Effect of fluorophenylalanine on indole-3-acetic acid oxidation in *Avena* coleoptiles

ANDREW B. MAKSYMOWYCH¹, JOSEPH A.J. ORKWISZEWSKI²

¹Department of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania 19010 and ²Department of Biology, Villanova University, Villanova, Pennsylvania 19085; U.S.A.

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Abstract

Effects of the amino acid analog D,L-p-fluorophenylalanine on auxin oxidizing activity from *Avena sativa* cv. Clintford have been examined. Incubation of etiolated *Avena* coleoptile apices with D,L-p-fluorophenylalanine, previously reported to promote elongation and depress phenolic metabolism, lowers auxin oxidizing activity. Temperature and pH profiles demonstrated that depression of oxidizing activity by D,L-p-fluorophenylalanine occurs over a relatively wide range. Incubation of coleoptiles in media containing L-phenylalanine alone or L-phenylalanine combined with D,L-p-fluorophenylalanine result in oxidizing activities which are, respectively, slightly higher than or restored to control levels. Thus, dark incubation of coleoptiles with D,L-p-fluorophenylalanine results in auxin oxidizing activity which differs from that of control and other treatment groups. Oxidizing activity from D,L-p-fluorophenylalanine pretreated coleoptiles has a Michaelis constant (70 μ M) and a maximum velocity (0.200) which are consistently lower than the control constants. These findings provide further clarification of the effects of D,L-p-fluorophenylalanine, on coleoptile elongation and auxin oxidizing activity in etiolated *Avena* coleoptiles.

Key words: auxin oxidation, *Avena* coleoptile, fluorophenylalanine, phenolic metabolism

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² To whom reprint requests should be sent.

ABBREVIATIONS: BSM — buffered sucrose medium; DCP — 2,4-dichlorophenol; IAA — indole-3-acetic acid; p-FPA — D, L-p-fluorophenylalanine; PAL — L-phenylalanine ammonia-lyase; Phe — L-phenylalanine

INTRODUCTION

Hopkins and Bonnell (1969) first reported that extension growth of excised *Avena* coleoptile apices in darkness was significantly enhanced by the amino acid analog D,L-p-fluorophenylalanine. This response was reversed by the inclusion of L-phenylalanine with D,L-p-fluorophenylalanine in the incubation medium. Growth enhancement induced by D,L-p-fluorophenylalanine preincubation was correlated with the finding that treatment of coleoptiles with the fluoro-analog results in depressed L-phenylalanine ammonia-lyase (E.C.4.3.1.5) activity and reduced *in vivo* chlorogenic acid levels (Hopkins and Orkiszewski 1971, Orkiszewski and Hopkins 1974, Orkiszewski et al. 1976).

Further studies have demonstrated that enhancement of elongation, depression of L-phenylalanine ammonia-lyase activity and regulation of phenolic levels measured by chlorogenic acid content are both isomer and substituent specific for D,L-p-fluorophenylalanine. In addition, preliminary data indicated that D,L-p-fluorophenylalanine may alter auxin oxidizing activity (Maksymowych and Orkiszewski 1987). These authors proposed that enhanced elongation in response to D,L-p-fluorophenylalanine treatment stems from a depression of L-phenylalanine ammonia-lyase activity bringing about lowered phenolic levels which, directly or indirectly, modify *Avena* auxin oxidizing activity. The purpose of this study is to further examine the effects of D,L-p-fluorophenylalanine pretreatment on indole-3-acetic acid oxidizing activity extracted from etiolated *Avena* coleoptiles. Data are presented suggesting a possible mechanism for D,L-p-fluorophenylalanine action on the growth of *Avena* coleoptiles.

MATERIAL AND METHODS

Plant material. Unhusked seeds of *Avena sativa* L., cv. Clintford (The Stanford Seed Co., Denver, Pennsylvania) were grown as described by Hopkins and Bonnell (1969). Plant material used were 8 mm apical coleoptile segments minus primary leaves, which were excised by hand from 4-day-old etiolated seedlings. All operations involving living tissue were conducted either in darkness or under a dim green safelight (Hopkins and Bonnell 1969).

