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Purification and characterization of acetyl coenzyme A: 10-hydroxytaxane *O*-acetyltransferase from cell suspension cultures of *Taxus chinensis*

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Abstract

An *O*-acetyltransferase that catalyzes the regiospecific acetylation of a range of taxanes possessing an unsubstituted 10-hydroxyl group was detected and purified to apparent electrophoretic homogeneity from a cytosolic fraction of *Taxus chinensis* cell cultures. The purification involved negative calcium phosphate adsorption, sephadex desalting, DEAE, AcA44 chromatography, HighQ, CHT II, HiTrap Blue, Phenylsepharose and Mimetic Green purification steps. The purified acetyltransferase was found to be a monomeric protein of 71 ± 1.5 kDa that is highly regio- and stereospecific towards the 10β-hydroxyl group of the taxane molecule and is also active towards 10-desacetylbaccatine III. The acetyltransferase reaction had a pH optimum of 9.0 with halfmaximal activities at pH 6.8 and 10.8, respectively. The temperature optimum was at 35°C and the isoelectric point at 5.6. The apparent K_m values for 10-desacetyltaxuyunnanine C and acetyl CoA were 23 and 61 μM, respectively. The turnover rate for the enzyme using both substrates was 0.2 mol mol^{-1} of enzyme. The kinetic optimum was determined to be $K_{cat}/K_m = 8.7$ s^{-1} L M^{-1}.

Keywords: Taxus chinensis; *O*-acetyltransferase; Cell culture; 10-Desacetyltaxuyunnanine C; 10-Desacetylbaccatine III

1. Introduction

Taxol® (**1**) and Taxotere® (**2**) are at present successful anticancer drugs of steadily increasing medical usage (Wani, Taylor, Wall, Coggon, & McPhail, 1971). The original limited supply of Taxol® (**1**) from natural resources (Wani et al., 1971) was overcome by a semisynthetic process allowing for the production of both Taxol® (**1**) and Taxotere® (**2**) from 10-desacetylbaccatine III (10-DAB III) (**3**, Figs 1 and 2), which is available in sufficient quantities (up to 0.2% dry weight) from needles of the European yew *Taxus baccata* L. (Sénilh, Guéritte-Voegelein, Guénard, Colin, & Potier, 1984; Guéritte-Voegelein, Sénilh, David, & Guénard, 1986). In order to secure a long-term supply of both anticancer drugs, the biosynthesis of this important precursor clearly needs to be studied with the aim of increasing the amount of **3** in the genus *Taxus* through molecular genetic approaches. At least two strategies can be considered to accomplish this; either to deregulate the biosynthesis of this precursor in differentiated plants and cell cultures or to clone and overexpress rate limiting enzymes in *Taxus* species to boost taxane production.

Studies on the biosynthesis of **1** and **3** can be divided into three different chapters. The first is the origin of the terpenoid precursor for the biosynthesis of the taxane carbon skeleton, which has been proven to follow the 1-desoxyxylulose and not the mevalonate pathway (Eisenreich, Menhard, Hylands, Zenk, & Bacher, 1996). The second point is the formation of the taxa-4(5),11(12)-diene ring system starting from geranylgeranyl-pyrophosphate as substrate, which was most elegantly shown recently (Koepp, Hezari, Zajicek, Stofer-Vogel, & La Fever, 1995; Hezari, Lewis, & Croteau, 1995; Wildung & Croteau, 1996; Hezari, Ketchum, Gibson, & Croteau, 1997). Third, the substitution of the tricyclic terpenoid by eight oxygenation reactions, two acetylations, one benzoylation and one sidechain addition at C-13 (Floss & Mocek, 1995; Hezari & Croteau, 1997).

* Papers with this heading have received accelerated publication, due to their particular significance in the field of phytochemistry.

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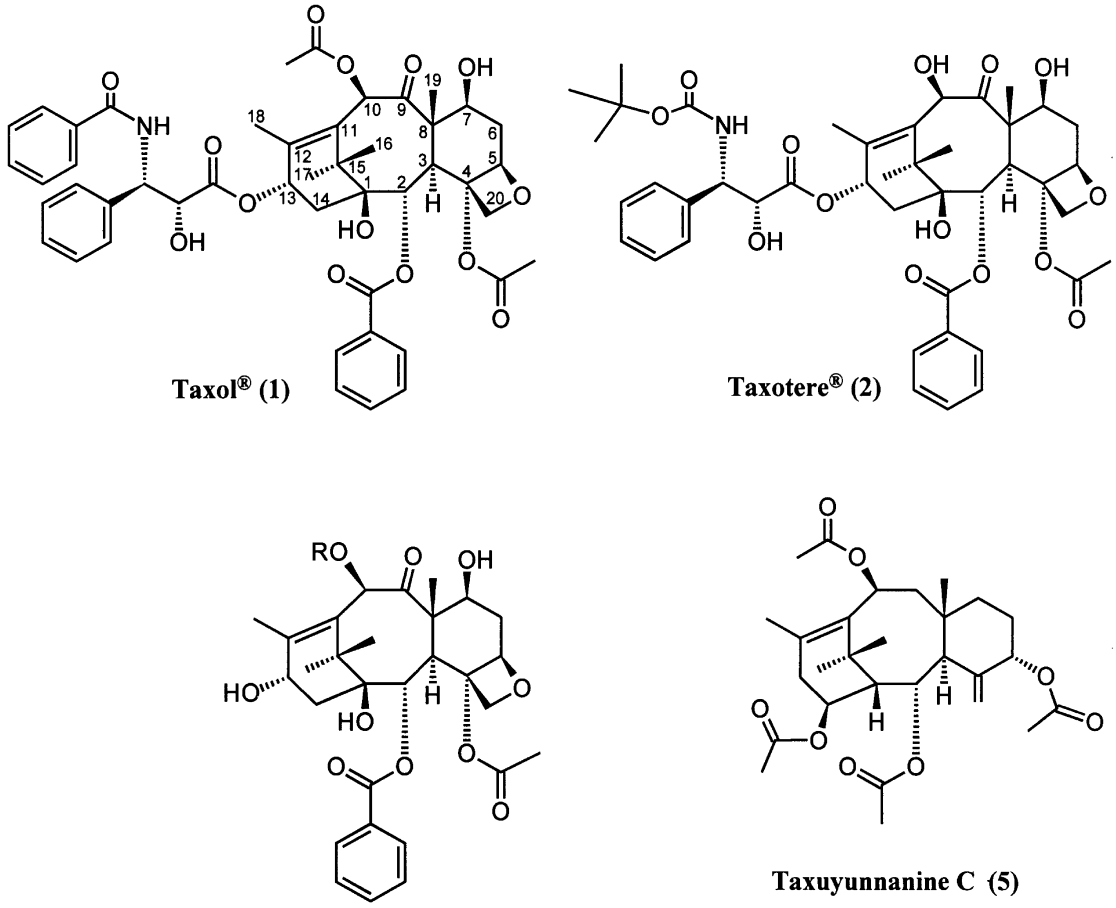


Fig. 1. Structures of naturally occurring taxanes.

