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KINETICS, REGULATION AND PURIFICATION OF L-PHENYLALANINE AMMONIA-LYASE FROM GERMINATING LETTUCE SEEDS (PHENYLPROPANOIDS, ENZYME, ALPHA-AMINOXY - BETA-PHENYLPROPIONIC ACID)

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KINETICS, REGULATION AND PURIFICATION OF L-PHENYLALANINE AMMONIA-LYASE FROM GERMINATING LETTUCE SEEDS (PHENYLPROPANOIDS, ENZYME, ALPHA-AMINOXY - BETA-PHENYLPROPIONIC ACID)

Abstract
The kinetics of L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) in germinating lettuce (Lactuca sativa cv. Grand Rapids) seeds was investigated. The Km of the enzyme was determined to be 4.2 x 10^-5 M. PAL in lettuce seeds did not show tyrosine ammonia-lyase activity. The nature of inhibition of PAL by various substrate analogues and phenylpropanoid compounds was studied. Substrate analogues like D-phenylalanine, p-fluorophenylalanine, (beta)-phenyllactic acid and tryptophan were found to inhibit PAL competitively whereas tyrosine did not show any inhibition of enzyme activity. Of the phenylpropanoids used, cinnamic acid was found to be a competitive inhibitor whereas chlorogenic acid showed mixed inhibition. Other phenylpropanoid compounds like p-coumaric acid, caffeic acid, coumarin, quercetin, ferulic acid etc. did not show any inhibition of PAL activity in vitro.

The regulation of PAL activity by various substrate analogues, intermediates and endproducts of the phenylpropanoid pathway in relation to the growth of the embryonic axes was studied. The substrate, L-phenylalanine, its D-isomer and (beta)-phenyllactic acid did not show any significant effect on PAL activity at low concentrations (50 and 100 uM), whereas at higher concentrations (500 uM) L-phenylalanine inhibited both PAL activity and radicle elongation. On the other hand, D-phenylalanine promoted both. Other substrate analogues like 2-aminoxy-3-phenylpropionic acid (AOPP), p-fluorophenylalanine and tryptophan showed a strong inhibition of PAL activity, the inhibition being concentration dependent. Another substrate analogue, tyrosine, stimulated both PAL activity and radicle elongation.

The phenylpropanoid compounds were inhibitors of PAL activity at higher concentrations. At lower concentrations, there was a concentration dependent inhibition of PAL activity and radicle elongation. In all these treatments, a strong correlation was observed between PAL activity and radicle length.

PAL was purified 37 fold from 40 h old lettuce seedlings. Excised radicles were used as a source of the enzyme for purification since more than 95% of the PAL activity was localized in the radicles. A combination of four techniques viz. ammonium sulfate precipitation, gel filtration, ion exchange and hydroxylapatite chromatography were used for the purification of PAL from lettuce seedlings.

Keywords
Biology, Plant Physiology

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KINETICS, REGULATION AND PURIFICATION OF
L-PHENYLALANINE AMMONIA-LYASE FROM
GERMINATING LETTUCE SEEDS

BY

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A DISSERTATION

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ABSTRACT

KINETICS, REGULATION AND PURIFICATION OF L-PHENYLALANINE AMMONIA-LYASE (PAL) FROM GERMINATING LETTUCE SEEDS

By

GEORGE JOSE KUDAKASSERIL
University of New Hampshire, December 1983

L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyses the elimination of ammonia from the aromatic amino acid L-phenylalanine to produce trans-cinnamic acid. The enzyme occupies an important position in the secondary metabolism of plants, being the first enzyme of the phenylpropanoid metabolism.

The kinetics of L-phenylalanine ammonia-lyase in germinating lettuce (Lactuca sativa cv. Grand Rapids) seeds was investigated. The \( K_m \) of the enzyme was determined from the Lineweaver-Burk plot to be \( 4.2 \times 10^{-5} \) M. PAL in lettuce seeds did not show any tyrosine ammonia-lyase activity. The nature of inhibition of PAL by various substrate analogues and some phenylpropanoid compounds was studied. Substrate analogues like D-phenylalanine, p-fluorophenylalanine, \( \beta \)-phenyllactic acid and tryptophan were found to inhibit PAL competitively whereas tyrosine did not show any inhibition of enzyme activity. The product of the enzyme reaction, cinnamic acid, was found to be a competitive inhibitor whereas chlorogenic acid, an endproduct of the phenylpropanoid pathway, was found to show mixed inhibition. Several other phenylpropanoid compounds such as p-coumaric acid, caffeic acid,
ferulic acid, coumarin and quercetin did not show any inhibition of PAL activity in vitro.

The regulation of PAL activity by various substrate analogues, intermediates and endproducts of the phenylpropanoid pathway in relation to the growth of the embryonic axis was studied. 200 seeds each were incubated in three different concentrations of each compound under light for 24 h and the enzyme extracted, dialysed and assayed. The length of the radicles from 10 randomly selected germinated seeds from each treatment was measured. The substrate L-phenylalanine, its D-isomer and β-phenylactic acid did not show any significant effect on PAL activity per seed at low concentrations (50 and 100 μM) whereas at higher concentrations (500 μM) L-phenylalanine inhibited the development of PAL activity. Growth of the radicle was also inhibited at this concentration. D-phenylalanine on the other hand promoted both the amount of PAL per seed as well as the elongation of the radicle. Other substrate analogues like L-2-aminoxy-3-phenylpropionic acid (L-AOPP), p-fluorophenylalanine and tryptophan showed a strong inhibition of PAL activity, the inhibition being concentration dependent. In contrast to other substrate analogues that inhibited PAL activity, AOPP produced a stimulation of radicle growth at concentrations that inhibited PAL activity. Another substrate analogue, tyrosine, was found to stimulate both PAL activity and radicle elongation.

Several intermediates and endproducts of the phenylpropanoid pathway acted as strong inhibitors of germination as well as the development of PAL activity at higher concentrations. At lower concentrations (50 and 100 μM) where germination did occur there was a
concentration dependent inhibition of PAL activity and a concomitant reduction in the radicle length. In all these treatments a strong correlation was observed between PAL activity and radicle length.

PAL was purified 37 fold from 40 h old lettuce seedlings. Since more than 95% of the PAL in germinating lettuce seeds was found to be localized in the radicles, excised radicles were used as the source of the enzyme for purification. A combination of four techniques viz. ammonium sulfate precipitation, gel filtration on Sephadex G-200, ion exchange chromatography on DEAE cellulose and hydroxylapatite chromatography on Bio Gel HTP were used for the purification of PAL.
INTRODUCTION

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyzes the elimination of ammonia from the aromatic amino acid L-phenylalanine to produce trans-cinnamic acid. This enzyme occupies a key position in the secondary metabolism of plants, being the first enzyme of the phenylpropanoid metabolism in higher plants. Trans-cinnamic acid, the product of the enzyme reaction, is a precursor to a variety of phenylpropanoid compounds like flavonoids, coumarins, chlorogenic acid, lignin etc. These compounds are either involved in protecting the plant against pathogens or in allelopathic interactions. The rate of PAL activity could determine the amount of phenylalanine available for protein biosynthesis and for phenylpropanoid biosynthesis. Therefore, the activity of this enzyme in the cells seems to be well regulated.

Phenylalanine ammonia-lyase is also one of the most extensively studied enzymes in plants. This enzyme was first isolated and characterised by Koukol and Conn (1961) from barley seedlings. Since then, it has been reported from a variety of higher plants as well as from algae and fungi. In microorganisms the enzyme functions in the catabolism of exogenously supplied amino acids, the products being used as a carbon source. There has been no report of the existence of PAL in animal systems.
Regulation of PAL

The regulation of phenylalanine ammonia-lyase in plants has received a great deal of attention because of its sensitivity to various external stimuli, such as, light, wounding, fungal and viral infections, hormones and various chemicals.

Light

Since the first report from Zucker (1965) on the induction of PAL in potato slices by light, the effect of light on the regulation of PAL levels has been studied in great detail in a variety of plant tissues. In most cases a typical time course of PAL response to light involves three phases; 1. a lag phase which is approximately 90 mins; 2. a phase of initially linear increase in enzyme activity lasting for 3-20 h followed by; 3. a phase of rapid loss of activity. The stimulatory effect of light seems to be a general one although exceptions are known where light has no effect on PAL (Nitsch and Nitsch, 1967; Walton and Sondheimer, 1968; Rov et al., 1969.) The photoreceptors involved in the PAL response to light vary among different plants.

Phytochrome involvement has been demonstrated in Sinapis alba (Durst and Mohr, 1966; Mohr et al., 1968; Dittes et al., 1971; Schopfer, 1971; Schopfer and Hock, 1971; Schopfer and Mohr, 1972; Acton and Schopfer, 1974; Attridge et al., 1974; Wellman, 1974; Tong and Schopfer, 1976; Mohr et al., 1979), Cucumis sativus (Engelsma, 1967, 1968; Attridge and Smith, 1974), Petroselinum hortense (Wellmann, 1974; Wellmann and Baron, 1974; Wellmann, 1975; Wellmann

On the other hand, continuous far red illumination increases PAL levels in etiolated tissues (Durst and Mohr, 1966; Attridge and Smith, 1967; Bellini and Van Poucke, 1970). According to Hartmann (1967) under constant far red light, 3-5% of the phytochrome in a given plant material is present in the active unstable (Pfr) form, the remainder being maintained in a stable but inactive form (Pr). The maintenance of the low but significant amount of Pfr is believed to be responsible for the photomorphogenetic effects of constant FR light. This hypothesis has been used by Durst and Mohr (1966) to explain the results on PAL activities in FR irradiated mustard and radish seedlings. More recently, however, Daines et al., (1983) have shown that lettuce seeds kept under continuous FR or germinated lettuce seeds transferred from white light to continuous FR do not produce significant levels of PAL.

In addition to exhibiting R-FR reversibility, some systems are sensitive to blue light. In gherkin seedlings, a long term irradiation
with blue light results in a great stimulation of PAL activity (Engelsma and Maljer, 1965; Engelsma, 1967). In this case there is no R-FR induction-reversion. Moreover, even tissue exposed to R or FR light will respond to blue light. The photoreceptor for blue light, the nature of which is not known at present, seems to be located in the cotyledons.

Wounding

An increased level of PAL upon wounding occurs in gherkin (Engelsma, 1968), excised bean axes (Walton and Sondheimer, 1968), sweet potato (Minamikawa and Uritani, 1968), citrus fruit peel (Riov et al., 1969), swedes (Rhodes and Wooltorton, 1971), and pea seedlings (Hyodo and Yang, 1971). In most cases this effect has been attributed to the production of ethylene in response to wounding (Imaseki et al., 1968; Riov et al., 1969; Hyodo and Yang, 1971; Chalutz, 1973).

Pathogens

PAL is also induced in response to various plant pathogens as in sweet potato infected with Ceratocystis fimbriata (Minamikawa and Uritani, 1965), soybean infected with Helminthosporium carbonum (Biehn et al., 1968), excised pea and bean pods inoculated with Fusarium solani and Fusarium phaseoli respectively (Hadwiger et al., 1970), in tobacco infected or with tobacco mosaic virus (Paynot et al., 1971), in wheat infected with Erysiphe graminis (Green et al., 1975), or potato infected with Phytophthora infestans (Smith and Rubery, 1981). This increase in PAL activity following infection is a host response associated with the synthesis of fungitoxic compounds like
phytoalexins (Hadwiger et al., 1970; Rahe et al., 1969; Loschke et al., 1981), or synthesis of lignin (Green et al., 1975; Kopp et al., 1983) and various phenolic compounds (Biehn et al., 1968; Smith and Rubery, 1981), implicated in defense mechanisms in plants.

**Growth Regulators**

The activity of PAL is induced by various hormones and growth regulators in various tissues. Treatment with gibberellic acid increased the specific activity of PAL in dwarf peas, corn, tomato and bean seedlings (Cheng and Marsh, 1968; Reid and Marsh, 1969). Ethylene also induced PAL in sweet potato (Imaseki et al., 1968), citrus fruit peel (Riov et al., 1969), swedes (Rhodes and Wooltorton, 1971), pea seedlings (Hyodo and Yang, 1971), and in lettuce leaves (Hyodo et al., 1978). Haddon and Northcote (1975) observed that the transfer of bean callus from a medium containing 2 mg l\(^{-1}\) 2,4-D to a medium containing 1 mg l\(^{-1}\) naphthaleneacetic acid (NAA) and 0.2 mg l\(^{-1}\) kinetin caused an increase in PAL activity. Dixon and Fuller (1976) found that the transfer of bean cells from a higher concentration of 2,4-D (2 \(\times\) 10\(^{-5}\) M) to a lower concentration of 2,4-D (2 \(\times\) 10\(^{-7}\) M) caused an increase in the specific activity of PAL. Benzyladenine (1 \(\mu\)M) caused an increase in PAL activity in cytokinin dependent tobacco callus (Kubol and Yamada, 1978). Bevan and Northcote (1979) have shown that NAA and kinetin stimulated PAL activity in bean suspension cultures. Recently Daines and Minocha (1983) have shown that ABA (0.02 mM) suppressed the appearance of PAL in germinating lettuce seeds. In addition, both benzyladenine and GA\(_3\) were also found to be inhibitory to the development of PAL in lettuce seeds.
**Phenylpropanoid compounds**

Various phenylpropanoid compounds have been shown to regulate PAL levels through feedback inhibition of the enzyme synthesis in gherkin hypocotyls (Engelsma, 1968; Johnson et al., 1975), Jerusalem artichoke (Durst, 1976), potato tuber slices (Lamb, 1979), and pea epicotyls (Shields et al., 1982). In pea epicotyls, exogenous supply of phenylpropanoid pathway intermediates like cinnamic acid, p-coumaric acid, ferulic acid and sinapic acid inhibited the initial development of PAL activity and if added at the time of high enzyme levels in the tissue, caused a rapid decrease in enzyme activity (Shields et al., 1982). Some of the endproducts of the phenylpropanoid pathway such as coumarins, flavonoids and chlorogenic acid have also been implicated in the regulation of PAL activity based on *in vitro* enzyme kinetics studies (Attridge et al., 1971; Iredale and Smith, 1974).