Auxin oxidase preparation. *Avena* coleoptiles were collected and randomized in buffer and distributed into 50 ml beakers containing 20 ml of 20 mM phosphate buffer, pH 6.2, with 1.5 % w/v sucrose (BSM). Coleoptile apices were incubated in BSM (control) or BSM with 5 mM p-FPA, Phe, or Phe + p-FPA, respectively, in darkness for 18 h at $26 \pm 1^\circ \text{C}$. After incubation, coleoptile tissue was drained on a strainer, thoroughly rinsed with type I water, rinsed with -20°C acetone and homogenized in cold acetone (-20°C) with a Waring blender in a pre-chilled

semi-micro jar for 60 s. 50 ml of acetone were used for each sample (approximately 200 coleoptile apices). The homogenate was filtered through Whatman No 1 filter paper under reduced pressure, washed several times with 50 ml aliquots of cold acetone and the residue dried in a vacuum desiccator at room temperature for 24 h. Thoroughly dried samples were ground to a finer powder with a mortar and pestle and stored at -20°C in tightly stoppered glass vials. Acetone powders could be stored for several weeks at -20°C without loss of enzyme activity (Maksymowych and Orkiszewski 1987). For preparation of soluble enzyme, acetone powders were suspended in 30 mM phosphate buffer (pH 6.2) at a rate of 10 mg powder per ml buffer and extracted by constant stirring at 3°C for 1/2 h. The resulting suspension was clarified by centrifugation at $25,000 \times g$, 3°C for 1/2 h. The clear supernatant was dialyzed against 15 l of assay buffer for 24 h, in Spectrapor-6 (MW cut-off 25,000) dialysis tubing. After 24 h, the dialyzed suspension was once again centrifuged at $25,000 \times g$, 3°C for 1/2 h. The resultant supernatant yields a partially purified enzyme solution with IAA oxidizing activity. This procedure was employed in all assays. A 1 ml aliquot of enzyme solution was removed for Lowry protein determination (Lowry et al. 1951). The remaining enzyme solution was held at 3°C and assayed immediately.

Enzyme kinetics. Kinetic data for auxin oxidizing activity were determined at 254 nm, 26°C , on a Gilford Response spectrophotometer in a constant temperature sample chamber. Reaction samples contained the appropriate substrate (IAA) concentrations for each study, 100 μgrams total protein from the respective samples, 40 nmol each of DCP and MnCl_2 in 30 mM phosphate buffer, pH 6.2, in a total volume of 1.2 ml. Reactions were monitored for 30 min and expressed as $\Delta A_{254} \times 20 \text{ min}^{-1}$. Reaction rates were stable and linear for the duration of the assay. Enzyme specific activities are expressed as $\Delta A_{254} \times \text{mg protein}^{-1} \times \text{h}^{-1}$.

RESULTS

Temperature and pH profile for auxin oxidizing specific activity. Figure 1(a) represents the effect of temperature on *Avena* auxin oxidizing activity and illustrates the specific activity of enzyme extracts from control and p-FPA pretreated coleoptiles. These data demonstrate that the depression of IAA oxidizing activity occurs throughout the temperature range studied and may be slightly more pronounced at higher assay temperatures. Figure 1(b) presents and extended pH vs activity profile for *Avena* auxin oxidizing activity. Data demonstrate decreased oxidizing specific activity for p-FPA pretreated coleoptiles between pH 5.3 and 6.8. According to this study the pH optimum and maximum depression of specific activity due to p-FPA pretreatment both occur at approximately pH 6.2. This is the pH at which coleoptiles were incubated and pretreated, resulting in the greatest degree of growth enhancement.

Fig. 1. Temperature and pH profiles for *Avena* auxin oxidizing activity. Enzyme specific activity was determined according to the assay described in Materials and Methods. Substrate concentrations were at saturation. Oxidation of IAA was determined with enzyme preparations from *Avena* coleoptiles preincubated in BSM (1) and *p*-FPA (2), respectively, for 18 h at $26 \pm 1^\circ\text{C}$ in darkness. A — Specific activity plotted versus the temperature at which enzyme activity was assayed. B — Specific activity plotted versus the pH at which reaction mixtures were assayed

Effect of Phe, *p*-FPA, Phe + *p*-FPA treatments on enzyme activity. Figure 2 illustrates the oxidative decarboxylation of IAA by auxin oxidizing activity extracted from coleoptiles pretreated for 18 h in the various growth media (*p*-FPA, Phe, Phe + *p*-FPA). Enzyme extracts from BSM incubated coleoptiles served as controls. Activity (ΔA_{254} vs time) for the enzyme extract from *p*-FPA pretreated coleoptiles was depressed below control (BSM) levels. On the other hand, incubation with Phe increased the rate of IAA oxidation above controls. Pretreatment of coleoptile apices with Phe + *p*-FPA reversed the effects of *p*-FPA and displayed activity similar to that resulting from incubation with Phe alone. Reaction rates in Figure 2 are plotted for 30 min, however the rates were linear for a minimum of 1 h.

