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PURIFICATION TO HOMOGENEITY AND SOME PROPERTIES OF L-PHENYLALANINE AMMONIA-LYASE OF IRRADIATED MUSTARD (*SINAPIS ALBA* L.) COTYLEDONS

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Summary

1. Lyase (L-Phenylalanine ammonia-lyase, EC 4.3.1.5) from far-red light-irradiated mustard cotyledons was purified to a single protein using ammonium sulphate fractionation, column chromatography on L-phenylalanyl-Sepharose 4B and on Sephadex G-200, isoelectric focusing and polyacrylamide gel electrophoresis.

2. The enzyme constituted 0.01% of total cellular protein, did not catalyse the deamination of L-tyrosine, had a pH optimum of pH 8.6 and an isoelectric point of pH 5.6.

3. The sedimentation coefficient was estimated as 11.3 S, the Stokes' radius 4.25 nm, and the molecular weight 240 000 ± 9000 (S.E.).

4. Electrophoresis on denaturing polyacrylamide gels gave a single stained protein band corresponding to a subunit molecular weight of 55 000 indicating a tetrameric structure of equal (or near-equal) size subunits.

5. Maximum velocity (V) for the purified lyase at 25°C was 3.83—4.10 nkat · l^{-1} enzyme and the K_m value 0.151—0.154 mM. Negative cooperativity (Hill coefficient, $n = 1.08$) was not detected over the substrate concentration range tested.

6. A putative non-diffusible inhibitor isolated from dark-grown gherkin hypocotyls inhibited the homogeneously purified mustard lyase.

Introduction

The allosteric enzyme lyase (L-phenylalanine ammonia-lyase, EC 4.3.1.5), which catalyses the non-oxidative deamination of L-phenylalanine to *trans*-

cinnamic acid, has attracted considerable attention because of its wide distribution [1,2] and its sensitivity to a variety of stimuli [1—3]. The enzyme has been purified to varying degrees from a number of higher plants [4—6].

Of notable interest has been the dramatic and transient increase in assayable levels of lyase activity mediated by light [7]. This phenomenon had led to the lyase becoming a tool used by many laboratories to study the mechanism of action of photomorphogenic pigments in the control of enzyme levels [8—13]. To date, no unifying hypothesis has emerged [2,14]. Given the complexity and variety of regulatory processes in eukaryotes, it would seem eminently feasible that photocontrol through synthesis de novo in parsley (*Petroselinum hortense* Hoffm.) [13,15] and through enzyme activation in radish (*Raphanus sativus* L.) [8] and gherkin (*Cucumis sativus* L.) [10] could coexist side by side. It remains our view, however, that evidence for the activation of the lyase is as yet insufficiently rigorous to be conclusive [11]. At the heart of present debate is phytochrome control of the lyase specifically in mustard cotyledons. In this plant labelling studies based on the measurement of differences [16] in deuteration of lyase protein between irradiated and dark-grown seedlings have produced conflicting and subsequently much criticized [9,11,12,17,18] data.

It seems clear to us that a sufficiently rigorous immunochemical study [19] should reveal once and for all whether or not phytochrome primarily acts by stimulating synthesis de novo of lyase protein, as the elegant deuterium-labelling study of Tong and Schopfer [12] indicates. With this specific aim in mind we have purified the mustard lyase down to a single protein. The present paper reports on the procedures used and on some properties of this particular enzyme.

Materials and Methods

Plant material. Mustard seedlings (*Sinapis alba* L., Asgrow, Company, Freiburg-Ebnet, F.R.G., 1972 harvest) were germinated in darkness for 36 h at 25° C and transferred to continous far-red light [20] for 20 h.