In an attempt to study the nature of regulation of PAL by cinnamic acid and other phenylpropanoid compounds, various competitive inhibitors of the enzyme e.g. substrate analogues, have been used *in vivo* to block the synthesis of these phenylpropanoids and examine their effect on PAL synthesis. Aminoxyacetic acid (AOA) and D and L-2-aminoxy-3-phenylpropionic acid (AOPP), two substrate analogues which are potent competitive inhibitors of the enzyme *in vitro* have been used *in vivo* to alter the phenylpropanoid metabolism. Of these, the effect of AOPP seems to be more specific than that of AOA. Noé and Seltz (1983) have recently shown that L-AOPP does not effect protein synthesis. Because of its specific effect on PAL, AOPP has become one
of the important tools in the physiological studies of the regulation of PAL and the metabolism of phenylpropanoids.

One of the most common effects of AOPP on many tissues is to cause a large increase in extractable PAL activity. This may be due to a decreased feedback inhibition of PAL synthesis because of the decreased levels of cinnamic acid and/or its derivatives (Duke et al., 1980). Both AOA and AOPP produced a superinduction of PAL in gherkin hypocotyls (Amhrein and Gerhardt, 1979). In some tissue, a 36-fold increase in PAL was observed in the presence of AOPP along with excision or wounding and light. In carrot cell suspension cultures, AOPP not only produced a superinduction of PAL activity (8 times higher than control), but also affected the time course of PAL appearance. In untreated cultures, maximum PAL activity was observed 24 h after inoculation. With AOPP (10^{-4} M) a second peak of PAL occurred after 96 h (Noé et al., 1980). Similarly, AOPP was found to prolong the phase of increase in PAL in potato tuber tissues (Lamb, 1982). In soybean seedlings, however, AOA and AOPP affected PAL activity differently. Whereas AOA linearly reduced extractable PAL activity in dark grown soybean seedlings (Hoagland and Duke, 1982), AOPP, on the other hand, increased PAL levels in this tissue (Duke et al., 1980). The extractable PAL activity in response to cold treatment in Sphagnum magellanicum was significantly increased in the presence of AOPP (Tutschek, 1982).

In contrast to above reports, Berlin and Vollmer (1979) found that AOPP did not affect extractable PAL activity in cell suspension cultures of tobacco. Furthermore, Havir (1981) reported that both AOA
and AOPP reduced the levels of extractable PAL from soybean cell suspension cultures. It was suggested that the apparent decrease in extractable PAL by AOPP could be due to its irreversible binding to PAL in the tissue leading to its inactivation.

Another competitive inhibitor of the enzyme which has been used to study the regulation of PAL is D-phenylalanine. In Jerusalem artichoke tuber tissue, D-phenylalanine caused a marked stimulation of extractable PAL activity when supplied in the culture medium (Durst, 1976). It was suggested that a decrease in the trans-cinnamate pool due to the competition of D-phenylalanine with L-phenylalanine for enzyme activity could have resulted in increased enzyme synthesis. Similar results have been reported in radish cotyledons (Huault and Klein-Eude, 1978) and potato tuber discs (Lamb, 1982). On the other hand, Walton (1968) did not observe any effect on PAL activity in excised bean axes in the presence of D-phenylalanine. The effect of p-fluorophenylalanine (another substrate analogue) on PAL activity has been investigated in excised bean axes (Walton, 1968) and oat coleoptiles (Hopkins and Orkwiszewski, 1971). In both cases an inhibition of PAL activity by this compound was observed.

Other Treatments

A wide variety of treatments such as DNA intercalating compounds, psoralen compounds, polyamines, herbicides etc., have been found to stimulate PAL activity in many tissues. In pea pods DNA intercalating agents like 9-aminoacridine, tacrine, DE-acridine, CDM-acridine, quinacrin, thionine, methylene blue and pyronine Y, have been reported to be inducers of PAL activity (Hess and Hadwiger, 1971; Hadwiger and
Schwochan, 1971). An analogue of thymidine, 5-Bromodeoxyuridine, was also found to induce PAL in pea pods (Sander and Hadwiger, 1979). Psoralen compounds like xanthotoxin induced PAL in pea pods in the presence of UV light (Hadwiger, 1972). The herbicide glyphosate was found to induce PAL in dark grown soybean seedlings (Hoagland et al., 1979). Polyamines like poly-L-lysine and poly-L-arginine (spermidine) have been found to induce PAL in pea pods (Hadwiger and Schwochan, 1970).

Transfer of callus tissues or cell suspension cultures to fresh media also resulted in the induction of PAL in parsley (Hahlbrock and Wellmann, 1973), dwarf French bean (Dixon et al., 1980) and pine (Lau et al., 1980). The composition of the medium also could affect PAL activity. In suspension cultures of tobacco PAL was induced following transfer to a phosphate free medium (Knobloch, 1982). The regulation of PAL by agents which cause water stress, eg. polyethylene glycol, has also been reported recently (Daines and Minocha, 1983).

Mechanism of PAL regulation

There are three principal ways in which an increase in extractable PAL activity observed in response to the above mentioned stimuli can be brought about; (1) activation or conversion of preexisting inactive enzyme protein, (2) stimulation of the rate of de novo enzyme synthesis, (3) decrease in the rate of enzyme degradation/inactivation. These processes are not mutually exclusive and, therefore, it is possible that the increases in PAL may be due to a combination of any two or all of the above effects.
Several attempts have been made to determine the specific mechanism for changes in PAL activity, especially in relation to the de novo enzyme synthesis. Many workers have employed inhibitors like actinomycin-D, 3'-deoxyadenosine (cordycepin), 7-methylguanosine triphosphate and cycloheximidide, which are believed to block transcription, mRNA transport, cap formation and ribosomal release in translation, respectively (Minamikawa and Uritani, 1965; Zucker, 1965; Engelsma, 1967; Scherf and Zenk, 1967; Walton and Sondheimer, 1968; Thorpe et al., 1971; Hahlbrock and Schroder, 1975; Dixon et al., 1980). Because of the lack of specificity of these transcription and translation inhibitors, the evidence for de novo synthesis of PAL based on these studies is not conclusive.

At present there are only three methods which can be reliably used to demonstrate the de novo synthesis of an enzyme; (1) radioactive labelling, (2) density labelling, and (3) immunology. Radioactive labelling has been used by Zucker (1969, 1970, 1971) to show that de novo synthesis of PAL occurs both in light and dark treated Xanthium leaf discs. Hahlbrock and Schroder (1975) have demonstrated light mediated increase in incorporation of $^{35}$S methionine into PAL purified from parsley suspension-culture cells. The density labelling technique with deuterium oxide ($D_2O$) has been used as evidence to support the view that de novo synthesis of enzyme occurs in response to light in mustard seedlings (Schopfer and Hock, 1971), potato tuber (Sacher et al., 1972), and several other tissues (Wellmann and Schopfer, 1975; Tong and Schopfer, 1976; Heinzmann and Seitz, 1977; Lamb et al., 1979). Density labelling technique was employed by
Duchesne et al., (1977) to show that de novo synthesis of PAL occurs in response to infection in tobacco leaves infected with tobacco mosaic virus. Immunological evidence for de novo synthesis of PAL in response to wounding has been reported in sweet potato (Tanaka and Uritani, 1977) and in pea pods in response to light and infection by Fusarium solani (Loschke et al., 1981).

There have been many efforts to demonstrate the existence of an inactive form of PAL which can be activated by light or other stimuli. Attridge and Smith (1973) have shown that PAL from light-grown pea seedlings was more unstable and more sensitive to inhibition by flavonoids like quercetin than PAL from dark grown seedlings. Since no isozymes for PAL exist in pea, the above differences were attributed to a light-mediated modification of a preexisting less active enzyme molecule. The cycloheximide-stimulated PAL increase in Cucumis seedlings (Attridge and Smith, 1973) was interpreted to arise from a pool of inactive PAL which was synthesized during or after imbibition (Iredale and Smith, 1973). In radish cotyledons, an inactive form of PAL was found to be synthesized in the dark which could be activated on exposure of the cotyledons to far red light (Klein-Eude et al., 1974). An inactive PAL protein was isolated from dark grown radish seedlings by Blondel et al., 1973. In gherkin, activation of preexisting PAL has been inferred from the inability of cycloheximide to inhibit an increase of PAL activity by cold treatment (Attridge and Smith, 1973).

In nearly all cases of light activation of PAL, the initial increase in enzyme activity is followed by a significant decline even
in the presence of the inducing stimulus such as continuous light. This loss of enzyme can be prevented by the application of cycloheximide at the point of maximum PAL activity (Engelsma, 1967; Zucker, 1968; Walton and Sondheimer, 1968; Klein-Eude et al., 1971). Engelsma (1970) suggested that the loss of activity may be due to the complexing of PAL with a proteinaceous inactivator. Engelsma and Van Bruggen (1971) reported a non-dialyzable substance which had leached from excised gherkin hypocotyls and which prevented the light-mediated increase in PAL when reapplied to hypocotyls. French and Smith (1975) have reported a similar non-dialyzable, heat-labile substance which reversibly inhibits PAL isolated from gherkin hypocotyls, whereas Creasy (1976) demonstrated the presence of a similar substance in sunflower leaf extracts which irreversibly inactivated PAL.

An alternative explanation for the decline in PAL activity based on proteolytic degradation of PAL was given by Zucker (1969) who noted the loss of radioactivity from labelled PAL during inactivation. Further studies in Xanthium revealed that synthesis of the PAL protein measured by the incorporation of labelled amino acids, occurred both in light during a net increase in activity and in darkness when the amount of activity was decreasing (Zucker, 1970, 1971). The influence of light, therefore, was suggested to be on the rate of inactivation rather than the rate of synthesis.

Creasy and Zucker (1974) suggested that induction of PAL by various stimuli may result in an increase in the rate of synthesis or activation of the enzyme which is followed by an increase in its inactivation system. A point is reached where synthesis is balanced
by inactivation and no net change in activity occurs until synthesis stops (removal of stimuli) and/or the rate of inactivation surpasses synthesis and a decline in total PAL activity is seen.

**Enzyme Purification and Kinetics**

PAL has been purified and characterized from a number of sources such as *Ustilago hordei* (Subba Rao et al., 1967), potato (Havir and Hanson, 1968), *Streptomyces verticillatus* (Emes and Vining, 1970), tobacco (O'Neal and Keller, 1970), mustard (Schopfer, 1971), *Rhodotorula glutinis* (Hodgins, 1971), *Sporobolomyces roseus* (Parkhurst and Hodgins, 1972), wheat (Nari et al., 1972), maize (Havir and Hanson, 1973), gherkin (Iredale and Smith, 1974), parsley (Zimmermann and Hahlbrock, 1975), sweet potato (Tanaka and Uritani, 1977), radish (Billet et al., 1978), spinach (Nizhizawa et al., 1979), mustard (Gupta and Acton, 1979), bean (Dudley and Northcote, 1979), pea (Loschke et al., 1981), soybean (Havir, 1981), and carrot (Noé and Seitz, 1982).

The enzyme is usually extracted in a borate or Tris buffer at a pH between 8 and 9. Various reducing agents such as mercaptoethanol and isoascorbate have been used in the extraction buffer. In addition either PVP or polyclar AT is generally added during extraction to reduce polyphenol oxidase activity. Fractionation of the crude extract with either cold acetone, acetic acid, heat or protamine sulphate has been used as one of the preliminary purification procedures in many schemes.

Ammonium sulphate precipitation has been used as an effective
procedure by several workers. It resulted in a 2-9 fold purification. In most instances, the enzyme precipitated between 30-60% saturation of ammonium sulphate. Sodium citrate has been used in some instances (Hodgins, 1971) in place of ammonium sulphate. The enzyme extract obtained from ammonium sulphate precipitation is either desalted on a column of Sephadex G-25 or dialysed.