Kinetic studies of auxin oxidizing activity. Further studies were performed employing a variety of substrate concentrations. Figure 3 presents double-reciprocal plots for enzyme activity. Enzyme extracted from *p*-FPA pretreated *Avena* coleoptile apices exhibits kinetics which differ from that of the control

Fig. 2. Effect of Phe, p-FPA and Phe + p-FPA pretreatment on *Avena* auxin oxidizing activity. Increase in absorbance (formation of product) is plotted vs time. Auxin oxidizing activity was extracted from coleoptile apices incubated for 18 h at $26 \pm 1^\circ\text{C}$ in darkness on BSM (1) with or without 5 mM p-FPA (2), Phe (3) or Phe + p-FPA (4). Assays were carried out according to procedures described in Materials and Methods. Substrate concentrations were at saturation. Each data point represents three replicate experiments ($n = 3$). Best straight lines were calculated by linear regression analysis. Specific activities ($\Delta A_{254} \cdot 0.1 \text{ mg protein}^{-1} \cdot \text{h}^{-1}$) calculated from data points are significantly different when compared to BSM (BSM vs p-FPA $p < 0.5\%$; BSM vs Phe + p-FPA $p < 1.0\%$; BSM vs Phe $p < 10.0\%$)

(BSM), Phe and Phe + p-FPA extracts. The relative direction of inhibition (p-FPA), stimulation (Phe) and reversal (Phe + p-FPA) are as described above. Linear regression analysis (R o s n e r 1982) of Lineweaver-Burk plots demonstrated that the K_m for p-FPA, Phe, Phe + p-FPA and BSM pretreatments were $70 \mu\text{M}$, $120 \mu\text{M}$, $140 \mu\text{M}$ and $100 \mu\text{M}$, respectively. Maximum velocities for p-FPA, Phe, Phe + p-FPA and BSM were 0.200, 0.313, 0.350 and 0.282, respectively. The K_m and V_{max} values determined from Eadie-Hofstee transformations were similar to the values listed above (D o w d and R i g g s 1965).

DISCUSSION

Pretreatment of coleoptiles with p-FPA increases coleoptile elongation, depresses extractable PAL activity and decreases *in vivo* chlorogenic acid levels as compared to controls (H o p k i n s and B o n n e l l 1969, H o p k i n s and O r k w i s z e w s k i 1971, O r k w i s z e w s k i and H o p k i n s 1974, O r k w i s z e w s k i et al. 1976, M a k s y m o w y c h and O r k w i s z e w s k i 1987). H o p k i n s and O r k w i s z e w s k i (1971) suggested that p-FPA promotes elongation by lowering PAL activity, which in turn decreases the synthesis of potentially phytotoxic low molecular weight phenols.

Fig. 3. Lineweaver-Burk transformations for auxin oxidizing activity extracted from Phe, p-FPA and Phe + p-FPA pretreated coleoptiles. Reactions were carried out according to procedures detailed in Materials and Methods. Oxidizing activity in protein extracts from coleoptile apices incubated with BSM (1), p-FPA (2), Phe (3), and Phe + p-FPA (4) for 18 h at $26 \pm 1^\circ\text{C}$ in darkness was assayed. Reciprocal values for increase in absorbance at 254 nm were plotted vs reciprocal substrate (IAA) concentrations. Each data point represents three replicate experiments ($n = 3$). Best straight lines were calculated by linear regression analysis

Orkiszewski et al. (1976) showed that Phe alone depressed elongation and increased *in vivo* phenolic levels. Pretreatment with p-FPA + Phe reverses the effect of p-FPA on elongation and demonstrates the same enhanced level of low molecular weight phenols as treatment with Phe alone (Orkiszewski et al. 1976, Maksymowych and Orkiszewski 1987). It is possible that Phe inhibits the elongation response resulting from p-FPA pretreatment by increasing the concentration of phenolics thus counteracting growth stimulation by p-FPA.

There are several growth mechanisms by which phenols may exert their effects. One explanation may be interaction of phenols with endogenous auxin oxidizing activity. Various phenolic compounds have been shown to influence the activity of IAA oxidation *in vitro* (Mumford et al. 1961, 1962, Zenk and Muller 1963, Lee 1977, Lee et al. 1982). Modification of oxidizing activity may be a growth regulatory mechanism since compounds which enhance IAA oxidation appear to inhibit growth whereas those which depress oxidizing activity promote growth (Galston 1967).