Enzymes and chemicals. Partially purified *Rhodotorula glutinis* L-phenylalanine ammonia-lyase (spec. act. 1.43 units · mg^{-1}) was obtained from PL-Biochemicals (Milwaukee, WI, U.S.A.). Catalase (EC 1.11.1.6) (beef liver), β-galactosidase (EC 3.2.1.23) (*Escherichia coli*) and phosphofructokinase (EC 2.7.1.11) (rabbit muscle) were obtained from Boehringer Mannheim (Melbourne, Australia); peroxidase (EC 1.11.1.7) (horseradish, A grade) from Calbiochem (Sydney, Australia) and alcohol dehydrogenase (EC 1.1.1.1) (Yeast), phosphorylase *a* (EC 2.4.1.1) (rabbit muscle), L-glutamate dehydrogenase (EC 1.4.1.2) (liver), lysozyme (EC 3.2.1.17) (egg white) and bovine serum albumin (99%) used for routine protein assay from Sigma, St. Louis, MO, U.S.A. Thyroglobulin, ferritin, catalase, lactate dehydrogenase (EC 1.1.1.27) and bovine serum albumin monomer used for marker proteins on polyacrylamide gradient gels were obtained as a kit from Pharmacia (Sweden) as were Pharmalyte, CNBr-activated Sepharose 4B and Sephadex. Ampholine and Ultrogel AcA 34 were purchased from LKB (Sweden); dichlorodimethylsilane from Ajax (Australia) and ultrapure $(NH_4)_2SO_4$ and sucrose for gradients from Mann Research Laboratories (Orangeburg, NJ). All other chemicals were of the highest grade commercially available.

Extraction and purification. All operations were performed at 3°C. Harvested cotyledons were homogenized in 50 mM Tris-HCl (pH 8.6)/10 mM isoascorbate/1 mM EDTA (1 ml/ten cotyledon pairs) in a Polytron PCU-2 at half-speed for 1 min, cell debris removed by centrifugation at 29 500 × g_{av} for 25 min and pooled samples applied to a Sephadex G-25 column (380 × 26 mm) previously equilibrated in homogenizing buffer. Protein-containing effluent was saturated to 30% with $(NH_4)_2SO_4$, the precipitate removed by centrifugation at 29 500 × g_{av} for 25 min, the resultant supernatant made to 45% saturation and the pellet formed by a repeat centrifugation was resuspended in 10 mM K_2HPO_4-KH_2PO_4 (pH 6.0)/0.55 M sorbitol/10 mM isoascorbate/0.5 mM EDTA (buffer 1) (2.25 mg protein · ml^{-1}). L-Phenylalanyl-Sepharose 4B [5] (270 × 15 mm column) was equilibrated in buffer 1; the extract bound at pH 6.0 (150 mg protein/column) and the column washed then with 70 ml buffer 1. A linear elution gradient (1—10 mM L-phenylalanine in 5 mM Tris-HCl (pH 9.0) (buffer 2) containing the same concentrations of sorbitol, isoascorbate and EDTA as buffer 1 was applied and the portion of gradient eluted corresponding to 2—6 mM L-phenylalanine collected. The extract was concentrated (Centriflo CF 50A membrane cones) to approx. 4—6 mg protein · ml^{-1} and applied to a column of Sephadex G-200 (380 × 26 mm) previously equilibrated in buffer 2 without sorbitol. The effluent between 65 and 120 ml was collected and concentrated. The concentrate (6—8 mg protein · ml^{-1}) was made to 1% (w/v) in glycine, 0.01% (v/v) in Triton X-100 and 1% (w/v) in Ampholine pH 4—6 (or Pharmalyte pH 4—6.5), applied to an isoelectric focusing column (LKB 8101 or 8102) and focused as described in the legend to Fig. 1. Peak fractions (corresponding to pH 5.4—5.7) were collected, concentrated to approx. 1—2 mg protein · ml^{-1} and layered onto preparative polyacrylamide (7% w/v) slab gels (7 × 5.5 × 0.3 cm thick) containing buffer and chemicals as described in the legend to Fig. 2. Electrophoresis was performed at 340 V/25 mA for 5 h at 3°C on a Gradipore (Australia) electrophoresis unit. Gels were then cut into 1 mm slices, each slice eluted (100 mM Tris-HCl (pH 8.6)/0.5 mM EDTA/0.5 mM β-mercaptoethanol) and resulting solutions assayed for L-phenylalanine ammonia-lyase activity. Peak fractions were pooled, concentrated, layered onto 5% (w/v) preparative polyacrylamide slab gels and electrophoresis repeated as above.

Assays. Protein and lyase activity as assayed by the spectrophotometric method were estimated as previously described [11]. Except where stated otherwise, lyase activity was determined by isotopic assay using ring-labelled L-phenylalanine and a 20 h incubation (25°C) as described by Attridge et al. [9] except that 100 mM Tris-HCl (pH 8.6) replaced the borate buffer. Lyase activity is expressed in katals [21]. β-Galactosidase activity was estimated as described previously [16], peroxidase by the method of Evans [22], alcohol dehydrogenase by the procedure of Racker [23] and catalase by the procedure of Aebi [24] modified to measure absorbance difference A_{240nm} minus A_{320nm} in a Hitachi 156 spectrophotometer. These enzymes were used as markers on sucrose gradients and/or AcA 34 chromatography columns. Peak positions were always determined by enzyme assay.

