

Application of Liquid Chromatography to a Study on 4-Coumarate: Coenzyme A Ligase Activity

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Z. Naturforsch. 36 c, 197–199 (1981); received December 9, 1980

Avena sativa, Poaceae, Ligase, *p*-Coumaroyl-CoA, Polyamide Column Chromatography, High Performance Liquid Chromatography

This report describes the separation of components from a 4-coumarate:CoA ligase assay by means of liquid chromatography. With the aid of polyamide column chromatography it is possible to enrich and isolate chromatographically and UV spectroscopically pure *p*-coumaroyl-CoA using as a solvent 0.01% NH₄OH in methanol subsequent to water and methanol alone.

High performance liquid chromatography on octadecylsilane-bonded silica stationary phase allows a discontinuous determination of ligase activity. All components – ATP, Coenzyme A, *p*-coumaric acid, and the products AMP and *p*-coumaroyl-CoA – can be separated and accurately quantified within 20 min using a water-acetonitrile gradient, containing 1% phosphoric acid. The presented HPLC method may be used to affirm the accuracy of optical tests.

Introduction

Developing primary leaves of oat (*Avena sativa*) accumulate high quantities of phenylpropanoids, esp. flavone derivatives, in the various tissues of this organ [1]. It is of great interest to investigate the biochemical potential of the individual leaf tissues: epidermis, mesophyll or protoplasts therefrom [2], and vascular elements [3].

In most of the cases reported so far the formation of thiolesters, catalyzed by CL, represents the principle reaction in phenylpropanoid metabolism [4]. In order to investigate this enzymatic activity in leaf tissues of oat, it is favorable to avoid partial purification of the instable CL and to work with crude enzyme extracts [5]. In the convenient photometrical determination of their enzyme activities [6], however, side reactions often interfere with the optical test. Thus, identification and quantification of the CL reaction product becomes necessary and the linearity of this reaction has to be proven.

Several methods of identification of hydroxycinnamoyl-CoA thiolesters have been described, including paper chromatography [7] and column chromatography on Sephadex [8, 9], DEAE-cellulose [10] or polyamide [11].

Abbreviations: CL, 4-coumarate:CoA ligase; pC, *p*-coumaric acid; pC-CoA, *p*-coumaroyl-CoA; HPLC, high performance liquid chromatography.

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0341-0382/81/0300-0197 \$ 01.00/0

In the present paper we offer a simple method for a large scale preparation of pC-CoA by polyamide column chromatography and a simple and effective HPLC technique for the determination of CL activity, including quantification of all relevant assay components.

Materials and Methods

Plant material and culture conditions are described elsewhere [12]. Protein preparations were obtained from 5 and 7 day old oat primary leaves. All steps were carried out at 0–4 °C. 1 g of fresh leaves were frozen in liquid nitrogen and homogenized in a mortar. The powder was mixed with 0.1 g dry Polyclar AT (Serva, Heidelberg), 0.1 g Dowex 1X2 (Cl⁻) (Serva), and 10–15 ml 0.1 M potassium phosphate buffer (pH 7.3), containing 40 mM dithiothreitol (Serva). After stirring for 20 min the suspension was squeezed through 4 layers of gauze and the filtrate centrifuged for 20 min at 38000 × *g*. The clear supernatant was taken as enzyme source. Spectrophotometrical determination of CL activity with pC (Roth, Karlsruhe) was carried out as described in ref. [6] by measuring the increase in absorbance at 333 nm (PMQ II, Zeiss).

The product pC-CoA was isolated from a 50-fold assay volume using polyamide column chromatography. The enzyme reaction was stopped at its equilibrium by the addition of HCOOH up to pH 3.0. Precipitated protein was removed by centrifugation and the clear incubation mixture was then



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lyophilized. The residue was redissolved in a small volume of 50% aq. methanol and transferred to the polyamide column, grain size <0.07 mm (1×16 cm) (Macherey, Nagel & Co., Düren). Elution was done with water, followed by methanol and 0.01% NH_4OH in methanol. The isolated pC-CoA was identified by UV spectroscopy and thin layer chromatography, taking chemically synthesized pC-CoA as reference [10].

