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AN *N*-ACYLAMINO ACID ACYLASE FROM *NICOTIANA TABACUM* LEAVES

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Abstract—An *N*-acylamino acid acylase was partially purified from tobacco (*Nicotiana tabacum*) leaves and some of its properties are described. It hydrolyses *N*-acetylarginine, *N*-acetylmethionine, *N*-acetylcysteine and to a lesser extent *N*-formylmethionine. It does not appreciably hydrolyse *N*-formyl peptides and is therefore unlikely to be involved in protein synthesis.

INTRODUCTION

N-FORMYLMETHIONINE initiates the synthesis of protein chains in prokaryotes, and the formyl and methionyl residues are separately removed as the chains grow.[1] The enzyme responsible for deformylating the peptides has been identified in extracts of *E. coli*[1,2] and extracts of *Bacillus subtilis*.[3] *N*-Formylmethionine also initiates protein synthesis on the 70*S* ribosomes of the organelles of plants,[4] and it may, likewise, be removed as formate and methionine. However, Lugay and Aronson[5] suggested that the formylmethionine may be removed intact and then hydrolysed by an *N*-acylamino acid acylase (E.C. 3.5.1.14); they pointed out that the specificity of the acylase from the seeds of *Parkinsonia aculeata* suits it to such a function.

Extracts of tobacco leaves deacylate *N*-acetylmethionine; this raised the possibility that the acylase responsible might resemble either the bacterial or the *Parkinsonia* enzyme, and may have a role in modifying nascent peptide chains.

RESULTS

Extraction and Partial Purification of the Acylase

The enzyme was extracted from acetone powders of tobacco leaves and partially purified by precipitation with ammonium sulphate, chromatography on Sephadex G200 and absorption onto DEAE-cellulose. Table 1 gives some details of the recoveries and specific activities obtained.

Precipitating all the protein in the initial extracts with ammonium sulphate allowed the first stages of the process to be done under N_2, conditions which minimized browning reactions although they did not increase the yield of enzyme. Some fractionation of the

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[2] FRY, K. T. and LAMBORG, M. R. (1967) *J. Mol. Biol.* **28**, 423.
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precipitated protein was obtained by extracting the precipitate in buffer containing successively less ammonium sulphate: 200 g/l. ammonium sulphate extracted about 96% of the recovered enzyme and only 40% of the total protein N.

TABLE 1. PARTIAL PURIFICATION OF *N*-ACYLAMINO ACID ACYLASE

	Stage	Activity recovered at each stage %	Sp. act. (units/mg protein N)
I	$(NH_4)_2SO_4$ precipitate redissolved in buffered $(NH_4)_2SO_4$ (200 g/l.)	—	7·1
II	Eluted from Sephadex G200	53	18
III	Eluted from DE 32	87	147
IV	Eluted from DE 32 again	86	1900

Enzyme was extracted from acetone powder (21 g) of tobacco leaves, and with *N*-acetyl-methionine as substrate, the recovery and activity was measured after each stage of purification.

Chromatography on G200 removed more UV absorbing material (80%) than the increase in specific activity (Table 1) suggests. This material emerged as a distinct peak near the void volume of the column (140 ml) but was only completely eluted by 1·4 bed-volumes of eluant. The acylase emerged as a single broad peak in about 230 ml, only partly separated from the *o*-diphenol oxidase of the extract which moved faster. The eluted activity was concentrated and further purified by absorption on to DEAE-cellulose. It was eluted as a sharp peak at a KCl concentration of 0·08 M. Little activity was lost in this process and it was repeated with a shallower KCl gradient to remove more of the inactive protein (Table 1).

Properties of the Acylase

At all stages of purification the enzyme was stable, surviving well (80–100%) 12 weeks at −15°. It was active over a wide range of alkaline pHs, the optimum depending on substrate and buffer. It was 8 for *N*-acetylmethionine in orthophosphate, but 9 for *N*-acetylarginine and 9 for *N*-formylmethionine in pyrophosphate. The enzyme was only about one-third as active in pyrophosphate as in orthophosphate.