Further steps in PAL purification have included ion exchange chromatography using DEAE cellulose or Sephadex, gel permeation on Sephadex G-200, agarose A-15 or sepharose 6B and hydroxylapatite chromatography (Havir and Hanson, 1968; Emes and Vining, 1970; Zimmermann and Hahlbrock, 1975; Tanaka and Uritani, 1977; Gupta and Acton, 1979; Loschke et al., 1981; Havir, 1981). Affinity chromatography using Sepharose 4B-L-Phe has been partially successful in the purification of PAL, yielding a 3-70 fold purification (Blondel et al., 1973; Tanaka and Uritani, 1977; Billet et al., 1978; Gupta and Acton, 1979). Billet et al., (1978) purified PAL from gherkin hypocotyls 60-70 fold using the affinity chromatography procedure of Blondel et al., (1973). The final stage of purification in most schemes includes techniques like preparative electrophoresis. The purity of the enzyme was confirmed by subjecting the purified sample to SDS gel electrophoresis.

Studies on Enzyme Kinetics

The enzyme has an approximate molecular weight of 300,000 daltons. The molecular weight ranges from 226,000 for Streptomyces verticillatus (Emes and Vining, 1970) to 330,000 as in potato (Havir
and Hanson, 1973), parsley (Zimmermann and Hahlbrock, 1975) and soybean (Havir, 1981). It has four identical subunits each of 83,000 daltons in potato and maize (Havir and Hanson, 1968, 1973) or of 55,000 or 60,000 each as in mustard (Gupta and Acton, 1979). But in wheat (Narla et al., 1972) the subunits are found to be dissimilar. There are two subunits of 75,000 daltons and two of 85,000 daltons each. Labelling experiments indicate that there are two active sites per tetramer.

The range of Km values at pH 8.7 for the enzyme from various sources is quite narrow (0.16-0.27 mM). The enzyme from microorganisms appears to show standard Michaelis Menten kinetics whereas with the enzyme from higher plants, negative cooperativity (Conway and Koshland, 1968) has been observed in several instances. For the potato enzyme (Havir and Hanson, 1968) the apparent Km at pH 8.7 increased from 0.038 to 0.26 mM and the apparent Vmax doubled as the substrate concentration increased. Similar results have been observed for PAL from maize (Havir et al., 1971; Havir and Hanson, 1973), pea seedlings (Attridge et al., 1971) and parsley cell suspension cultures (Zimmermann and Hahlbrock, 1975). The same effect is observed when L-tyrosine is a substrate for the maize and barley enzymes (Kindl, 1970; Havir et al., 1971).

The enzyme from soybean cell suspension culture exhibited negative cooperativity before hydroxylapatite chromatography during purification and positive cooperativity following this step (Havir, 1981).
This is the first example of positive cooperativity observed for phenylalanine ammonia-lyase.

The enzyme from *Streptomyces verticillus* could not utilize D-phenylalanine as a substrate (Emes and Vining, 1970), whereas PAL from yeast (Hodgins, 1971) was capable of using this analogue as a substrate when high concentrations of the enzyme were used. The Vmax was much lower than that for the L-isomer. Similar results were obtained for potato PAL (Havir and Hanson, 1968). D-phenylalanine was not a good substrate for PAL from sweet potato (Tanaka and Uritani, 1977), and tobacco (O'Neal and Keller, 1970).

The ability of PAL to deaminate other aromatic amino acids has been studied. Tyrosine ammonia-lyase activity of PAL from many sources, especially from grasses, has been reported by several workers (Nelsh, 1961; Young et al., 1966; Havir et al., 1971; Parkhurst and Hodgins, 1971; Ruis and Kindl, 1971; Jangaard et al., 1974). Koukol and Conn (1961) showed that PAL from barley seedlings could deaminate L-tyrosine and DL-m-tyrosine besides L-phenylalanine. But the enzyme showed no activity towards L-histidine, L-alanine, DL-aspartic acid, DL-leucine, glycine, L-cysteine and L-tryptophan.

Various analogues of phenylalanine besides D-phenylalanine have been tested as substrates for PAL. In *Avena* DL-m, DL-o and DL-p-fluorophenylalanine served as substrates for the enzyme. But there was a marked difference in the behaviour of the meta from that of the para and ortho species. The meta form was a better substrate (Hopkins and Orkiewszewski, 1971; Orkiewszewski et al., 1976). Walton (1968)
reported that in excised bean axes, p-fluorophenylalanine was
deaminated at about one half the rate of L-phenylalanine.

PAL from wheat (Young and Nelsh, 1966) showed activity towards 3-
hydroxyphenylalanine and 3,4-dihydroxyphenylalanine. It was reported
that substitution at the ortho-position on the benzene ring produced
inactive substrates whereas the meta substituted compounds were quite
active. Thus 2-hydroxyphenylalanine was not deaminated whereas 3-
hydroxyphenylalanine was more readily deaminated than tyrosine.

Various substrate analogues have been used in *in vitro* studies
to see the nature of inhibition of PAL if any by these compounds. Of
these analogues, D-phenylalanine has been found to inhibit the enzyme
from potato (Havir and Hanson, 1968), bean (Walton, 1968), yeast
(Hodgins, 1971), gherkin (Iredale and Smith, 1974) and tobacco (O'Neal
and Keller, 1970). No such inhibition was observed in the enzyme from
Streptomyces (Emes and Vining, 1970) and sweet potato (Tanaka and
Urutan, 1977). The inhibition was competitive at least in potato
(Havir and Hanson, 1968) and yeast (Hodgins, 1971).

The enzyme from bean (Walton, 1968), barley (Hopkins and
Orkwiszewski, 1971), *Streptomyces* (Emes and Vining, 1970) and wheat
(Young and Nelsh, 1966) is inhibited by p-fluorophenylalanine. The
inhibition has been shown to be competitive in barley (Hopkins and
Orkwiszewski, 1971) and sweet potato (Minamikawa and Tanaka, 1965).
Other substrate analogues like β-phenyllactic acid inhibit the enzyme
from *Rhodotorula* and *Sporobolomyces* (Parkhurst and Hodgins, 1972).
The enzyme from tobacco was inhibited by phenylbutyric acid (O'Neal
and Keller, 1970) and that from yeast by phenylpropiolic acid (Hodgins, 1971).

Much more significant inhibition was observed with two other substrate analogues, viz. 2-aminoxyacetic acid (AOA) and D and L-2-aminoxy-3-phenylpropionic acid (D-and L-AOPP) (Amrhein et al., 1976). Both compounds act as potent competitive inhibitors of the enzyme in vitro in the micromolar and nanomolar range respectively. Inhibition was usually reversible but L-AOPP was found to irreversibly inactivate PAL from soybean (Havir, 1981). These compounds have proved to be important tools in studying the phenylpropanoid metabolism and the role of PAL in this metabolism (Amrhein and Godeke, 1977; Amrhein and Hollander, 1979; Tutschek, 1982; Lamb, 1982).

Other aromatic amino acids besides phenylalanine have also been used in in vitro studies on enzyme kinetics. Tyrosine was found to inhibit the enzyme activity from Sporobolomyces (Parkhurst and Hodgins, 1972), gherkin (Iredale and Smith, 1974), wheat (Young and Neish, 1966), tobacco (O'Neal and Keller, 1970) and barley (Koukol and Conn, 1961). Tryptophan inhibited the enzyme activity from tobacco (Innerarity et al., 1972), whereas, histidine inhibited the enzyme from Streptomyces (Emes and Vining, 1970).

The effect of various metabolites of the shikimic acid pathway and the phenylpropanoid pathway have also been tested for their effect on in vitro deamination of L-phenylalanine. Of these compounds, cinnamic acid was found to be a competitive inhibitor of the enzyme from potato (Havir and Hanson, 1968), barley (Koukol and Conn, 1961) sweet potato (Minamikawa and Tanaka, 1965), bean (Walton, 1968),
tobacco (O'Neal and Keller, 1970), oak (Boudet et al., 1971), yeast (Parkhurst and Hodgins, 1972), *Streptomyces* (Emes and Vining, 1970) and gherkin (Iredale and Smith, 1974). P-coumaric acid was an inhibitor of the enzyme showing tyrosine ammonia-lyase activity. O-coumaric acid competitively inhibits the enzyme from gherkin (Iredale and Smith, 1974). Phenolic acids such as caffeic acid, ferulic acid etc. are inhibitors of the enzyme from tobacco and gherkin. Other phenolic inhibitors of PAL from various sources include coumarin, quercetin, kaempferol, catechol, benzoic acid and gallic acid (Iredale and Smith, 1974; Walton, 1968; Parkhurst and Hodgins, 1972). Most of these compounds showed mixed inhibition, suggesting that they may be acting as allosteric modulators (Iredale and Smith, 1974). No inhibition was observed with shikimic acid, salicylic acid, chlorogenic acid, quinic acid and vanillic acid for enzyme from gherkin seedlings (Iredale and Smith, 1974).

The present studies were undertaken as a continuation of the work done on phenylalanine ammonia-lyase in germinating lettuce seeds in this laboratory (Daines, 1981, Daines and Minocha, 1983, Daines et al., 1983). It has been reported that PAL in lettuce seeds was induced only when germination is occurring. The enzyme could be detected in these seeds within 4 h of inhibition under white light. The specific activity of PAL increased rapidly during the next 12-16 h of inhibition. Far-red light completely suppressed germination as well as the development of PAL. Gibberellic acid (GA$_3$) and benzyladenine (0.1 mM) retarded radicle elongation and produced a concomittant decrease in PAL activity. Abscisic acid (ABA) and polyethylene glycol
(PEG-35%) completely inhibited germination of the seed under white light and caused a parallel inhibition of PAL activity. Effects of ABA were not reversed by GA$_3$ and only partially reversed by BA both for germination as well as for PAL. Transfer of germinated seeds from white light to far-red, or to ABA or to PEG resulted in the loss of enzyme activity. The loss of PAL caused by PEG was readily reversible on removal of the osmotica. The following conclusions were drawn from these studies: (1) PAL activity in lettuce seeds is induced only when germination is occurring. (2) PAL activity per seed is strongly related to the extent of growth of the radicle and (3) PAL activity is inhibited by any physical or chemical treatment that inhibits radicle growth.

The main objectives of the present research were (i) to study the kinetic properties of the L-phenylalanine ammonia-lyase from germinated lettuce seeds, (ii) to study the regulation of the enzyme activity by various substrate analogues, intermediates and endproducts of the phenylpropanoid pathway and (iii) to purify the enzyme using various techniques of protein purification.
MATERIALS AND METHODS

Source of Chemicals

$^{14}$C(U)-Phenylalanine was obtained from New England Nuclear, Boston, Mass. U.S.A. Chemicals used for inhibition studies like L-phenylalanine, D-phenylalanine, DL-$\beta$-phenyllactic acid, p-fluorophenylalanine, tryptophan, tyrosine, trans-cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, coumarin, quercetin etc., and other chemicals such as polyvinylpolypyrrolidone and ascorbic acid were obtained from Sigma Chemicals Co., St. Louis, Mo., U.S.A. 2-aminoxy-3-phenylpropionic acid (L-AOPP) was a gift from Dr. N. Amrhein, Bochum University, W. Germany. The protein assay dye, was obtained from Bio Rad Laboratories, Richmond, CA.

Plant Material

Lettuce seeds (achenes of Lactuca sativa L. cv Grand Rapids) supplied by the Ferry Morse Seed Company (Mt. View, CA) were used throughout this investigation. The dry seeds were stored in a refrigerator at 4°C.

Growth Conditions

Batches of approximately 200 seeds (measured by volume) were incubated in 125 ml Erlenmeyer flasks with 50 ml of distilled de-ionised water or an appropriate treatment solution. The flasks were placed on a gyratory shaker (125 rpm) under fluorescent light (120 $\mu$ E s$^{-1}$ m$^{-2}$) and maintained at 25± 2°C during incubation.
Enzyme Extraction

After appropriate periods of incubation, the seeds were collected on filter papers, blotted dry and homogenized in a mortar and pestle with 5 ml of cold extraction buffer (0.1M borate + 0.5% ascorbate, pH 8.8). The grinding mixture also contained 100 mg of glass beads and 100 mg of insoluble PVP (polyvinylpolypyrrolidone). The homogenate was centrifuged at 20,000 g at 4°C for 20 minutes. In most cases the resulting supernatant was directly used for enzyme assay.

