ELSEVIER

Journal of Chromatography A, 752 (1996) 123–130

JOURNAL OF CHROMATOGRAPHY A

Isocratic high-performance liquid chromatography of coenzyme A esters involved in the metabolism of 3*S*-hydroxy-3-methylglutaryl-coenzyme A Detection of related enzyme activities in *Catharanthus roseus* plant cell cultures

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Received 1 February 1996; revised 23 May 1996; accepted 28 May 1996

Abstract

For studies on enzyme activities involved in the metabolism of 3*S*-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) in *Catharanthus roseus* plant cell cultures, an isocratic HPLC system was developed for the separation of coenzyme *A* (CoASH), acetyl-CoA, acetoacetyl-CoA, HMG-CoA, 3-methylglutaconyl-CoA (MG-CoA), and their respective 3′-dephospho-derivatives. Using an RP-18 column (250×4.6 mm, 5 μm) retention in eluents containing 0.2 *M* sodium phosphate buffer (pH range 4.7–6.7) supplemented with methanol (12.5–20 ml per 100 ml of buffer) was studied. Baseline separation was obtained with an eluent consisting of buffer pH 5.0–methanol (100:17, v/v). The linearity for the quantitative determination of the esters (range 0.5–10 nmol) was tested. Detection limits were found to be between 2.5 and 6 pmol. Guanosine was used as an internal standard. In cell-free preparations of *C. roseus* cell cultures, the enzyme activities of HMG-CoA lyase (EC 4.1.3.4) and MG-CoA hydratase (HMG-CoA hydrolyase, EC 4.2.1.18) were detected.

Keywords: *Catharanthus roseus*; Coenzyme A esters; Enzymes; Hydroxymethylglutaryl-coenzyme A

1. Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) is a central intermediate in a number of essential metabolic processes, as sterol biosynthesis, ketogenesis and leucine metabolism. HMG-CoA is directed into these pathways by specific enzymes (Fig. 1). HMG-CoA reductase (HMGR, EC 1.1.1.34), catalyzing the formation of mevalonate from HMG-CoA, is a regulatory enzyme in (chole)sterol biosynthesis. HMG-CoA lyase (HMGL, EC 4.1.3.4) and 3-methylglutaconyl-CoA hydratase (MGH, EC 4.2.1.18) are involved in ketogenesis and in the metabolism of the branched-chain amino acid leucine. HMG-CoA is formed from three molecules of acetyl-CoA in two successive condensation steps, catalyzed by acetoacetyl-CoA thiolase (AACT, EC 2.3.1.9) and HMG-CoA synthase (HMGS, EC

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0021-9673/96/$15.00
PII S0021-9673(96)00526-2

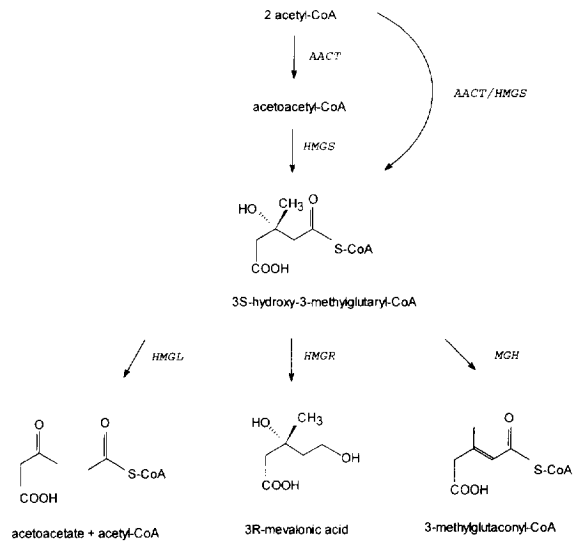

Fig. 1. Reactions catalyzed by enzymes involved in the metabolism of HMG-CoA. AACT: 2 acetyl-CoA (**4**) ↔ acetoacetyl-CoA (**3**) + CoASH (**1**); HMGS: acetoacetyl-CoA (**3**) + acetyl-CoA (**4**) → HMG-CoA (**2**) + CoASH (**1**); HMGR: HMG-CoA (**2**) +2 NADPH → mevalonate + CoASH (**1**); HMGL: HMG-CoA (**2**) → acetyl-CoA (**4**) + acetoacetate; MGH: HMG-CoA (**2**) ↔ MG-CoA (**5**) + H_2O; phosphatase (non-specific conversion of CoA-esters (**1–4**)): X-CoA (**1–4**) → *X*-3′-dephospho-CoA (**1A–4A**).