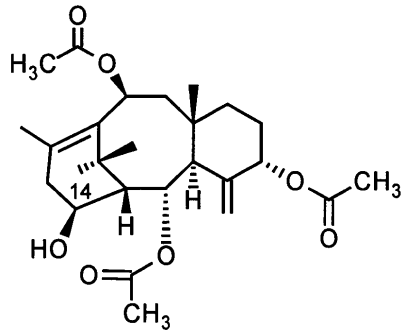
One of the late steps in the biosynthesis and semi-synthesis of Taxol® (**1**) is the acetylation at the C-10 position of 10-DAB III (**3**) to yield baccatine III (**4**). This compound is assumed to be the immediate precursor of **1** (Floss & Mocek, 1995). This acetylation reaction has recently been described in insufficient form using acetyl CoA as a cosubstrate and a crude enzyme mixture from needles of *T. baccata* and lacking controls (Zocher et al., 1996). A specific enzymic 10-acetylation of **3** would be of considerable interest, because it potentially circumvents the introduction of a protecting group at C-7 in the chemical acetylation reaction during the synthesis of **1** (Guéritte-Voegelein et al., 1986).

During our investigation on the origin of the terpenoid precursor of the taxane carbon skeleton (Eisenreich et al., 1996) we used a cell culture of *T. chinensis* (Pilger) Rehd., which produced up to 70 mg l^{-1} of 2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadiene (taxuyunnanine C (**5**)). This yield could be increased up to consistent levels of 150 mg l^{-1} by medium optimization (Menhard, Eisenreich, Hylands, Bacher, & Zenk, 1998). In order to investigate the specificity of the 10-acetylation reaction, we set out to purify and characterize this enzyme activity using **5** as a substrate. It should be clearly noted that taxuyunnanine C (**5**) possesses a 14-hydroxyl group and no substitution at C-13. This is at variance with **1** and **3**, which possess the C-13 oxygen function and are unsubstituted at C-14. In this study we describe the isolation and characterization of an acetyl coenzyme a:acetyltransferase from a *T. chinensis* cell culture that is highly regio- and stereospecific for the 10β-hydroxyl group of **7** but surprisingly also accepts 10-DAB III (**3**) and congeners as substrates.

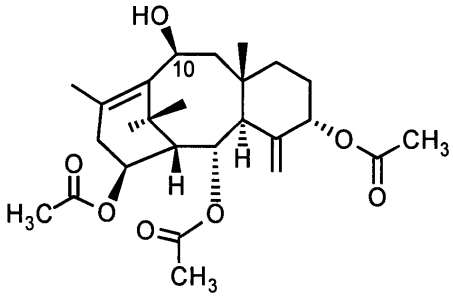
2. Results

2.1. Preparation of substrates

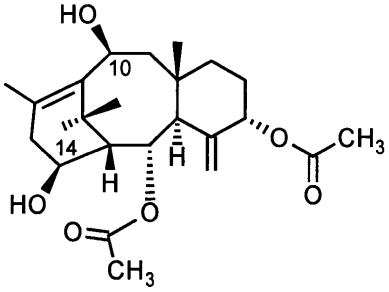
Readily available **5** isolated from the optimized *T. chinensis* cell culture (Eisenreich et al., 1996) was used for the preparation of select desacetylation products. Figure



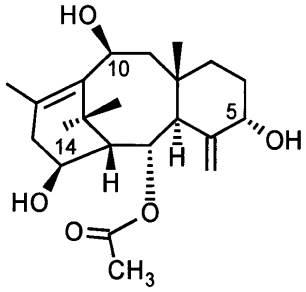
14-Desacetyl-taxuyunnanine C (6)



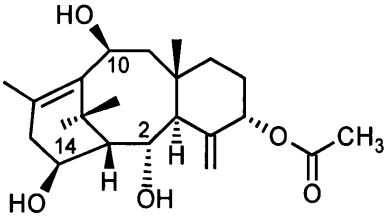
10-Desacetyl-taxuyunnanine C (7)



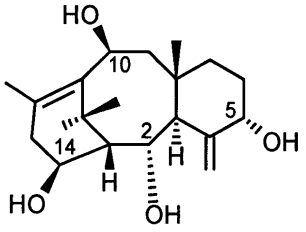
10,14-Desacetyl-taxuyunnanine C (8)



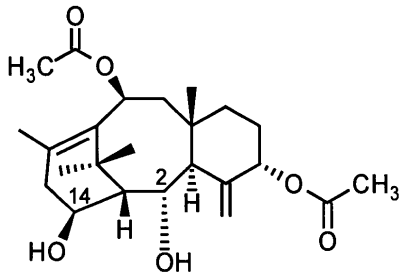
5,10,14-Desacetyl-taxuyunnanine C (9)



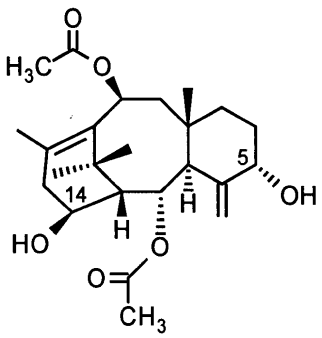
2,10,14-Desacetyl-taxuyunnanine C (10)



2,5,10,14-Desacetyl-taxuyunnanine C (11)



2,14-Desacetyl-taxuyunnanine C (12)



5,14-Desacetyl-taxuyunnanine C (13)

Fig. 2. Compounds generated by desacetylation of taxuyunnanine C (**5**).

