Volume 292, number 1,2, 293–297 FEBS 10368 November 1991

ADONIS 001457939101068R

The formation of 3α- and 3β-acetoxytropanes by *Datura stramonium* transformed root cultures involves two acetyl-CoA-dependent acyltransferases

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Received 21 August 1991; revised version received 18 September 1991

Tropine (tropan-3α-ol) is an intermediate in the formation of hyoscyamine. An acyltransferase activity that can acetylate tropine using acetyl-coenzyme A as cosubstrate has been found in transformed root cultures of *Datura stramonium*. A further acyltransferase activity that acetylates pseudotropine (tropan-3β-ol) with acetyl-coenzyme A is also present. These two activities can be partially resolved by anion-exchange chromatography, some fractions containing only the pseudotropine-utilizing activity. The basic properties of these two enzymes are reported and their roles in forming the observed alkaloid spectrum of *D. stramonium* roots discussed.

Acyltransferase; Acetyl-CoA; Tropine; Pseudotropine; Transformed root; *Datura stramonium*

1. INTRODUCTION

The pharmaceutical, hyoscyamine (atropine), is the major tropane alkaloid produced by a wide range of solanaceous genera [1,2]. The biosynthesis of this alkaloid involves the esterification of tropine (tropan-3α-ol), a putrescine-derived amine, with a unit of phenylalanine-derived tropic acid (Fig. 1A). The reaction by which the ester is formed is yet to be elucidated. Some early reports claimed that hyoscyamine could be made in vitro by incubating tropine and tropic acid with root extracts [3,4] but these claims have failed to be substantiated. More recently, a number of laboratories have tried this reaction replacing tropic acid with tropyl-CoA, again without success [5] (T. Hashimoto, personal communication).

Transformed root organ cultures of *Datura stramonium* competent in the biosynthesis of hyoscyamine have been established [6]. During experiments in which precursors were fed to these cultures, intermediates up to and including tropine were found to accumulate to levels considerably above those of the control tissues. However, no additional accumulation of hyoscyamine was found. It is therefore apparent that tropine esterification is a potential, if not always an actual, limitation to hyoscyamine formation. Another ester of tropine, the 3-acetyl derivative (Fig. 1B), was, however, found to accumulate to high levels (up to 3 mM) in response to feeding precursors [6]. 3-Acetyl-tropine is present in several transformed root cultures at low levels, accompanied by traces of the 3-acetyl derivative of pseudotropine (tropan-3β-ol) (Fig. 1B) [2]. The apparently high capacity to make 3-acetyltropine has been exploited in order to determine the conditions under which the enzymatic esterification of tropine might be achieved. In the course of this work, it was found that an activity esterifying pseudotropine was also present.

Tropine is synthesized enzymically by the NADPH-dependent reduction of the corresponding 3-one, tropinone, by a stereospecific enzyme, TR I (Fig. 1B) [7,8]. A second stereospecific activity, TR II, reduces tropinone to pseudotropine [9]. There was prolonged uncertainty as to whether the reduction of tropinone represented one or two separate proteins [7–9].

In view of this, we wish to report that two separable acetyl-CoA:tropan-3-ol acyltransferases are present in root cultures of *D. stramonium*. Furthermore, we report that an activity acetylating pseudotropine can be obtained free of any acetyl-CoA:tropine acyltransferase activity.

2. MATERIALS AND METHODS

2.1. *Root cultures*

Transformed root cultures of *D. stramonium* line D15/5 were grown as described previously [6]. For the assays described, roots were subcultured at about 0.5 g per flask (50 ml medium) and harvested at 10

Abbreviations: CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; TEPP, tetraethyl pyrophosphite; TR, tropinone reductase

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days old. Following blotting to remove surface medium, the roots were frozen in liquid N_2 and stored at −70°C until required. No apparent deterioration was found after several months under these conditions.

2.2. *Enzyme extraction*

To obtain a crude extract, roots were taken directly from liquid N_2, crushed and mixed with Polyclar AT (4:1 ratio w/w). To this coarse powder, ice-cold buffer (200 mM KH_2PO_4; 20 mM EDTA·K_2; 3 mM 1,4-dithio-L-threitol; 125 mM sucrose; pH 7.0) was added (3 ml/g) and the slurry homogenized (Ultraturrax; 180 V; 30 s). Following filtration (Miracloth), a clear supernatant was obtained by centrifugation (15 000 × *g*; 20 min; 4°C).

