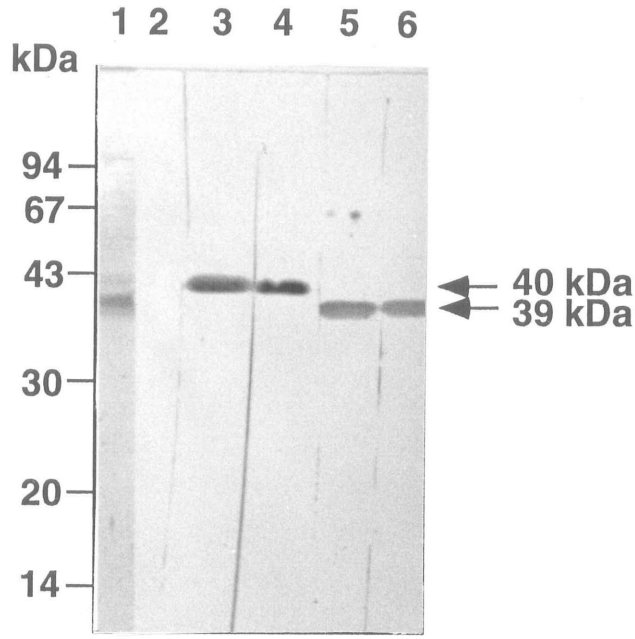

Fig. 14.

Immunoblott analysis of the ABP fraction containing ABP40 and ABP39 using the sera against ABP40 and ABP39. Lane 1, Amido black staining; lane 2, nonimmuneserum; lanes 3,4, immunostaining with the anti-ABP40 serum; lanes 5, 6, immunostaining with the anti-ABP39 serum.

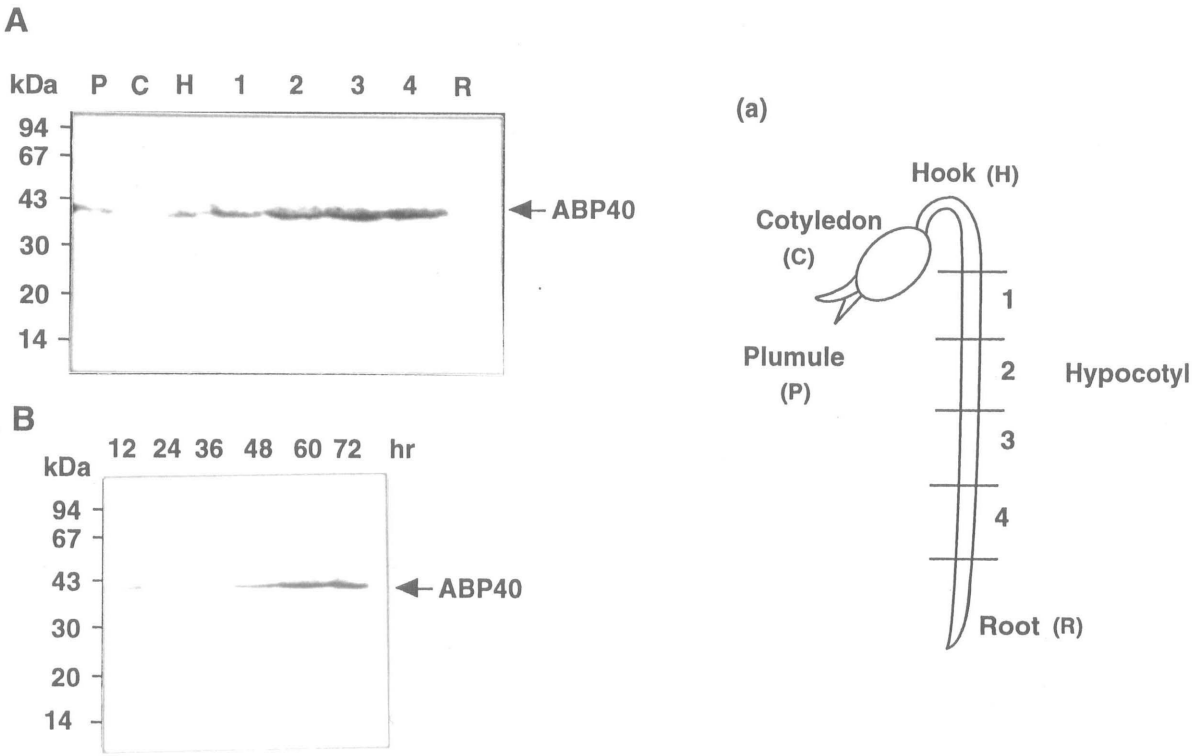

Fig. 15.