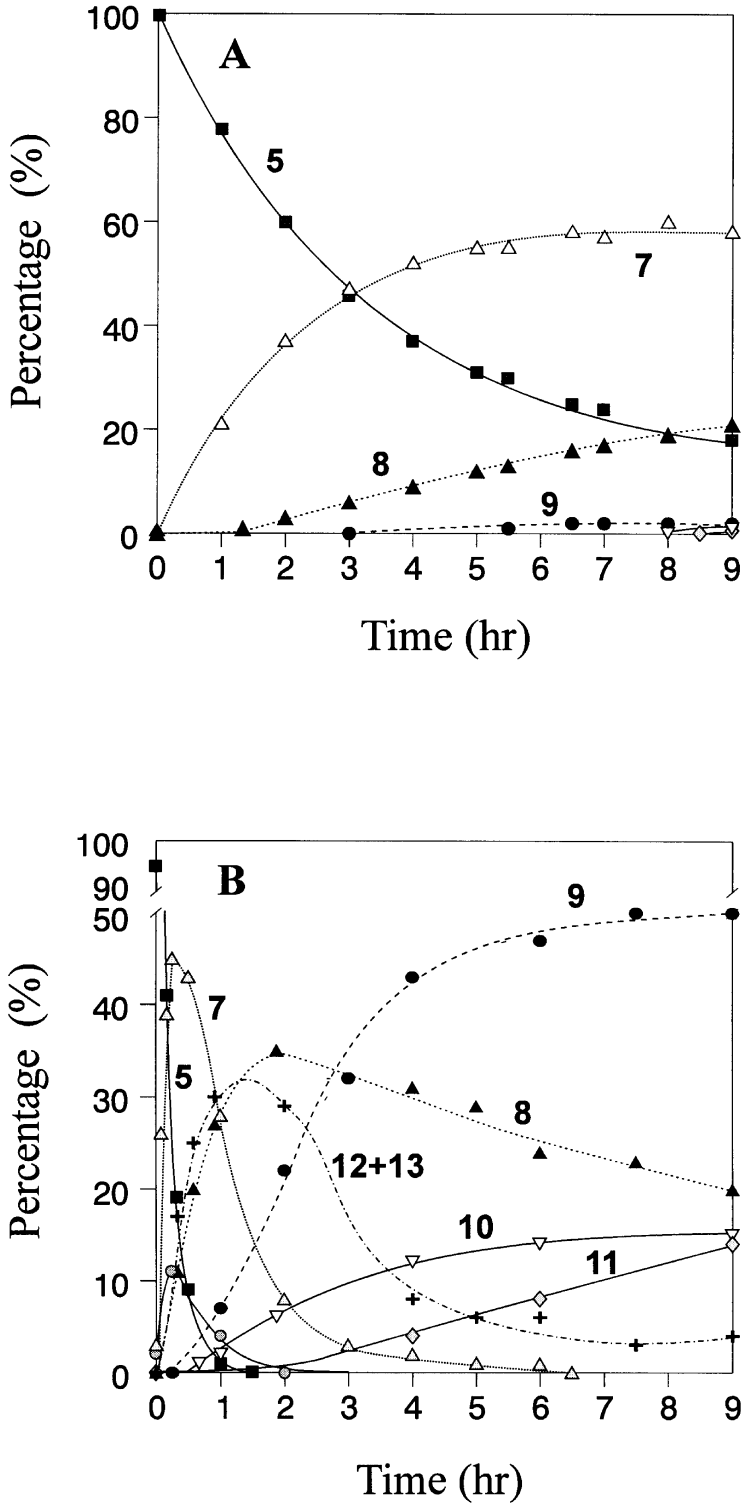


Fig. 3. Kinetics of the desacetylation of taxuyunnanine C (**5**) by incubation with (a) 0.1 M NaOH and (b) 1 M NaOH. This mixture was incubated at 30°C. The progress of the reaction was monitored by analyzing aliquots by HPLC. Structures of the compounds in question are shown in Fig. 2.

3a shows the kinetics of the desacetylation reaction using 0.1 M NaOH at 30°C. Under these conditions, **7** was synthesized in 60% yield. The compound was isolated using semipreparative HPLC and its structure was verified by MS and NMR. **7** was the main substrate used for the detection and isolation of the acetyltransferase. Desacetyltaxuyunnanine derivatives containing two, one or no acetyl group, were isolated from hydrolysis experiments using **5** but with more severe conditions such as 1 N NaOH at 30°C (Fig. 3b).

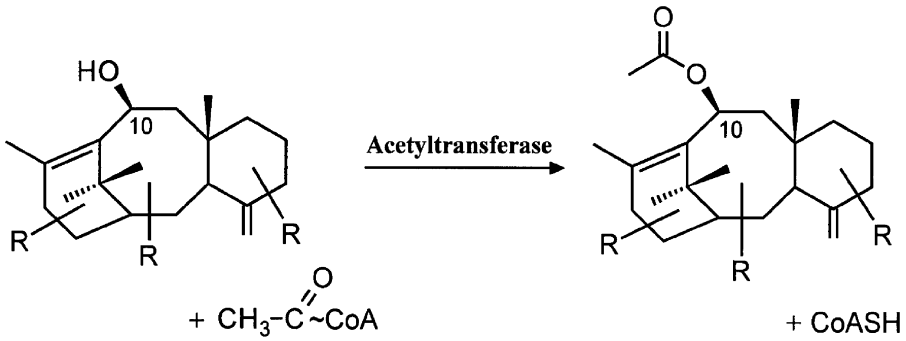


Fig. 4. Reaction sequence catalyzed by acetyl CoA:10-hydroxytaxane *O*-acetyltransferase.

2.2. Enzyme assay and enzyme detection

The enzyme in question was thought to be an acetyl CoA-dependent acetyltransferase, catalyzing the acetyl transfer from the thiolester onto the only free hydroxyl group in position 10 of **7** (Fig. 4). A radioactive assay was chosen for a reliable quantitative and sensitive testing.