The hydrolysis of acetylmethionine was stimulated by cobalt chloride (mM) as was the activity of seed acylases.[6,7] The stimulation depended on the nature of the preparation; with cruder extracts it was less (50–100%) than with stage II or IV preparations (300–400%), whereas the activity in preparations of leaf organelles was completely dependent on added cobatlt chloride. The activity was completely abolished by *p*-chloromercuribenzoate (0·5 mM) but not by iodoacetate (1·5 mM) or EDTA (20 mM). It was also inhibited by *N*-acetylmethionine at concentrations above 17 mM. Its K_m for this substrate, estimated from extrapolation of Lineweaver–Burk plots, was 16 mM, almost the same as for the non-inhibitory *N*-acetyl arginine.

The migration of the enzyme on Sephadex G200, compared to that of reference proteins, suggested that its MW is *ca.* 160 000 approximately twice that of the *Parkinsonia* enzyme.[6]

[6] LUGAY, J. C. and ARONSON, J. N. (1969) *Biochim. Biophys. Acta* **191**, 397.
[7] OZAKI, K. and WETTER, L. R. (1961) *Can. J. Biochem. Physiol.* **39**, 843.

Subcellular Location of Acylase

About two-thirds of the acylase activity that could be extracted from tobacco leaves into a buffered-sucrose medium,[8] was sedimented by differential centrifugation. It was usually more concentrated in the 'mitochondrial' and 'microsomal' fractions than in the

TABLE 2. LOCATION OF THE ACYLASE

Fraction	Acylase (units)	Acylase (%)	Protein N (mg)	Protein N (%)	Chlorophyll (mg)	Chlorophyll (%)
Extract	163	100	60	100	23	100
'Chloroplast' (1000 *g* for 10 min)	40·5	25	17·6	30	18	78
'Mitochondrial' (10 000 *g* for 30 min)	74	45	2·8	4·7	1·5	6·5
'Microsomal' (50 000 *g* for 120 min)	42	26	1·4	2·4	0·22	1·0
Supernatant	—	—	25	42	1·3	5·2
Concentrated supernatant	52·5	32	—	—	—	—

Tobacco leaves (55 g) were disrupted in a buffered sucrose-medium, fractionated as previously described[8] and dialysed overnight against phosphate buffer (0·05 M; pH 7). The supernatant was precipitated with ammonium sulphate (600 g/l.), redissolved and dialysed.

chloroplasts (Table 2). Unsedimented enzyme was demonstrated after it had been concentrated from the supernatant by precipitation with ammonium sulphate. Activity in all fractions was completely dependent on added cobalt chloride.

TABLE 3. HYDROLYSIS OF *N*-ACYLATED AMINO ACIDS

Substrate	Relative activity (%) Stage IV	Relative activity (%) Stage I		Relative activity (%) Stage IV	Relative activity (%) Stage I
N-Acetyl-DL-methionine	100	100	*N*-Acetyl-L-proline	0	—
N-α-Acetyl-L-arginine	140	130–180	*N*-Acetyl-DL-tryptophan	0	0
N-Acetyl-L-cysteine	55–100	50	*N*-Acetyl-L-tyrosine ethyl ester	—	0
N-Acetyl-DL-leucine	6	4	*N*-Acetyl-DL-valine	0	0
N-Acetylglycine	3	0	*N*-α-Acetyl-DL-lysine	—	0
N-Acetyl-L-aspartate	0	0	*N*-ε-Acetyl-L-lysine	—	0
N-Acetyl-L-glutamate	0	0	α,ε-Diacetyl-DL-lysine	0	2
N-Acetyl-L-glutamine	—	0	*N*-Formyl-DL-methionine	12	10–15
N-Acetyl-L-histidine	0	0	*N*-Formyl-glycine	0	3
N-Acetyl-D-methionine	0	0	*N*-Formyl-L-leucine	0	—
N-Acetyl-L-ornithine	0	0			

Each substance (20–30 μmol) was tested as a substrate with 0·1 ml of a stage IV preparation (1·9 units) and with 0·04 ml (2·0 units) of a stage I preparation. The hydrolysis of acetylmethionine was measured in each experiment.