4.1.3.5), respectively. These processes and enzymes have been well documented from mammals and yeast. Except for the enzyme HMGR, little is known of HMG-CoA metabolism in plants (reviewed in [1]).

In our studies on the regulation of biosynthetic pathways leading to the formation of plant secondary metabolites (e.g., terpenoid indole alkaloids, triterpenes and anthraquinones), part of the research is now focused on the early steps of terpenoid biosynthesis, in particular on the enzymes involved in HMG-CoA metabolism in *Catharanthus roseus* cell cultures [2,3]. For these studies we have now developed an analytical procedure to separate the different CoA-esters, being the substrates and/or products of these enzyme reactions.

Various methods have been described for the analysis of short chain acyl-CoA derivatives in (mammalian) tissue extracts by HPLC (reviewed in [4]). However, none of these methods met our requirements for our specific set of CoA-esters: UV detection instead of radiochemical detection, isocratic instead of gradient elution and separation from 3′-dephospho-CoA-derivatives (dPCoA-derivatives, which are formed by phosphatase activity present in protein extracts from the cultured plant cells [2]). Here we report on isocratic separations, using UV detection at 254 nm, of CoASH (**1**), dPCoASH (**1A**), HMG-CoA (**2**), HMG-dPCoA (**2A**), acetoacetyl-CoA (**3**), acetoacetyl-dPCoA (**3A**), acetyl-CoA (**4**), acetyl-dPCoA (**4A**) and 3-methylglutaconyl-CoA (MG-CoA) (**5**); enzyme activities, related with these compounds were detected in cell-free preparations of *C. roseus* cell cultures.

2. Experimental

2.1. Chemicals

Guanosine hydrate was from Aldrich (Axel, Netherlands). CoA, acetyl-CoA, acetoacetyl-CoA (all sodium salts), polyvinylpolypyrrolidone and 3′-ribonucleotide dephosphohydrolase (EC 3.1.3.6) were from Sigma (Bornem, Belgium), while 3(*R/S*)-HMG-CoA was from Fluka (Bornem, Belgium). 1,4-Dithiothreitol was from Merck (Darmstadt, Germany). Leupeptin was from ICN (Zoetermeer, Netherlands). Triton X-100 was from J.T. Baker (Deventer, Netherlands). All other chemicals were of analytical grade. Solvents were distilled prior to use. MG-CoA was obtained through incubation of HMG-CoA with partially purified MG-CoA hydratase (see Section 2.2 below). HPLC-eluents were filtered through a 0.45-μm RC55 membrane filter (Schleicher and Schuell, Dassel, Germany) and degassed by vacuum. The HPLC column was a Hypersil ODS (5 μm, 250×4.6 mm, Shandon, Zeist, Netherlands), with a 20×2 mm guard column (Upchurch, Oak Harbor, Washington, USA), filled with LiChrosorb RP18 (15–25 μm) (Merck).

2.2. Preparation of dPCoA-esters

A 20-μl volume of 10 m*M* CoASH (**1**) or CoA-ester (**2–4**) in 25 m*M* sodium phosphate buffer pH 4.5 was mixed with 930 μl 50 m*M* Tris HCl buffer pH 7.5 and 0.4 U 3′-ribonucleotide dephosphohydrolase (100 μl of a 4 U/ml solution in 50 m*M* Tris HCl pH 7.5). After incubation for 10 min at

37°C, the reaction was stopped by adding 250 μl 1 *M* H_3PO_4. At this stage about 70% of the CoA-esters was converted. The mixtures of CoA-ester and its dPCoA derivative were used for the determination of the *k'* values in the different eluents. Injection volumes were 20–40 μl. The mixtures (pH about 3.5) were stored in 100 μl aliquots at −15°C; under these conditions no decomposition of the samples was noticed during the course of our experiments.