Phenylalanine ammonia-lyase (PAL) activity was assayed by the method described by Minocha and Halperin (1976) (Fig. 1). Enzyme activity was determined by measuring the rate of formation of 14C-cinnamic acid from L-14C(U) phenylalanine. The reaction mixture contained in a total volume of 2 ml, 0.3 ml borate buffer (0.1M borate, pH 8.8), 0.2 ml of 600 µM L-Phe, 0.06 µCi 14C-Phe (0.5 ml) (sp.act. 450 mCi/mM) and 1 ml of the enzyme extract. The reaction mixture was incubated at 38°C for one hour. The reaction was stopped by adding 0.2 ml of 8N HCl. The reaction product 14C cinnamic acid was separated from the remaining radioactive Phe by partitioning with 5 ml toluene. Two ml of the toluene layer was removed and mixed with 8 ml of a toluene based scintillation fluid (OCS-Amersham). Radioactivity was counted on a Beckman LS 7000 liquid scintillation counter at 98% efficiency. One unit of PAL activity is defined as 1 nmol of cinnamic acid formed per hour.
Fig. 1. Flow diagram. PAL extraction and assay.
200 SEEDS
GRIND (0.05 M BORATE + 0.5% ASCORBATE, pH 8.8 + PVP) + GLASS BEADS

EXTRACT
CENTRIFUGE (20,000 x g, 20 minutes)

PELLET SUPERNATANT
(ASSAY)

ASSAY MIXTURE:

$^{14}$C-Phe - 0.06 μCi - 0.5 ml
Phe - 60 μM - 0.2 ml
BORATE BUFFER - 0.1 M - 0.3 ml
ENZYME EXTRACT - 1 ml
(TOTAL VOLUME - 2 ml)

INCUBATE 60 min - 38°C

ADD 0.2 ml, 8 N HCl

ADD 5 ml, TOluene

MIX, CENTRIFUGE

REMOVE 2 ml FROM TOluene PHASE

ADD 8 ml SCINTILLATION FLUID

COUNT FOR $^{14}$C.
Protein Assay

The concentration of protein in the extract was determined by the Bradford assay using Bio Rad dye reagent (Bradford 1976). A standard curve for the protein assay was prepared using dilutions of a protein standard (bovine serum albumin) containing from 0.2 to about 1.4 mg/ml. The unknowns were read from this standard curve. The assay was performed as follows.

1 ml of the appropriately diluted (x10 or x100) enzyme extract was placed in dry test tubes (2 replicates). Only sample buffer was used in blank test tube. To each tube 3 ml of the diluted protein assay dye (1 part of dye in 4 parts of water) was added. The tubes were vortexed gently. After a period of 10 minutes absorbance was measured at 595 nm using a Bausch and Lomb Spectronic 21 spectrophotometer.

Kinetics of Enzyme Activity

1. Determination of Km.

Ammonium sulfate precipitated (see details below) and desalted or dialysed enzyme extract was used to determine the Km. The enzyme activity was determined over a wide range of substrate concentrations (15-600 μM Phe) and a saturation curve was prepared. The Km was determined from the double reciprocal plot according to Lineweaver and Burk (1934).

2. Inhibition Studies

The effects of various substrate analogues, intermediates and endproducts of the phenylpropanoid pathway on PAL activity in vitro were determined using partially purified enzyme obtained by
ammonium sulfate precipitation (60% saturation) of the crude extract. Two different concentrations of each compound (viz 100 and 500 μM) were used with five different substrate concentrations: 60.6, 90.9, 121.2, 151.5 and 181.8 μM.

The substrate analogues used included D-phenylalanine, DL-p-fluorophenylalanine, β-phenyllactic acid, tyrosine and tryptophan. Phenylpropanoid compounds such as trans-cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, coumarin, quercetin, etc., were also used. The nature of PAL inhibition, if any by these compounds was determined by double reciprocal plots according to Lineweaver and Burk (1934). The plotting was done using a combination of two computer programs (Minitab and U-Plot).

**Regulation of PAL Activity**

Batches of approximately 200 seeds (2 replicates) were incubated in 125 ml Erlenmeyer flasks containing 50 ml of either water or the treatment solution. Three different concentrations of each compound (viz 50, 100, and 500 μM) were used. The conditions of incubation were as described earlier. The supernatant from 20,000 g centrifugation of the crude extract was dialyzed against the extraction buffer for 24 hours before performing the enzyme assay. For measurement of radicle length 10 germinated seeds were randomly selected from each flask and the lengths of the radicles measured under a dissecting microscope to the nearest mm. Each experiment was repeated at least two times.

In some experiments (see details later) the crude extract was
precipitated with ammonium sulphate and the precipitate resuspended in the extraction buffer and dialysed against the same buffer for 24 hours before use for the enzyme assay.

**Transfer Experiments**

One hundred seeds each were incubated in water for 12 hours and then transferred to either water (control) or the appropriate treatment solutions. The enzyme was extracted at various times after transfer as described later. Enzyme activity was assayed after dialysis of the crude extract overnight. The lengths of the radicles were also measured at each of these times.

**Ethylene Production**

Batches of 100 seeds each were incubated in water or appropriate treatment solution in 125 ml flasks sealed with one-holed rubber stoppers through which three inch long glass tubes were inserted. These tubes were closed tightly by rubber caps. At appropriate times, 2 ml gas samples were withdrawn from each flask using a 5 ml hypodermic syringe and injected into the gas chromatograph. Quantities of ethylene were measured in these gas samples using a Perkin Elmer (model 990) gas chromatograph equipped with 1.22m x 3.2mm stainless steel columns of 80/100 mesh Poropak N (Supelco Inc., Bellefonte, Pennsylvania) and flame ionization detectors. The carrier was pre-purified nitrogen at a flow rate of 40 ml/min. Detection gases were hydrocarbon free air and ultra high purity hydrogen. (Airco Inc.). Operating temperatures for the injection block and manifold were 50° and 120°C, respectively. The column oven was programmed for one
minute at 50°C increasing to 120°C at 24°C/min and remaining at 120°C for 4 minutes. Signals were processed by a CR1-B Chromatopac (Shimadzu Inc., Columbia, MD). Maximum sensitivity was to 5 pl of ethylene. Retention time for ethylene in this system was 49 ± 6 sec. An external standard of 1 ml/l ethylene in nitrogen was obtained from Matheson Gas Products Inc., East Rutherford, NJ.

Localisation of PAL in Seed Parts

One hundred seeds incubated in water for 24 hours were dissected into 4 parts each. (1) seed coat, (2) endosperm, (3) cotyledons and plumule and (4) radicle. The seed parts were ground separately and the enzyme activity was assayed in each case. The protein concentration was also determined in each part.

Purification of PAL

a. Extraction of the enzyme

Radicles of 40 h old germinated lettuce seeds were used as the source of the enzyme. About 15,000 seeds of lettuce (15 gms) were germinated on a plastic screen placed on a plastic tray under light so that all the radicles would grow through the screen. After germination, the radicles were excised with a razor blade. Approximately 25 gms of radicle obtained this way were ground with a mortar and pestle with 60 ml borate ascorbate buffer (pH 8.8) containing 2 mM dithiothreitol (DTT) along with 8 gms of glass beads and 8 gms of PVP. The enzyme extract was centrifuged at 20,000 g for 20 mins. The supernatant was saved and the pellet was re-extracted with the same buffer (30 mls) and centrifuged again. The supernatants from the two
extractions were pooled and filtered through cheesecloth. The concentration of the protein and the enzyme activity were determined in the extract which was used for further purification.

**Ammonium Sulfate Precipitation**

The crude extract was subjected to ammonium sulfate precipitation. Sufficient quantities of ammonium sulfate were added slowly to the extract to obtain 30% saturation. The solution was stirred at low speed during the addition of ammonium sulfate. The extract was kept undisturbed for an hour at 4°C and then it was centrifuged at 25,000 g for 30 min. The precipitate was discarded. To the supernatant more ammonium sulfate was added with constant stirring to achieve 55% saturation. The precipitation was allowed to continue for an hour at 4°C, after which the extract was centrifuged at 20,000 g for 30 min. This time the precipitate was saved and dissolved in 2.5 ml of 0.1 M borate buffer, pH 8.8.

**Desalting**

The enzyme extract was desalted on a column of Sephadex G-50. For the preparation of the column, about 20 g of Sephadex G-50 (Sigma Chemical Co., St. Louis, Mo. U.S.A.) were added to 500 ml of 0.1 M borate buffer, pH 8.8, while gently stirring the buffer. The suspension was allowed to stand overnight. About 400 ml of the supernatant buffer was decanted and the remaining slurry was poured gently into a column of 52 cm x 3 cm. The column was allowed to pack to a bed height of 36 cm. About 100-150 ml of the borate buffer containing 2 mM DTT was allowed to flow through the column to stabilize the absorbance
of the eluent. When the column had drained and the buffer just reached the bed surface, the enzyme solution (2.5 ml) was slowly layered on the column and allowed to move into the Sephadex. The column was eluted with the same buffer at a flow rate of 3 ml/min and the eluent monitored constantly for absorbance at 280 nm using an ISCO (Lincoln, Nebraska) UA5 absorbance monitor. Fractions of 8 ml each were collected using a Gilson Micro Fraction Collector. The fractions were analysed for total protein and for enzyme activity. Fractions showing high enzyme activity were pooled together and used for further purification.

Concentration

The desalted enzyme sample was transferred to a dialysis tubing (1 x 20 cm) and covered with crystalline sucrose for 4 h. When the sucrose became wet, it was replaced by fresh sample. Alternately, polyethylene glycol or Sephadex G-200 were also used, but sucrose proved to be the most effective material. The concentrated enzyme was dialysed against 0.1 M borate containing 2mM DTT overnight and then used for further purification.

Gel Filtration (Sephadex G-200)

The dialysed sample was subjected to gel filtration on a Sephadex G-200 column. (Sigma Chemical Co., St. Louis, Mo. U.S.A.). About 10 gms of Sephadex G-200 were added to 500 ml of 0.1 M borate buffer, pH 8.8, while gently stirring the buffer. The suspension was allowed to stand overnight. About 300 ml of the supernatant buffer was decanted and the remaining slurry was poured gently into a column of 55 x 3 cm.
The column was allowed to pack to a bed height of 42 cm. About 150 ml of the borate buffer (0.1M) containing 2mM DTT was allowed to flow through the column to stabilize its absorbance. The enzyme solution (4.5 ml) was slowly layered on the column bed and allowed to move into the sephadex. The column was then eluted with the same buffer at a flow rate of 0.5 ml/min. The absorbance at 280 nm was monitored as before. Fractions of 8 ml each were collected and analysed for protein. Those with protein were assayed further for PAL activity. Fractions showing high enzyme activity were pooled together, concentrated with sucrose and used for further purification.

Ion-Exchange Chromatography

The concentrated sample from Sephadex G-200 was dialysed against 0.01 M borate buffer and was subjected to ion-exchange chromatography on a DEAE cellulose column. Twenty gms of preswollen DEAE cellulose (DE.52, Whatman Inc., Clifton, NJ) was stirred with 0.2M borate buffer, pH 8.8, for 5 min. The slurry was allowed to stand and the supernatant was decanted. The ion exchanger was redispersed in more buffer and decantation repeated. The remaining slurry was used to pack a column of 10 x 3 cm. The column was equilibrated with approximately 600 ml of 0.01 M borate buffer (pH 8.8) containing 2mM DTT. The enzyme sample (17ml) was slowly layered on the column. The column was washed with about 100 ml of 0.01 M borate buffer at a flow rate of 2 ml/min. The column was then eluted with a step wise gradient of sodium chloride in 0.2M borate buffer containing 2mM DTT and the eluent monitored constantly at 280 nm. Sodium chloride concentrations used were 0.05, 0.3 and 0.5 M. Fractions of 8ml each were collected.
The fractions were analysed for protein and those which contained protein were analysed for PAL activity. The fractions showing enzyme activity were pooled and concentrated on sucrose and used for further purification.

**Hydroxylapatite chromatography**

The concentrated enzyme from DEAE cellulose chromatography was dialysed against 0.01 potassium phosphate (monobasic) buffer pH 8.8 containing 2mM DTT and then subjected to hydroxylapatite chromatography on a Bio Gel HTP column. Five gms of Bio Gel HTP (Bio Rad Laboratories, Richmond, CA) were stirred with 0.01M potassium phosphate (monobasic), pH 8.8 for 5 min and then allowed to settle. The supernatant containing the fine particles was decanted. The hydroxylapatite was redispersed in more of the buffer and decanted again. The remaining slurry was used to pack a column of 4 x 3 cm. The column was washed with 200 ml of 0.01M potassium phosphate buffer at a flow rate of 1.5 ml/min. The enzyme sample (15 ml) was layered on the column and more of the starting buffer was passed through the column. Then the column was eluted with a step wise gradient of potassium phosphate (0.03, 0.05, 0.1, 0.2 M) and eluent monitored constantly at 280 nm. Fractions of 8 ml each were collected. They were analyzed for protein and PAL activity. Those with PAL activity were pooled and concentrated on sucrose.
Fig. 2. Saturation curve (A) and Lineweaver Burk plot (B) for PAL from germinating lettuce seeds at various concentrations of phenylalanine. An ammonium sulfate precipitated sample of PAL was used in this experiment. Each point represents an average of 2 replicates.
1. **Effect of substrate analogues**

The effect of various analogues of phenylalanine such as D-phenylalanine, p-fluorophenylalanine, β-phenyllactic acid, tyrosine and tryptophan on PAL activity *in vitro* was determined. The combined results are shown in Table 1 and figs. 3-5. All of these compounds, except tyrosine, were found to inhibit PAL activity. The strongest inhibition was observed in the presence of p-fluorophenylalanine (92% at 500 μM) and the lowest with β-phenyllactic acid (27% at 500 M). Lineweaver-Burk plots (Double reciprocal plots) of substrate concentration vs reaction velocity in the presence of the inhibitor were made using the computer programs mentioned earlier. The nature of inhibition of PAL by these substrate analogues was determined by seeing their effects on Km and Vmax of this enzyme. D-Phenylalanine, p-fluorophenylalanine, β-phenyllactic acid and tryptophan were found to be competitive inhibitors of PAL as judged from the straight lines in reciprocal plots intersecting at a point on the Y axis (Figs. 3, 4, and 5).