Specificity of the Acylase

Of the *N*-acyl amino acids tested (Table 3), only acetylarginine, acetylmethionine, acetylcysteine and formylmethionine were appreciably hydrolysed. Their relative susceptibilities

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were the same with enzyme preparations at different stages of purification. The hydrolysis of acetyl-DL-methionine was probably decreased a little by the presence of the inhibitory D-isomer. The hydrolysis of formylmethionine varied from one experiment to another, but was never more than 25% of that of acetylmethionine when tested in a variety of conditions.

Chromatographic examination of reaction mixtures showed methionine as the only ninhydrin-reactive product from acetylmethionine. Similarly arginine was produced from acetylarginine, but no acetylornithine as would have resulted if arginase were present. Both cysteine and cystine were produced from acetylcysteine, as well as a substance whose reaction to ninhydrin, nitroprusside and nitroprusside plus cyanide, suggested it to be *N*-monoacetylcystine. This substance (R_f 0·25 in butanol–acetic acid–H_2O) was produced non-enzymically when *N*-acetylcysteine was incubated with cysteine at pH 7.

TABLE 4. HYDROLYSIS OF PEPTIDES AND DERIVATIVES

Substrate	Relative activity (%) Stage IV	Stage I	Stage 0	Products
N-Acetyl-DL-methionine	100	100	100	MET
N-Acetyl-L-methionyl-L-alanine	6	76	60	—
N-Formyl-L-methionyl-L-alanine	3	104	180	MET-ALA; MET; ALA (Stage I and 0 Only)
N-Formyl-glycyl-DL-methionine	16	20	20	MET
Glycyl-DL-methionine	+	+	+	MET; GLY
L-Methionylglycine	—	+	+	MET; GLY
L-Methionyl-L-alanine	—	+	+	MET; ALA
Acetylated histone (5 mg/ml)	0	—	—	

Each substance was tested as in Table 3 against Stage IV and State I enzyme preparations, and also against an extract of acetone powder which has been precipitated with ammonium sulphate but otherwise unfractionated (Stage 0; 2·4 units; 6 units/mg protein N).

Three acylated dipeptides were, apparently, deacylated to a small extent by the most purified enzyme preparations (Table 4). However, the products formed, and the more extensive hydrolysis of these substances by cruder preparations suggest that the acylase under study was not responsible. Thus methionine, not glycylmethionine is split from formylglycylmethionine, by an enzyme which is purified along with the acylase and which probably hydrolyses glycylmethionine. Crude extracts also contain an enzyme which hydrolyses methionylglycine and methionylalanine, and, judging from the formation of methionylalanine from formylmethionylalanine (Table 4), one which deformylates peptides; both are largely removed from stage IV preparations, but remaining traces of either could produce the small observed hydrolysis of acylated methionylalanines.

Adding cobalt chloride (mM) to the reaction mixture caused a little hydrolysis of some substances such as acetylglycine which were not appreciably affected in its absence; but it did not significantly alter the relative rates of hydrolysis of the acylated arginine, methionine and dipeptide compounds from those listed in Tables 3 and 4,

DISCUSSION

Although the amino acid acylase is probably located, partially at least, in leaf organelles, it is unlikely that it deformylates peptides produced on 70*S* ribosomes. Its activity is

limited to acyl amino acids, and in particular it does not appreciably hydrolyse formylmethionylalanine. In both these respects, as well as others, it differs from the bacterial deformylases.[1-3] Moreover, its activity against formylmethionine is less than one-tenth of that against acetylarginine, suggesting that even if this substance is split off peptide chains,[5] it is not likely to be the natural substrate of the acylase. There appears to be another enzyme in crude extracts of tobacco leaves which deformylates formylmethionylalanine (Table 4), and which is separated as the amino acylase is purified. This may have a role in protein synthesis.