Localization and developmental changes of ABP40. A, Localization of ABP40. Proteins were extracted from different regions of etiolated mung bean seedlings as shown in (a). Equal amounts of protein of each fraction were resolved by SDS-PAGE. B, Quantitative changes in ABP40 during the development of etiolated mung bean seedlings. Numbers indicate the time (h) after sawing on the agar-gel bed.

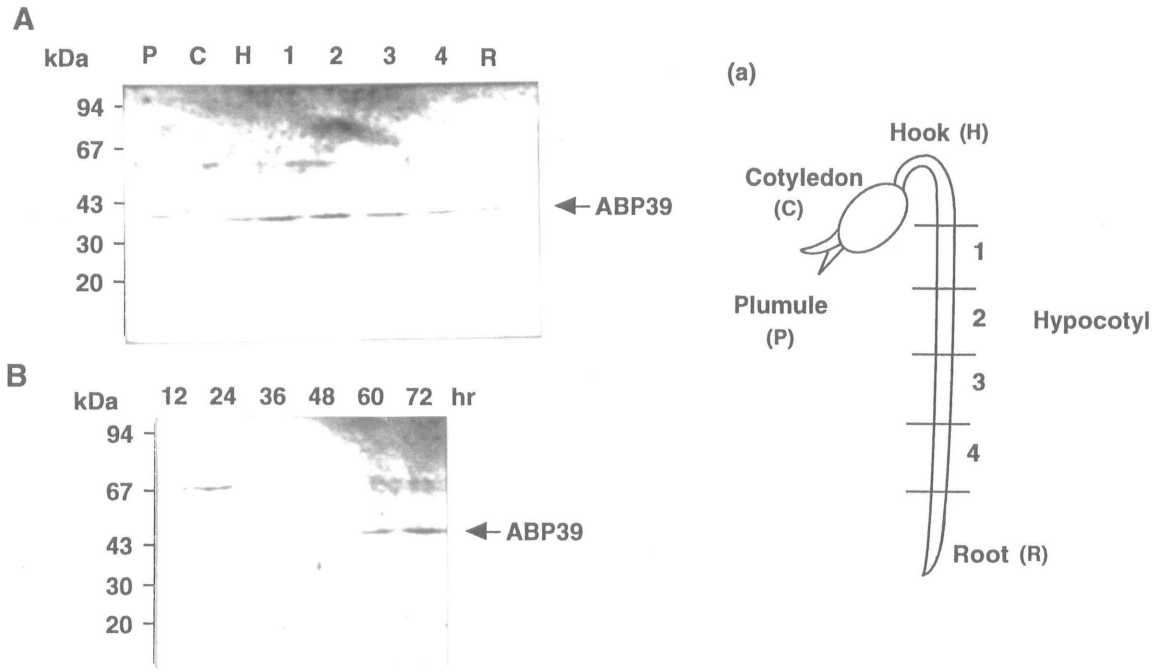

Fig. 16.

Localization and developmental changes of ABP39. A, Localization of ABP39. Proteins were extracted from different regions of etiolated mung bean seedlings as shown in (a). Equal amounts of protein of each fraction were resolved by SDS-PAGE. B, Quantitative changes in ABP39 during the development of etiolated mung bean seedlings. Numbers indicate the time (h) after sawing on the agar-gel bed.