2. **Effect of phenylpropanoid compounds**

The effect of various intermediates and endproducts of the phenylpropanoid pathway on the deamination of phenylalanine *in vitro* by PAL was determined. Once again two concentrations of each compound were used in the presence of various concentrations of phenylalanine. The results shown in Table 2 and Fig. 6 show that the product of the enzyme reaction, trans-cinnamic acid was a strong competitive inhibitor of this enzyme (74% inhibition at 500 μM). The inhibition was competitive as judged by the Lineweaver Burk plot. Other
Table 1. Nature and percentage of inhibition of PAL in germinating lettuce seeds *in vitro* by various substrate analogues. Each compound was used at 500 μM concentration.

<table>
<thead>
<tr>
<th>Substrate Analogues</th>
<th>Nature of Inhibition</th>
<th>Percentage of Inhibition (at 500 μM Conc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-phenylalanine</td>
<td>Competitive</td>
<td>66</td>
</tr>
<tr>
<td>P-fluorophenylalanine</td>
<td>Competitive</td>
<td>92</td>
</tr>
<tr>
<td>Phenyllactic Acid</td>
<td>Competitive</td>
<td>27</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Competitive</td>
<td>47</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>--</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 3. Lineweaver Burk plots of the data on the inhibition of PAL by D-phenylalanine (▲) 100μM and (■) 500μM. (●) denotes activity in the control at various substrate concentrations. An ammonium sulfate precipitated sample of PAL was used.
Fig. 4. Lineweaver Burk plots of the data on the inhibition of PAL by p-fluorophenylalanine, (▲) 100μM and (■) 500μM. (●) denotes activity in the control at various substrate concentrations. An ammonium sulfate precipitated sample of PAL was used in this experiment.
Fig. 5. Lineweaver Burk plots of the data on the inhibition of PAL by L-tryptophan (▲) 100μM and (■) 500μM. (●) denotes activity in the control at various substrate concentrations. An ammonium sulfate precipitated sample of PAL was used in this experiment.
Table 2. Nature and percentage of inhibition of PAL in germinating lettuce seeds *in vitro* by various phenylpropanoid compounds. Each compound was used at 500 μM concentration.

<table>
<thead>
<tr>
<th>Phenylpropanoid Compounds</th>
<th>Nature of Inhibition</th>
<th>Percentage of Inhibition (at 500 μM Conc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans-cinnamic Acid</td>
<td>Competitive</td>
<td>74.2</td>
</tr>
<tr>
<td>P-coumaric Acid</td>
<td>---</td>
<td>0</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>---</td>
<td>0</td>
</tr>
<tr>
<td>Ferulic Acid</td>
<td>---</td>
<td>0</td>
</tr>
<tr>
<td>Coumarin</td>
<td>---</td>
<td>0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>---</td>
<td>0</td>
</tr>
<tr>
<td>Chlorogenic Acid</td>
<td>Mixed</td>
<td>61.7</td>
</tr>
</tbody>
</table>
Fig. 6. Lineweaver Burk plots of the data on the inhibition of PAL by trans-cinnamic acid (▲) 100μM and (■) 500μM. (●) denotes activity in the control of various substrate concentrations. An ammonium sulfate precipitated sample of PAL was used for these experiments.
Fig. 7. Lineweaver Burk plots of the data on the inhibition of PAL by chlorogenic acid (▲) 100µM and (■) 500µM. (●) denotes activity in the control at various substrate concentrations. An ammonium sulfate precipitated sample of PAL was used in this experiment.
Intermediates of the phenylpropanoid pathway like p-coumaric acid, caffeic acid and ferulic did not show any inhibition of PAL activity \textit{in vitro} (Table 2). Of the endproducts of the phenylpropanoid pathway used in these studies, only chlorogenic acid showed strong inhibition of the enzyme activity (61% inhibition at 500 \textmu M). The inhibition was found to be mixed as is evident from the Lineweaver Burk plots (Fig. 7). Both the \( K_m \) and the \( V_{max} \) were changed in the presence of this inhibitor. Two other endproducts of the pathway, coumarin and quercetin, did not show any effect on PAL activity.

\textbf{Regulation of PAL in Relation to Growth}

The regulation of the levels of PAL in germinating lettuce seeds was studied in the presence of various substrate analogues and some intermediates and endproducts of the phenylpropanoid pathway. Lettuce seeds were incubated for 24 h in three different concentrations of each compound (viz. 50, 100 and 500 \textmu M) under light. The supernatant obtained after centrifugation of the crude extract was dialysed and used for enzyme assay. For growth analysis radicles from ten randomly selected germinated seeds from each treatment were measured.

1. \textbf{Effect of Substrate Analogues}

Results on the effect of D and L-phenylalanine, p-fluorophenylalanine, \( \beta \)-phenyllactic acid, L-tyrosine and L-tryptophan on PAL activity and the growth of embryonic axes in 24 h old germinated lettuce seeds are shown in table 3. The substrate L-phenylalanine and its D-isomer did not have a significant effect on PAL activity nor did they
Table 3. Effect of substrate and various substrate analogues on radicle elongation and PAL activity in germinating lettuce seeds. Observations were taken after 24 h of incubation in the presence of various compounds. The radicle length in control seeds was 5.2 ± 0.27 mm and PAL activity per seed was 1.35 ± 0.09 units.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (µM)</th>
<th>% Germ.</th>
<th>Radicle lgth. (% control)</th>
<th>PAL activity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-phenylalanine</td>
<td>50</td>
<td>100</td>
<td>88</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>81**</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>100</td>
<td>77**</td>
<td>87**</td>
</tr>
<tr>
<td>D-phenylalanine</td>
<td>50</td>
<td>100</td>
<td>120**</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>125**</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>100</td>
<td>131**</td>
<td>113*</td>
</tr>
<tr>
<td>DL-p fluorophenylalanine</td>
<td>50</td>
<td>100</td>
<td>91</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>66**</td>
<td>60**</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>2**</td>
</tr>
<tr>
<td>α-phenyllactic acid</td>
<td>50</td>
<td>100</td>
<td>109</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>111*</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>100</td>
<td>116**</td>
<td>103</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>50</td>
<td>100</td>
<td>117**</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>137**</td>
<td>110**</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>100</td>
<td>158**</td>
<td>118**</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>50</td>
<td>100</td>
<td>43**</td>
<td>71**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>50</td>
<td>18**</td>
<td>28**</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>0.6***</td>
</tr>
</tbody>
</table>

1 unit of enzyme activity = nmol cinnamic acid formed per hour.
* significant at 0.05 level.
** significant at 0.025 level.
*** significant at 0.01 level.
affect the elongation of radicle at low concentrations (50 and 100 
μM), but at higher concentration (500 μM), a slight inhibition of PAL 
activity was observed. A corresponding decrease in radicle growth was 
also seen in the presence of L-phenylalanine. D-phenylalanine, on the 
other hand, caused a slight stimulation of both PAL activity as well 
as radicle growth at 500 μM concentration. Another substrate analogue, 
p-fluorophenylalanine was found to be a strong inhibitor of 
extractable PAL activity in lettuce seeds (98% inhibition at 500 μM). 
Germination was also inhibited at that concentration. At lower con­
centrations, where germination was not inhibited, the elongation of 
the radicle was inhibited, inhibition being concentration dependent. 
Phenylalanine ammonia-lyase activity per seed was significantly lower 
in the presence of 50 and 100 μM p-fluorophenylalanine. Tyrosine, 
another substrate analogue, and a substrate for PAL from some sources, 
and tryptophan, a related aromatic amino acid, showed different ef­
facts. The former did not affect the development of PAL activity in 
lettuce seeds when used at lower concentrations. At high concentra­
(500 μM) there was a significant promotion of radicle elongation and 
also that of PAL activity. On the other hand, tryptophan, strongly 
inhibited PAL activity as well as radicle elongation in germinated 
seeds. There was a complete suppression of germination as well as PAL 
activity at 500 μM tryptophan. Lower concentrations of tryptophan 
only partially inhibited PAL activity.

2. Effect of Intermediates of the Phenylpropanoid Pathway

Table 4 shows the combined data on the effect of various interme­
diates of the phenylpropanoid pathway, like trans-cinnamic acid, p-
Table 4. Effect of various intermediates of the phenylpropanoid pathway on radicle elongation and PAL activity in germinating lettuce seeds. Observations were taken after 24 h of incubation in the presence of various compounds. The radicle length in control seeds was 5.8 ± 0.25 mm and PAL activity per seed was 1.43 ± 0.02 units.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (μM)</th>
<th>% Germ.</th>
<th>Radicle lgth. (% control)</th>
<th>PAL activity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans-cinnamic acid</td>
<td>50</td>
<td>100</td>
<td>79***</td>
<td>64***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>45***</td>
<td>44***</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>7***</td>
</tr>
<tr>
<td>P-coumaric acid</td>
<td>50</td>
<td>100</td>
<td>59***</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>38***</td>
<td>77**</td>
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<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>38***</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>50</td>
<td>100</td>
<td>82**</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>60***</td>
<td>84***</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>50</td>
<td>20***</td>
<td>20***</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>50</td>
<td>100</td>
<td>85***</td>
<td>87***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>61***</td>
<td>84***</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>40</td>
<td>33***</td>
<td>20***</td>
</tr>
</tbody>
</table>

1Unit of enzyme activity = nmol cinnamic acid formed per hour.

* significant at 0.05 level.
** significant at 0.025 level.
*** significant at 0.01 level.
coumaric acid, caffeic acid, and ferulic acid on PAL activity and radicle growth in lettuce seeds germinated for 24 h. Seeds failed to germinate in the presence of cinnamic acid at a concentration of 500 µM. But at lower concentrations (50 and 100 µM), where germination was not affected, there was a concentration dependent inhibition of extractable PAL activity per seed. Radicle elongation was also significantly inhibited at low concentrations of cinnamic acid. On the other hand, the other intermediates of the phenylpropanoid pathway did not inhibit PAL activity to the same extent as cinnamic acid at comparable concentrations. Nevertheless, all these compounds were strong inhibitors of PAL activity as well as radicle elongation. At a concentration of 100 µM the order of potency of inhibition of PAL was cinnamic acid > p-coumaric acid > ferulic acid > caffeic acid.

Cinnamic acid caused an inhibition of PAL/seed even if supplied after the seeds had already germinated. When seeds incubated in water for 12 h were transferred to a solution of 10 or 100 µM cinnamic acid and PAL activity measured for the next 24 h, a rapid decline in PAL per seed was observed. The growth of the radicle was also retarded by cinnamic acid (fig. 8).

3. Effect of the Endproducts of the Phenylpropanoid Pathway

The endproducts of the phenylpropanoid pathway used in this study, such as coumarin, quercetin and chlorogenic acid, were found to be strong inhibitors of the enzyme (Table 5). Coumarin inhibited extractable PAL per seed by 98%, quercetin by 99%, and chlorogenic acid by 98% at 500 µM. Germination of seeds was also inhibited by these compounds at this concentration. At lower concentrations, where
Fig. 8. Effect of cinnamic acid on the time course of PAL activity. The seeds were germinated in water for 12 h under white light and then transferred to cinnamic acid at (▲) $10^{-5}$ and (■) $10^{-4}$ M. (●) represents activity in control seeds. Each point represents an average of four replicates.
Table 5. Effect of various endproducts of the phenylpropanoid pathway on radicle elongation and PAL activity in germinating lettuce seeds. Observations were taken after 24 h of incubation in the presence of various compounds. The mean radicle length for controls was 5.6±0.23 mm and PAL activity per seed was 1.40±0.09 units¹.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (µM)</th>
<th>% Germ.</th>
<th>Radicle Lgth. (% control)</th>
<th>PAL activity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td>50</td>
<td>80</td>
<td>28***</td>
<td>84***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10</td>
<td>23***</td>
<td>34***</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>2***</td>
</tr>
<tr>
<td>Quercetin</td>
<td>50</td>
<td>100</td>
<td>60***</td>
<td>77***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>50</td>
<td>33***</td>
<td>40***</td>
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<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>0.7***</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>50</td>
<td>100</td>
<td>78</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>50***</td>
<td>78**</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>0***</td>
<td>2***</td>
</tr>
</tbody>
</table>

¹Unit of PAL activity = nmol cinnamic acid formed per hour.
* significant at 0.05 level.
** significant at 0.025 level.
*** significant at 0.01 level.
germination did occur, the inhibition of PAL activity was proportional to the reduction of radicle growth in each case (Table 5).

When radicle lengths from all the above experiments were plotted against corresponding values for PAL activity, a strong correlation was obtained between the two (Fig. 9). Radicle length was regressed on PAL activity. The regression was highly significant ($R^2=0.86$, $p>0.001$). In order to get further insight into this apparent correlation between the radicle length and the PAL activity per seed it was decided to see the distribution of PAL activity in various parts of the germinated seed. Twenty-four h old germinated lettuce seeds were dissected into four parts, (1) seed coat, (2) endosperm, (3) cotyledons + plumule and (4) radicle. Phenylalanine ammonia-lyase activity as well as the concentration of protein were determined in each seed part separately. The results presented in Table 6 show that most of the protein in the germinated seed was in the cotyledons and plumule, whereas, very little protein was present in the radicle. On the other hand, more than 90% of the PAL activity of the seed was confined to the radicle. There was very little PAL activity in other parts of the seed.