Results

Purification

The resultant L-phenylalanine ammonia-lyase preparation constituted approx. 0.01% of total extractable cell protein and represented a final purification of 200-fold (Table I). A notable feature of the purification procedure was our inability, despite numerous attempts [25] (but see Discussion) to obtain better than a three-fold purification on our affinity column (Table I). A second unexpected finding was a minor peak at pH 5.3 on the isoelectric focusing column (Fig. 1). Only protein at the major peak of lyase activity (p*I* at pH 5.6) was carried forward to preparative gel electrophoresis (see legend to Fig. 1). It seems unlikely that the appearance of an additional protein, capable of eliminating an ammonium ion from L-phenylalanine, is induced by phytochrome since the focusing pattern of lyase extracted from etiolated cotyledons of the same age was identical (Ref. 26 and unpublished data).

Criteria for purity

Single bands, representing the total protein running on the gel, were obtained by polyacrylamide gel electrophoresis without (Fig. 2) and with (data not presented) sodium dodecyl sulphate. Both the stained protein band on the non-denaturing gel (R_F 0.18) (Fig. 2) and the protein band on the sucrose gradient following centrifugation (Fig. 3) (final density position at 1.0610 $kg \cdot l^{-1}$) coincided exactly with single and sharp peaks of lyase activity (R_F 0.18, 1.0610 $kg \cdot l^{-1}$).

Physical properties

The sedimentation coefficient of mustard lyase, calculated from the rate of sedimentation relative to catalase marker according to the procedure of Martin and Ames [28] and assuming a partial specific volume of 0.725 $l \cdot kg^{-1}$ for each

TABLE I

PURIFICATION OF L-PHENYLALANINE AMMONIA-LYASE FROM MUSTARD COTYLEDONS

Seeds were germinated in darkness and seedlings grown in darkness for 36 h and then transferred to continuous far-red light for 20 h prior to harvesting.

Step	Protein		Activity		Purification	
	kg ($\times 10^6$)	%	pkat	nkat/kg	% yield	-fold
Homogenate	1140	100	2060	1.81	100	1.0
30—45% $(NH_4)_2SO_4$ saturation	362	32.0	1348	3.72	65	2.1
L-Phenylalanyl-Sepharose 4B	36.0	3.16	344	9.56	17	5.3
Sephadex G-200	15.6	1.37	263	16.9	13	9.3
Isoelectric focusing	0.88	0.08	119	135	6	75
Preparative gel electrophoresis						
(i) 7% polyacrylamide	0.18	0.02	63.6	353	3	195
(ii) 5% polyacrylamide *	0.10	0.01	38.4	384	2	212

* The second cycle of electrophoresis failed to improve purification substantially but was required to remove minor protein contaminants.