No enzyme activity could be detected in crude extracts of *T. chinensis* suspension cells, which was most likely due to an excessive amount of phenols in the crude extract. This extract was, therefore, treated with calcium phosphate gel, subjected to gel filtration and then, with the resulting protein fraction still showing no enzyme activity, passed through a DEAE-Sephacel column. The brownish colored protein fraction that was not absorbed again did not show any enzyme activity but 84% of protein from the crude cell extract had been removed by the three previous steps. The protein fraction eluting from the DEAE column was applied to a column containing Ultrogel AcA44. A total of 60 fractions (Fig. 5a) were collected and for the first time enzyme activity could be detected. If this protein fraction was assayed in the presence of **7** and [1-^{14}C]acetyl CoA, the product of the reaction was clearly **5** as shown by TLC and HPLC analysis. When, however, 2,5,10,14-desacetyltaxuyunnanine C (**11**) was used as a substrate, the sole product formed was also **5**. This clearly showed that in the semipurified extract either one unspecific enzyme that acetylated all four possible hydroxyl groups of the taxane used as substrates was present or that the extract contained a mixture of at least four different and regiospecific acetyltransferases that sequentially acetylated the compound **11** in a position-specific matter. To address this question, **7** was used as a sole substrate furtheron for the subsequent purification of the target enzyme by anion exchange chromatography (HighQ), hydroxylapatite, HiTrap Blue, Phenylsepharose and Mimetic Green as summarized in Fig. 5b–e. These chromatographic steps resulted in a 280-fold increase in specific activity with a 0.1% recovery. The enzyme was obviously purified to apparent homogeneity as determined by SDS-PAGE. The purification procedure is summarized in Table 1.

2.3. pH optimum, temperature optimum and isoelectric point

The pH optimum of the enzyme was measured under standard assay conditions. The enzyme showed a pH optimum at 9.0 with half maximal activities at 6.8 and 10.8, respectively. The optimal temperature for the reaction was at 35°C. Half maximal activities were at 16 and 43°C, respectively.

The isoelectric point was determined by chromatofocusing on a polybuffer exchanger (Mono P HR) column to be at pH 5.6.

The molecular mass of the acetyltransferase was determined by two methods. Using a previously calibrated Bioselect SEC 250-5 FPLC column, the enzyme activity was eluted at a volume corresponding to a protein of approximately 72 kDa. Under denaturing conditions the purified enzyme showed (after Ag^+ staining) a protein band at 70.9 kDa. The results indicate that the acetyltransferase is a monomeric protein with a molecular mass of 71 ± 1.5 kDa.

2.4. Product identification

The purified acetyltransferase catalyzed the transfer of the acetyl moiety of acetyl CoA to **7**. In order to characterize the reaction product of this transfer, [1,2-$^{13}C_2$]acetyl CoA was incubated with unlabelled **7**. After incubation for 1 h at 35°C, the product of the reaction was extracted with ether and purified first by TLC and subsequently by HPLC. A single compound was isolated that had the same R_f and R_t value as well as the same UV spectrum as authentic **5**. The compound was subjected to MS analysis and showed a clear and unequivocal $[M+1]^+$ peak of 507 (CI mode; Fig. 6b) in contrast to the **5** reference showing an $[M+1]^+$ at 505. The latter spectrum is shown in Fig. 6a. Clearly, only one [1,2-$^{13}C_2$]acetyl group was transferred to **7**, and since the substrate taxane

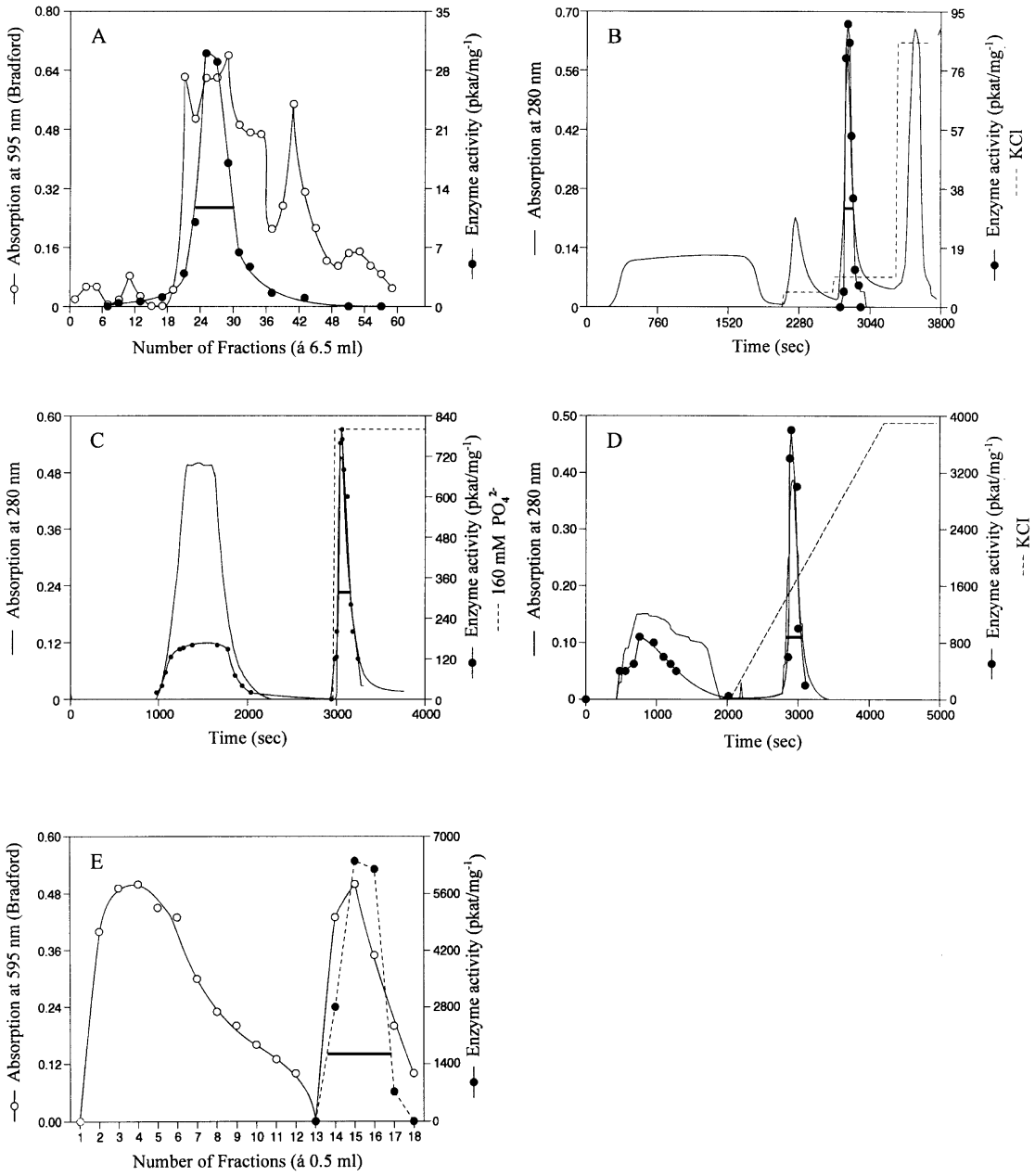


Fig. 5. Purification of 10-hydroxytaxane *O*-acetyltransferase. (a) Elution profile of an AcA 44 column. (b) Elution profile of a HighQ column. (c) Elution profile of a CHT II column. (d) Elution profile of a HiTrap Blue column. (e) Elution profile of a phenylsepharose column. Collected fractions were assayed with the standard enzyme assay. The bars indicate the combined fractions, which were used for subsequent enzyme purification.

was unequivocally characterized as **7**, the purified enzyme must have catalyzed the stoichiometric transfer of one acetyl group from acetyl CoA to the 10-position of 1 mol of the acceptor molecule.

