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A SPECIFIC AND REVERSIBLE MACROMOLECULAR INHIBITOR OF PHENYLALANINE AMMONIA-LYASE AND CINNAMIC ACID-4-HYDROXYLASE IN GHERKINS

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Summary

A non-dialysable inhibitor of phenylalanine ammonia-lyase (L-phenylalanine ammonia-lyase, EC 4.3.1.5) has been partially purified from dark-grown gherkin hypocotyls. On extraction of tissue it is found both in the soluble (106 000 $\times g$ supernatant) and microsomal (106 000 $\times g$ pellet) fractions and can be extracted from the microsomal membranes with 10 mM sodium cholate and 1 M KCl. The soluble and microsomal fractions have similar properties, suggesting the presence of the same active component.

The inhibitor is small ($M_r < 20\,000$), thermolabile, sensitive to proteolytic digestion, and apparently hydrophobic. Purification of the inhibitor was achieved by chromatography on DEAE-cellulose followed by gel filtration on Sephadex G-50. The inhibitor preparations inhibit phenylalanine ammonia-lyase isolated from a number of plant tissues and also cinnamic acid-4-hydroxylase (*trans*-cinnamate, NADPH:oxygen oxidoreductase (4-hydroxylating), EC 1.14.13.11) from gherkins and peas, but not a wide range of other enzymes. The evidence suggests that inhibition of the two enzymes is due to the same substance, but this has not yet been confirmed. Kinetic experiments show that the inhibitor is competitive with phenylalanine for the lyase and that its association with the lyase is reversible. Further, a mixture of inhibitor and lyase can be separated on non-denaturing polyacrylamide gels without loss of lyase activity.

The activities of phenylalanine ammonia-lyase and cinnamic acid 4-hydroxylase are often concurrently regulated and both have regulatory roles in phenol metabolism; it is suggested that the inhibitor may be specifically involved in controlling their activities in vivo.

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Introduction

The regulation of enzyme activity by specific macromolecular inhibitors whose action is reversible has aroused increasing interest in recent years [1—5], and it is thought [6] that such inhibitors may have physiological significance. It is known that the activity of L-phenylalanine ammonia-lyase (EC 4.3.1.5) in etiolated gherkin hypocotyls fluctuates in response to external stimuli, in particular blue light treatment [7—9]. Evidence suggests that, at least in gherkins, light-mediated changes in activity in vivo are due to activation/inactivation of the enzyme, with the lyase, in dark grown tissues existing in an inactive form which is activated by blue light [10,11]. In preliminary experiments [12] we reported the presence of an apparently heat-stable reversible inhibitor of phenylalanine ammonia lyase in gherkin hypocotyls. The preparations used previously were unpurified and in this paper we examine the purification and properties of the inhibitor in greater detail.

Methods

Plant material

Gherkin seedlings (*Cucumis sativus* cv. Venlo Pickling) were grown in darkness at 25°C ± 1°C for 4 days. The upper 2 cm of hypocotyl including the pulmular hook were used both as a source of inhibitor and of lyase. Inhibitor preparations were extracted from dark grown tissue and lyase from dark grown plants pretreated with blue light ($1 \cdot 1\ W \cdot m^{-2}$) for 4 h [7—9] before extraction.

Buffers

Buffer A: 0.17 M ammonium acetate (pH 6.0). Buffer B: 50 mM Hepes/0.3 M mannitol/1 mM EDTA/0.1% (w/v) bovine serum albumin/5 mM dithiothreitol (pH 7.5). Buffer C: 10 mM Tris · HCl (pH 8.0). Buffer D: 50 mM Tricine/0.3 M mannitol/1 mM EDTA/0.1% (w/v) bovine serum albumin (pH 7.5). Buffer E: 0.3 M mannitol/10 mM potassium phosphate/5 mM $MgCl_2$/10 mM KCl/1.5 mM β-mercaptoethanol/15% (v/v) glycerol (pH 7.5).

Phenylalanine ammonia-lyase purification and assay

All operations were performed at 4°C unless stated otherwise. Tissue was ground in liquid N_2, taken up in Buffer A, filtered through 1 layer of Miracloth and the filtrate centrifuged at 30 000 × *g* for 15 min. Protein in the supernatant which precipitated between 40% and 70% $(NH_4)_2SO_4$ was recovered, dissolved in Buffer A and desalted on a Sephadex G-25 column (2.5 × 35.0 cm) pre-equilibrated with the same buffer. The desalted protein was then purified by affinity chromatography on a column (1.5 × 4.0 cm) of Sepharose 4B L-phenylalanine conjugate [13] pre-equilibrated with Buffer A. The lyase bound to this conjugate at pH 6.0 and was eluted with 0.2 M borate (pH 7.9). The lyase was purified 60- to 70-fold following the above procedure.