2.3. *Enzyme assay procedures*

All extracts were treated on a PD10 column (Pharmacia, Milton Keynes, UK) prior to the assay of activity. Columns were pre-equilibrated and eluted using half-strength extraction buffer.

2.3.1. Radiometric

Activity was determined by following the incorporation of radiolabel from [1-^{14}C]acetyl-CoA into 3-acetyltropine. The incubation mix contained (250 μl): extract (various volumes diluted to 100 μl in extraction buffer); acetyl-CoA (10 μl of 2.1 mM, 1.05 kBq [1-^{14}C]acetyl-CoA); tropine (20 μl of 75 mM); TEPP (10 μl of 50 mM in EtOH); glycine buffer (50 μl of 1 M, pH 9.0); water (50 μl). All solutions were freshly prepared. Blank reactions lacked tropine. After 45 min at 37°C, the reaction was stopped with 0.1 ml 20% KOH solution and 1.0 ml ethyl acetate. Following vigorous shaking and centrifugation, the organic phase was removed. The ethyl acetate extraction was repeated and the radioactivity in the combined organic phases determined by liquid scintillation counting.

2.3.2. Extrelute extraction and gc analysis

Activity was assessed by separating the alkaloids from the incubation mixture on Extrelute and determining the products by gc. The incubation mix was as described above, except that only unlabelled acetyl-CoA (2 mM) was added. Boiled enzyme was used as a blank. The reaction was stopped by adding 0.1 ml 35% NH_3 solution and applying the sample to the top of a small Extrelute 1 column. After 15 min the column was eluted with 16 ml $CHCl_3$, the solvent removed at 30°C in vacuo and the residue taken into 50 μl MeOH. Between 1 and 5 μl was applied to a DB-1 capillary column and analysed against standards as described previously [6]. Recoveries of products were calculated on the assumption that these were the same as that determined for the substrates.

2.4. *Protein determination*

Protein was determined by the dye-binding method, using Bio-Rad dye reagent and following the Bio-Rad protocol.

2.5. *Chemicals and materials*

[1-^{14}C]Acetyl-CoA was purchased from Amersham International (Amersham, UK) at an initial specific activity of 185 MBq/mmol; acetyl-CoA from Sigma (Poole, UK). All other chemicals were of the highest grade available. Extrelute 1 (Merck product No. 15371) was purchased from Francis Mason Diagnostics (Poole, UK) and a DB-1 capillary column (0.32 mm i.d./30 m/0.25 μm film thickness, J&W Scientific) from Fisons (Loughborough, UK).

3. RESULTS

3.1. *Identification of the products of the reactions*

Following incubation, product was extracted from the incubation mix using Extrelute columns and subjected to gc/ms analysis. The product of incubation with tropine displayed a retention time and mass spectrum [*m/z* (relative intensity): 183 (M^+, 21), 140 (8), 124 (100), 96 (24), 94 (34), 83 (45), 82 (72), 67 (30), 42 (55)] identical [11] to that of an authentic synthetic standard of acetyltropine. Similarly, the product of incubation with pseudotropine was identical [*m/z* (relative intensity): 183 (M^+, 21), 140 (4), 124 (100), 96 (21), 94 (38), 83 (45), 82 (89), 67 (30), 42 (53)] to an authentic synthetic standard of acetylpseudotropine.

3.2. *Rate as a function of time and protein content*

The rates of acetylation of tropine and pseudotropine, using either assay method, were both linear for up to 60 min incubation but thereafter linearity was lost rapidly. Similarly, the reaction rate observed with both methods was linear over the range 20–200 μg protein per assay but fell sharply at higher protein levels.

3.3. *Effect of enzyme inhibitors*

The possibilities that acetyl-CoA and/or the products of the acetylations were being degraded by esterase activity during incubation were tested using a range of esterase inhibitors. Extracts were made in buffer with dithiothreitol and sucrose only (see section 2.2 above). From Table I it can be seen that the inclusion of EDTA and TEPP was beneficial. EDTA was therefore included routinely in the extraction buffer and TEPP in the enzyme incubation mixture.