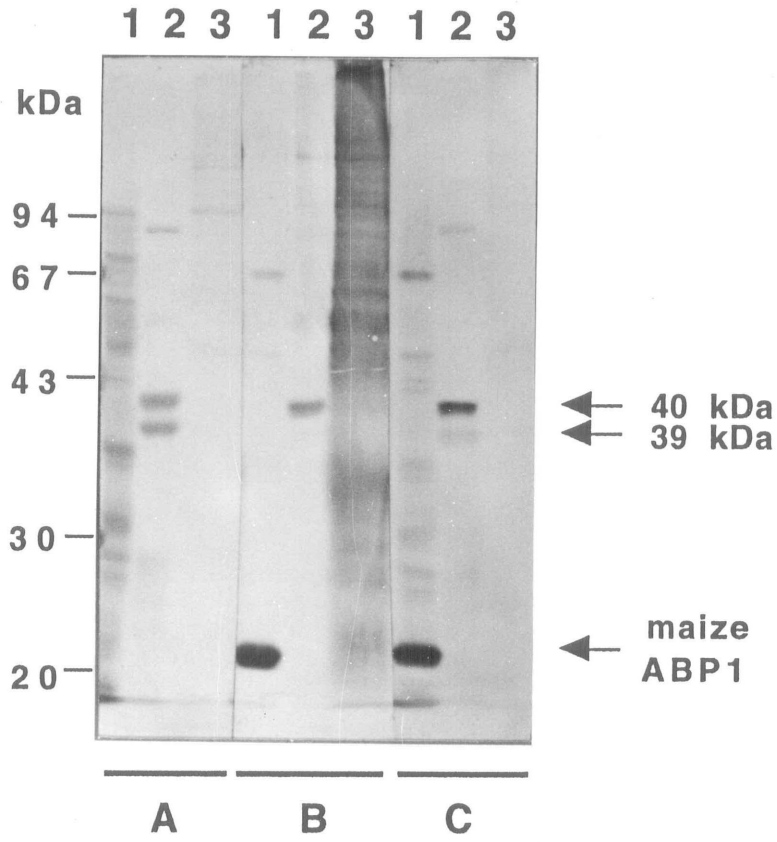

Fig. 17.

Immunoblott analysis of the ABP fraction obtained by column chromatography on 2,4-D-linked Sepharose 4B using the antiserum against the oligopeptide-KLH conjugate. A, Amido black staining. B, Immunostaining with the antiserum against the oligopeptide-KLH conjugate. C, Immunostaining with the antiserum that was previously incubated with 0.5% (w/v) KLH. Lane 1, membrane fraction from maize coleoptiles; lane 2, ABP fraction; lane 3, KLH.

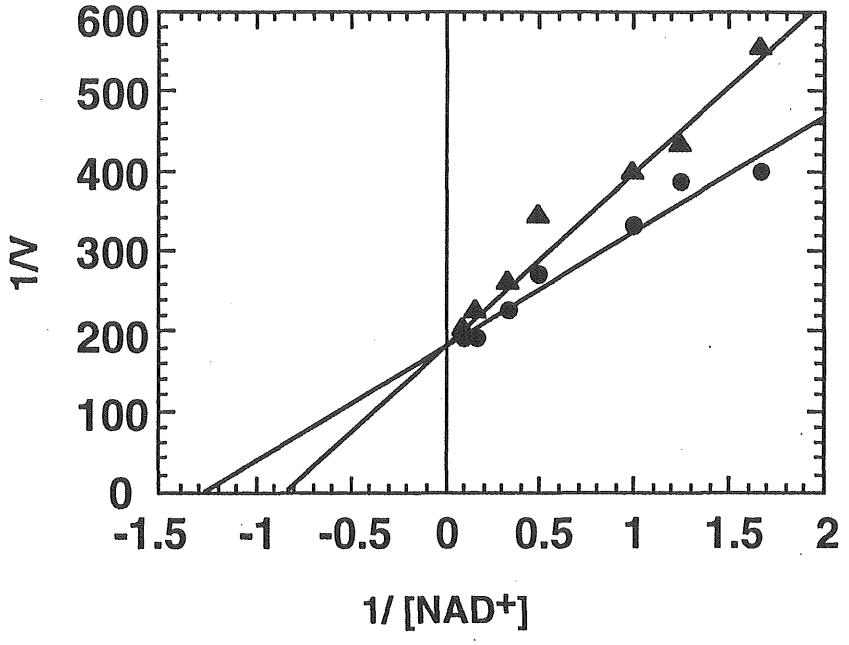

Fig. 18.

Inhibition of FDH activity by 2,4-D. The enzyme activity was measured in the absence (●) and presence of 1 mM 2,4-D (▲).