Lyase activity in 0.1 ml aliquots of the partially purified enzyme preparation was assayed by incubation with 1.0 ml 0.1 M borate (pH 8.8) containing 10 μM L-[4-^{3}H]phenylalanine (10 Ci/mol) (Radiochemical Centre, Amersham). After incubation, normally for 1 h at 25°C, the reaction was stopped and product

formation measured as described previously [14]. For the inhibitor assays one unit of phenylalanine ammonia-lyase was defined as that amount of enzyme which produced 0.75 nmol cinnamic acid per h.

The lyase was also extracted as described above from light-treated mustard cotyledons [15], pea apical buds [16] and radish cotyledons [17].

Preparation of cellular fractions

All operations were performed at 4°C. Tissue was homogenised with a pestle and mortar in 1.5 vol of Buffer B. Routinely the homogenate was filtered through 1 layer of Miracloth, centrifuged at 21 000 $\times g$ for 15 min, and the supernatant centrifuged at 106 000 $\times g$ in a Ti 60 rotor (Beckman L2-65B centrifuge) for 60 min. If a 500 $\times g$ pellet was required (Table I) the initial homogenate was centrifuged at 500 $\times g$ for 15 min; in this case the 500 $\times g$ supernatant was then centrifuged at 21 000 $\times g$. All pellets were suspended in Buffer C using a TRI-R (California) Teflon hand homogeniser. The 500 $\times g$ and 21 000 $\times g$ pellets were further homogenised in a steel hammer press. Protein in the 106 000 $\times g$ supernatant was concentrated by 0—90% $(NH_4)_2SO_4$ precipitation and redissolved in Buffer C. All cellular fractions were dialysed extensively against Buffer C before assaying for inhibitory activity.

Cinnamic acid-4-hydroxylase extraction and assay

Cinnamic acid-4-hydroxylase (*trans*-cinnamate, NADPH:oxygen oxidoreductase (4-hydroxylating), EC 1.14.13.11) was extracted from terminal buds of dark grown 8-day old pea seedlings (*Pisum sativum* cv. Alaska) which had been irradiated with white fluorescent light for approx. 5 min, 12 h before harvest [18]. Tissue was homogenised with a pestle and mortar in 1.5 vol Buffer D containing 10% (w/w, fresh weight) polyclar AT (Sigma). Sand (approx. 0.25 g/g fresh weight) was added to aid extraction. The homogenate was filtered through 1 layer of Miracloth, centrifuged at 21 000 $\times g$ for 15 min, and the supernatant centrifuged at 106 000 $\times g$ in the Ti 60 rotor for 60 min. The 106 000 $\times g$ pellet (microsomes), which contained the hydroxylase, was resuspended to approx. 15 mg/ml protein in Buffer E and stored in 1 ml aliquots in liquid N_2. Cinnamic acid-4-hydroxylase was measured after radiochemical method 1 of Russell [18]. Incubation mixtures contained 50 μl microsomal suspension, 100 μM *trans*-[3-^{14}C]cinnamic acid, potassium salt (3 Ci/mol, C.E.N.S., France), 0.5 mM β-mercaptoethanol, 20 mM KH_2PO_4 (pH 7.5) and an NADPH regenerating system (1.5 mM NADP, 2.0 mM glucose-6-*P* and 0.1 units glucose-6-phosphate dehydrogenase) in a final volume of 0.5 ml. The NADPH regenerating system was incubated for 5 min at 25°C before being added to the assay. The complete assay mixture was incubated at 25°C for a further 20 min and the reaction terminated by the addition of 100 μl 6 M HCl. The protein was removed by centrifugation, washed once with 0.5 ml water, and the supernatant and wash solutions combined before extracting twice with 1.0 ml ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness, and the resulting residue taken up in 100 μl ethyl alcohol containing approx. 1 mM unlabelled *p*-coumaric acid and 80 μl then chromatographed on plastic fluorescent 0.25 mm silica gel plates (Camlab, Cambridge) using C_6H_6/acetic acid/H_2O (2 : 2 : 1, v/v), upper phase. The *p*-coumarate spots were detected

under ultra violet light, cut directly into scintillation vials and counted. The NADPH regenerating system was omitted from control incubations.