We decided to test this hypothesis using p-FPA in an attempt to induce changes on *in vivo* phenolic levels and then to monitor for possible effects on auxin oxidizing activity *in vitro*. Since the effects of the ortho-, para- and meta-isomers of FPA have been examined and para-isomer specificity for the fluorinated analog has been established (Orkiszewski and Hopkins 1974, Orkiszewski et al. 1976, Maksymowych and Orkiszewski 1987), this investigation was limited to p-FPA. Maksymowych and

Orkwiszewski (1987) presented preliminary evidence that pretreatment of coleoptile apices for 18 h with p-FPA results in depressed auxin oxidizing activity. Based on the demonstration of enhanced elongation accompanied by a depression of IAA oxidizing activity, further characterization of auxin oxidizing activity was undertaken. Data (Fig. 1a, b) indicate that the depression of oxidizing specific activity is not temperature dependent and appears to be most pronounced at our incubation pH of 6.2.

IAA oxidizing activity may be due to a single protein (Sequeira and Mineo 1966, Sirois and Miller 1972), a protein complex (Ray 1960, Ricard et al. 1972), or isoenzymes (Frenkel 1972). Differing activities (Fig. 2) may be due to relative differences in the amount of oxidizing protein present in the total protein in respective assay mixtures. On the other hand, the kinetic properties of the oxidizing protein may also be altered by p-FPA treatment.

Data from kinetic experiments were plotted according to the Lineweaver-Burk transformation (Fig. 3). The enzyme system extracted from p-FPA pretreated coleoptiles exhibits kinetics different from that of the control and maintains the relative direction of inhibition (p-FPA), stimulation (Phe) and reversal (Phe + p-FPA) of enzyme activity. These data provide presumptive evidence for the role of the oxidizing activity and its modulation by p-FPA pretreatment in *Avena* coleoptile elongation and are consistent with growth and metabolic data presented by Orkwiszewski et al. (1976).

Both the K_m (70 μ M) and V_{max} (0.200) for the extracts from p-FPA pretreated coleoptiles are 30% lower than control values. Differences in enzyme activity resulting from p-FPA pretreatment may be due to several possibilities. The lowering of K_m for oxidizing activity may indicate an increase in the affinity of the oxidizing protein for IAA, suggesting a change in protein structure or a change in the spectrum of proteins responsible for IAA oxidation. On the other hand, the reduced V_{max} points to a diminished capacity of the extract for IAA decarboxylation. At the present a distinction cannot be made as to whether the difference in IAA oxidizing activity resulting from p-FPA pretreatment is due to structural changes in protein, a change in protein profile or both. The other pretreatments (Phe, Phe + p-FPA) appear to stimulate enzyme activity. A direct cause for this stimulation of activity is not known at present.

Modification of oxidizing activity may be a growth regulatory mechanism (Galston 1967, Hopkins and Bonnell 1969, Hopkins and Orkwiszewski 1971, Orkwiszewski and Hopkins 1974, Orkwiszewski et al. 1976, Maksymowych and Orkwiszewski 1987). Modulation of clearance and, therefore, the endogenous supply of free IAA, up to (or down to) a threshold constitutes a possible mechanism by which the rate of coleoptile elongation may be controlled.

Our results clearly warrant further investigation. A tool (p-FPA) and an experimental system (etiolated *Avena* coleoptile apices) are available which may be used to further investigate growth regulation by IAA. We propose a mecha-

nism which may account for p-FPA induced extension growth in *Avena* coleoptiles. Data presented in this report are consistent with the hypothesis that p-FPA pretreatment modifies auxin oxidizing activity resulting, directly or indirectly, in enhanced extension growth.