The liquid chromatograph used was from Spectra-Physics (Santa Clara, Calif., USA) and included two Model 740 B pumps with 740 B pump control units, a 714 pressure monitor, a 744 solvent programmer, and injection was done *via* a Rheodyne rotary valve with a 20 μl loop. Detection was achieved with a Schoeffel SF 770 UV-VIS spectroflow monitor. Quantitative calculations were obtained with an Autolab System I computing integrator (Spectra-Physics). The chromatographic column (200×4 mm) was prepacked with Nucleosil 5 C_{18} (5 μm). Elution system is described in the legend of Fig. 1.

Results and Discussion

Fig. 1 demonstrates the separation of a standard mixture of ATP, AMP, CoASH, pC-CoA (*trans* and *cis*), and pC (1 a) and the analysis of a CL assay after 4 h of incubation (1 b). This system had been improved for resolving components of CL assays. In order to obtain good separation of pC and its thioester, it is essential to use a water-acetonitrile gradient and 1% phosphoric acid in both solvents. If a rapid separation of ATP and ADP is required, we recommend ammonium dihydrogen phosphate (pH 6.0) with a water-methanol gradient as described in ref. [13]. An excellent HPLC of pmol amounts of CoASH using an anion-exchange resin is described in ref. [14].

The HPLC analysis shown in Fig. 1 b confirmed the photometrical determination of CL activity, obtained from 5 day old primary leaves of oat. After 4 h of incubation HPCL separation resulted in an amount of 0.25 nmol of pC-CoA in 20 μl , which were injected onto the column; from the optical test