Cinnamic acid-4-hydroxylase was similarly extracted from gherkin cotyledons harvested from 4-day old dark-grown plants pre-treated with blue light for 4 h, but the assay mixture contained 0.1 mM glutathione and 22 μM *trans*-[3-^{14}C]cinnamic acid (50 Ci/mol).

Other enzymes

Nitrate reductase was extracted from maize scutella and mustard cotyledons and assayed as described by Wallace [19]. Commercial β-galactosidase (*Escherichia coli* Grade IV) and β-glucosidase (*Prunus amygdalus*) were assayed using *o*-nitrophenylglucoside and *o*-nitrophenylgalactoside, respectively, as substrates [20]; glucose-6-phosphate dehydrogenase (Baker's Yeast, Type VII) and α-amylase (*Bacillus subtilus* Type 11-A) were assayed as described previously [21,22]. NADPH- and NADH-cytochrome *c* reductase were extracted from gherkin cotyledons and maize scutella, by preparing microsomes from these tissues as for the hydroxylase, and assayed as by Johnson and Wallace [23]. All enzyme assays were carried out at 25°C.

Protein

Protein was determined by the method of Lowry et al. [24] with bovine serum albumin as standard.

Protease treatment

Inhibitor preparations were treated with soluble pronase (*Streptomyces griseus*, B grade, Calbiochem, 1 μg/mg inhibitor protein) and trypsin (bovine pancreas, Type III, 10 μg/mg inhibitor protein) in 10 mM Tris · HCl (pH 8.0), 0.1% Tween 20, at 25°C for 2 h. Residual inhibitor activity was then assayed for 15 min with partially-purified phenylalanine ammonia-lyase. Controls contained boiled inhibitor extracts and protease solutions and correction was made for proteolytic digestion of the lyase during the assay. Controls were included containing *Rhodotorula glutinis* phenylalanine ammonia-lyase (P-L Biochemicals Inc.) to ensure that the proteases were active.

Gel electrophoresis

Proteins were fractionated under non-denaturing conditions on 12 cm long polyacrylamide (5% w/v) gels at 2°C using a discontinuous system and no stacking gel. The separating gel contained a 20 : 0.53 ratio of acrylamide to *N,N'*-methylene bisacrylamide in 0.4 mM Tris · HCl (pH 8.5). The reservoir buffer contained 50 mM Tris, 380 mM glycine, and 0.4 mM β-mercaptoethanol. Gels were run at 4 mA/gel until the bromophenol blue marker had reached the end of the gel (approx 3 h). The gels were then frozen using dry ice and sliced into 2 mm thick fractions before assaying for lyase activity.

Source of chemicals and commercial enzymes

Sepharose 4B was obtained from Pharmacia and ion exchange resins from Whatman. Commercial enzymes and all other biochemicals were obtained from Sigma unless stated otherwise.

Results

Assay of inhibitor(s)

Extracts of dark-grown gherkin hypocotyls contain certain nondialysable components which inhibit gherkin phenylalanine ammonia-lyase when incubated with partially-purified enzyme preparations in the presence of 10 μM [^{3}H]phenylalanine. Inhibition of the lyase is linear with increasing concentrations of these components, up to at least 50% inhibition. An inhibitor unit is therefore defined as the volume of the preparation required to inhibit one unit of lyase by 50%; this allows inhibitor activity to be expressed as inhibitor units per ml.

Intracellular localisation of inhibitor(s)

Differential centrifugation of dark-grown gherkin hypocotyl extracts produced four fractions, in which the inhibitor was distributed at the following levels of activity: 500 $\times g$ pellet, 3.7 units/mg protein; 20 000 $\times g$ pellet, 1.3; 106 000 $\times g$ supernatant, 6.8; 106 000 $\times g$ pellet, 11.4. Phenylalanine ammonia-lyase was found exclusively in the 106 000 $\times g$ supernatant at an activity of 0.18 nmol cinnamate $\cdot$ min^{-1} $\cdot$ mg^{-1} protein. Neither microsome nor soluble preparations from other plant tissues (for example, dark-grown mustard cotyledons and maize scutella), inhibit the gherkin lyase.