The K_m value of acetyl CoA was calculated from the Lineweaver–Burk plot to be 61 μM, while the K_m value for **7** was determined to be 23 μM for this enzyme. The turnover with these substrates was 0.2 kat mol^{-1} at optimal conditions using the homogenous enzyme. The kinetic optimum (k_{cat}/K_m) for the reaction was calculated to be 8.7 s^{-1} L M^{-1}.

2.5. Substrate specificity

A crucial test was whether this acetyltransferase catalyzed the regiospecific acetylation only at the 10-position of desacetyltaxuyunnanine C derivatives or whether this enzyme catalyzed unspecifically all four or select hydroxyl-groups in 2,5,10,14-desacetyltaxuyunnanine C (**11**). Incubation of the latter substrate with the pure enzyme and [2-^{14}C]acetyl CoA showed that only one hydroxyl group was acetylated, which ruled out that the target enzyme was not selective and acetylated all four or

Table 1
Purification of acetyl CoA:10-hydroxytaxane *O*-acetyltransferase from *Taxus chinensis* cell cultures

Purification step	Total protein (mg)	Total activity (pkat)	Specific activity (pkat mg^{-1})	Recovery (%)	Purification-(fold)
Raw extract	372	–	–	–	–
$Ca_3(PO_4)_2$	194	–	–	–	–
Sephadex G25	78	–	–	–	–
DEAE	59	–	–	–	–
AcA44	25.7	724	28	100	1
HighQ	8.8	796	90.5	110	3.2
CHT II	0.73	578	792	80	2β
HiTrap Blue	0.05	168	3360	23	120
Phenylsepharose	0.001	6.3	6300	0.87	225
Mimetic Green	0.0001	0.7	7000	0.1	250

several free hydroxyl groups in that substrate molecule. The substrates 10,14- (**8**), 2,10,14- (**10**), as well as 5,10,14-desacetyltaxuyunnanine C (**9**), were singly acetylated as determined by radioTLC and radioHPLC on comigration with authentic standards. A crucial experiment was whether 10-DAB III (**3**) can act as an acetyl acceptor for the transferase. Indeed **3** was also singly acetylated. Incubation of **3** in the presence of enzyme and [1,2-$^{13}C_2$]acetyl CoA gave a product that was indistinguishable in its chromatographic behavior (TLC, HPLC) from baccatine III (**4**). Mass spectral analysis of the labelled product yielded in the CI-mode a clear $[M+1]^+$ peak at 589 *m/z* (Fig. 7b) in comparison to the reference compound (587 $[M+1]^{+1}$; Fig. 7a). Interestingly, the 10-epi-10-DAB III that has the α-configuration at the 10-hydroxyl group is not at all acetylated, indicating that the enzyme is not only regio-, but also stereoselective. The finding that 2,5-desacetyl-10,14-desacetoxy-taxuyunnanine C, which lacks the hydroxyl group recognized by the acetyl transferase, is inactive as a substrate underscores the regioselectivity of the enzyme. The results of the substrate specificity are shown in Table 2. The new enzyme is named acetyl coenzyme A:10-hydroxytaxane *O*-acetyltransferase and catalyzes the reaction shown in Fig. 4. It is a soluble enzyme and membranous fractions are devoid of this enzyme activity. This enzyme was found to occur in cell cultures of a number of Taxaceae including *T. baccata*, *T. media* and *Torreya taxifolia.*

3. Discussion

To this point only two acetyltransferases involved in the biosynthesis of naturally-occurring taxanes had been reported in the literature and neither of these enzymes had been extensively purified or characterized. One transferase was claimed to occur in roots of *T. baccata* (Zocher et al., 1996). This enzyme was reported to regiospecifically acetylate **3** at the 10-position with acetyl CoA as acetyl donor. The reaction product, however, was characterized only by TLC, HPLC and radioscanning and no rigorous spectrometric proof of its identity as baccatine III was presented. This is insufficient evidence to base the assertion that a regiospecific acetyltransferase had been isolated.

The second acetyltransferase is specific for the 5-hydroxyl group of the taxane nucleus and has been reported only in preliminary form (Hezari & Croteau, 1997). This enzyme was partially purified and shown to be a 50 kDa monomeric protein with an isoelectric point of $\sim$4.7. Both characteristics as well as the regiospecificity of the enzyme are clearly different from the enzyme discovered and characterized in this study.

There is no doubt that in *T. chinensis* cell cultures there are different acetyltransferases with different regiospecificity towards the different hydroxyl groups of the taxane ring system, as represented by the substrate **11**. The first fraction (AcA44 eluate) showing acetyltransferase activity in our purification scheme Table 1 acetylated in the presence of acetyl CoA all four hydroxyl groups of the taxane, yielding taxuyunnanine C (**5**) as a reaction product. In contrast, the purified enzyme that was isolated using **7** as the only substrate, is absolutely regiospecific for the 10-position. Furthermore, not only is 10-desacetyltaxuyunnanine C (**7**) monoacetylated, but other partly desacetylated taxuyunnanine C derivatives are also acetylated only at the 10-position Fig. 4. Moreover, 10-DAB III (**3**) as well as 19-hydroxy-10-DAB III is only acetylated at this 10-hydroxyl position.

We have demonstrated for this first time a regio- and stereoselective acetyltransferase that selectively acetylates the 10 β-hydroxyl position in a number of taxanes. This shows clearly that functionalization of the diterpenoid ring system requires highly specific enzymes.