**Effect of AOPP on PAL Activity and Growth**

2-Aminoxy 3-phenylpropionic acid (AOPP) is a well known specific inhibitor of PAL from many sources. It has also been shown to cause a superinduction of PAL in some plant tissues (Amrhein and Gerhardt, 1979; Noé et al., 1980; Tutschek, 1982). When lettuce seeds were incubated in two different concentrations of L-AOPP ($10^{-5}$M and $10^{-4}$M) for 24 h and enzyme activity measured in the crude extract, very
Fig. 9. Correlation between PAL activity and radicle length in germinating lettuce seeds exposed to various compounds. Data from table 3, 4, and 5. (Radicle length was regressed on PAL activity. The regression was highly significant. \(R^2 = 0.86, p > 0.001\).)
Table 6. Distribution of PAL activity and total protein in different parts of germinating lettuce seeds. After 24 h of incubation in water under white light, 100 seeds were dissected and different parts assayed for PAL activity and protein content.

<table>
<thead>
<tr>
<th>Seed Part</th>
<th>Protein (µg Seed)$^{-1}$</th>
<th>Units PAL (mg Protein)$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed Coat</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Endosperm</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Cotyledons and Plumule</td>
<td>210</td>
<td>2</td>
</tr>
<tr>
<td>Radicle</td>
<td>23</td>
<td>57</td>
</tr>
</tbody>
</table>
little PAL was detected. Even after dialysis of the crude extract there was more than 65\% inhibition of PAL activity at $10^{-5}$M AOPP and more than 80\% inhibition at $10^{-4}$M AOPP in dialysed samples. In contrast to the effect of other substrate analogues that inhibited PAL activity and caused a concomitant inhibition of the radicle growth, with AOPP, there was a slight stimulation of the radicle growth (Table 7).

In order to test whether the inhibition of PAL in seeds germinated in the presence of L-AOPP could be due to the synthesis of a low molecular weight proteinaceous inhibitor, ammonium sulfate-precipitated enzyme (60\% saturation) obtained from AOPP treated seeds was used for enzyme assay. Enzyme activity per seed was still as low as in the original crude extract showing that ammonium-sulfate precipitation did not remove the inhibitor, if there was any.

**Transfer Experiments to AOPP**

In order to further analyse the effects of AOPP on the development of PAL activity, experiments were conducted where AOPP was provided to the seeds after germination and also during the extraction process. In one experiment, seeds were germinated in water for 12 h and transferred to two different concentrations of L-AOPP ($10^{-5}$M and $10^{-4}$M). Enzyme activity as well as radicle length of the seeds were determined at the time of transfer and again at various times after transfer. Results presented in Fig. 10 show that there was a rapid decline in PAL activity on transfer of seeds to AOPP. This decline continued until at least 36 h of incubation. Seeds incubated in $10^{-5}$ M AOPP, however, seemed to slightly overcome the inhibition at 36 h.
Table 7. Effect of AOPP (10$^{-5}$ and 10$^{-4}$ M) on PAL activity\(^1\) and radicle length in 24 h old germinating lettuce seeds. The enzyme was assayed before dialysis, after dialysis and after ammonium sulfate precipitation followed by dialysis. Each value for PAL activity represents an average of 4 replicates of 100 seeds each. Each value for radicle length represents an average of 4 replicates of 10 seeds each.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Undialyzed</th>
<th>Dialyzed</th>
<th>(NH$_4$)$_2$SO$_4$ precipitated</th>
<th>Radicle length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44.89</td>
<td>48.43</td>
<td>41.31</td>
<td>3.1</td>
</tr>
<tr>
<td>10$^{-5}$ M AOPP</td>
<td>7.57</td>
<td>16.66</td>
<td>14.34</td>
<td>5.7</td>
</tr>
<tr>
<td>10$^{-4}$ M AOPP</td>
<td>7.44</td>
<td>8.64</td>
<td>8.84</td>
<td>4.8</td>
</tr>
</tbody>
</table>

\(^1\)Unit of PAL activity = nm of CA formed per hr.
Fig. 10. Effect of AOPP on the time course of PAL activity (top) and radicle length (bottom). The seeds were germinated in water for 12 h under white light and then transferred to AOPP at (▲) $10^{-5}$ M and (■) $10^{-4}$ M. (●) represents activity in control seeds. Each volume for PAL activity represents an average of 4 replicates of 100 seeds. Each value for radicle length represents an average of 10 measurements.
As noted earlier, there was a progressive stimulation of radicle length in the presence of AOPP till 24 h.

**Effect of Extracting PAL in a Buffer containing AOPP**

As mentioned earlier, ammonium sulfate precipitation and dialysis of the crude extract from the seeds grown in AOPP did not restore PAL activity. One of the possible explanations for this could be that the inhibition of PAL observed in the presence of AOPP could be due to the irreversible binding of AOPP to PAL leading to its inactivation. To test this hypothesis, 24 h old germinated seeds were homogenized in an extraction buffer containing $10^{-4}$M AOPP. The enzyme was assayed before and after dialysis of the centrifuged extract. A substantial decrease in enzyme activity was observed when the seeds were extracted in the presence of AOPP (Table 8). Furthermore, dialysis did not result in full recovery of the active enzyme indicating that the binding of L-AOPP to PAL may be irreversible.

When the above experiment was done using various substrate analogues mentioned earlier in the inhibition studies, it was observed that dialysis resulted in a complete recovery of the enzyme activity (Table 8), indicating that the binding of these compounds to PAL was reversible.

**Effect of AOPP on Ethylene Production**

Amrhein (personal communication) suggested that stimulation of radicle elongation in response to the low concentrations of AOPP ($10^{-5}$ M) could be secondary effect of AOPP due to its inhibition of ethylene synthesis. In order to test this hypothesis the production of
Table 8. Effect of the presence of various inhibitors of PAL (100 μM) in the extraction buffer on enzyme activity. The extract was dialyzed and the enzyme was assayed. Each value represents an average of 4 replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAL Activity¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70.8</td>
</tr>
<tr>
<td>P-fluorophenylalanine</td>
<td>70.8</td>
</tr>
<tr>
<td>D-phenylalanine</td>
<td>65.8</td>
</tr>
<tr>
<td>β-phenyllactic acid</td>
<td>68.3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>65.6</td>
</tr>
<tr>
<td>α-aminooxy-β-phenyl propionic acid</td>
<td>4.4</td>
</tr>
</tbody>
</table>

¹ Unit of PAL activity = nmol CA formed h⁻¹
ethylene by germinating lettuce seeds in the presence of AOPP over a period of 72 h was monitored. Seeds were incubated in two different concentrations of AOPP (10^{-5}M and 10^{-4}M) in flasks sealed with rubber stoppers. At various time intervals, 2 ml gas samples from the flasks were analysed for ethylene using a gas chromatograph. No ethylene could be detected in the flask for the first 6 h of imbibition. But at 18 h when ethylene could be detected, a slight inhibition of ethylene production which was concentration dependent was observed in the presence of AOPP (fig 11). The magnitude of inhibition of ethylene synthesis by AOPP was found to increase with time. There was 57% inhibition of ethylene production at 10^{-4}M AOPP and 12% inhibition at 10^{-5}M AOPP after 72 h.

Purification of PAL

As shown in Table 6, most of the PAL activity is confined to the radicles of germinating lettuce seeds whereas most of the protein is present in the cotyledons and plumule. Therefore, it was only logical to use radicles as the source of the enzyme for purification. Approximately 15,000 seeds (15 gms by weight) were germinated on a plastic mesh soaked in water. After 40 h of incubation the radicles were excised and used for the extraction of the enzyme.

The enzyme was extracted in a borate ascorbate buffer, pH 8.8, as described earlier, with the exception that 2 mM dithiothreitol (DTT) was added to the extraction buffer. After the first centrifugation the pellet was re-extracted in the same buffer. The combined extract was filtered through a cheesecloth and subjected to ammonium sulfate
Fig. 11. Effect of AOPP on the time course of ethylene production in germinating lettuce seeds incubated for various time periods in (▲) $10^{-5}$ M and (■) $10^{-4}$ M AOPP. At different intervals 2 ml gas samples from each flask were analyzed for ethylene. Each point represents an average of three replicates.
precipitation. It was found that most of the enzyme precipitated between 30 and 55% saturation with ammonium sulfate (Table 9).

The ammonium sulfate precipitated enzyme was dissolved in a 0.1 M borate buffer containing 2 mM DTT and desalted on a Sephadex G-50 column. The column was eluted with the same buffer and the effluent monitored continuously at 280 nm. The elution profile from Sephadex G-50 column is shown in Fig. 12. Fractions containing protein were assayed for enzyme activity. This step yielded a three fold purification of the enzyme (Table 10). Even though the data in Table 10 show a recovery of only 60% of the original enzyme activity present in the crude extract, for this preparation, some preparations did show much better yields of activity (as high as 90%).

Alternately, the precipitate was dissolved in 0.01 M borate buffer and dialysed against the same buffer for 16-24 h. Dialysis always resulted in significantly reduced recovery as compared to desalting through Sephadex G-50 column, at the same time reducing the fold purification.

The desalted enzyme had to be concentrated before further fractionation. Three methods were tested for the concentration of enzyme and the recovery of the enzyme compared. These methods utilized sucrose, polyethylene glycol and Sephadex G-200. The enzyme solution was placed in a dialysis bag and the bag covered with these materials for 4-5 h. Of the three methods used, concentration with sucrose was found to be the most suitable. About 10 fold concentration could be achieved in 4-5 hrs. The concentrated sample was dialysed against 0.1 M borate buffer containing 2 mM DTT and subjected to gel filtration on
Table 9. Percent saturation of ammonium sulfate at which PAL precipitates from a crude extract. Solid ammonium sulfate was used.

<table>
<thead>
<tr>
<th>Percent Saturation [(NH₄)₂SO₄]</th>
<th>Enzyme Activity (% of total recovered)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-30</td>
<td>1.9</td>
</tr>
<tr>
<td>30-40</td>
<td>11.6</td>
</tr>
<tr>
<td>40-55</td>
<td>85.1</td>
</tr>
<tr>
<td>55-65</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Fig. 12. Elution profile for PAL activity (O) and total protein (●) from a column of Sephadex G-50. Fractions of 8 ml each were collected and each sample analyzed for PAL activity and protein content. The PAL activity is expressed as CPM in cinnamic acid obtained when 0.1 ml sample from each fraction was assayed. Protein content is expressed as A595 in 0.1 ml sample from each fraction. (Column size = 52 x 3 cm).
Table 10. Purification of phenylalanine ammonia-lyase from the radicles of germinating lettuce seeds. Changes in total protein and PAL activity during various steps of purification. About 15,000 seeds germinated for 40 h were used.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (nm cinn./hr)</th>
<th>Specific activity</th>
<th>Recovery (% of original)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Crude extract</td>
<td>60</td>
<td>1740</td>
<td>10860</td>
<td>6.2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2) Amm. sulfate</td>
<td>50</td>
<td>300</td>
<td>6550</td>
<td>21</td>
<td>60</td>
<td>3.4</td>
</tr>
<tr>
<td>precipitation - Sephadex G-50</td>
<td>17</td>
<td>19</td>
<td>2103</td>
<td>110</td>
<td>19.2</td>
<td>17.7</td>
</tr>
<tr>
<td>3) Sephadex G-200</td>
<td>17</td>
<td>19</td>
<td>2103</td>
<td>110</td>
<td>19.2</td>
<td>17.7</td>
</tr>
<tr>
<td>4) DEAE Cellulose</td>
<td>15</td>
<td>7</td>
<td>1032</td>
<td>147</td>
<td>9.41</td>
<td>23.7</td>
</tr>
<tr>
<td>5) Hydroxylapatite</td>
<td>9</td>
<td>0.92</td>
<td>210</td>
<td>232</td>
<td>1.88</td>
<td>37.4</td>
</tr>
</tbody>
</table>
a Sephadex G-200 column. The proteins were eluted in a broad trailing peak and the enzyme came out in the first few fractions (Fig. 13). This step proved to be quite effective in separating the enzyme from other proteins. The resulting enzyme was purified five fold (Table 10). The recovery from this column was 32% of the amount loaded.

The fractions from Sephadex G-200 showing PAL activity were pooled together and concentrated with sucrose as described above. The concentrated solution was dialysed against 0.01 M borate buffer containing 2mM DTT and subjected to ion exchange chromatography on a DEAE cellulose column. The column was eluted with a stepwise gradient of sodium chloride in a 0.2 M borate buffer containing 2mM DTT. Two other kinds of gradients (concave and convex) obtained by a peristaltic pump were also tested, but the step wise gradient was found to be more suitable. The proteins came out in three different peaks at sodium chloride concentrations of 0.05, 0.3 and 0.5 M (Fig. 14). Most of the enzyme activity was associated with the third peak eluting at a concentration of 0.5 M NaCl (Fig. 14). This technique resulted in the increase in specific activity of PAL from 110 to 147 units / mg protein. The recovery of PAL from this column was approximately 49% (Table 10).