Specificity of inhibitor(s)

The gherkin soluble and microsome fractions inhibit phenylalanine ammonia-lyase isolated from gherkin hypocotyls and also from other plant tissues; for example, mustard cotyledons, radish roots and pea buds. These fractions do not inhibit a range of other enzymes including mustard and maize nitrate reductase, gherkin and maize NADPH: cytochrome *c* reductase and NADH: cytochrome *c* reductase, and commercial sources of α-amylase, glucose-6-phosphate dehydrogenase, β-galactosidase and β-glucosidase. They do, however, inhibit cinnamic acid-4-hydroxylase isolated from gherkin hypocotyls and pea buds in addition to the lyase. The specificity of the inhibitor for the lyase and the hydroxylase is retained even after solubilisation from microsomal membranes (see below). It is not certain whether a single component in these extracts inhibits both the lyase and the hydroxylase but the results are compatible with this interpretation.

Solubilisation of inhibitor from microsomal membranes

When microsomes are subjected to treatments which alter the electrostatic environment of membranes (e.g. low ionic strengths with or without chelating agents) [25] no significant release of inhibitor from microsomes occurs. If microsomes are treated with detergents, on the other hand, at least 90% of the inhibitor is released. Removal of non-ionic detergents following treatment of microsomes is difficult; the ionic detergent sodium cholate, however, gives good inhibitor solubilisation and is easy to remove by desalting. Treatment of microsomes with cholate alone is most effective if the microsomes are sonicated prior to the detergent treatment (Table I). If cholate treatment is undertaken in the presence of 1 M KCl, sonication does not improve solubilisation. Treat-

TABLE I

RELEASE OF INHIBITOR FROM MICROSOMES FOLLOWING VARIOUS TREATMENTS WITH THE NON-IONIC DETERGENT SODIUM CHOLATE

The microsomal fraction was resuspended in Buffer C containing 10 mM sodium cholate ± 1 M KCl before stirring at 2°C for 15 min. The treated microsome suspensions were then centrifuged at 106 000 × *g* for 60 min. The resultant pellet was resuspended in Buffer C. Both the resultant pellet and the supernatant were desalted on a Sephadex G-25 column pre-equilibrated with Buffer C containing 0.3% Tween-20 before assaying with phenylalanine ammonia-lyase for inhibitory activity. In some cases the original resuspended microsome pellets were sonicated (3 × 20 s bursts, 1 μm amplitude on M.S.E. ultrasonic disintegrator, 100 watt model) before the addition of cholate ± 1 M KCl.

Treatment	Fraction	Units inhibitor per g fresh wt	Units inhibitor per mg protein
None	microsome	6.6	10.1
10 mM Cholate	pellet	9.9	41.4
	supernatant	1.8	15.7
10 mM Cholate + sonication	pellet	6.8	33.7
	supernatant	4.9	37.8
10 mM Cholate + 1 M KCl	pellet	1.4	14.4
	supernatant	11.5	38.5
10 mM Cholate + 1 M KCl + sonication	pellet	1.5	16.0
	supernatant	11.5	45.8

ment with cholate doubles the total inhibitor recovery. Sonication alone only released approx. 20% of the inhibitor from the microsomal membranes. Cholate treatment in the presence of 1 M KCl, without sonication, is used to solubilise the inhibitor in the present work.

These results suggest that the inhibitor is a component of the membranes, rather than being just loosely associated, since only treatments which alter the hydrophobic properties of membranes [25] allowed its satisfactory solubilisation. In the rest of this paper the inhibitor which has been solubilised from the microsomal membranes is referred to as the "microsomal inhibitor". The inhibitor found in the 106 000 × *g* supernatant following the original tissue fractionation is referred to as the "soluble inhibitor", but it may represent fragments of membrane not sufficiently dense to pellet under the conditions used.

Properties of the microsomal and soluble inhibitors

The microsomal and soluble inhibitors have similar, perhaps identical properties, suggesting that they contain the same active component. Both are non-dialysable and susceptible to digestion by trypsin and pronase indicating that the inhibitor is a protein. The inhibitor was treated at 25°C for 2 h with trypsin and pronase; the residual inhibitor activity was then assayed. The remaining inhibitor activities, compared to that of an untreated preparation (18.5 units/ml) were: trypsin-treated, 2.8; pronase-treated, 5.6; trypsin + pronase-treated, 6.4. The presence of polyclar AT during extraction or purification of the inhibitor did not affect its recovery. Heating at 90°C for 10 min, at 70°C for 30 min and at 50°C for 70 min destroyed 50% of the inhibitor activity. The heat stability previously observed [12] was due to the presence of phenolic substances in the crude extracts.