4. Experimental

4.1. Plant material

Cell cultures of *T. chinensis* were as given in (Menhard et al., 1998). The cells were cultivated in 1 l Erlenmeyer

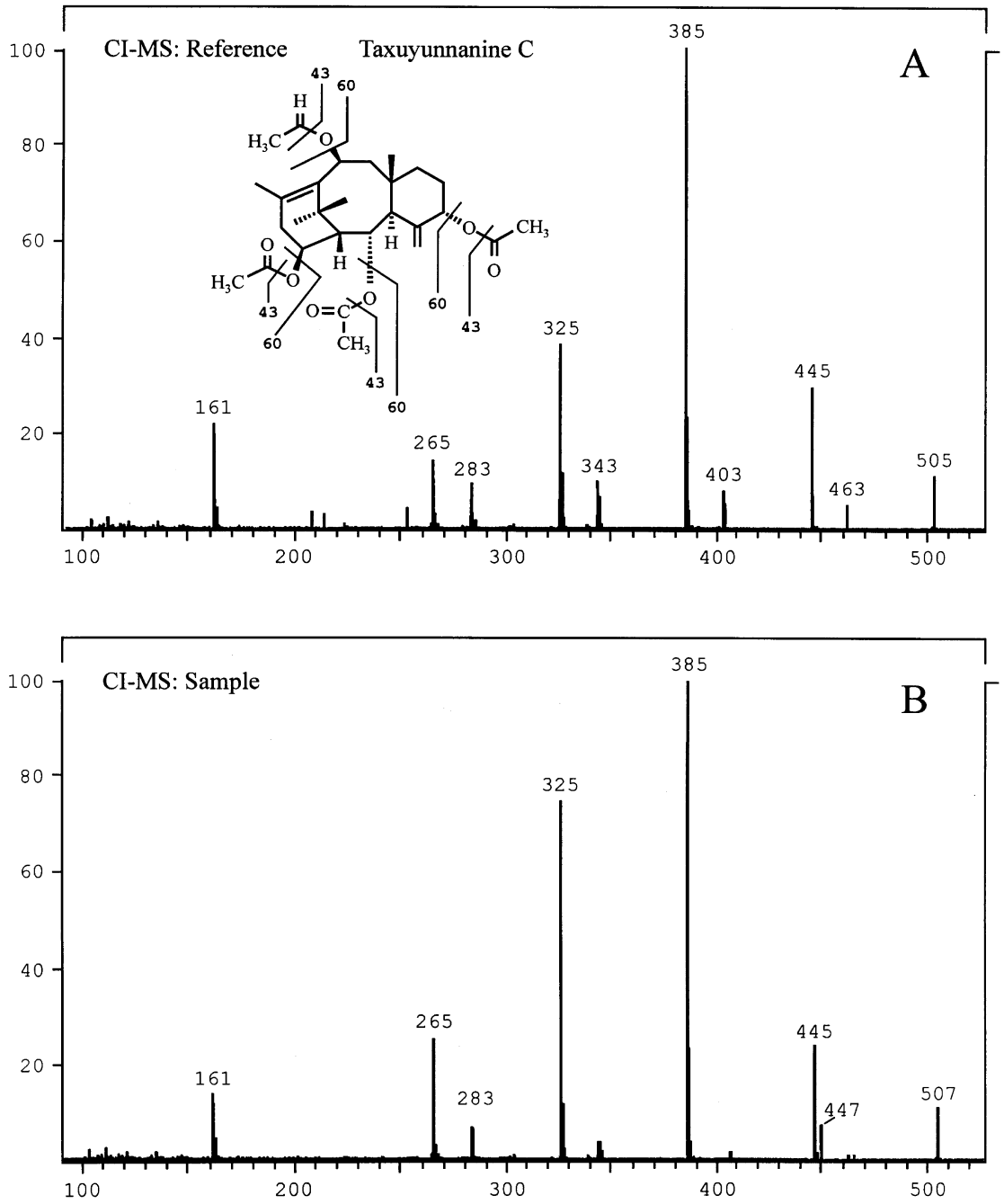


Fig. 6. CI-MS spectrum of taxuyunnanine C (**5**) as reference (a) and the product (b) synthesized from **7** and [1,2-^{13}C]acetyl CoA by the 120-fold purified acetyltransferase in the standard enzyme incubation system.

flasks containing 400 ml cell suspension over a period of 14 days at 24°C on a gyratory shaker (100 rpm) under constant illumination (650 lux). An inoculum of 170 ml suspension was added to 230 ml of fresh medium and three days later elicited with 30 mM methyl jasmonate (Serva). After seven days of cultivation the cells were harvested by suction filtration yielding 70 g fresh mass. The tissue was shock frozen with liquid nitrogen and could be stored at −20°C for up to one month.

4.2. Chemicals

[2-^{14}C]NaAc and [1-^{14}C]acetyl CoA were purchased from Amersham. ATP, acetyl CoA and acetyl CoA synthetase were obtained from Boehringer–Mannheim. [1,2-$^{13}C_2$]NaAc was purchased from Isotec. Tert-butylmethylether was obtained from Fluka. Materials for chromatography and prefilled columns were purchased from Pharmacia (HiTrap Blue, Phenylsepharose fast