3.4. *Effect of pH*

The pH optimum for tropine acetylation was examined using both assay procedures with acetate, phosphate, Tris and borate as buffers (0.2 M) over the range 5–10 pH units. No activity was detected at pH 6 or below. As shown in Fig. 2, there is a rapid increase in activity between 7 and 8 and a broad optimum around pH 8 to 9. The activity with pseudotropine was examined using the Extrelute/gc method only and over the range 7–10 pH units. Activity with this substrate showed a similar response to pH, although the activity at pH 7 was somewhat higher.

3.5. *Effect of substrate concentrations on rate*

The effects on the rate of reaction of varying the tropine concentration in the presence of 0.1 mM acetyl-CoA and of varying the acetyl-CoA concentration in the

Table I

The effect on the rate of acetyl-CoA:tropine acyltransferase reaction of inhibitors of esterase activity

Inhibitor	Concentration (mM)	Rate of reaction[a] (% of control)
None		100
Eserine	0.02	102
EDTA	1.0	330
TEPP	0.02	170

[a]Determined by the radiometric method.

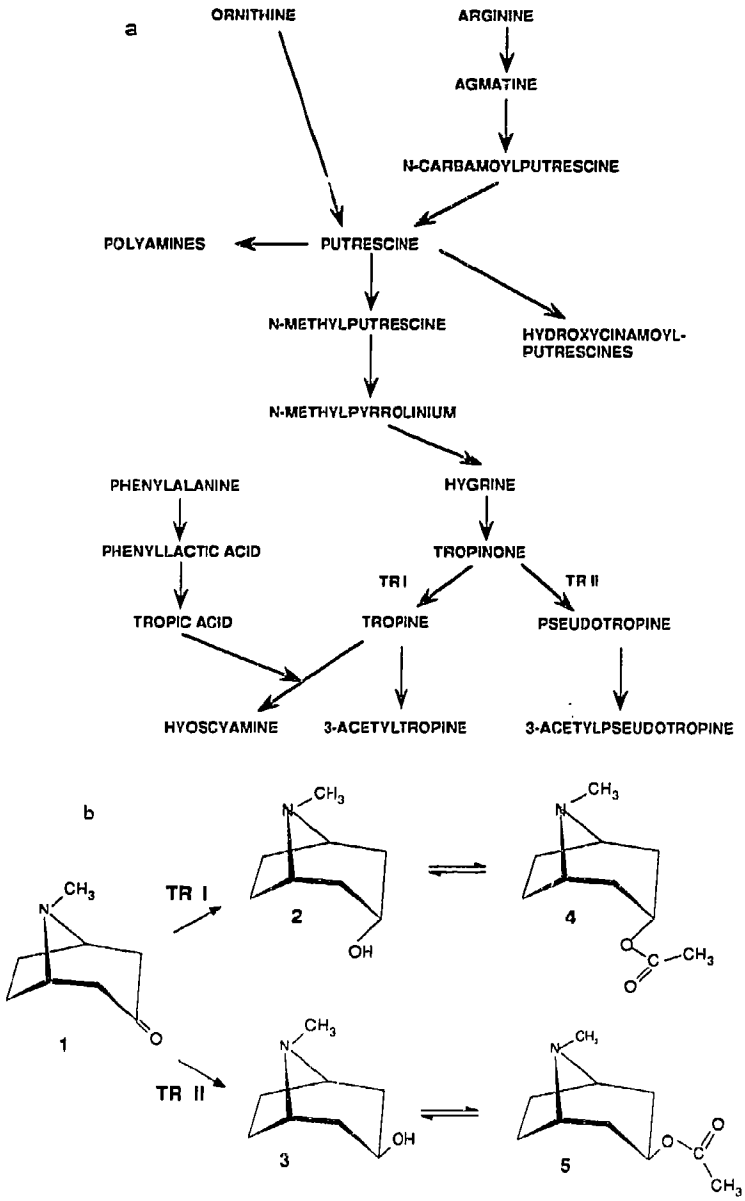

Fig. 1. (A) The pathway of biosynthesis of hyoscyamine, acetyltropine and acetylpseudotropine. (B) The reduction of tropinone (**1**) to tropine (**2**) or pseudotropine (**3**) and their respective acetyl derivatives (**4**,**5**).

presence of 8.2 mM tropine were examined. Preliminary data gave linear double-reciprocal plots, from which apparent K_m values of 0.24 mM for tropine and 0.22 mM for acetyl-CoA could be calculated. It should be noted that acetyl-CoA was not saturating during these assays. No data for pseudotropine are yet available.