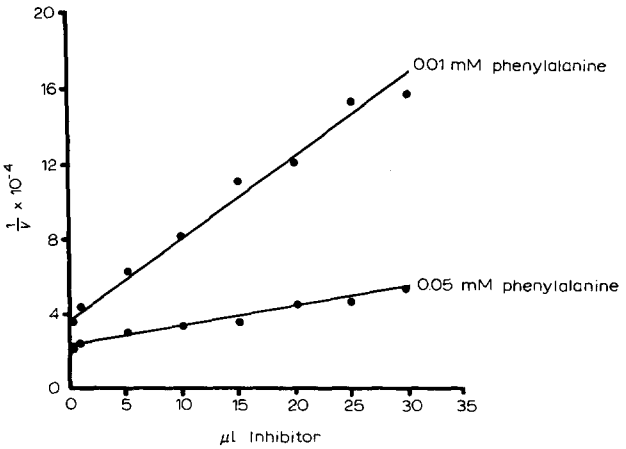

Fig. 1. The effect of inhibitor concentration on phenylalanine ammonia-lyase activity. The lyase and inhibitor were incubated with either 0.1 mM or 0.05 mM phenylalanine.

Gherkin phenylalanine ammonia-lyase exhibits negative cooperativity towards its substrate, phenylalanine, and thus two values for K_m (8.30 and $1.03 \cdot 10^{-5}$ M) and V (1.78 and 0.78 nmol cinnamate $\cdot$ min$^{-1} \times 10^{-3}$) can be determined. In the presence of the inhibitor, V is unaffected (1.80 and 0.83 nmol cinnamate $\cdot$ min$^{-1} \times 10^{-3}$) but the calculated values of K_m at both high and low substrate concentrations are increased (10.70 and $2.78 \cdot 10^{-5}$ M). This shows that the inhibitor is competitive with the substrate for the lyase. The Dixon plot (Fig. 1) relating lyase activity and amount of inhibitor added is a straight line, indicating a freely reversible and freely dissociable interaction between enzyme and inhibitor [27]. The slope of the line is reduced as the substrate concentration increases, again indicating the competitive nature of the inhibitor. Since the inhibitor $\cdot$ lyase complex has a high dissociation constant, changes in enzyme concentration, at a constant inhibitor concentration, have no effect on percentage inhibition.

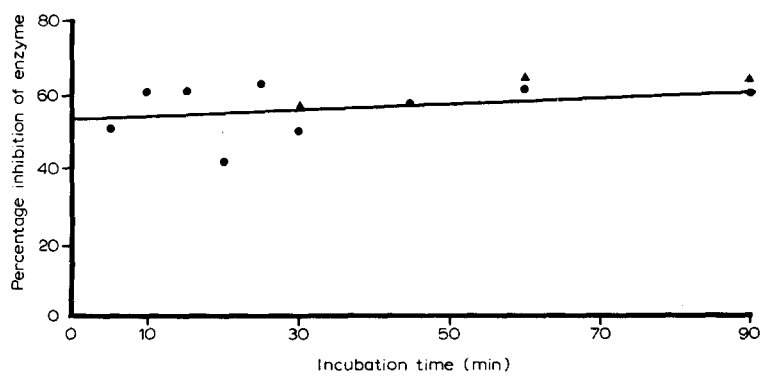

Fig. 2. Inhibition of phenylalanine ammonia-lyase during incubation at 25°C with microsomal inhibitor. The plotted lines were derived from a regression analysis of the results. Inhibition during incubation at 37°C in the presence of the inhibitor is also included (▲, at 30 min, 60 min and 90 min).