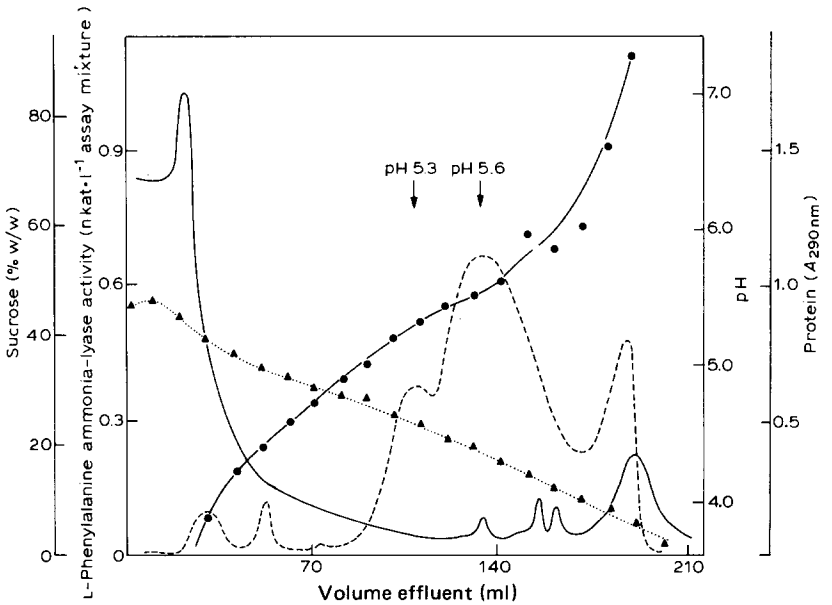

Fig. 1. Isoelectric focusing of L-phenylalanine ammonia-lyase. Extract from the G-200 column (Table I) (8—10 mg protein) was applied to the centre of an LKB 8101 column previously coated with dichlorodimethylsilane and focused for 9 h (1500 V, 3 mA final settings) (3°C) in a pH 4—6 Ampholine gradient (●) in a 0—40% (w/w) sucrose gradient (▲) containing 5 mM Tris-HCl (pH 8.6)/1% (w/v) glycine/0.01% (v/v) Triton X-100. The anode is on the left. Lyase (- - - - - -) was assayed by increase in absorbance at 290 nm in the presence of L-phenylalanine and protein (———) by absorbance at 290 nm in the absence of L-phenylalanine.

protein, was 11.3 S. In fact the mustard lyase and catalase peaks coincided exactly (1.0610 kg · l^{-1}) (Fig. 3). Partially purified *R. glutinis* lyase was run as an internal standard throughout the procedures described in this paper since the characteristics of the highly purified enzyme have been well-defined [29,30]. We confirm the finding of Hodgins [29] that this yeast lyase has a higher sedimentation coefficient (11.5 S in our hands) than catalase.

Chromatography of marker enzymes on Ultrogel AcA 34, showed the expected linear relationship between $(K_D)^{1/3}$ and Stokes' radius [34] (data not presented). Values for Stokes' radii are those recently obtained on the same chromatographic column by Burgoyne and Skinner [31]. These are smaller than earlier estimates (see e.g. Ref. 32). We estimate the Stokes' radii of mustard and *R. glutinis* lyases to be 4.25 and 4.33 nm, respectively, at pH 8.6 in 100 mM Tris-HCl buffer.

The molecular weight of mustard lyase was calculated from the relationship $M_1/M_2 = s_1a_1/s_2a_2$ where M, s and a represent molecular weight, sedimentation coefficient ($S_{20,w}$) and Stokes' radius, respectively. Applying the values M_1 and s_1 for catalase given in the legend to Fig. 3, we obtain a molecular weight of 250 000. This agrees closely with our molecular weight determinations by gel exclusion chromatography and electrophoresis in a polyacrylamide gel gradient of 255 000 and 225 000, respectively. The molecular weight of the single subunit was calculated as 55 000 on calibrated polyacrylamide gels. This is

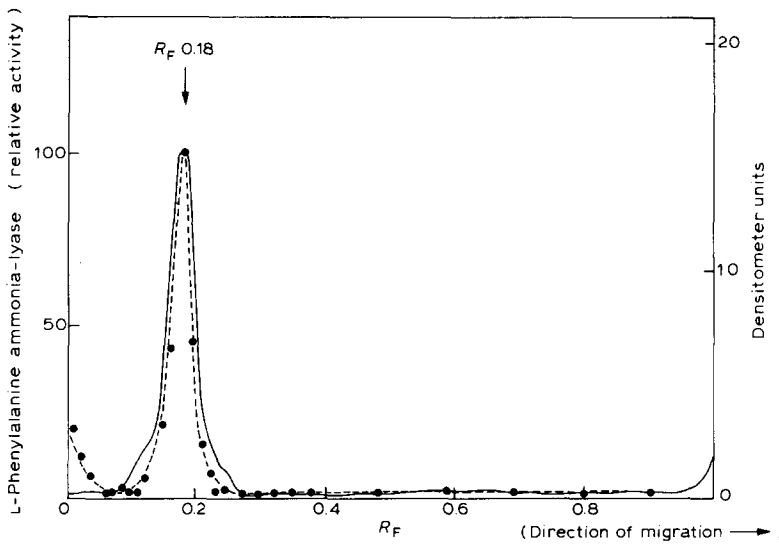

Fig. 2. Polyacrylamide gel electrophoresis of homogeneously purified L-phenylalanine ammonia-lyase in the absece of sodium dodecyl sulphate. Lyase eluted from the 5% (w/v) preparative polyacrylamide gel (Table I) was applied to 7% (w/v) analytical polyacrylamide gels (15—20 μg protein/gel) (gel made up in 3.0 M Tris-HCl (pH 8.9) containing *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethyl-1,2-diaminoethane and ammonium persulphate as described in Ref. 27). Electrophoresis (migration to anode) was carried out for 3 h at 2 mA (constant current)/tube at 3°C. Gels were either stained (0.2% (w/v) Coomassie brilliant blue R) and scanned on densitometer (———) or cut into 1.0 mm slices and assayed for lyase activity (•). Data are from gels run at the same time. Both lyase activity and the protein band were localised at R_F 0.18.