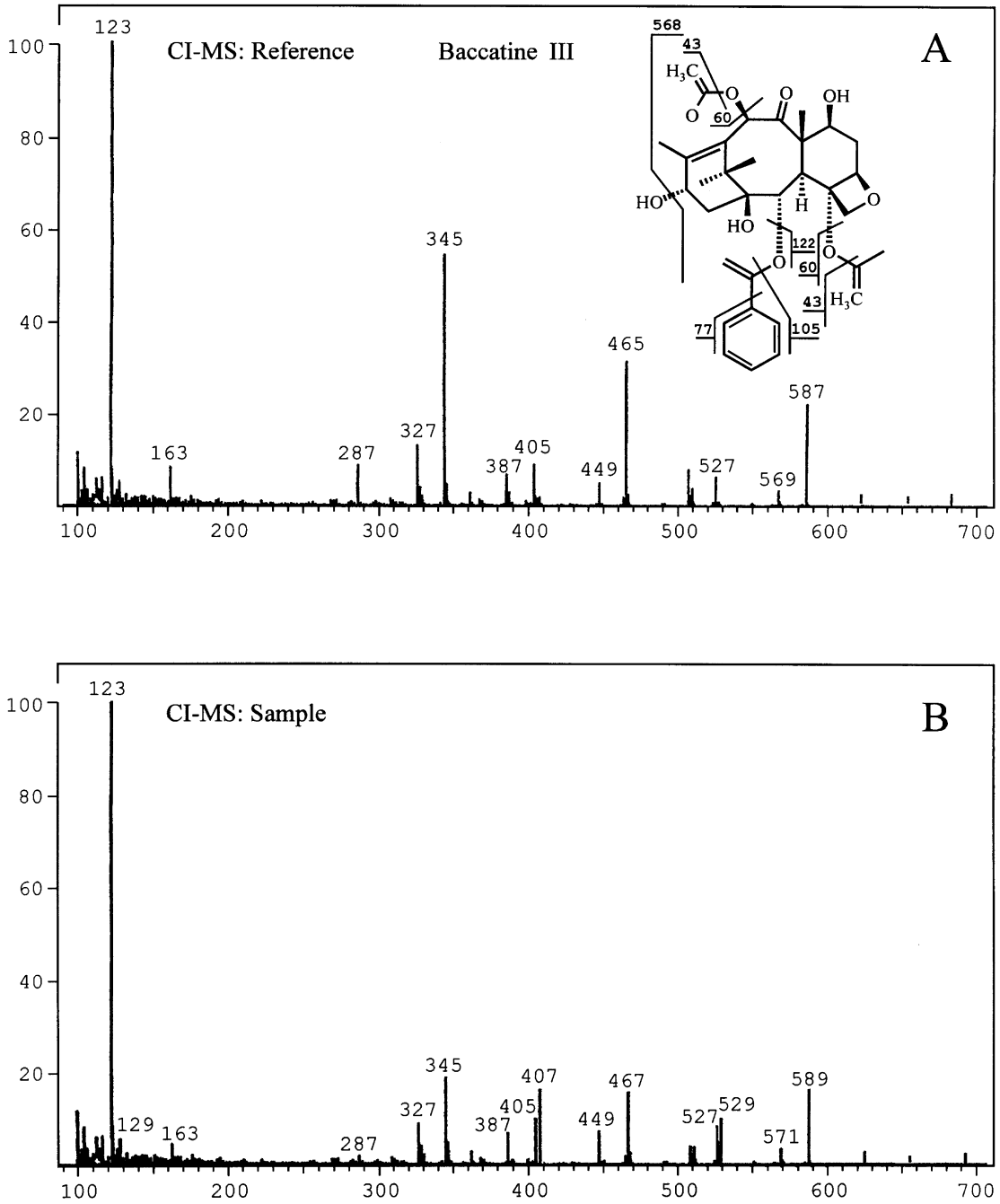


Fig. 7. CI-MS spectrum of baccatine III (**4**) as reference (a) and the product (b) synthesized by the 120-fold purified acetyltransferase from 10-desacetylbaccatine III (**3**) and [1,2-^{13}C]acetyl CoA.)

flow, Mono P HR 5/20, Sephadex G25, DEAE-Sephacel, PD10), Biorad (HighQ, CHT II, Bioselect SEC 250-5), Affinity Chromatography Ltd. (Mimetic Green 1A6 XL) and Serva (AcA44 Ultrogel). Calcium phosphate gel was prepared as described by (Colowick, 1955).

Labelled acetyl CoA was prepared by incubating the following substances for 3 h: 2 ml 5 mM Tris, pH 7.5, 20 mmol $MgCl_2$ (4.06 mg), 15 mmol ATP (9.08 mg), 9.3 mmol CoA–SH (7.64 mg), 3 U acetyl CoA synthetase, 9.3 mmol NaAc (0.78 mg) and 0.5 μCi [2-^{14}C]NaAc (50 mCi mmol^{-1}). The purification and isolation of the resulting acetyl CoA was done by paper chromatography using Whatman paper No. 3 and the solvent system BuOH–AcOH–H_2O (5:2:3). Acetyl CoA showed a R_f value of 0.26. [1,2-$^{13}C_2$]Acetyl CoA was prepared from [1,2-$^{13}C_2$]NaAc as given above.

Table 2
Rate of acetylation of different taxanes in the presence of acetyl CoA:10-hydroxytaxane *O*-acetyltransferase (100% = 6.3 nmol · s^{-1} · mg protein^{-1})

Substrate	Relative activity (%)
10-Desacetyltaxuyunnanine C (**7**)	100
10,14-Desacetyltaxuyunnanine C (**8**)	102
5,10,14-Desacetyltaxuyunnanine C (**9**)	88
2,10,14-Desacetyltaxuyunnanine C (**10**)	81
10-Desacetylbaccatine III C (**3**)	80
2,5,10,14-Desacetyltaxuyunnanine C (**11**)	76

For the synthesis of desacetylated derivatives of **5**, in each of five Eppendorf® caps **5** (15 mg) was dissolved in methanol (500 µl). NaOH (1 ml, 0.1 N or 1 N) was added. These mixts were incubated at 30°C for various times and then extracted into tert-butylmethylether. The organic layers were combined, dried over Na_2SO_4 and evapd to dryness. The residue was sepd using semipreparative HPLC (Vertex RP-18, 5 mm, 16 × 250 mm, Knauer). The solvent system A consisted of MeCN–H_2O (2:8) and solvent system B of MeCN. Gradient: 0 min 20% B; 30 min 70% B, 45 min 70% B; flow rate: 5 ml min^{-1}; detection: 227 nm). By this method, eight different desacetylated derivatives of **5** could be isolated. Their structures were determined by NMR spectroscopy.

The following substances were kind gifts of Indena Comp., Milan: 10-DAB III (**3**) baccatine III (**4**), 19-OH-10-DAB III, 10-epi-DAB. 2,5-Desacetyl-10,14-desacetoxy-taxuyunnanine C was prepared from **5** by Dr. U. Maier of this laboratory.

4.3. Enzyme assay

The activity of the acetyl CoA:10-hydroxytaxane *O*-acetyltransferase was measured using an incubation mixture consisting of a sufficient amount of enzyme, 50 µl 0.8 M Tris–HCl, pH 8.5, acetyl CoA (5 nmol, containing 0.02 µCi [1-^{14}C]acetyl CoA) and **7** (15 nmol) dissolved in 5 µl DMSO in a total volume of 185 µl. Assays with boiled enzyme or DMSO instead of **7** served as controls. After incubation for 30 min at 35°C, the reaction was stopped by the addition of 600 µl tert-butylmethylether and 20 µl 10% H_2SO_4 and the mixture was shaken for 10 min. After centrifugation, the amount of **5** formed was measured in a 500-µl aliquot of the organic layer. This aliquot was dried by a stream of air and the radioactivity measured using a liquid scintillation counter or analyzed by radio TLC (solvent system $CHCl_3$–MeCN, 7:3).