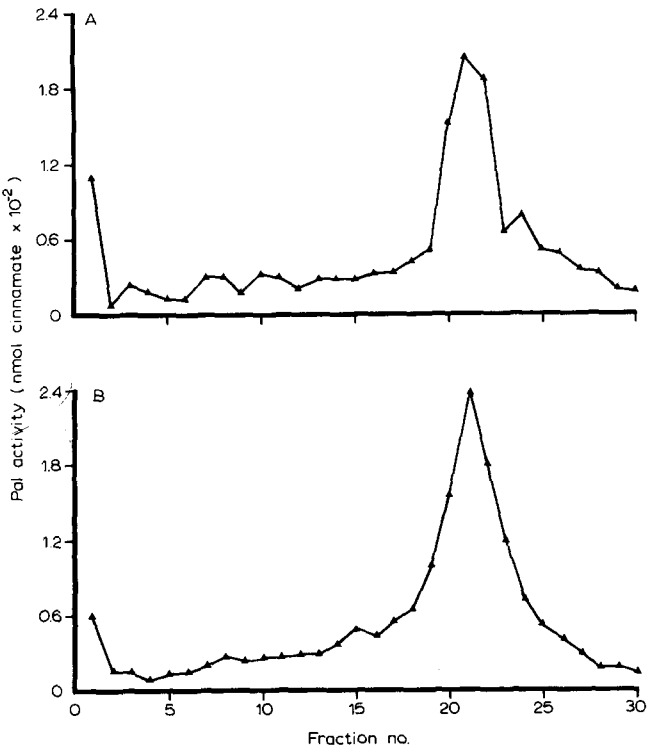

Fig. 3. Non-denaturing polyacrylamide gel electrophoresis of phenylalanine ammonia-lyase in the absence (A) and presence (B) of 100 μl microsomal inhibitor. In B the lyase was pre-incubated for 5 min with inhibitor at 25°C before loading. After running the gels, lyase activity was measured along their lengths by incubating gel sections with 0.1 mM L-[4-^{3}H]phenylalanine (1 mCi/mol) for 12 h.

The reversible nature of the lyase-inhibitor interaction was confirmed by further experiments. Inhibition of the lyase by the inhibitor in the presence of 10 μM phenylalanine (a substrate which allows marked inhibition) is immediate and constant throughout a 90 min incubation (Fig. 2). Inhibition is independent of the assay temperature in the range 25°C—37°C (Fig. 2). This non-progressive inhibition indicates that the inhibitor is not degrading the enzyme. Upon electrophoresis of a mixture of lyase and inhibitor, lyase activity was recovered in full; i.e. the inhibition was reversed when lyase and the inhibitor were separated (Fig. 3).

Purification of inhibitor

(a) Gel filtration. Both the soluble and microsomal inhibitor preparations gave unusual elution profiles following gel filtration on Sepharose 4B. Substantial amounts of both the soluble and microsomal inhibitors, in the presence or absence of sodium cholate or Tween-20, are excluded from Sepharose 4B, with heterogeneous peaks of inhibition extending to V_t (Fig. 4). It thus appears that the inhibitor is aggregated to a variable extent under these elution conditions. Extraction and fractionation at lower pH values (pH 7.0 or 6.0 instead of the

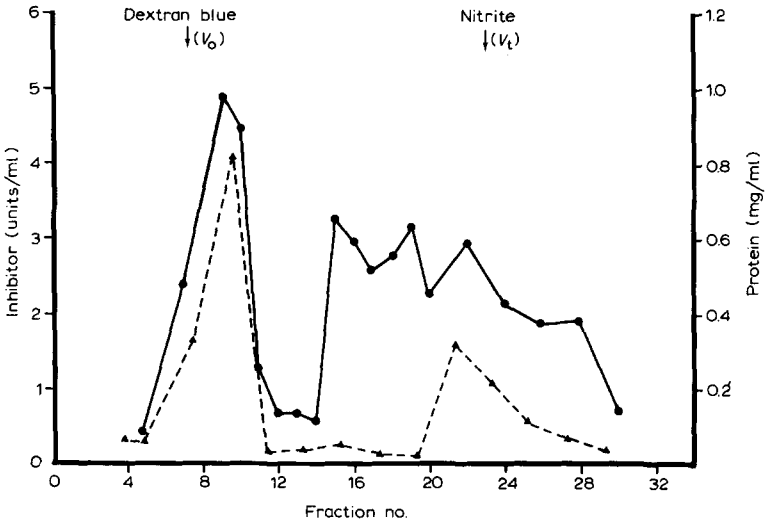

Fig. 4. Gel filtration of microsomal inhibitor on Sepharose 4B in Buffer C containing 0.5 mM sodium cholate. ●———●, inhibitor (units per ml); ■- - - - -■, phenylalanine ammonia-lyase activity (nmol per h); ▲- - - - -▲, protein (mg per ml). The column (1 X 22 cm) was eluted at the rate of 0.20 ml per min and 1 ml fractions collected and aliquots assayed with partially purified lyase.