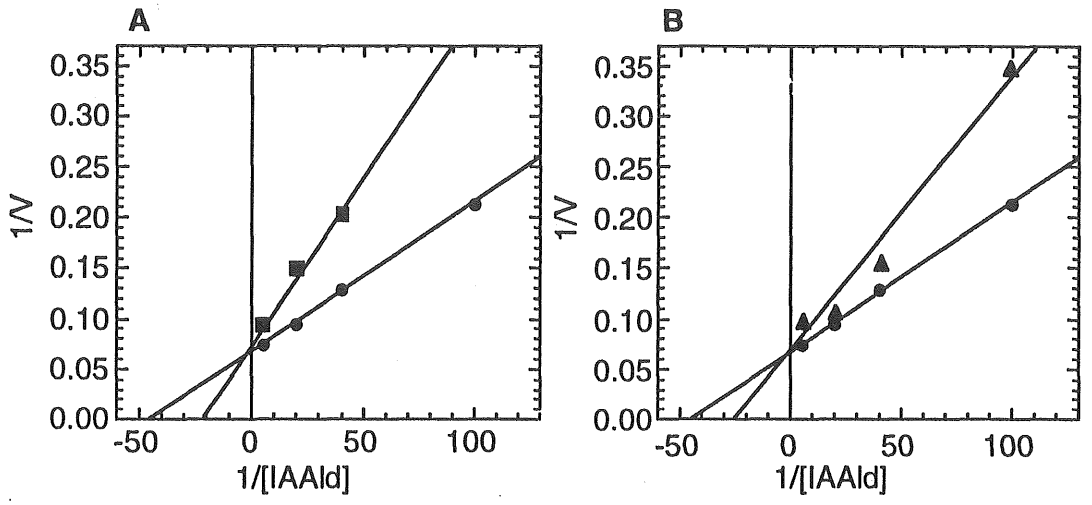

Fig. 19.

Inhibition of IAAld reductase activity by auxins. The enzyme activity was measured in the absence (●) and either the presence of 1 mM IAA (A) (■) or 2,4-D (B) (▲). Data represent Linewever-Berg plots.