consistent with a lyase composed of four subunits of identical or very similar (see Ref. 33) size and having a molecular weight of 220 000. Thus, the average molecular weight of the homogeneously purified mustard lyase as 240 000 ± 9000 (S.E.). Accuracy of this determination is further verified by the average value for the internal standard lyase of 278 000 ± 8000 (S.E.), which is identical (within given limits of error) to the estimate of 275 000 by Hodgins [29].

Effect of pH on mustard lyase activity

Using three different buffer systems (potassium phosphate, sodium barbital, glycine/NaOH, all at 50 mM) the homogeneously purified lyase gave the broad response curve to pH (see e.g. Refs. 5 and 34) characteristic of this enzyme (data not presented). The optimum at 25° C was at pH 8.6.

L-Tyrosine ammonia-lyase activity

Some purified preparations of L-phenylalanine ammonia-lyase catalyse the deamination of L-tyrosine to *trans-p*-coumaric acid [1,35,36]. We tested our homogeneously purified mustard preparation for L-tyrosine ammonia-lyase activity. None was detected by the spectrophotometric assay of Havir et al. [37] using a final L-tyrosine concentration of 1.33 mM in 100 mM Tris-HCl at pH 8.6 (data not presented).

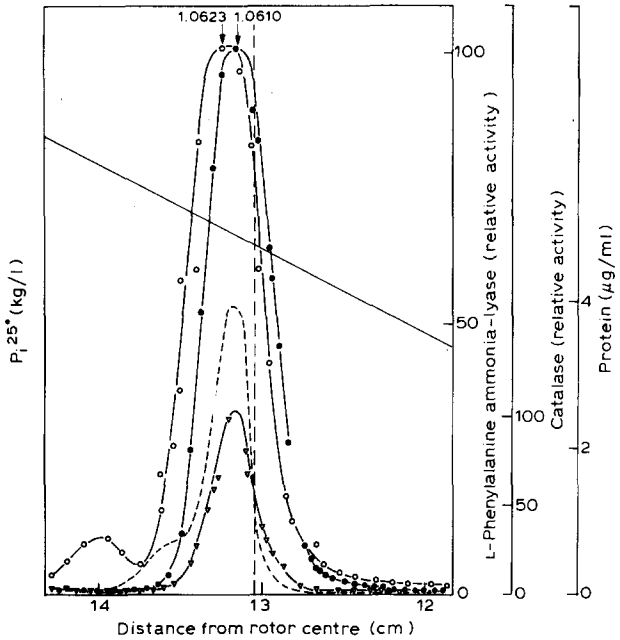

Fig. 3. Rate-zonal centrifugation of intact homogeneously purified L-phenylalanine ammonia-lyase. Enzymes were layered onto linear 5—20% (w/w) sucrose gradients (5.3 ml) in 100 mM Tris-HCl (pH 8.6)/0.5 mM EDTA/0.5 mM β-mercaptoethanol, tubes filled with paraffin, centrifuged (SW-41Ti rotor at −2°C; integrated field time $8.6 \cdot 10^7$ g · min at r_{av} 12.5 cm), gradients unloaded as previously described [11] and enzymes assayed. Data are from three gradients run in parallel: gradient (i) containing purified mustard lyase (50 μg protein) for lyase assay (•) and β-galactosidase (gradient marker enzyme); gradient (ii) containing *R. glutinis* lyase (○), catalase (▽) (marker enzyme of known molecular weight (244 000) and sedimentation coefficient (11.3 S)) and β-galactosidase; gradient (iii) containing purified mustard lyase (50 μg protein) for protein assay (- - - - - -) only. Gradients (i) and (ii) were overlayered at the lower density peak of the galactosidase (— —), gradient (iii) was aligned without assitance of a gradient marker protein. Gradient is indicated by the unbroken line. Corrected densities attained at end of run were 1.0623 kg · l^{-1} for *R. glutinis* lyase run as an internal standard and 1.0610 kg · l^{-1} for mustard lyase, catalase and protein.