The tobacco leaf acylase resembles the acylases purified from seeds of *Parkinsonia*[6] and *Brassica campestris*:[7] it has a similar stability and responds in a similar way to pH changes, added cobalt and inhibitors. Although their specificities are different, they overlap, and all three enzymes are active against acetylmethionine. Many other plant extracts hydrolyse acetylmethionine,[9,10] and may well contain similar acylases. An acylase in tulip bulbs hydrolyses propanil derivatives,[11] and probably confers resistance to these herbicides on some plants.[12] The tobacco leaf enzyme, however, did not split acetyl-propanil (3,4-dichloroacetanilide); moreover it was not inhibited by sevin (1-naphthyl-*N*-methylcarbamate), a herbicide synergist which inhibits the herbicide-hydrolysing enzyme from rice.[12]

EXPERIMENTAL

Extraction of acylase. Acetone powder of leaves of *Nicotiana tabacum* (var. Xanthi-nc) was extracted with 50× its wt of phosphate buffer (0·1 M; pH 7·2) containing benzene sulphinic acid (0·05 M), an an N_2-filled Glove Bag. The extract was centrifuged at 3000 rpm for 30 min, and the supernatant poured directly onto enough ammonium sulphate to bring the concentration to 600 g/l. After shaking and standing in the cold (5°) overnight, the precipitated protein was collected by centrifugation and extracted successfully with phosphate buffer (0·05 M; pH 7) containing about 360, 300, 200, 150 and 0 g/l. $(NH_4)_2\ SO_4$. The extracts were dialysed for 24 hr against phosphate buffer (0·05 M; pH 7). Active extracts were freeze-dried, suspended in H_2O (1–5 ml) and dialysed against phosphate buffer (0·05 M; pH 7). They were chromatographed in this buffer on a column (usually 4 × 29 cm) of Sephadex G200. Eluant was collected at about 20 ml/hr and monitored on a Uvicord I. The active fractions from the G200 column were dialysed against phosphate buffer (0·01 M; pH 7), and absorbed onto a column (2·2 × 10·5 cm) of Whatman microgranular DE 32 which had been 'precycled' and equilibrated in the same buffer. The column was then washed with this buffer and absorbed protein (up to 16 mg) eluted with a linear gradient of KCl buffered at pH 7 (phosphate 0·01 M).

Acylase activity. was estimated by incubating the extract in 0·5–0·6 ml of solution containing substrate (0·1 ml; 0·2 M) and pH 7 phosphate buffer (0·1 ml; 0·2 M). After 2 hr at 30°, 1 ml trichloroacetic acid (10% w/v) was added, the solution cooled, centrifuged at 2500 rpm for 15 min and the supernantant neutralized and diluted to 5 ml. Control tubes were incubated without substrate or without extract. When cobalt chloride (0·01 ml; 0·05 M) was present in the reaction media, it was also added to all control tubes immediately before the trichloroacetic acid. Liberated amino groups were estimated in samples (0·5 ml) of the supernatants by adding 1·0 ml of pH 8 phosphate buffer (0·4 M), 1 ml of 2,4,6-trinitrobenzenesulphonic acid (TNBS; 0·1% w/v) and H_2O to 3 ml. The yellow colour produced after incubating for 90 min at 40° was measured at 430 nm. Standards containing an amino acid, usually methionine, were included in each estimation, and gave a colour (av. $\epsilon = 1{\cdot}37 \times 10^4$) close to that expected from the data of Goldfarb.[13] In these conditions, the release of up to 4 μmol of methionine was linear with respect to time and amount of enzyme present. A unit of activity was defined as that which liberates 1 μmol in 2 hr. Reaction with TNBS is not suitable for detecting all liberated amino acids. Proline, and cyst(e)ine in the presence of acetylcysteine, were estimated by the ninhydrin procedure of Moore and Stein.[14] Ornithine in the presence of acetylornithine was estimated by the acid-ninhydrin reagent of Vogel and Bonner.[15] Any lysine liberated

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from monoacetylated lysine was sought for chromatographically. Reaction products were separated directly from reaction mixtures by PC in *n*-BuOH–HOAc–H_2O (12:3:5) or *n*-PrOH–NH_4OH–H_2O (6:3:1). They were located with ninhydrin (0·2% w/v in acetone), nitroprusside reagents[16] or *p*-benzoquinone in dimethyl sulphoxide.[17] Protein N and chlorophyll were estimated as previously described.[8] *N*-Acetyl and *N*-formyl derivatives of peptides were prepared and freed from unreacted peptide as described by Adams.[1] Histone was acetylated by the method of Candido and Dixon.[18] α-ε-Diacetyl-DL-lysine was given by Dr. A. Benoiton.

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