3.6. *Concentration and purification of the activities*

No activity could be detected in the crude extract. Low activity could be measured once small-molecular-weight contaminants had been removed by filtration on a PD10 column (Pharmacia, Milton Keynes, UK) or by diafiltration through an Amicon YM10 molecular separation membrane (Amicon, Stonehouse, UK). Activity was retained by this 10 000-Da cut-off membrane when extract was ultrafiltered. An overall decrease in activity occured above about a fivefold concentration, presumably due to protein aggregation.

3.6.1. Ammonium sulphate fractionation

Extract was fractionated with ammonium sulphate and each fraction assayed separately for the acetylation of tropine or pseudotropine using the Extrelute/gc method. The activities transferring an acyl group from acetyl-CoA to one or the other of these substrates could be partially resolved between the 20/40%- and the 40/55%-saturated fractions (Table II).

3.6.2. Anion-exchange fractionation

A 40 to 55% saturated ammonium sulphate fraction,

Table II

The partial resolution of acetyl-CoA:tropine acyltransferase and acetyl-CoA:pseudotropine acyltransferase activities by ammonium sulphate fractionation

Ammonium sulphate concentration (%)		Total protein (mg)	Specific activity[b] (pkat/mg protein)	Yield (%)	Purification (fold)
Initial	Final				
A. Tropine					
Crude[a]		93	13.2	100	1.0
0	20	3.8	10.5	3.3	0.8
20	40	15.8	35.8	46.2	2.71
40	55	30.5	1.9	4.7	0.14
55	75	29	0.33	0.8	0.03
B. Pseudotropine					
Crude[a]		93	31.6	100	1.0
0	20	3.8	12.7	1.7	0.4
20	40	15.8	7.5	4.1	0.23
40	55	30.5	36.3	37.7	1.15
55	75	29	2.0	2.0	0.06

[a]After elution from a PD10 column.
[b]Determined by the Extrelute/gc method.

enriched in the pseudotropine-acetylating activity, was fractionated by anion-exchange chromatography on a MonoQ FPLC 5/5 column (Pharmacia, Milton Keynes, UK). As shown in Fig. 3, the activity with pseudotropine was largely not retained by the column under the conditions used (see Fig. 3 legend), whereas the activity with tropine was entirely retained by the column and mostly eluted between 0.12 and 0.22 M KCl. Thus, fractions 2 to 10 contain activity against pseudotropine that is free of any tropine-acetylating activity. Fractions 13 to 18, obtained during the gradient, still show activity with both substrates but are greatly enriched in the tropine-acetylating activity. Complete resolution of the tropine-acetylating activity from the pseudotropine-acetylating activity has yet to be achieved.

4. CONCLUSION

From the data presented, it can be concluded that extracts of transformed root cultures of *D. stramonium* contain two acetyl-CoA-dependent acyl transferase activities that transfer an acetyl group to 3-hydroxytropanes. We have demonstrated that it is possible by ammonium sulphate fractionation followed by anion-exchange chromatography to resolve a protein that acetylates exclusively pseudotropine and is therefore free of any tropine-acetylating activity. Whether the tropine-acetylating activity can be similarly resolved from the pseudotropine-acetylating activity has yet to be clarified. A further activity, which transfers a tiglyl moiety from tiglyl-CoA to pseudotropine, is also present in the 40%- to 55%-saturated ammonium sulphate fraction. Whether this represents a further separable protein is under investigation.

Both acetylating activities show optimal activity well into the alkaline pH range. This may indicate that the substrate is uncharged tropine or pseudotropine, the deprotonation of the nitrogen being strongly favoured at pH's above 8.5. Some other acetyl-CoA-utilising enzymes involved in alkaloid biosynthesis, such as vinorine synthase from *Rauwolfia serpentina* [12] and acetyl-CoA:deacetylvindoline *O*-acetyl transferase from *Catharanthus roseus* [13], have also been found to have pH optima in this range.