2.3. HPLC equipment

Two HPLC systems were used. System A consisted of an LKB 2150 HPLC-pump (LKB, Bromma, Sweden), a Rheodyne 7125 sample injector equipped with a 20- or 100-μl loop (Rheodyne, Cotati, CA, USA), an LKB 2158 Uvicord SD UV-detector, set at 254 nm and a Waters 741 data module (Waters Associates, Milford, MA, USA). System B consisted of an LKB 2150 HPLC-pump, a Waters Wisp 710B autosampler, a Waters 990 photodiode array detector and a Waters 5200 printer plotter.

2.4. Methods

All HPLC analyses were carried out at ambient temperature at a flow-rate of 1.5 ml/min. *k'* values were determined from at least two sample injections, the hold-up time was determined by the retention time of the injection peak. Dilutions for the linearity studies were prepared in duplicate, each sample was injected twice.

2.5. Extraction of enzymes

Catharanthus roseus (L.)G. Don cell cultures were routinely grown at 25°C on gyratory shakers (type G10, New Brunswick Scientific, Edison, NJ, USA) at 120 rpm under continuous light (2800 lux, Philips 58W33, Philips, Eindhoven, Netherlands). Cells were subcultured every week by 1:4 dilution in an MS-medium [5] containing 3% sucrose (no growth regulators were added).

Biomass (200 g FW) of a 5-day-old *C. roseus* cell culture was frozen in liquid nitrogen and homogenised in a Waring blender (Waring, New Hartford, CT, USA). The homogenate was thawed in the presence of 100 ml cold (4°C) extraction buffer (0.1 *M* potassium phosphate buffer pH 7.5, containing 1 *M* sucrose, 2 m*M* Na_2EDTA, 10 m*M* dithiotreitol (DTT), 10 μ*M* leupeptin and 1% Triton X-100). Ten gram polyvinylpolypyrrolidone was also added. After thawing the homogenate was squeezed through Miracloth (Calbiochem-Novabiochem, La Jolla, CA, USA) and the filtrate was centrifuged for 30 min at 20 000 *g* at 4°C. By ultrafiltration (ProVario-3 system equipped with a filter with a nominal molecular mass average of $30 \cdot 10^{-3}$, Filtron Technology, Terheijden, Netherlands) 190 ml of the supernatant was concentrated to 48 ml while the buffer was changed to 20 m*M* Tris HCl pH 7.5, containing 3% glycerol and 3 m*M* DTT (buffer A). A 2-ml volume of this concentrate was injected into an FPLC system (Pharmacia, Uppsala, Sweden, equipped with a Mono-Q column (HR5/5, Pharmacia). Elution of proteins was monitored by UV detection (280 nm). The eluate was collected in fractions of 2 ml after 30 ml linear gradient elution of 0–0.5 *M* NaCl in buffer A at a flow-rate of 0.5 ml/min. MGH activity eluted at about 0.25 *M* NaCl.

2.6. Preparation of MG-CoA (5)

The Mono-Q fraction containing MGH activity was desalted over Sephadex G-25 columns (PD-10, Pharmacia), which were equilibrated with incubation buffer (0.2 *M* Tris HCl pH 8.2, 20 m*M* $MgCl_2$, 0.2 m*M* EDTA). A 500-μl volume of the desalted sample was mixed with 100 μl 10 m*M* 3(*R/S*)-HMG-CoA (in 25 m*M* phosphate buffer pH 4.5) and 1400 μl incubation buffer. After incubation for 10 min at 30°C the reaction was stopped by addition of 230 μl of a 326 μ*M* guanosine hydrate solution in H_3PO_4 (8.5%, v/v). At this stage about 3.5% of the substrate was converted into MG-CoA (probably, MG-CoA hydratase is specific for 3S-HMG-CoA, so 7% of this isomer is thus converted). The mixture was used for the determination of the *k'* values in the different eluents. Injection volumes were 50 μl.