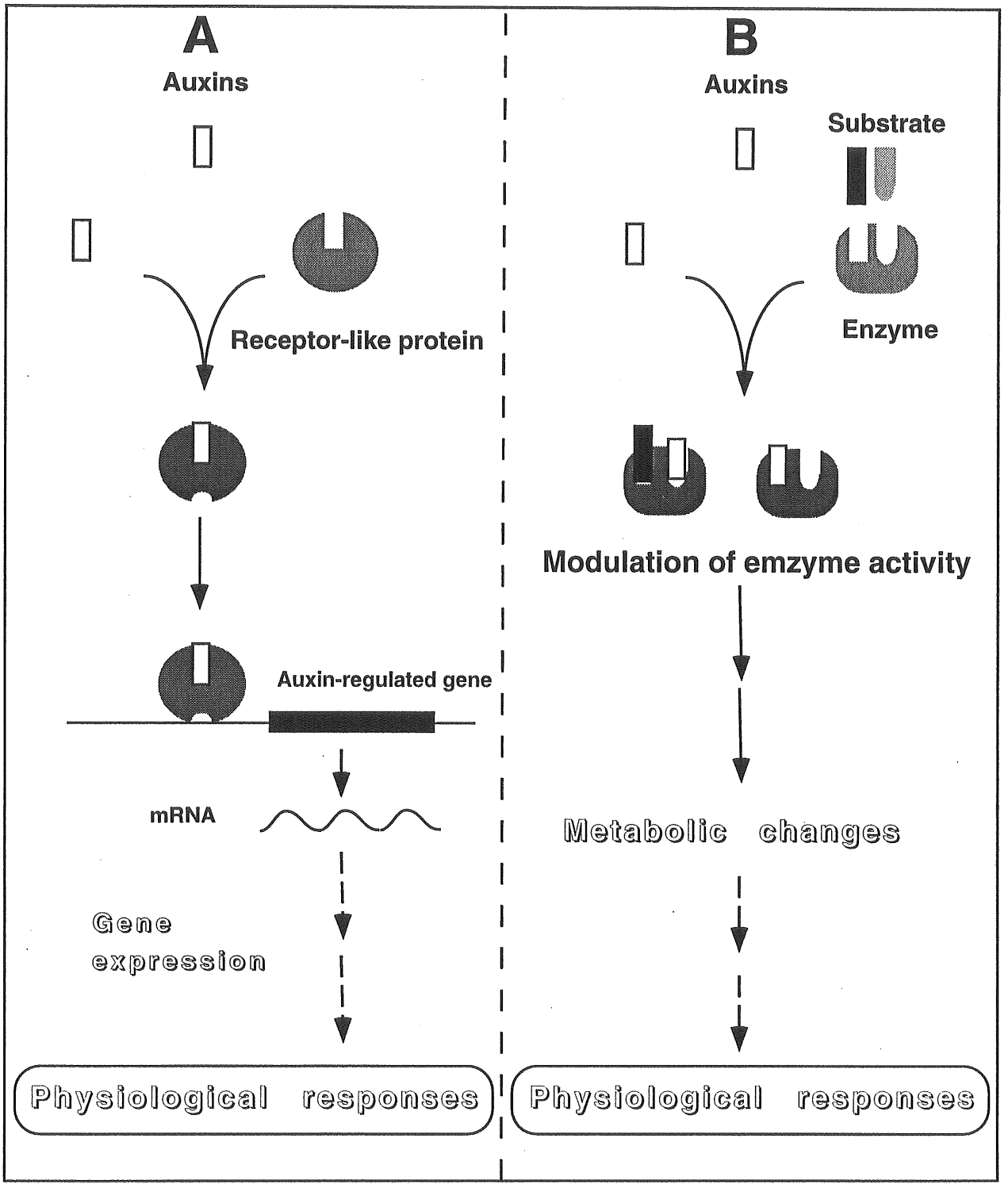

Fig. 20.

Schematic diagrams of the model of the molecular mechanisms in auxin actions. (A) The model of the mechanism predicted by previous reports (Theologis 1986, Libbenga 1986). This mechanism involves receptor-like proteins. (B) The model of the mechanism demonstrated by the results of this study.

Table 1.

Relative effects of auxins and derivatives on [^{14}C]-2,4-D binding by ABP40

Compound (10 μM)	Inhibition
2,4-D	100
IAA	39
NAA	55
PCIB	100
Benzoic acid	19
Tryptophan	0

* 100% inhibition: Extant of relative inhibition in the presence of 10 μM 2,4-D (59% inhibition in absolute terms)

Table 2. Purification of ABP40

Step	Total protein (mg)	Activity (units)	Specific activity (units/μg)	Degree of purification	Yield (%)
60 % $(NH_4)_2SO_4$	602	2.00	0.003	1	100
DEAE	256	2.37	0.010	2.8	117
2,4-D Sepharose	18	1.96	0.11	33	97
Hydroxyapatite	8.5	1.78	0.21	63	87
Mono Q	0.31	0.39	1.3	380	20
Mono P	0.11	0.23	2.1	620	11

Table 3. Substrate specificity of ABP40

Substrate	Activity (units/mg)*
Formaldehyde	2.1
Octanol	0.14

*Values were based on an initial velocity measured at 100 mM substrate.

Table 4. Purification of ABP39

Step	Total protein (mg)	Activity (units)	Specific activity (units/mg)	Degree of purification	Yield (%)
60 % $(NH_4)_2SO_4$	957	256	0.33	1	100
DEAE	128	43	0.33	1	17
2,4-D Sepharose	34	10.1	0.29	0.9	0.04
Hydroxyapatite	11.0	32.5	2.97	9	0.13
Mono Q	0.78	14.4	18.5	56	0.06
Mono Q rechromatography	0.27	6.0	22.5	68	0.004
Superose 12	0.014	0.4	35.7	108	0.002

Table 5. Substrate specificity of ABP39

Substrate	% of control activity
Indole-3-acetaldehyde	100
Indole-3-aldehyde	23
Phenylacetaldehyde	125
Benzaldehyde	94
Propionealdehyde	73
Acetaldehyde	86

All substrates were used at a concentrations of 200 μM in the reaction mixture.