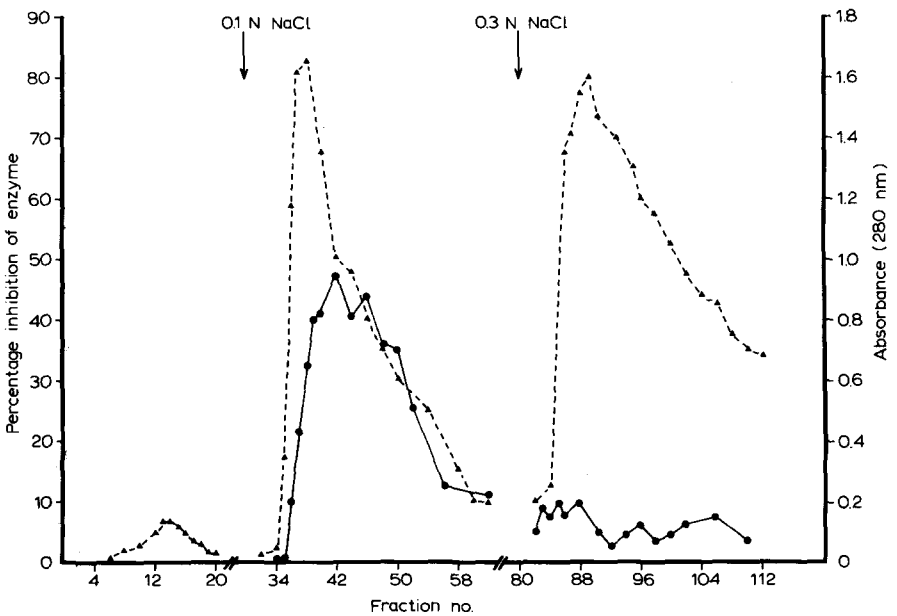

Fig. 5. DEAE-cellulose chromatography of microsomal inhibitor. The inhibitor fraction was desalted on Sephadex G-25 pre-equilibrated with Buffer C containing 0.1% Tween-20. This desalted inhibitor was loaded on to DEAE-cellulose column (7.0 X 1.5 cm) pre-equilibrated with Buffer C containing 0.1% Tween-20. After elution of non-retained protein the retained protein was eluted step-wise with NaCl at the concentrations indicated. 2-ml fractions were collected and aliquots assayed with partially purified phenylalanine ammonia-lyase. ●———●, inhibitor; ▲- - - - -▲, absorbance 280 nm.

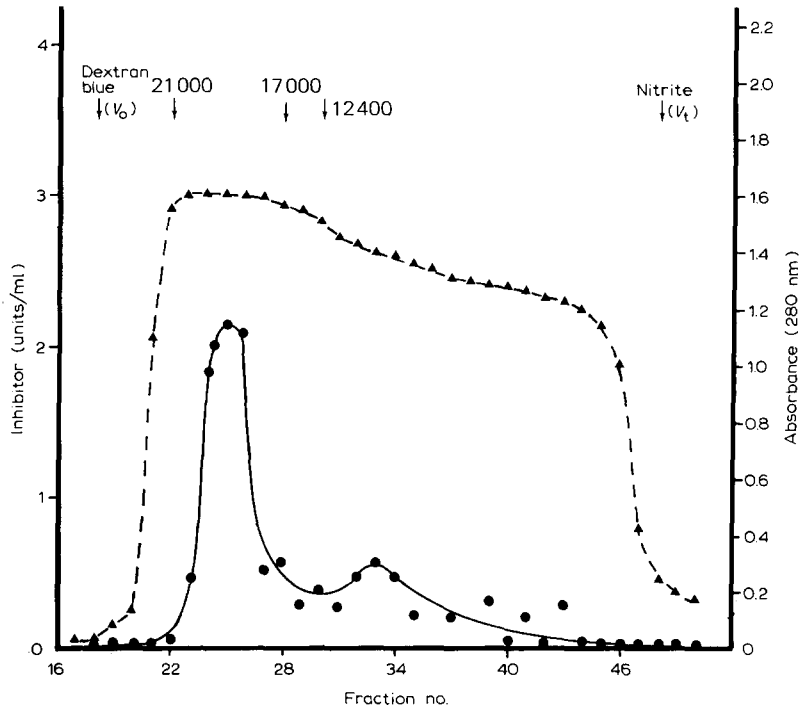

Fig. 6. Gel filtration of inhibitor fraction bound to DEAE-cellulose on Sephadex G-50-80 in Buffer C containing 0.3% Tween-20. Inhibitor fraction bound to DEAE-cellulose was eluted with 0.1 M NaCl (Fig. 5), concentrated by ultrafiltration and loaded on to the column. The column (1 X 22 cm) was eluted at the rate of 0.5 ml/min and 0.3 ml fractions collected and assayed with partially purified phenylalanine ammonia-lyase. Results expressed as lysate activity (cpm/h) in the presence of column fractions. Column was calibrated with trypsin inhibitor T_1 (M_r = 21 000, soybean, Boehringer), myoglobin (M_r = 17 000, horse heart Type 111) and cytochrome *c* (M_r = 12 400, horese heart Type 111). ●———●, lyase activity; ▲- - - - -▲, absorbance 280 nm.

usual pH 8.0) or fractionation in the presence of 0.3 M KCl did not alter the elution properties of the inhibitor.