2.7. Cell-free conversion of HMG-CoA (2)

A 500-μl volume of the concentrated extract (see Section 2.5 was mixed with 100 μl 10 m*M* HMG-CoA (in 25 m*M* phosphate buffer pH 4.5) and 1400 μl incubation buffer, to which was added 2 m*M*

DTT. After incubation for 10 min at 30°C the reaction was stopped by addition of 230 μl of a 326 μM guanosine hydrate solution in H_3PO_4 (8.5%, v/v).

3. Results and discussion

3.1. Choice of organic solvent and its concentration in the eluent

For the development of an analytical system for the various CoA-ester substrates and products of the enzymes involved in the metabolism of HMG-CoA, an HPLC system described for the analysis of 17 short chain CoA-esters [6] was taken as a starting point. The separation was obtained using an octadecylsilica column and gradient elution with a sodium phosphate buffer and acetonitrile. Using the eluent 0.2 M sodium phosphate buffer pH 5.0–acetonitrile (100:5, v/v) in isocratic mode, we obtained resolution of (**1–4**) within 22 min, though acetyl-CoA (**4**) and acetoacetyl-CoA (**3**) were not baseline separated. Including also the dPCoA esters (**1A–4A**) in the mixtures to be analysed, resolution was not satisfactory. By using methanol instead of acetonitrile as organic solvent in the eluent, peak shapes improved and complete resolution of acetoacetyl-CoA (**3**) and acetyl-CoA (**4**) could be obtained. It is noteworthy that, comparing acetonitrile and methanol, the elution sequence of acetoacetyl-CoA (**3**) and acetyl-CoA (**4**), is inversed. Using an eluent consisting of buffer pH 5.0–methanol (100:20, v/v), k' values of 1.3 and 3.8 were found for CoA (**1**) and acetyl-CoA (**4**), respectively. Separation of (**1–4**) was obtained within 10 min (flow-rate 1.5 ml/min). Furthermore the methanol containing eluent showed to be more stable: small variations in organic solvent content do not cause significant shifts in retention times, as was observed for the acetonitrile containing eluents.

The full set of CoA-esters, including MG-CoA (**5**) and the dPCoA-esters (**1A–4A**), could be separated within 25 min in the eluent buffer pH 5.0–methanol (100:17, v/v). Fig. 2 shows the relation between the k' values of the CoA-esters (**1–5**, **1A–4A**) and the volume of methanol (in ml) added to 100 ml 0.2 M sodium phosphate buffer pH 5.0. In Fig. 3 a separation of a mixtures of (**1–4**) and (**1A–4A**) is given.