References

Berger, J., and Avery, G. S. (1943) The mechanism of auxin action. Science, 98: 454-455.

Bilang, J., Macdonald, H., King, P.J. and Sturm, A. (1993) A soluble auxin-binding protein from *Hyoscyamus muticus* is a glutathione S-transferase. Plant Physiol., 102: 29-34.

Boysen-Jensen, P. (1910) Uber die Leitung des phototropischen reizes in *Avena*-keimpflanzen. Ber. Deut. Bot. Ges., 28, 118-120.

Brown, H. M. and Purves, W. (1976) Isolation and characterization of indole-3-acetaldehyde reductase from *Cucumis sativus.* J. Biol. Chem., 251; 907-913.

Brown, H. M. and Purves, W. K. (1980) Indoleacetaldehyde reductase of *Cucumis sativus* L. Kinetic properties and role in auxin biosynthesis. Plant Physiol. 65; 107-113.

Campos, N., Bako, L., Feldwisch, J., Schell, J. and Palme, K. (1992) A protein from maize labeled with azide-IAA has novel β-glucosidase activity. Plant J., 2: 675-684.

Cleland, R. (1965) Auxin-induced cell wall loosening in the presence of actinomycin D. Plant Physiol., 40: 595-600.

Davies, P. J. (1973) Current theories on the mode of action of auxin. Bot. Rev., 39: 139-171.

Darwin, C. (1896) The Power of Movement in Plants. D Appleton and Co., New York.

Deutscher, M. (1990) Guide to protein purification. Method in Enzymology. 182: 610-611.

Dohrmann, U., Hertel, R. and Kowalik, H. (1978) Properties of auxin-binding sites in different subcellular fractions from maize coleoptiles. Planta, 140: 97-106.

Evans, M. L. (1974) Rapid responses to plant hormones. Annu. Rev. Plant Physiol., 25: 195-223.

Giese, M., Bauer-Doranth, U., Langebartel, C. and Sandermann, H. (1994) Detoxification of formaldehyde by the spider plant *(Chlorophytum comosum L.)* and by soybean (*Glycine max* L.) cell-suspension cultures. Plant Physiol., 104: 1301-1309.

Gutheil, W., Holmquist, B. and Vellee, B. (1992) Purification, characterization, and partial sequence of the glutathione-dependent formaldehyde dehydrogenase from *Escherichia coli:* a class III alcohol dehydrogenase. Biochemistry, 31: 475-481.

Haagen-Smit, A. J., Dandiker, W.B., Wittwer, S. H. and Murneek, A. E. (1946) Isolation of 3-indoleacetic acid from immature corn kernels. Amer. J. Bot., 33, 118-120.

Hansch, C., Muir, R. M., Fujita, T., Malony, P., Geiger, F. and Streich, M. (1963) The correlation of biological activity of plant growth regulators and chloromycetin derivatives with Hammett constants and partition coefficients. J. Amer. Chem. Soc., 85, 2817-2824.

Hertel, R., Thomson, K. and Russo, V. (1972) *In vitro* auxin binding to particulate cell fractions from corn coleoptiles. Planta, 107: 325-340.

Hesse, T., Feldswisch, J., Balshusemann, D., Bauw, G., Puype, M., Puype, M., Vandekerckhove, J., Löbler, M., Klämbt, D., Schell, J. and Palme, K. (1989) Molecular cloning and structural analysis of a gene from *Zea mays* (L.) coding for a putative receptor for the plant hormone auxin. EMBO J., 8: 2453-2461.

Hicks, G. R., Rayle, D. L. and Lomax, T. L. (1989) The diageotropica mutant of tomato lacks high specific activity auxin binding sites. Science, 245: 52-54.

Hicks, G. R., Rice, M. S. and Lomax, T. L. (1993) Characterization of auxin-binding proteins from zucchini plasma membrane. Planta, 189: 83-90.

Inohara, N., Shimomura, S., Fukui, T. and Futai, M. (1989) Auxin-binding protein located in the endoplasmic reticulum of maize shoots : Molecular cloning and complete primary structure. Proc. Natl. Acad. Sci. USA, 86: 3564-3568.