4.4. Preparation of crude enzyme extracts

All steps were performed at 4°C. Frozen cells of *T. chinensis* suspension cultures (200 g fr. wt) were homogenized in the presence of 20 g PVPP, 350 ml 100 mM boric acid/NaOH, pH 8.5, 20 mM MeSH, 20% glycerol (buffer A) until the mixture was thawed. This homogenate was filtered through cheese cloth and centrifuged at 10,000*g* for 10 min. The supernatant was mixed with $Ca_3(PO_4)_2$ (1.9 g dry wt according to Colowick (1955), suspended in 50 ml buffer A). After 10 min, this suspension was centrifuged at 3000*g* for 5 min and the supernatant used for further purification.

4.5. Ammonium sulfate precipitation

The supernatant was subjected to $(NH_4)_2SO_4$ precipitation (0–70%). The ppt was dissolved in 30 ml 50 mM Tris–HCl, pH 8.5, 20 mM MeSH, 20% glycerol (buffer B) and subsequently desalted using a Sephadex G25 column (2.7 × 7 cm). Subsequently, colored impurities were partly removed by a DEAE-Sephacel column (2.5 × 5 cm). Both columns had been previously equilibrated with buffer B. The protein-containing fractions were collected and concd in a stirring cell (Amicon) to approximately 5 ml by ultrafiltration (PM10 membrane).

4.6. AcA44 gel filtration

The concentrate was applied to an AcA44 column (2.8 × 100 cm) previously equilibrated with buffer B. This buffer was also used for fractionation at 20 ml h^{-1} Fractions of 6.5 ml were collected and assayed for protein and enzyme activity.

4.7. HighQ ion exchange chromatography

The fractions of the gel filtration step containing the major activity were pooled (60 ml) and applied to a HighQ column (5 ml) that had been equilibrated with buffer B. The column was operated at 2 ml min^{-1} and washed with 70 ml buffer B prior to protein elution. The protein was eluted with a 0.07–1 M KCl gradient in buffer B (0.07 M for 10 min, 0.14 M for 10 min, 1 M for 15 min). Fractions of 1 ml were collected and assayed for protein and enzyme activity.

4.8. CHT II (hydroxylapatite)

Active fractions (4 ml) of the HighQ column were pooled and applied to a CHT II (5 ml) column previously equilibrated with 10 mM NaP_i buffer pH 6.8, 20 mM MeSH, 20% glycerol and operated at 0.5 ml min^{-1} The column was washed with 20 ml of the same buffer and eluted with 15 ml of 160 mM NaP_i buffer, pH 6.8, 20 mM MeSH, 20% glycerol. Fractions of 0.5 ml were collected and assayed for protein and enzyme activity.

4.9. HiTrap Blue affinity chromatography

The pooled active fractions (1.5 ml) were applied to a HiTrap Blue column (1 ml) that had been equilibrated with buffer B. The loading was done at 0.5 ml min^{-1}. The column was washed with 15 ml buffer B before eluting the protein using a linear 0–1 M KCl gradient at 0.5 ml min^{-1} (total volume 15 ml). Fractions of 0.5 ml were collected and assayed for protein and enzyme activity.

4.10. Phenylsepharose hydrophobic interaction chromatography

Active fractions (1.5 ml) of the HiTrap Blue step were pooled. With a solution of 1 M $(NH_4)_2SO_4$ in buffer B this pool was adjusted to 0.5 M $(NH_4)_2SO_4$ and then applied to a Phenylsepharose column (1×1.3 cm). The column was operated by gravity and had previously been equilibrated with 0.5 M $(NH_4)_2SO_4$ in buffer B. It was washed with 7 ml 0.5 M $(NH_4)_2SO_4$ in buffer B and the protein was eluted with 0.1 M $(NH_4)_2SO_4$ in buffer B (10 ml). The eluate was collected in fractions of 0.5 ml and assayed for protein and enzyme activity.

4.11. Mimetic Green 1A6 XL affinity chromatography

The fractions of the hydrophobic interaction chromatography containing the major activity were pooled (2.5 ml) and desalted using a PD10 column and buffer B. Subsequently, the desalted protein was applied to a Mimetic Green column (1×1.2 cm) which was again operated without pump. This column had been equilibrated with buffer B and was washed with the same buffer (3 ml). Elution with 3 ml 0.5 M acetyl coenzyme A gave an eluate containing exclusively the acetyl CoA:10-hydroxytaxane *O*-acetyltransferase as judged by SDS/PAGE.

4.12. Electrophoretic analysis of enzyme purity

SDS/PAGE of the protein bands obtained from the Mimetic Green step was performed as described by Laemmli (1970). After electrophoresis, protein was detected by silver nitrate staining (Blum, Beier, & Gross, 1987). Molecular mass standards for SDS/PAGE (rainbow markers, Amersham) were used in order to estimate the molecular mass of acetyl CoA:10-hydroxytaxane *O*-acetyltransferase under denaturating conditions.

4.13. Native molecular mass determination by gel filtration chromatography

The molecular mass of the native enzyme was determined by gel filtration chromatography on a Bioselect SEC 250-5 column at 0.2 ml min^{-1}. The following proteins were used as standards: chymotrypsinogen A (25 kDa), bovine serum albumin (67 kDa), aldolase rabbit (160 kDa), catalase bovine (240 kDa), ferritine horse (450 kDa).

4.14. Isoelectric point

The isoelectric point of acetyl CoA:10-hydroxytaxane *O*-acetyltransferase was determined by chromatofocussing on a Mono P HR 5/20 column (0.5 cm × 20 cm) performed at 0.7 ml min^{-1}. Equilibration was done with 25 mM imidazole/HCl, pH 7.4, containing 20 mM MeSH. The adsorbed protein was eluted with polybuffer 74-H_2O (1:8, Pharmacia) adjusted to pH 4 and containing 20 mM MeSH. Fractions of 1 ml were collected and assayed for enzyme activity.

4.15. Protein determination

Protein concs were determined by the method of Bradford (1976) with bovine serum albumin as a standard.

4.16. MS and NMR spectroscopy

MS spectroscopy was done using a Finnigan MAT SSQ 70 (70 eV, isobutane). NMR spectroscopy was performed with a Bruker DRX 500 spectrometer. For measurement the taxanes were dissolved in $CDCl_3$.

4.17. HPLC system

Merck-Hitachi (pump L-6200; autosampler AS-4000; detector L-4250).

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