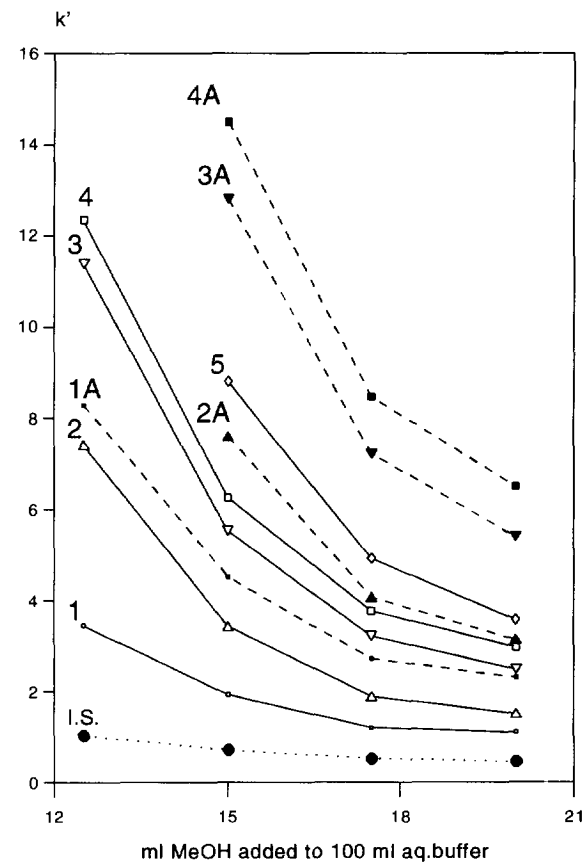

Fig. 2. Capacity factors (k') of CoA (**1**), dPCoA (**1A**), HMG-CoA (**2**), HMG-dPCoA (**2A**), acetoacetyl-CoA (**3**), acetoacetyl-dPCoA (**3A**), acetyl-CoA (**4**), acetyl-dPCoA (**4A**), 3-methylglutaconyl-CoA (MG-CoA) (**5**) and guanosine (IS) versus the ratio of methanol in the eluent (expressed as the volume of methanol (in ml) added to 100 ml 0.2 M phosphate buffer pH 5.0). Column RP-18 (5 μm).

3.2. pH of the eluent

CoASH (**1**) is a fairly strong acid with pK_a-values of 9.6 (thiol), 6.4 (secondary phosphate) and 4.0 (adenine NH_3) [7]. At pH 5.0 both the secondary phosphate and the adenine amino group are partly protonated and thus retention behaviour will be influenced by changes in pH. Fig. 4 gives the relation

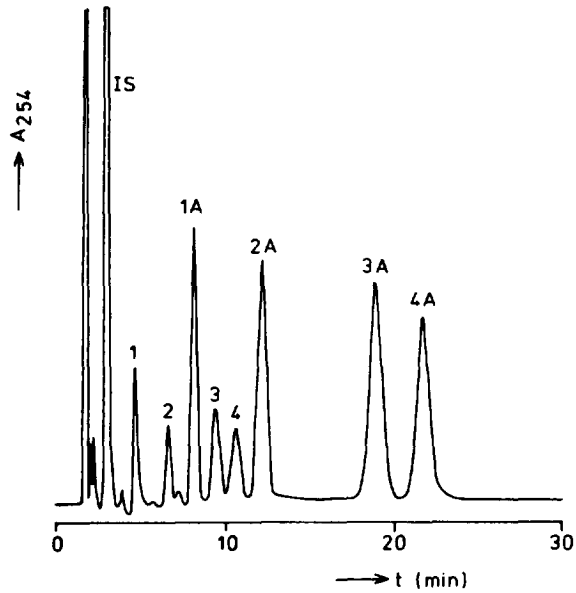

Fig. 3. Separation of CoA-esters (**1–4**) and dPCoA-esters (**1A–4A**) and IS guanosine. HPLC-system A. Eluent, 0.2 *M* phosphate buffer pH 5.0–methanol (100:17, v/v). Column, RP-18 (5 μm); flow-rate, 1.5 ml/min. UV detection at 254 nm. *y*-axis full scale=0.016 AU. Amounts injected: (**1–4**) circa 0.5 nmol; (**1A–2A**) circa 1 nmol; (**3A–4A**) circa 2 nmol; injection volume, 100 μl. (combined acidified incubationreaction mixtures) Peak identification: CoA (**1**), dPCoA (**1A**), HMG-CoA (**2**), HMG-dPCoA (**2A**), acetoacetyl-CoA (**3**), acetoacetyl-dPCoA (**3A**), acetyl-CoA (**4**), acetyl-dPCoA (**4A**) and guanosine (IS).