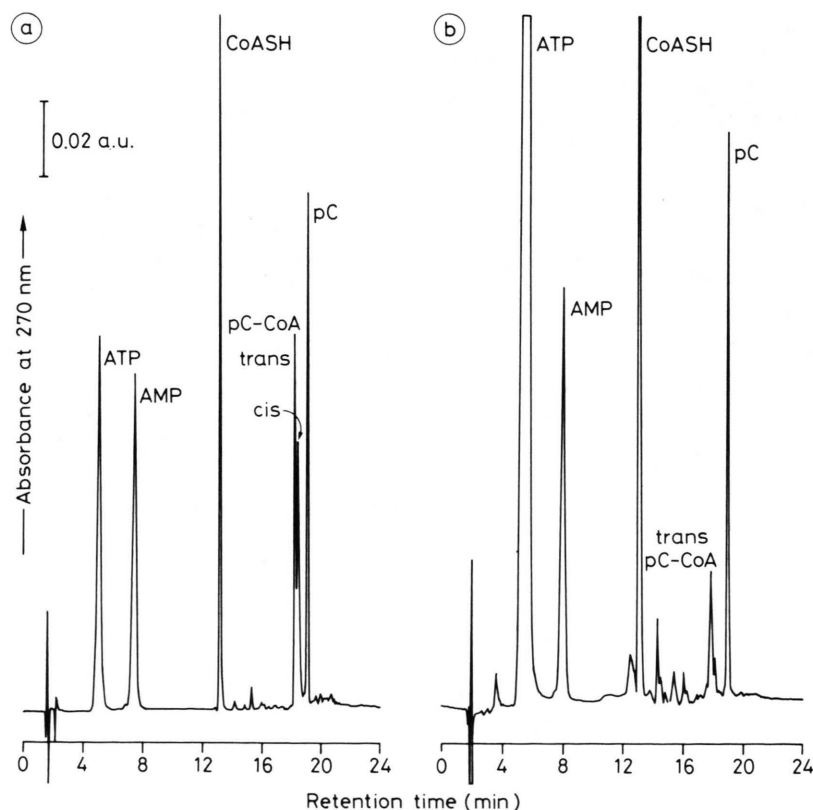


Fig. 1. HPLC analyses (a) of a standard mixture of ATP (1.75 nmol), AMP (1.75 nmol), CoASH (1.75 nmol), pC-CoA (1.57 nmol), and pC (3.51 nmol); (b) of a CL assay using a protein preparation from 5 day old primary leaves from oat on Nucleosil 5 C_{18} (5 μm , 200×4 mm) with a linear gradient from solvent A (1% *ortho*-phosphoric acid in water) to 50% solvent B (1% *ortho*-phosphoric acid in acetonitrile) in A + B (1 ml/min). After 4 h of incubation 20 μl were directly injected without any pretreatment.

0.27 nmol were calculated for this volume. Table I lists the quantities of all the assay components, calculated from HPLC at t_0 and after 4 h of incubation. It shows that CoASH is only consumed in the synthesis of the thiolester, whereas pC undergoes some additional unidentified reactions. The consumption of ATP corresponds with the formation of AMP, but the quantity of accumulated AMP exceeds that of the thiolester. This was not observed during the linear phase of the reaction (10–15 min), but has to be expected at the stage of equilibrium. In the back reaction the thiolester probably is split without resynthesis of ATP. In the reference assay (without CoASH) we also observed the formation of AMP. This might be due to hydrolysis of the intermediate acyladenylate which cannot react with CoASH.

HPLC analysis of a CL assay using a crude protein extract from 7 day old leaves revealed that the optical test did not reflect the total amount of synthesized thiolester. Furthermore, we found that crude protein preparations from epidermal tissues of 7 day old leaves do not contain any CL activity, as might be deduced from photometrical tests.

A method for quantification and isolation of pC-CoA in a preparative scale is represented by polyamide column chromatography. Fig. 2 shows a separation of a 50-fold assay with protein from 5 day old leaves.

Table I. Quantitative composition of a CL assay with a protein preparation from 5 day old primary leaves from oat. Values are expressed in nmol/20 μ l, calculated from HPLC analysis.

	Time [h]	pC	ATP	AMP	CoASH	pC-CoA
Sample:	0	4.11	20.06	—	4.03	—
	4	3.40	17.71	2.25	3.75	0.25
Reference:	4	4.08	18.60	1.53	—	—

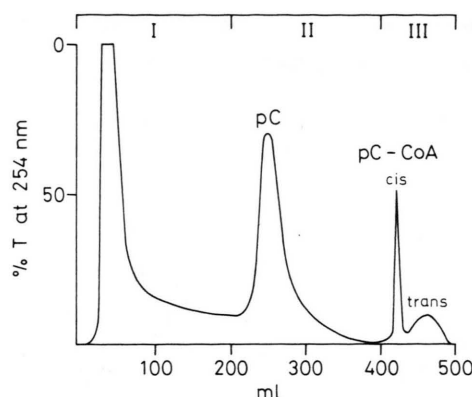


Fig. 2. Polyamide column elution profile of a CL assay (50-fold) with a protein preparation from 5 day old primary leaves from oat after 4 h of incubation. Flow-rate was 1–2 ml/min. Transmission was monitored at 254 nm. Solvents: I, H₂O; II, CH₃OH; III, 0.01% NH₄OH in CH₃OH.

Using 0.01% NH₄OH in methanol as solvent, subsequent to water- and methanol-elution, pC-CoA was isolated in an UV spectroscopically and chromatographically pure form. Before usage, the column was thoroughly washed with all the solvents for subsequent chromatography. The elution profile of the thiolester exhibits separation of the trans- and cis-form. UV spectra gave the typical absorption maxima at 333 and 260 nm [10]. TLC on microcrystalline cellulose (Avicel) in three solvent systems gave the expected R_F -values [7].

The liquid chromatographic procedures described in this paper offer efficient and reliable methods for analyzing CL activity.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledgement.

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