Fractions collected from the above column that showed PAL activity were pooled and concentrated with sucrose and dialyzed against 0.01 M K$_2$HPO$_4$ buffer, pH 8.8, before subjecting it to hydroxyapatite chromatography on a Bio Gel HTP column. The column was eluted with a step wise gradient of K$_2$HPO$_4$. In this case also concave and convex gradients of phosphate were tested, but stepwise gradient
Fig. 13. Elution profile for PAL activity (○) and total protein (●) from a column of Sephadex G-200. Fractions of 8 ml were collected and each sample assayed for PAL activity and protein content. PAL activity is expressed as CPM in cinnamic acid obtained when 0.1 ml sample from each fraction was assayed. The protein content is expressed as A595 in 0.1 ml samples from each fraction (Column size - 52 x 3 cm)
Fig. 14. Elution profile for PAL activity (○) and total protein (●) from a column of DEAE cellulose. Fractions of 8 ml each were collected. PAL activity is expressed as CPM in cinnamate obtained when 0.1 ml sample from each fraction was assayed. Similarly protein content is expressed as A595 in 0.1 ml sample from each fraction. The column was eluted with a stepwise gradient of NaCl (----) expressed in molarity of NaCl used (Column size - 10 x 3 cm).
was more effective. Most of the enzyme was eluted in the first few fractions at a concentration of 0.3 M phosphate. This method of separation increased the specific activity from 147 to 232 units/mg protein (Table 10). The recovery from this column was however only 20%.
Fig. 15. Elution profile of PAL activity (○) and protein (●) from a column of Bio Gel HTP. Fractions of 8 ml each were collected and analyzed for PAL activity and protein content. PAL activity is expressed as CPM in cinnamic acid obtained when 0.1 ml sample from each fraction was assayed. The protein content is expressed as A595 in 0.1 ml sample from each fraction. The column was eluted with a stepwise gradient of K₂HPO₄. (Column size - 4 x 3 cm).
DISCUSSION

Kinetics

As is evident from the hyperbolic saturation curve and the Line-weaver-Burk Plot for PAL activity at various substrate concentrations, PAL in germinating lettuce seeds shows typical Michaelis-Menten kinetics (Fig. 2). This type of kinetics has been reported for PAL mostly from microorganisms (Emes and Vining, 1970; Hodgins, 1970; Parkhurst and Hodgins, 1972) and only from one higher plant species viz. mustard (Gupta and Acton, 1979). This situation is quite different from that of PAL from potato (Havir and Hanson, 1968), pea (Attridge et al., 1971), parsley (Zimmermann and Hahlbrock, 1971) and maize (Havir and Hanson, 1973), where kinetics showing negative co-operativity have been reported. No indication of co-operativity was observed with lettuce PAL over a wide range (15-600 \text{\mu M}) of concentration of phenylalanine.

The $K_m$ of the enzyme from lettuce seeds was found to be $4.2 \times 10^{-5}$ M. This value compares well with values reported for wheat (Narl et al., 1972), potato (Havir and Hanson, 1973), and gherkin (Iredale and Smith, 1974). The $K_m$ values for PAL reported in the literature range from $3.8 \times 10^{-5}$ M to $1.7 \times 10^{-3}$ M. Phenylalanine ammonia lyase from lettuce seeds does not show any tyrosine ammonia-lyase (TAL) activity as is evident from the lack of inhibition of enzyme activity by tyrosine or p-coumaric acid, the product of tyrosine ammonia-lyase.
Most of the cases where PAL exhibits TAL activity are limited to monocots.

Various analogues of the substrate were used in enzyme kinetic studies to examine the nature of inhibition of PAL by these compounds. Of the five substrate analogues tested, D-phenylalanine, showed competitive inhibition of the enzyme activity in lettuce seeds (Fig. 3). In the past D-phenylalanine has been reported to inhibit PAL from potato (Havir and Hanson, 1968), bean (Walton, 1968), tobacco (O'Neal and Keller, 1970), yeast (Hodgins, 1971) and, gherkin (Iredale and Smith, 1974), whereas it did not inhibit PAL activity from Streptomyces (Emes and Vining, 1970) and sweet potato (Tanaka and Uritani, 1977). At comparable concentrations, the strongest inhibition of PAL in lettuce seeds occurred in the presence of p-fluorophenylalanine (92% inhibition at 500 μM). On further analyses, the inhibition was found to be competitive. A similar situation has been reported for PAL from wheat (Young and Neish, 1966), bean (Walton, 1968), and Streptomyces (Parkhurst and Hodgins, 1972). Of the two aromatic amino acids used in these studies, tyrosine did not show any effect on PAL activity, whereas tryptophan was found to be a competitive inhibitor (Table 1). Tryptophan has been reported to inhibit the enzyme from tobacco (Innerarity et al., 1972). Tyrosine generally inhibits PAL activity in cases where this enzyme shows TAL activity (Koukol and Conn, 1961).

Of the phenylpropanoid compounds used for kinetic studies, the product of the enzyme reaction cinnamic acid, was found to be a potent competitive inhibitor of the enzyme from lettuce seeds (Fig. 6). At the highest concentration used in these studies (500 μM), it inhibited
PAL activity by 75% (Table 2). Similar results have been reported earlier for PAL from barley (Koukol and Conn, 1961), sweet potato (Minamikawa and Tanaka, 1965), bean (Walton, 1968), potato (Havlir and Hanson, 1968), tobacco (O'Neal and Keller, 1970), oak (Boudet et al., 1971), yeast (Parkhurst and Hodgins, 1972) and gherkin (Iredale and Smith, 1974). Cinnamic acid not only inhibited enzyme activity in vitro, it also played an important role in the regulation of PAL levels in this tissue (see discussion later). P-coumaric acid, one of the intermediates of the phenylpropanoid pathway, did not show any inhibition of PAL from lettuce seeds. Generally it has been reported to inhibit PAL from only sources that show TAL activity (Neilsh, 1961; Young et al., 1965; Havlir et al., 1971; Hodgins, 1971). Other phenylpropanoid pathway intermediates like caffeic acid and ferulic acid also did not inhibit PAL from lettuce seeds. In tobacco (O'Neal and Keller, 1970) and gherkin (Iredale and Smith, 1974) caffeic acid did not show any inhibition whereas ferulic acid did inhibit PAL activity. In sweet potato, on the other hand, caffeic acid was found to inhibit the enzyme competitively (Minamikawa and Uritani, 1965).

Endproducts of the phenylpropanoid pathway like coumarin, quercetin, kaempferol etc. have been shown to be strong inhibitors of this enzyme from bean (Walton, 1968), tobacco (O'Neal and Keller, 1970), yeast (Parkhurst and Hodgins, 1972) and gherkin (Iredale and Smith, 1974). In gherkin the inhibition by these compounds was shown to be mixed, thereby suggesting that the inhibitor is binding at a site other than the catalytic site on the enzyme molecule. In lettuce seeds, however, no inhibition was observed in the presence of two of
Regulation of PAL

One of the main objectives of the present research was to examine the nature of regulation of PAL in germinating lettuce seeds by various substrate analogues, and some intermediates and endproducts of the phenylpropanoid pathway especially those that interfere with the enzyme activity in vitro. Phenylalanine ammonia-lyase, being the first enzyme in the phenylpropanoid pathway, is an important regulatory enzyme controlling the flux of phenylalanine into the synthesis of various phenolic compounds. These compounds are involved in the protection of the plant against fungal and bacterial pathogens or in allelopathic interactions (Camm and Towers, 1972). It seems that the activity of PAL is precisely regulated in the cells by concentrations of (1) the substrate L-phenylalanine (Walton, 1968); (2) the product of the enzymatic reaction, cinnamic acid (Johnson et al., 1975; Shields et al., 1982); (3) various intermediates of the phenylpropanoid pathway such as p-coumaric acid, caffeic acid and, ferulic acid etc. (Attridge et al., 1971). Some of these compounds regulate the amount of enzyme in the cells whereas others act mainly through their effects on enzyme activity per se.

Externally supplied L-phenylalanine, the natural substrate for this enzyme, did not affect PAL levels in 24 h old germinating lettuce seeds when used at a concentration below 100 μM. However, at 500 μM,
a significant inhibition of PAL / seed was well as PAL / mg protein was observed (Table 3). Exogenously applied L-phenylalanine has been reported to inhibit extractable PAL activity in many tissues (Zucker, 1965; Walton, 1968; Creasy, 1971). The mechanism of inhibition has been suggested to be due to the increased production of cinnamic acid as a result of high substrate levels (see discussion later). However an increase in cinnamic acid level in the presence of high substrate levels has not been demonstrated clearly. But the fact that exogenous cinnamic acid inhibits the levels of PAL in several tissues, makes this a reasonable assumption. On similar lines, James et al. (1980) observed that low concentrations of phenylalanine (5 x 10^{-5} M) increased extractable PAL activity in Jerusalem artichoke callus culture.

D-phenylalanine, a competitive inhibitor of the enzyme from germinating lettuce seeds, did not show any significant effect on the levels of PAL when used at concentrations of 50 and 100 µM although at 500 µM a slight but significant stimulation of the enzyme activity / seed was observed. This is similar to the situation in Jerusalem artichoke tuber tissue, where exogenous D-phenylalanine was found to cause a marked stimulation of extractable PAL when supplied in the culture medium (Durst, 1976). It was suggested that the depletion of the cinnamate pool due to the competition of D-phenylalanine with L-phenylalanine for enzyme activity could have resulted in increased enzyme levels. This suggestion is based on the assumption that cinnamate is a feedback inhibitor of PAL (Zucker, 1965). Similar results were reported for radish cotyledons by Huault and Klein-Eude (1978).
On the other hand, Walton (1968) did not observe any effect of D-phenylalanine on PAL activity in excised bean axes. P-fluorophenylalanine, another substrate analogue, was found to be a strong inhibitor of PAL activity in germinating lettuce seeds (98% inhibition at 500 µM). At lower concentrations, there was a concentration dependent inhibition of both PAL activity and the radicle growth in the presence of this compound. Walton (1968) and Hopkins and Orkwiszewski (1971) found a similar inhibition of PAL in excised bean axes and oat coleoptiles, respectively, by p-fluorophenylalanine. No mechanism of this inhibition was suggested by these authors.

Tyrosine has been shown to be a competitive inhibitor of PAL from tissues where this enzyme can utilize tyrosine as a substrate (Koukol and Conn, 1961). In these tissues, most of them grasses, tyrosine also inhibits the cellular PAL levels. Creasy (1971) found that exogenous tyrosine inhibited PAL activity and the accumulation of cinnamic acid as well as flavonoids in strawberry leaf discs (PAL in this case shows TAL activity). Walton (1968), however, observed a stimulation of PAL by tyrosine in excised bean axes, a tissue which does not show any TAL activity. Our results agree with the findings of Walton in that a slight but significant promotion of PAL activity/seed was observed with 500 µM tyrosine. As with other compounds that stimulated PAL a corresponding stimulation of the radicle growth was also observed. It should also be noted that PAL in germinating lettuce seeds does not show any TAL activity. On the other hand, tryptophan, another related aromatic amino acid strongly inhibited PAL activity as well as radicle elongation in the present study. In
lettuce seeds there was complete suppression of germination at 500 μM in presence of tryptophan. But at lower concentrations, where germination was not inhibited, elongation of the radicle was inhibited in proportion to the concentration of this compound. At the same time, there was a consistent decrease in the activity of extractable PAL per seed. A similar inhibition of PAL by exogenous tryptophan was reported earlier in tobacco suspension cultures (Innerarity et al., 1972). Phenylactic acid, another analogue of phenylalanine had no visible effects on germination or PAL activity in lettuce seeds.

It has been shown that high cellular concentrations of various intermediates of the phenylpropanoid pathway can regulate PAL levels through feedback inhibition of the enzyme in gherkin hypocotyls (Engelsma, 1968; Johnson et al., 1975), Jerusalem artichoke (Durst, 1976), potato tuber slices (Lamb, 1979) and pea epicotyls (Shields et al., 1982). In pea epicotyls, exogenous supplies of the pathway intermediates like trans-cinnamic acid, p-coumaric acid, ferulic acid and sinapic acid were shown to inhibit the initial development of PAL activity. If added at the time when high enzyme levels in the tissue had already developed, these compounds caused a rapid decrease in enzyme activity (Shields et al., 1982). Lamb (1979) had earlier shown that the modulation of PAL by cinnamic acid and p-coumaric acid occurred by a rapid post-transcriptional mechanism. The inhibitory effect of exogenous cinnamic acid, the immediate product of the enzyme reaction, is expressed quite rapidly in pea epicotyl tissue (Shields et al., 1982) where it exerts a dual control both over the production and the removal of PAL. The authors have suggested that the dual
control over production and the removal of an enzyme by a product may be of general importance in slow growing tissues for the rapid removal of biosynthetic enzymes no longer required by the cell. Durst (1976) found that the increase in endogenous cinnamate levels by the inhibition of cinnamic acid hydroxylase (for which cinnamic acid is a substrate) by anaerobiosis caused a rapid decline in PAL activity. Upon return to air, the accumulated cinnamate was rapidly metabolised and PAL resumed its previous activity. These results clearly indicate that the endogenous levels of cinnamate may modulate cellular levels of PAL.