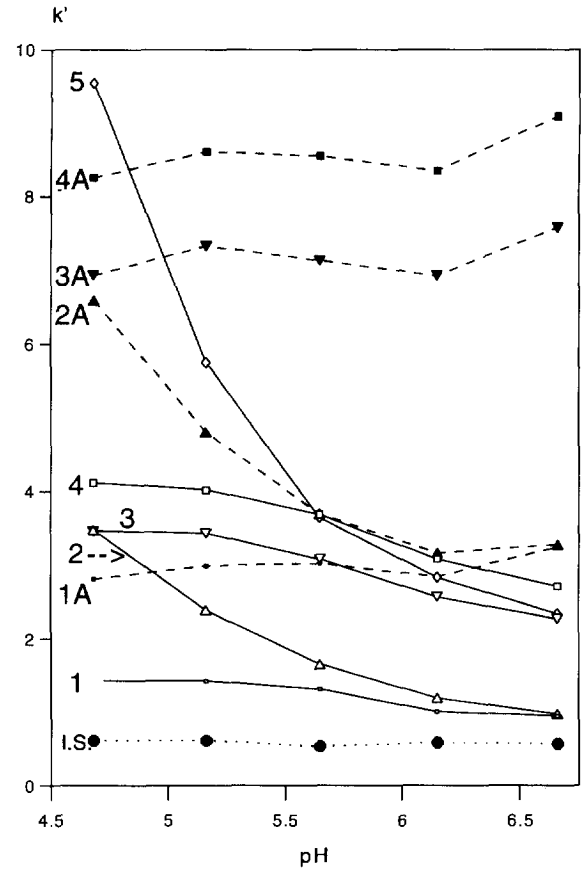

Fig. 4. Capacity factors (k') of CoA (**1**), dPCoA (**1A**), HMG-CoA (**2**), HMG-dPCoA (**2A**), acetoacetyl-CoA (**3**), acetoacetyl-dPCoA (**3A**), acetyl-CoA (**4**), acetyl-dPCoA (**4A**), 3-methylglutaconyl-CoA (MG-CoA) (**5**) and guanosine (IS) versus the pH of the buffer in the eluent containing 17.5 ml methanol added per 100 ml 0.2 *M* phosphate buffer. Column RP-18 (5μm).

between pH and the retention of the CoA-esters (**1–5** and **1A–4A**). Within the pH range 4.6–6.7 retention of HMG-CoA (**2**), HMG-dPCoA (**2A**) and MG-CoA (**5**) is susceptible to changes in pH due to the presence of an additional carboxyl-group. Over the pH range tested, all compounds eluted as symmetrical peaks. The peaks tended to be sharper at higher pH values, however at pH values above 5.5 no satisfactory separation was obtained. Reading the Figs. 2 and 4 it should be noted that the pH of the buffer is increased by about 0.25 units when 17–20% methanol is added.

The influence of the negatively charged phosphate group on the retention behaviour was illustrated by the k' values of the dPCoA esters as compared to those of their phosphorylated counterparts. Due to the absence of phosphate, the k' increased with a factor 2.1–2.4, as determined in eluents with decreasing concentrations of methanol (Fig. 2). When changing the pH in the range 4.7 to 6.7, the influence of the phosphate group is stronger: k'-values of the dPCoA esters shifted up to a factor 3.4 (Fig. 4).

3.3. Temperature

Selectivity was slightly affected by temperature, as the dk'/dT was not the same for all esters. Increase of temperature decreased k'; the factor $k'(27°)/$

$k'(22°)$ ranged from 0.85 for CoASH (**1**) to 0.94 for acetyl-CoA (**4**).

3.4. Selection of internal standard

Guanosine was selected as internal standard, in all eluents tested it eluted before the CoA-esters and separated from the injection peak (Figs. 2 and 4). Adenosine and adenine, compounds more related to the CoA structure, did not meet these requirements. The retention of the internal standard guanosine was not affected by changes in pH (Fig. 4).

3.5. Calibration curves and detection limits

The relation between injected concentration of the CoA-esters (**1–4**) and the detector output, was studied by injection of 0.5 up to 10 nmol of each ester (Table 1). A linear relation was found between 0.5 and 10 nmol. Detection limits, as determined at peak heights of 3 times the signal-to-noise ratio (in system A), are 2.5 pmol for CoA (**1**), 4 pmol for HMG-CoA (**2**) and 6 pmol for acetoacetyl-CoA (**3**) and acetyl-CoA (**4**).