Substrate concentration

The values for K_m and maximum velocity (V) estimated from the linear Eadie-Hofstee single-reciprocal plot (not presented) were 0.154 mM and 4.10 nkat · l^{-1} enzyme, respectively, at 25°C. A Lineweaver-Burk double-reciprocal plot (not presented) of the same data also gave a straight line and values of 0.151 mM for K_m and 3.83 nkat · l^{-1} enzyme for V. The Hill coefficient [38] for the lyase reaction was calculated as 1.08. Thus, negative cooperativity, if it existed over the 0.1—1.8 mM substrate concentration range tested, was not appreciable at 25°C.

Effect of gherkin lyase inhibitor

Homogeneously purified mustard lyase was inhibited by 39% and by 48% by high concentrations of microsomes and a microsomal extract respectively prepared from gherkin hypocotyls (Table II). However, we found inhibition difficult to detect when inhibitor and lyase proteins were incubated in equal

TABLE II

INHIBITION BY MICROSOMES AND MICROSOMAL EXTRACT FROM GHERKIN HYPOCOTYLS OF HOMOGENEOUSLY PURIFIED L-PHENYLALANINE AMMONIA-LYASE FROM MUSTARD

Freshly prepared and dialysed microsomal fraction and 10 mM sodium cholate/1 M KCl microsomal extract, prepared as described by Billett et al. [4], were incubated (37°C) at ten times and five times the protein concentration, respectively, as the mustard lyase. Rates calculated from time course assay run over 2 h using 10 μM L-phenylalanine in 100 mM Tris-HCl (pH 8.6). The inhibitor units are as defined by Billett et al. [4]: one unit of inhibitor is the amount of gherkin preparation required to inhibit 375 pkat mustard lyase.

Additions	Lyase activity (nkat · l^{-1} enzyme)	% inhibition	Units inhibitor	
			g fresh wt.	mg gherkin protein
None (control)	5.83	—	—	—
Microsomes	3.56	39	5.09	41.1
10 mM cholate/1 M KCl extract from microsomes	3.05	48	4.98	105.9

amounts (data not presented), suggesting that the interaction could be relatively unspecific.

Discussion

The mustard lyase preparation is judged pure on the basis that contaminating protein was not detected on either polyacrylamide zymograms or sucrose gradients, and that the lyase constituted approx. 0.01% of soluble cell protein [5,39]. In line with recent highly purified [34] or homogeneous [5] preparations, L-tyrosine ammonia-lyase activity was not apparent.

The molecular weight estimate of 240 000 is based on marker proteins (Fig. 3 and previously cited data) and would be judged as accurate on the basis of a molecular weight of 275 000 [29] for the *R. glutinis* lyase run as an internal standard. However, we must add the constraint that protein-protein interactions in the partially purified standard, which gave in excess of ten protein bands on our zymograms, could introduce error into the inbuilt check. In addition, if the conventional marker proteins are ignored and values determined solely from the *R. glutinis* enzyme at the more recent molecular weight estimate of 332 000 [30], values of 280 000 and 60 000 would be obtained for undissociated mustard enzyme and subunit, respectively. Thus, either way, the mustard lyase is relatively small [1,2,39]. Similarly, the substantially low value [28,32,34] for the Stokes' radius (4.25 nm) cannot entirely be explained on the basis of updated (and lower) data [31] for the marker proteins. Together, these data raise the serious question of proteolytic damage during purification. Unfortunately, we cannot completely exclude this possibility. However, rigorous attempts to obtain larger sized subunits by including protease inhibitors (e.g. phenylmethylsulphonylfluoride at 1.0 mM) at purification failed (e.g. subunit molecular weight 53 500 obtained). A high and single K_m value (K_m 0.154 mM; lack of negative homotropic interactions) obtained for the mustard lyase must be viewed in light of the constraint that weak negative cooperativity

of the type reported for the sweet potato enzyme at 40°C [5] would go undetected. In conclusion, it could be argued against the view of Havir and Hanson [30] that, taken as a whole, our data for molecular size, shape and relatively low affinity for substrate fit a general pattern which is reminiscent of a lyase from a fungus [29,34,36].