(b) Ion-exchange chromatography. In the presence of 0.1% Tween-20, all of the inhibitor normally binds to DEAE-celluose (Fig. 5); on occasions, however, a small proportion of the inhibitor remained unbound. The bound inhibitor elutes following a step addition of 0.1 M NaCl (Fig. 5) and when subsequently fractionated on Sepharose 4B, is not excluded. Indeed, the inhibitor eluted from DEAE-cellulose is retained on precalibrated Sephadex G-50 with an elution profile corresponding to a molecular weight of approx. 19 000 (Fig. 6).

Discussion

The macromolecular inhibitor isolated from dark grown gherkin hypocotyls is a small ($M_r \approx 19\,000$) thermolabile protein which is sensitive to proteolytic digestion. It inhibits not only phenylalanine ammonia-lyase but also cinnamic acid-4-hydroxylase, both isolated from several plant sources. On the other

hand, it does not inhibit a wide range of other enzymes, including β-galactosidase and β-glucosidase which are assayed by incubation with aromatic substrates (*o*-nitrophenyl galactoside and *o*-nitrophenyl glucoside respectively), suggesting that the specificity to the lyase and the hydroxylase is not solely based on the aromatic ring structure of their substrates.

Cinnamic acid-4-hydroxylase immediately follows phenylalanine ammonia-lyase in the phenylpropanoid biosynthetic pathway and both enzymes are thought to have regulatory roles in phenol metabolism [28,29]. Furthermore, the activities of the lyase and the hydroxylase are often concurrently regulated in response to different external stimuli [30—34]. The inhibitor may thus be specifically involved in controlling the activities of two important, consecutive enzymes in the central pathway of phenylpropanoid synthesis. This suggestion is supported by the intracellular distribution of the inhibitor and of the two enzymes. In crude extracts, half of the total inhibitory activity is found in the microsomal pellet, and the remainder in the supernatant. This may indicate that the inhibitor is a mobile component communicating between the cytosolic and the membrane phase of the cell. The ready aggregation of the "soluble" inhibitor suggests that it is lipophilic, or still has lipid material associated with it. The inhibitor precipitates over a wide range of ammonium sulphate concentrations and is difficult to re-dissolve; furthermore, butanol extraction leads to a loss of inhibitory activity. Both of these observations suggest the inhibitor has a lipophilic nature [25]. The lyase is predominantly a cytosolic enzyme [35,36], whilst the hydroxylase is found exclusively on the endoplasmic reticulum [18,37—39]. Concurrent regulation of the activities of spatially separated enzymes may be entirely fortuitious; alternatively, a specific integrating factor may be involved, and both cinnamic acid and *p*-coumaric acid have been proposed for this role [40]. The results described here suggest, as a further possibility, the involvement of a macromolecular inhibitor of both enzymes in this concurrent control. Environmental stimuli might, for example, change the intracellular distribution of the inhibitor and/or its orientation within the membrane, resulting in correlated changes in the activities of both the lyase and the hydroxylase.

If such an inhibitor is to have a potential physiological role, its effect must be reversible. The kinetic behaviour of the inhibitor, coupled with the fact that the lyase can be separated from the inhibitor on polyacrylamide gels without loss of activity, indicates that the lyase-inhibitor interaction is freely reversible. The properties of the inhibitor, therefore, are consistent with the postulated existence of inactive phenylalanine ammonia-lyase in dark grown hypocotyls and its activation by light [10,11]. Inactive forms of the lyase have also been shown to be present in dark grown radish cotyledons by immunochemical techniques [41] and in mustard cotyledons by density labelling [14]. An inhibitor of the lyase has been postulated in aged potato discs [42] and another isolated from sunflower cotyledons [43], but the sunflower inhibitor is of unknown specificity and may be a protease, i.e. its effect is progressive and not reversible. Recently, activation of pre-existing inactive enzyme has been proposed to account for a 4- to 10-fold increase in hydroxycinnamoyltransferase activity in tomatoes following chilling [44]. It is worth noting, however, that the gherkin inhibitor reported here is the only inhibitor which specifically inhibits enzymes of the phenylpropanoid pathway in a reversible manner.

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