At present it is not clear what specific role these two enzymes play in alkaloid biosynthesis in *Datura*. Acetyltropine formation in whole plants only occurs at trace levels and it has previously been suggested [2] that the higher levels found in root cultures are due to an excess capacity for tropine formation over that for hyoscyamine biosynthesis. The formation of the acetyl ester has been proposed as a shunt mechanism to regulate the concentration of tropine in tissues [6]. That it is the

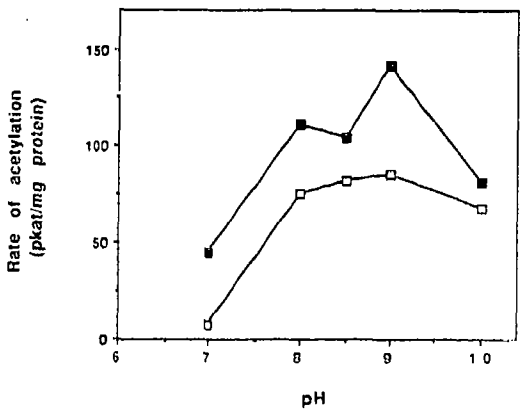

Fig. 2. The effects of pH on the activities of acetyl-CoA:tropine acyltransferase (□) and acetyl-CoA:pseudotropine acyltransferase (■).

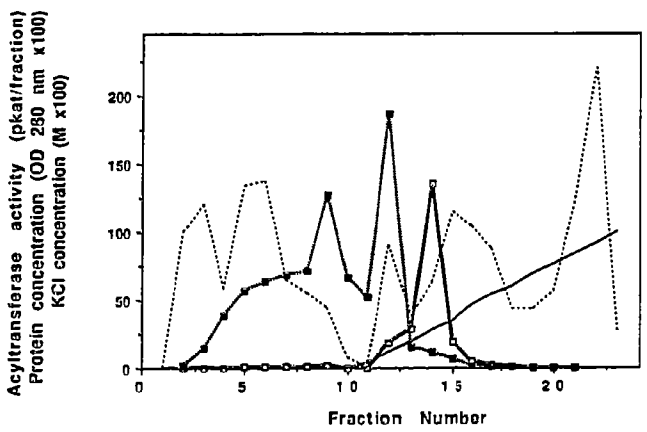

Fig. 3. The profiles of elution of acetyl-CoA:tropine acyltransferase (□), acetyl-CoA:pseudotropine acyltransferase (■) and u.v. absorption at 280 nm (---) from a MonoQ anion-exchange column eluted with a gradient of KCl (—). The column was pre-equilibrated in buffer A (50 mM Tris, 5 mM EDTA, 1 mM 1,4-dithio-L-threitol, 50 mM sucrose, pH 7.8) and loaded at 0.25 ml/min. Elution was by linear gradient at 0.5 ml/min with buffer B (= 1.0 M KCl in buffer A). Fractions of 2.0 ml were collected prior to the start of the gradient and of 1.0 ml during the gradient of KCl. Each fraction was assayed, 0.5 ml being used in a single incubation for acyl transferase activities with both tropine and pseudotropine present as substrates.

acetyl ester that is formed, rather than some of the other esters of tropine identified in this tissue [2], may reflect the abundance of acetyl-CoA relative to other CoA-activated acids. What is surprising is that the ability to form acetylpseudotropine is as great as that to form acetyltropine. Pseudotropine occurs in *D. stramonium* transformed root cultures at about one-thirtieth of the level of tropine and the acetyl-derivatives occur in about the same ratio. Thus, the accumulation of acetyl-pseudotropine apparently reflects the relative activities of TR I and TR II in the tissue, TR I being present at 22 times the level of TR II (T. Hashimoto and K. Nakajima, personal communication), rather than the relative abilities to acetylate the two products of these enzymes. Why there is such a high ability to acetylate pseudotropine remains to be elucidated.

Acknowledgements: We are most grateful to Dr T. Hashimoto for discussions during part of this work and for the use of facilities in Kyoto. In Norwich, we are grateful to Drs N.J. Walton and A.J. Parr for discussions, to John Eagles for running gc/ms and to Abigael Peerless for growing and harvesting the root cultures. Some of the pseudotropine used was the kind gift of Dr B. Dräger (Münster, Germany). R.J.R. is particularly grateful to the British Council (Tokyo) and The Royal Society (London) for grants enabling him to visit and work in the Kyoto laboratory. P.B. wishes to acknowledge support from a Fellowship from the Japan Society for the Promotion of Sciences.

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