A number of differences with earlier reports on mustard L-phenylalanine ammonia-lyase are worthy of especial note. Firstly, the enzyme did not aggregate in 5 mM Tris buffer at pH 8.6 in our expeirments, as in those of Schopfer [40]. Secondly, the molecular weight estimate is somewhat lower than the 300 000 of the same enzyme [40]. Thirdly, and most importantly, we were unable to obtain the 60—70-fold purification of mustard lyase by affinity chromatography recently reported by Billett et al. [4] using the protocol of Blondel et al. [41] which involves binding of L-phenylalanine to the matrix through the amino group (CNBr-Sepharose). Furthermore, no better than a three-fold purification was obtained on the same conjugate using the procedure of Tanaka and Uritani [5] (Table I). We have already expressed the opinion that some other process, possibly ion exchange, is responsible for the generally poor performance (Mack, J., personal communication and Rollin, P., personal communication) of L-phenylalanyl-Sepharose columns * [25]. This viewpoint is strictly in line with the theoretical considerations of expecting an enzyme, which eliminates an ammonium ion, and which is highly specific for substrate [5], recognizing the immobilized ligand substituted at that ammonium ion.

It is puzzling that while mustard does not contain a proteinaceous lyase inhibitor [4], homogeneously purified mustard lyase is more sensitive to the gherkin inhibitor than is the gherkin enzyme (Table II) [4]. Prompted by a referee's suggestion, we considered the possibility of phenolic compounds becoming adsorbed during cell rupture (microsomes are subject to adsorption artefacts [42]) onto proteins, one (or a group) of which had associated boundary lipid. An artefactual aromatic lipoprotein complex of this type could meet the requirements [4] for extraction from microsomal-enriched pellets, and sensitivity to heat would be conferred through free radical chain reactions of lipid peroxidation [43]. We could find no overwhelming reason for dismissing this concept in either our own, or the earlier study [4]. On the contrary, both the wide distribution of the assumed lyase inhibitor amongst subcellular cell fractions and its strange heterogeneity in size [4] would be compatible with such a concept. Furthermore, the presence of unattached phenolic compounds (i.e. not an aromatic lipoprotein complex) in crude extracts have on one occasion already led Smith's group [4,44] to misinterpret heat-stable inhibition in terms of the existence of a reversible and specific lyase inhibitor. The best that can be said is that assigning a physiological role to the putative proteinaceous gherkin inhibitor [4] is both highly speculative and premature.

Finally, two points relating to phytochrome control of L-phenylalanine ammonia-lyase arise. Firstly, no difference in purification patterns were

* Binding the ligand through the carboxyl group (AH-Sepharose) did not improve capacity of the conjugate for lyase protein, nor prevent non-lyase protein being eluted by the L-phenylalanine gradient.

observed when the lyase was extracted from dark-grown (instead of irradiated) mustard cotyledons (data not presented). Thus, our data are consistent with the claim by Schopfer [40] that phytochrome does not induce synthesis of a novel lyase having distinguishing physical properties. Secondly, physical differences exist between our mustard enzyme and the gherkin lyase, albeit impure, preparations of Smith's group [4,45]. We would therefore caution the drawing by Smith and coworkers [4,46] of analogies with respect to photocontrol of these two enzymes which assumes a degree of uniformity in these two proteins, and which relies heavily on the least tenable set [9] (see Ref. 11) of the contradictory density-labelling data.