Jacobsen, H. -J., Hajek, K., Mayerbacher, R. and Herber, B. (1987) Soluble auxin-binding: Is there a correlation between growth-stage dependent high-affinity auxin-binding and auxin competence? In Plant Hormone Receptors (D. Klämbt, ed.) Springer-Verlag, Berlin, Heidelberg., 63-69.

Jones, A.M. (1994) Auxin-binding proteins. Annu. Rev. Plant Physiol. Plant Mol.Biol., 45: 393-420.

Julia, P., Pares, X. and Jornvall, H. (1988) Rat liver alcohol dehydrogenase of class III. Primary structure, functional consequences and relationships to other alcohol dehydrogenases. Eur. J. Biochem., 172: 73-83.

Kaiser, R., Holmquist, B., Hempel, J., Vallee, B. and Jornvall, H. (1988) Class III human liver alcohol dehydrogenase: a novel structural type equidistantly related to the class I and class II enzymes. Biochemistry, 27: 1132-1140.

Kaiser, R., Holmquist, B., Valee, B. L. and Jornvall, H. (1989) Characteristics of mammalian class III alcohol dehydrogenase, an enzyme less variable than the traditional liver enzyme of class I. Biochemistry, 28: 8432-8438.

Key, J. L. (1964) Ribonucleic acid and protein synthesis as essential processes for cell elongation. Plant Physiol., 39: 365-370.

Kikuchi, M. Imaseki, H. and Sakai, S. (1989) Modulation of gene expression in isolated nuclei by auxin-binding proteins. Plant Cell Physiol., 30: 765-773.

Klämbt, D. (1990) A view about the function of auxin-binding proteins at plasma membranes. Plant Mol. Biol., 14: 1045-1050.

Laemmli, U. K. (1970) Cleavage of structual proteins during the assembly of the head of bacteriophage T4. Nature, 224: 680-685.

Libbenga, K. R., Mann, A. C., van der Linde, P. C. G. and Mannes. A. (1986) Auxin receptors. *In* Hormones, receptors and cellular interactions in plants, ed. Chadwick, C. M. & Garrod, D. R. Cambridge Univercity Press, Cambridge, 1-68.

Löbler, M. and Klämbt, D. (1985) Auxin-binding protein from coleoptile membrane of corn (*Zea mays* L.). I. Purification by immunological methods and characterization. J. Biol. Chem., 260: 9848-9853.

Löbler, M. and Klämbt, D. (1985) Auxin-binding protein from coleoptile membrane of corn (*Zea mays* L.). II. Localization of a putative auxin receptor. J. Biol. Chem., 260: 9854-9859.

Ludwig-Müller, J. and Hilgenberg, W. (1989) Purification of NADPH-specific indole-3-acetaldehyde reductase from *Cucumis sativus* by two-dementional native polyacrylamide gel electrophoresis. Physiol. Plant., 77: 613-616.

Ludwig-Müller, J., Schramm, P. and Hilgenberg, W. (1990) Indole-3-acetaldehyde reductase in *Phycomyces blakesleeanus*. Characterization of enzyme. Physiol. Plant., 80; 472-478.

Ludwig-Müller, J. and Hilgenberg, W. (1990) Identification of indole-3-acetaldehyde and indole-3-acetaldehyde reductase in Chinese cabbage. Physiol. Plant., 80; 541-548.

Macdonald, H., Jones, A. and King, P. (1991) Photoaffinity labeling of soluble auxin-binding proteins. J. Biol. Chem., 266: 7393-7399.

Masuda, Y., Setterfield, G. and Bayley, S. T. (1966) Ribonucleic acid metabolism and cell expansion in oat coleoptile. Plant Cell Physiol., 7: 243-262.

Paal, A. (1914) Uber phototropische Reizleitung. Jahrb. wiss. Bot. 32, 499-502.

Penny, P. and Galston, A. W. (1966) The kinetics of inhibition of auxin-induced growth in green pea stem segments by actinomycin D and other substances. Amer. J. Bot., 53: 1-7.

Pope, D. and Black, M. (1972) The effect of indole-3-acetic acid on coleoptile extension growth in the absence of protein synthesis. Planta, 102: 26-36.

Porter, W. L. and Thimann, K. V. (1965) Molecular requirements for auxin action. Phytochem. 4, 229-243.