Noé et al., (1980) on the other hand, suggested that the effect of trans-cinnamic acid on PAL may not be specific. They found that cinnamic acid at a concentration of $10^{-4}$ M was toxic to the growth of carrot cells in culture. Furthermore, a reduction in the content of soluble proteins at that concentration was also observed. The same authors recently reported that trans-cinnamic acid, in fact, interferes with protein synthesis in vitro as well as in vivo. Considering the toxic effects of cinnamic acid and other hydroxycinnamic acids (Noe et al., 1980), one would expect the cells to turn over the cinnamic acids rapidly in order to avoid these detrimental effects. Since these substances are not accumulated in the cells to any great extent, (Braun and Seitz, 1975), their role in PAL regulation is, therefore, questionable.

The inhibition of germination of lettuce seeds at 500 μM cinnamic acid (Table 4) is not surprising in light of the fact that it has been reported to be an inhibitor of germination in many seeds (Rasmussen
and Einhellig, 1979). However, at lower concentrations (50 and 100 µM) where germination was not inhibited, there was a concentration dependent inhibition of PAL activity. A significant inhibition of radicle elongation was also observed in the presence of cinnamic acid at these concentrations. Cinnamic acid caused an inhibition of PAL in seed even if supplied after the seeds had already germinated and developed high levels of PAL. When seeds incubated in water for 12 h were transferred to cinnamic acid (10 or 100 µM), there was a rapid decline in PAL activity for the next 24 h. The kinetics of the decline in PAL levels in presence of cinnamic acid differ from those reported earlier in the presence of abscisic acid (ABA) and polyethylene glycol (Dalnes 1981; Dalnes and Minocha, 1983), in that the rate of decline with cinnamic acid was much slower than with ABA or PEG.

A number of other intermediates of the phenylpropanoid pathway did not inhibit PAL activity to the same extent as cinnamic acid at comparable concentrations. The order of effectiveness of inhibition of PAL by four of the compounds at 100 µM concentration was cinnamic acid > p-coumaric acid > ferulic acid > caffeic acid. This is similar to the situation in dwarf French bean suspension cultures (Dixon et al., 1980) and pea epicotyls (Shields et al., 1982). This may reflect the metabolic proximity of these compounds to PAL in the phenylpropanoid pathway.

Some of the endproducts of the phenylpropanoid pathway like coumarins, flavonoids, chlorogenic acid etc. have been implicated in the regulation of PAL activity based on their nature of in vitro
inhibition of enzyme activity. Most of these compounds showed mixed inhibition \textit{in vitro} indicative of an allosteric modulation of the enzyme \textit{in vivo} (Attridge et al., 1971; Iredale and Smith, 1974). In the present study, of the endproducts of the phenylpropanoid pathway used, only chlorogenic acid showed mixed inhibition of PAL \textit{in vitro}. But when supplied exogenously to the seeds, these compounds were all found to inhibit germination as well as PAL activity at 500 µM (Table 5). At lower concentrations, where germination did occur, the inhibition of PAL activity was proportional to the reduction in radicle growth in the presence of these compounds. Amrhein et al., (1976) did not see much effect on PAL activity in buckwheat in the presence of endproducts of the phenylpropanoid pathway.

In all the above treatments, a strong correlation was observed between PAL activity and the growth of the radicle. These results generally support the earlier observations from this laboratory that whenever the growth of the radicle of germinating lettuce seeds is affected by either chemical factors (eg. phytohormones) or physical factors (eg. far red light, high osmoticism) (Daines, 1981; Daines and Minocha, 1983; Daines et al., 1983) there is a concomitant effect on PAL activity per seed. An important question arising out of these studies is, "what is the causative relationship between the changes in PAL activity and the growth of the radicle?" The inhibition of radicle elongation in lettuce seeds in the presence of various phenylpropanoid compounds could be due to their inhibitory effect on various growth processes and most likely not due to their effect on the inhibition of PAL activity. In fact, phenolic compounds are known to be
natural inhibitors of germination and radicle growth in several cases (Rasmussen and Einhellig, 1979). Some of them are known to interfere with indole biosynthesis (Kefeli and Kutacek, 1976). Whereas others, such as trans-cinnamic acid have been reported to have anti-auxin properties (Van Overbeek et al., 1951).

In an attempt to explain the high correlation observed between PAL activity and radicle elongation, the distribution of PAL activity in different seed parts was examined. It was observed that 95% of the extractable PAL activity was in the radicles of germinating lettuce seeds (Table 6). This restricted localization of PAL in the radicle is quite consistent with the strong correlation of PAL activity and radicle length observed in all the treatments given to the seeds. These results are also similar to the earlier reports of the presence of high levels of PAL in the roots of etiolated buckwheat (Amrhein and Zenk, 1971), etiolated radish (Bellini and Van Poucke, 1970) and etiolated Impatiens seedlings (Weissenbock, 1972).

The effect of PAL inhibition on growth has been studied in the past in excised bean axes (Walton, 1968) and oat coleoptiles (Hopkins and Orkwiszewski, 1971). Walton (1968) observed an increase in the fresh weight of excised bean axes following inhibition of PAL activity. Hopkins and Orkwiszewski (1971) also found that reduced levels of PAL were accompanied by an increased elongation in oat coleoptile. The authors explained this relationship of PAL to growth in terms of the reduced production of phenylpropanoid compounds which would have inhibited growth. A similar stimulation of radicle growth
was observed in germinated lettuce seeds in the presence of α-aminoxy-β-
-phenylpropionic acid (AOPP) a specific inhibitor of PAL (Table 7).

When lettuce seeds incubated in water for 12 h were transferred
to AOPP a rapid decline in PAL activity was observed which continued
through 36 h. There was a slight stimulation of radicle elongation in
the presence of AOPP in contrast to the effect of other substrate
analogues that inhibited PAL activity (Fig. 10). One of the most
common effects of AOPP is a substantial increase in extractable PAL
activity in its presence (Amrhein and Gerhardt, 1979; Duke et al.,
1980; Noé et al., 1980; Noé and Seitz, 1982; Tutschek, 1982). This
increase in PAL activity was attributed to the decreased feedback
inhibition of PAL synthesis by cinnamic acid and / or its derivatives
(Duke et al., 1980). In contrast to these reports, in the present
study, AOPP caused a strong inhibition of extractable PAL activity in
germinating lettuce seeds (Table 7). Even dialysis of the crude
extract could not restore the enzyme activity. In order to test
whether this inhibition of PAL could be due to the synthesis of a low
molecular weight proteinaceous inhibitor, ammonium sulphate precipi-
tated enzyme obtained from AOPP treated seeds was used for enzyme
assay. Enzyme activity per seed was still as low as in the original
extract.

One of the possible explanations for this inhibition of PAL
activity by AOPP could be that AOPP could bind to PAL irreversibly
leading to its inactivation. To test this hypothesis, 24 h old germi-
nated seeds were homogenised in an extraction buffer containing AOPP.
The enzyme was assayed before and after dialysis of the extract. A
substantial decrease in enzyme activity was observed in both cases, indicating that the binding of AOPP to PAL may be irreversible (Table 8). A similar effect of AOPP on PAL has been reported in soybean cell culture suspension cultures (Havir, 1981), where AOPP was found to bind to PAL irreversibly leading to its inactivation.

Even though AOPP is considered to be a specific inhibitor of PAL activity, it has also been shown to inhibit phenylalanine transaminase (Amrhein et al., 1976). The potency of inhibition was, however, far less pronounced than in the case of phenylalanine deamination. AOPP has also been reported to inhibit other transaminases (John et al., 1978). This inhibition of transamination by AOPP could explain the inhibition of ethylene synthesis observed in the presence of AOPP in some tissues (Amrhein and Wenker, 1979). Yu et al. (1979) suggested that the site of inhibition of ethylene synthesis by aminooxyacetic acid (AOA), another substrate analogue closely related to AOPP, could be 1-aminocyclopropane carboxylate synthase, a pyridoxal phosphate containing enzyme like transaminases. Amrhein (personal communication) suggested that the stimulation of radicle elongation observed in germinating lettuce seeds in the presence of AOPP could be a secondary effect of inhibition of ethylene synthesis by this compound. To check this possibility the production of ethylene by germinating lettuce seeds over a period of 72 h was monitored. It was found that ethylene synthesis was indeed inhibited by concentration of AOPP, that caused a promotion of radicle growth (Fig. 11).
Purification of PAL

Most of the PAL activity in germinating lettuce seeds was found to be confined to the radicles whereas most of the protein was present in the cotyledons and the plumule. Hence the radicles were used as the source of the enzyme for purification. Since excised radicles were used as the source of the enzyme for purification instead of the whole seedlings, it was possible to start with a sample of relatively high specific activity (6.2 units of PAL/mg protein). The initial specific activity of PAL from most other sources is generally quite low. Such a high specific activity in the radicles of lettuce seedlings indicates a relatively high proportion of PAL in this tissue as compared to most other tissues.

The first step in the purification of PAL from lettuce seeds, like most other schemes, was ammonium sulphate precipitation. In other studies on PAL purification this step resulted in a two to nine fold purification of the enzyme (Havir and Hanson, 1968; Emes and Vining, 1970; Zimmermann and Hahlbrock, 1975; Tanaka and Uritani, 1977; Gupta and Acton, 1979; Loschke et al., 1981; Havir, 1981). In lettuce seeds ammonium sulphate precipitation yielded a three fold increase in the specific activity of the enzyme (Table 10). The enzyme in this case, like in most previous studies, precipitated between 30 and 55% saturation of ammonium sulphate (Table 9). This is in contrast to the enzyme from potato (Havir and Hanson, 1968), which was precipitated between 27 and 40% saturation, and also from mustard cotyledons (Gupta and Acton, 1979) where it precipitated between 30 and 45% saturation with ammonium sulphate. As high as 60-90% of the
original activity in the crude extract could be recovered after desalting of the ammonium sulphate precipitated protein on Sephadex G-50. Dialysis to remove ammonium sulphate generally resulted in lower recoveries of enzyme activity.

The second step in the purification of PAL from germinating lettuce seeds employed Sephadex G-200 column chromatography. This technique proved to be quite effective and yielded a 5 fold purification of the enzyme (Table 10). This is comparable to the 3-8 fold purification through gel permeation that other authors have reported using Sephadex G-200 or Sepharose 6 B (Havir and Hanson, 1968; Zimmermann and Hahlbrock, 1975; Tanaka and Uritani, 1977; Loschke et al., 1981; Havir, 1981). Tanaka and Uritani used two consecutive gel filtration steps for purification of PAL from sweet potato, one with Sephadex G-200 and the second with Sepharose 6 B. The first step resulted in a 6 fold and the second a further 2 fold purification of the enzyme (Tanaka and Uritani, 1977).

The third step in the purification of PAL from lettuce seeds was ion exchange chromatography using DEAE cellulose. This step removed a good proportion of the unwanted proteins. But it also resulted in a substantial loss of enzyme activity. This method has been used in the past in the purification of PAL from potato (Havir and Hanson, 1968), Streptomyces (Emes and Vining, 1970), oak (Boudet et al., 1972), parsley (Zimmermann and Hahlbrock, 1975), spinach (Nizhizawa et al., 1979) and pea (Loschke et al., 1981). Two forms of this enzyme were distinguishable by DEAE cellulose chromatography in oak (Boudet et al., 1972). They differed principally in their optimum pH and allo-
teric properties. Minamikawa and Uriltani (1965) also reported two forms of PAL separable by DEAE cellulose chromatography in sweet potato, but later studies from the same laboratory mention only one form of the enzyme (Tanaka and Uriltani, 1977). Three forms of PAL separable by DEAE cellulose chromatography have been reported in spinach leaves, two of them coming, one being from the chloroplast and one from the cytoplasm (Nizhizawa et al., 1979). In lettuce seeds, however, there was no indication of the presence of more than one form of PAL.

Following DEAE cellulose chromatography, hydroxylapatite chromatography on a Bio Gel HTP column was used. This technique has been used in the past for the purification of PAL from potato (Havir and Hanson, 1968), wheat (Nari et al., 1972), parsley (Zimmermann and Hahlbrock, 1975), spinach (Nizhizawa et al., 1979) and soybean (Havir, 1981) where it yielded a 2-3 fold purification. In the purification of PAL from lettuce seeds this technique increased the specific activity from 147 to 232 units / mg protein (Table 10).

Thus using a combination of four different techniques, PAL from germinating lettuce seeds was purified 37 fold yielding a specific activity of 232. Since the starting material had a relatively high specific activity the final specific activity obtained in the present case is comparable to that obtained for other purification schemes. The final yield is only about 2% of the original extract. This could be partly due to the fact that more emphasis was given to achieve a high degree of purification rather than a high yield of the enzyme.
and, therefore, only the fractions possessing reasonably high specific activity for the enzyme were pooled after each step.
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