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References

1 Camm, E.L. and Towers, G.H.N. (1973) Phytochemistry 12, 961—973
2 Huault, C., Faye, L., Klein-Eude, D., Fourcroy, P., Blondel, J.-D. and Rollin, P. (1976) Etudes de Biologie Végétale (Jacques, R., ed.), pp. 575—600, Gauthier-Villars, Paris
3 Schopfer, P. (1977) Annu. Rev. Plant Physiol. 28, 223—252
4 Billett, E.E., Wallace, W. and Smith, H. (1978) Biochim. Biophys. Acta 524, 219—230
5 Tanaka, Y. and Uritani, I. (1977) J. Biochem. (Tokyo) 81, 963—970
6 Betz, B., Schäfer, E. and Hahlbrock, K. (1978) Arch. Biochem, Biophys. 190, 126—135
7 Mohr, H. and Schopfer, P. (1977) Nucleic Acids and Protein Synthesis in Plants (Bogorad, L. and Weil, H., eds.), pp. 239—260, Plenum, New York
8 Faye, L. (1977) Biochimie 59, 345—350
9 Attridge, T.H., Johnson, C.B. and Smith, H. (1974) Biochim. Biophys. Acta 343, 440—451
10 Attridge, T.H. and Smith, H. (1974) Biochim. Biophys. Acta 343, 452—464
11 Acton, G.J. and Schopfer, P. (1975) Biochim. Biophys. Acta 404, 231—242
12 Tong, W.-F. and Schopfer, P. (1976) Proc. Natl. Acad. Sci. U.S. 73, 4017—4021
13 Schröder, J. (1977) Arch. Biochem. Biophys. 182, 488—496
14 Smith, H. (1978) Trends Biochem. Sci. N204—N206
15 Wellmann, E. and Schopfer, P. (1975) Plant Physiol. 55, 822—827
16 Acton, G.J., Drumm, H. and Mohr, H. (1974) Planta 121, 39—50
17 Lamb, C.J. and Rubery, P.H. (1976) Biochim. Biophys. Acta 421, 308—318
18 Johnson, C.B. and Smith, H. (1978) Phytochemistry 17, 667—670
19 Tanaka, Y. and Uritani, I. (1977) Eur. J. Biochem. 73, 255—260
20 Acton, G.J. (1978) Photochem. Photobiol. 28, 361—367
21 Hoffmann-Ostenhof, O. (1974) Eur. J. Biochem. 45, 1—3
22 Evans, J.J. (1970) Plant Physiol. 45, 66—69
23 Racker, E. (1955) Methods Enzymol. 1, 500—503
24 Aebi, H. (1974) Methods in Enzymatic Analysis (Bergmeyer, H.V., ed.), 2nd edn., Vol. 2, pp. 673—684, Academic Press, London and New York
25 Gupta, S. (1978) Proc. Aust. Biochem. Soc. 11, 22
26 Acton, G.J. and Gupta, S. (1977) Proc. Aust. Biochem. Soc. 10, 20
27 Gabriel, O. (1971) Methods Enzymol. 22, 576
28 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
29 Hodgins, D.S. (1971) J. Biol. Chem. 246, 2977—2985
30 Havir, E.A. and Hanson, K.R. (1975) Biochemistry 14, 1620—1623
31 Burgoyne, L.A. and Skinner, J.D. (1979) J. Cell Sic., in the press
32 Havir, E.A. and Hanson, K.R. (1968) Biochemistry 7, 1896—1903
33 Nari, J., Mouttet, Ch., Pinna, M.H. and Ricard, J. (1972) FEBS Lett. 23, 220—224
34 Emes, A.V. and Vining, L.C. (1970) Can. J. Biochem. 48, 613—622
35 Minamikawa, T. and Uritani, I. (1965) J. Biochem. (Tokyo) 58, 53—59

36 Parkhurst, J.R. and Hodgins, D.S. (1972) Arch. Biochem. Biophys. 152, 597—605
37 Havir, E.A., Reid, P.D. and Marsh, H.V., Jr. (1971) Plant Physiol. 48, 130—136
38 Hill, A.J. (1913) Biochem. J. 7, 471—480
39 Zimmermann, A. and Hahlbrock, K. (1975) Arch. Biochem. Biophys. 166, 54—62
40 Schopfer, P. (1971) Planta 99, 339—346
41 Blondel, J.-D., Huault, C., Faye, L., Rollin, P. and Cohen, P. (1973) FEBS Lett. 36, 239—244
42 Amar-Costesec, A., Beaufay, H., Feytmans, E., Thinès-Sempoux, D. and Berthet, J. (1969) Microsomes and Drug Oxidations (Gillette, J.R., Conney, A.H., Cosmides, G.J., Estabrook, R.W., Fouts, J.R. and Mannering, G.J., eds.), pp. 41—58, Academic Press, New York and London
43 Friend, J. and Acton, G.J. (1966) Brookhaven Symp. Biol. 19, 485—490
44 French, C.J. and Smith, H. (1975) Phytochemistry 14, 963—966
45 Iredale, S.E. and Smith, H. (1974) Phytochemistry 13, 575—583
46 Smith, H., Attridge, T.H. and Johnson, C.B. (1976) Perspectives in Experimental Biology (Sunderland, N., ed.), Vol. 2, pp. 325—336, Pergamon, Oxford and New York