Roy, P. and Biswas, B. B. (1977) A receptor protein for indoleacetic acid from plant chromatin and its role in transcription. Biochem. Biophys. Res. Commun., 74: 1597-1606.

Prasad, P.V. and Jones, A. M. (1991) Putative receptor for the plant growth hormone auxin identified and characterized by anti-ideiotypic antibodies. Proc. Natl. Acad. Sci. USA, 88: 5479-5483.

Sakai, S. and Hanagata, T. (1983) Purification of an auxin-binding protein from etiolated mung bean seedlings by affinity chromatography. Plant Cell Physiol., 24: 685-693.

Sakai, S. (1984) Characterization of 2,4-D binding to the auxin-binding protein purified from etiolated mung bean seedlings. Agric. Biol. Chem., 48: 257-259.

Sakai, S. (1985) Auxin-binding protein in etiolated mung bean seedlings: Purification and properties of auxin-binding protein-II. Plant Cell Physiol., 26: 185-192.

Sakai, S. (1992) Regulatory functions of soluble auxin-binding proteins. Int. Rev. Cytol., 135: 239-267.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. New York Acad. Sci., 51: 660-672.

Schutte, H., Flossdorf, J., Sahm, H. and Kula, M-R. (1976) Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidnii*. Eur. J. Biochem., 62: 151-160.

Shimomura, S., Sotobayashi, T., Futai, M. and Fukui, T. (1986) Purification and properties of an auxin-binding protein from maize shoot membranes. J. Biochem., 99: 1513-1524.

Theologis, A. (1986) Rapid gene regulation by auxin. Annu. Rev. Plant Physiol., 37: 407-438.

Tillmann, U., Viola, G., Kayser, B., Siemeister, G., Hesse, T., Palme, K., Löbler, M. and Klämbt, D. (1989) cDNA clones of the auxin-binding protein from corn coleoptiles (*Zea mays* L.): isolation and characterization by immunological methods. EMBO J., 8: 2463-2467.

Uotila, L. and Koivusalo, M. (1974) Formaldehyde dehydrogenase from human liver. J. Biol. Chem., 249: 7653-7663.

Uotila, L. and Koivusalo, M. (1979) Purification of formaldehyde and formate dehydrogenase from pea seeds by affinity chromatography and S-formylglutathione as the intermediate of formaldehyde metabolism. Arch. Biochem. Biophys., 196: 33-45.

Vanderhoef, L. N. and Stahl, C. A. (1975) Separation of two responses to auxin by means of cytokinin inhibition. Proc. Natl. Acad. Sci. USA, 72: 1822-1825.

Venis, M.A., Napier, R.M., Barbier-Brygoo, H., Maurel, C., Perrot-Rechenmann, C. and Guern, J. (1992) Antibodies to a peptide from the maize auxin-binding protein have auxin agonist activity. Proc. Natl. Acad. Sci. USA, 89: 7208-7212.

Wehner, E., Rao, E. and Brendel, M. (1993) Molecular structure and genetic regulation of SFA, a gene responsible for resistance to formaldehyde in *Saccharomyces cerevisiae*, and characterization of its protein product. Mol. Gen. Genet., 237: 351-358.

Went, F. W. (1926) On growth-accelelerating substances in the coleoptile of *Avena sativa*. Proceedings of the section of sciences. Koninklijke Akademie van Wetenschappen te Amsterdam, 30: 10-19.

Went, F. W. and Thimann, K. V. (1937) Phytohormones. The MacMillan Co., New York.

Zettl, R., Feldwisch, J., Boland, W., Shell, J. and Palme, K. (1992) 5'-Azido-[3,6^{3}H]-1-naphthylphthalamic acid, a photoactivatable probe for naphthylphthalamic acid receptor proteins from higher plants: Identification of a 23-kDa protein from maize coleoptile plasma membranes. Proc. Natl. Acad. Sci. USA, 89: 480-484.

Zettl, R., Schell, J. and Palme, K. (1994) Photoaffinity labeling of *Arabidopsis thaliana* plasma membrane vesicles by 5-azido-[7-^{3}H] indole-3-acetic acid: identification of a glutathione S-transferase. Proc. Natl. Acad. Sci. USA, 91: 689-693.