The UV spectra, as obtained by diode-array detection, are sufficiently characteristic for identification of each CoA-ester (Fig. 5). The spectra of the dPCoA-esters are identical to their parent CoA-esters, but the pairs are well separated as the phosphate is important in the retention behaviour (see Section 3.6, below).

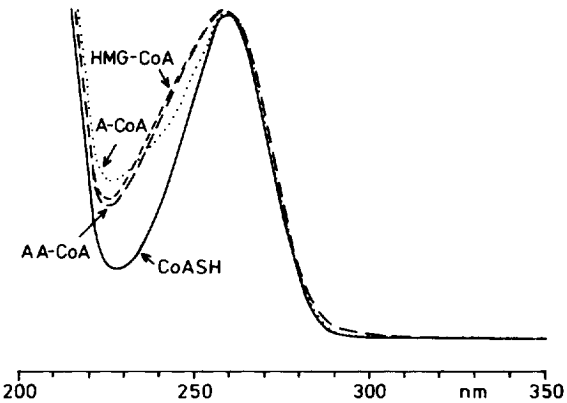

Fig. 5. UV absorption spectra of CoASH, HMG-CoA, acetyl-CoA (A-CoA) and acetoacetyl-CoA (AA-CoA) as recorded by photodiode array detection. Eluent, 0.2 *M* sodium phosphate buffer pH 5.0–acetonitrile (100:5).

3.6. Detection of enzyme activities involved in the metabolism of HMG-CoA in cell-free preparations of Catharanthus roseus cell cultures.

The HPLC system for the analysis of CoA-esters was applied to the detection of enzymes from *Catharanthus roseus* cell cultures, metabolizing HMG-CoA. Fig. 6 shows the chromatogram of an incubation experiment of HMG-CoA (**2**) with a protein extract of these cells as described in Section 2.7. Three enzyme activities can be detected simultaneously:

1. HMGL, which catalyses the formation of acetyl-CoA (**4**) from HMG-CoA (**2**).

Table 1
Calibration curves for the CoA-esters (**1–4**) and the IS guanosine, following the equation: peak area (254 nm)=slope·nmol CoA-ester+constant.

Compound	Peak area (254 nm) range (arbitrary units)	n^a	Slope	Constant	Standard error of regression	Correlation coefficient (r)
Guanosine	68–700	10	626	6.0	8.7	0.9995
CoASH (**1**)	300–6000	12	559	45.3	25.6	0.9999
HMG-CoA (**2**)	300–6000	12	511	35.1	23.0	0.9999
Acetoacetyl-CoA (**3**)	300–6000	12	586	19.5	44.8	0.9998
Acetyl-CoA (**4**)	300–6000	12	571	28.3	27.3	0.9999

Injected amounts 0.5, 1, 2, 4, 7.5 and 10 nmol per CoA-ester. Eluent: 0.2 *M* phosphate buffer pH 5.0–methanol (100:17.5); HPLC system A; injection volume 20 μl.

[a] Number of data points; each data point is the mean of two injections of the same sample.