REFERENCES

- Dowd J.E., Riggs D.S., 1965. A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. *J. Biol. Chem.* 240: 863-869.
- Frenkel C. 1972. Involvement of peroxidase and indole-3-acetic acid oxidase isozymes from pear, tomato and blueberry fruit in ripening. *Plant Physiol.* 49: 757-763.
- Galston A.W., 1967. Regulatory systems in higher plants. *Amer. Sci.* 55: 144-160.
- Hopkins W.G., Bonnell K.F., 1969. *Avena* coleoptile elongation: Stimulation by fluorophenylalanine. *Plant Physiol.* 44: 281-286.
- Hopkins W.G., Orkiszewski J.A.J., 1971. Inhibition of phenylalanine ammonia-lyase and enhancement of *Avena* coleoptile segment elongation by fluorophenylalanine. *Can. J. Bot.* 49: 129-135.
- Lee T.T., 1977. Role of phenolic inhibitors in peroxidase mediated degradation of indole-3-acetic acid. *Plant Physiol.* 59: 372-375.
- Lee T.T., Starrat A.N., Jevnikar J.J., 1982. Regulation of enzymic oxidation of indole-3-acetic acid by phenols: Structure-activity relationships. *Phytochem.* 21: 517-523.
- Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Maksymowych A.B., Orkiszewski J.A.J., 1987. Studies of fluorophenylalanine induced *Avena* coleoptile elongation: Possible modulation of auxin oxidase activity. *Acta Soc. Bot. Pol.* 56: 325-336.
- Mumford F.E., Smith D.H., Castle J.E., 1961. An inhibitor of indoleacetic acid oxidase from pea tips. *Plant Physiol.* 36: 724-752.
- Mumford F.E., Stark H.M., Smith D.H., 1962. A naturally occurring cofactor for indoleacetic acid oxidase. *Plant Physiol.* 37: suppl. XIV.
- Orkiszewski J.A.J., Hopkins W.G., 1974. Effect of fluorophenylalanine on phenylalanine ammonia-lyase activity in *Avena* coleoptiles. *Amer. J. Bot.* 61: 433-436.
- Orkiszewski J.A.J., Koroly M.J., DiMichele L., Weber D.M., 1976. Similarities between L-phenylalanine and m-fluorophenylalanine as substrates for L-phenylalanine ammonia-lyase. *Physiol. Plant.* 37: 288-292.
- Ray P.M., 1960. The destruction of indoleacetic acid. III. Relationship between peroxidase action and indoleacetic acid oxidation. *Arch. Biochem. Biophys.* 87: 19-30.
- Ricard J., Mazza G., Williams R.J.P., 1972. Oxidation reduction potentials and ionization states of two turnip peroxidases. *Eur. J. Biochem.* 28: 566-578.
- Rosner B.A., 1982. *Fundamentals of biostatistics*. Duxbury Press. PWS Publishers. Boston, pp. 344-368. ISBN 0-87872-254-8.
- Sequeira L., Mineo L., 1966. Partial purification and kinetics of indoleacetic acid oxidase from tobacco roots. *Plant Physiol.* 41: 1200-1203.
- Sirois J.C., Miller R.W., 1972. The mechanism of the scopoletin-induced inhibition of the peroxidase catalyzed degradation of indole-3-acetate. *Plant Physiol.* 49: 1012-1018.
- Zenk M.H., Muller G., 1963. *In vitro* destruction of exogenously applied indolyl-3-acetic acid as influenced by naturally occurring phenolic acids. *Nature* 200: 761-763.

Wpływ fluorofenylalaniny na właściwości utleniające kwasu indolo-3-octowego w liściach Avena

Streszczenie

Badano wpływ D,L-p-fluorofenylalaniny, analogu aminokwasu, na aktywność utleniającą auksyny pochodzącej z *Avena sativa* cv. Clintford. Wcześniej wykazano, że inkubacja etiolowanych stożków wzrostu liścieni *Avena* z D,L-p-fluorofenylalaniną przyspiesza wydłużanie liścieni, natomiast upośledza metabolizm fenolowy. Obecnie stwierdzono, że również zmniejsza aktywność utleniającą auksyny. Uzależnienie tego procesu od temperatury i pH wykazało, że ograniczenie aktywności utleniającej D,L-p-fluorofenylalaniny występuje w stosunkowo wąskim zakresie tych czynników. Inkubacja liścieni w środowisku tylko z L-fenylalaniną lub w połączeniu z D,L-p-fluorofenylalaniną wywołała, odpowiednio, zwiększenie aktywności utleniającej lub zahamowanie jej na poziomie kontroli. Natomiast zupełnie inny efekt, nieporównywalny z kontrolą i innymi wariantami doświadczalnymi, otrzymano po hodowli koleoptyli w ciemności z D,L,-p-fluorofenylalaniną. Aktywność utleniająca pochodząca z koleoptyli uprzednio traktowanych D,L,-p-fluorofenylalaniną charakteryzowała się stałą Michaelisa ($70 \mu\text{M}$) i maksymalnym zwielokrotnieniem (0,200) wyraźnie mniejszymi niż wartości dla kontroli. Wyniki tych badań zbliżają nas do wyjaśnienia wpływu D,L-p-fluorofenylalaniny na wydłużanie koleoptyli i na aktywność utleniającą auksyny w etiolowanych liściach *Avena*.