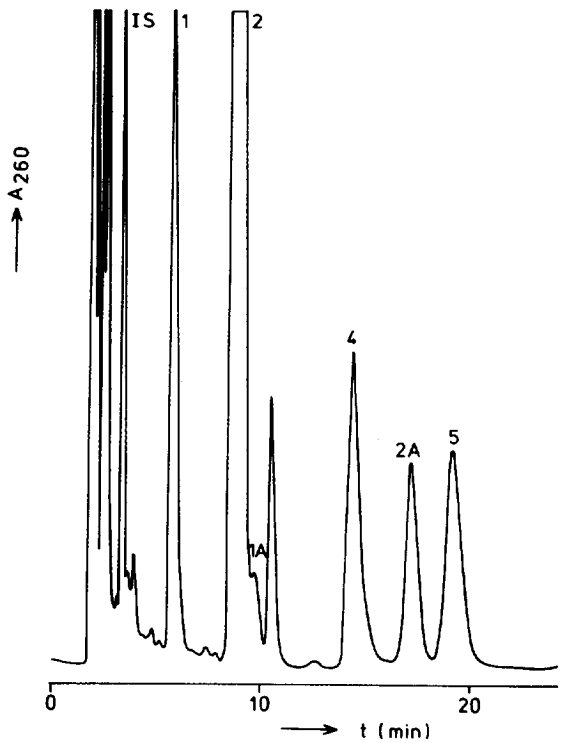

Fig. 6. Chromatogram obtained after cell-free conversion of HMG-CoA with a protein extract from *Catharanthus roseus* cell cultures (see Section 2.7). HPLC system B. Eluent: 0.2 *M* phosphate buffer pH 5.0–methanol (100:15, v/v). Column RP-18 (5 μm); flow-rate, 1.5 ml/min. UV detection, 260 nm; *y*-axis full scale 0.05 AU; injection volume, 50 μl. Peak identification: CoA (**1**), dPCoA (**1A**), HMG-CoA (**2**), HMG-dPCoA (**2A**), acetyl-CoA (**4**), acetyl-dPCoA (**4A**), 3-methylglutaconyl-CoA (MG-CoA) (**5**) and guanosine (IS). Before incubation the sample contained 1.7 nmol guanosine (IS) and 22.5 nmol of HMG-CoA (**2**) of which about 25% was converted by the three enzymes HMGL, MGH and 3′-nucleotidase.

2. MGH, catalysing the formation of MG-CoA (**5**) from HMG-CoA (**2**). (In all experiments racemic HMG-CoA (**2**) was used, HMGL and MGH accept only one isomer, probably the 3*S*-isomer).
3. Phosphatase (3′-nucleotidase) activity, a non-specific enzyme yielding HMG-dPCoA (**2A**) and dPCoASH (**1A**), which is derived from a CoASH (**1**) impurity of the substrate.

This example illustrates the advantage of HPLC in assaying enzyme activities. Side-reactions, which may be overlooked by spectrophotometrical or radiochemical methods, can be rapidly identified. This HPLC system is now used in our laboratory in the process of characterization of the enzymes involved in the metabolism of HMG-CoA in plant cell cultures.

4. Conclusion

An isocratic HPLC system has been developed for the separation of some short-chain CoA-esters which are detected by UV absorbance. The relative short run time makes this system attractive for the development of a series of HPLC assays for metabolic important enzymes (Fig. 1) as HMG-CoA lyase. Besides the absence of radio-labelled compounds in these methods, a further advantage is the possibility to quantify both substrate consumption and product formation of the enzyme reactions.

For the separation of the full set of CoA-esters (**1–5, 1A–4A**) 15–17 ml methanol per 100 ml 0.2 *M* sodium phosphate buffer pH 5.0 are required. In case the sample is free from dPCoA-derivatives, 20 ml methanol per 100 ml buffer allows an efficient separation of CoA-esters (**1–5**).

Abbreviations

AACT	acetoacetyl-CoA thiolase EC 2.3.1.9)
CoA(SH)	coenzyme A (reduced form)
$(CoAS)_2$	coenzyme A (oxidized form)
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMGL	HMG-CoA lyase (EC 4.1.3.4)
HMGR	HMG-CoA reductase (EC 1.1.1.34)
HMGS	HMG-CoA synthase (EC 4.1.3.5)
MG-CoA	3-methylglutaconyl-coenzyme A
MGH	MG-CoA hydratase (EC 4.2.1.18)
dPCoA	3′-dephospho-coenzyme A.

Acknowledgments

We wish to thank Mrs V. de Boer-Hlupá, Mr A.G.M Goosen and Mr K. Weisz for technical support. Financial support by the "Van Leersum

Fonds" is gratefully acknowledged. The research of RvdH has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

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