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Short communication

High performance liquid chromatography method for the determination of cinnamyl alcohol dehydrogenase activity in soybean roots

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Abstract

This study proposes a simple, quick and reliable method for determining the cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) activity in soybean (*Glycine max* L. Merr.) roots using reversed-phase high performance liquid chromatography (RP-HPLC). The method includes a single extraction of the tissue and conduction of the enzymatic reaction at 30 °C with cinnamaldehydes (coniferyl or sinapyl), substrates of CAD. Disappearance of the substrates in the reaction mixture is monitored at 340 nm (for coniferaldehyde) or 345 nm (for sinapaldehyde) by isocratic elution with methanol/acetic acid through a GLC-ODS (M) column. This HPLC technique furnishes a rapid and reliable measure of cinnamaldehyde substrates, and may be used as an alternative tool to analyze CAD activity in enzyme preparation without previous purification.

Keywords: Cinnamyl alcohol dehydrogenase; Coniferaldehyde; High performance liquid chromatography; Sinapaldehyde; soybean

1. Introduction

Lignin is the main structural component of secondarily thickened plant cell walls. It performs a relevant role in evolution, growth and resistance of the terrestrial vascular plants and in the global carbon cycle. Additionally, it is of great interest in agricultural and industrial research due its economic relevance. In plants it imparts mechanical support and efficient conduction of water and solutes over long distances within the vascular systems [1]. Structurally, lignin is a heteropolymer of hydroxylated and methoxylated phenylpropane units, derived from the oxidative polymerization of different hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl and sinapyl) connected by labile ether bonds and/or resistant carbon–carbon linkages [2]. In dicotyledonous angiosperms lignin is built from coniferyl and sinapyl alcohols, which are incorporated, respectively, as guaiacyl (G) and syringyl (S) units to form heterogeneous G–S polymers. In the last step of monolignol biosynthesis, coniferaldehyde and sinapaldehyde are converted into their corresponding alcohols by cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) and probably by sinapyl alcohol dehydrogenase (SAD, a CAD homolog from aspen) in a NADPH-dependent reaction (Fig. 1). Data related to the down-regulation of CAD by genetic engineering or to the properties of CAD mutants support its role as a specific marker [3,5–9,11].

CAD activity has typically been assayed by spectrophotometry [5,6,8,12] and occasionally by radiometry [1]. In spectrophotometric assays, CAD activity has been measured in the reverse reaction by following the oxidation of the appropriate hydroxycinnamyl alcohol at 400 nm (formation of the respective aldehyde) or at 340 nm (formation of NADPH). Enzyme activity has also been determined in the forward reaction by following the oxidation of NADPH (at 340 nm) due to reduction of the appropriate hydroxycinnamyl aldehyde. For a long period, high performance liquid chromatography (HPLC) has been firmly established as a favorable and reliable technique for separation and determination of traces from a wide range of compounds. Efficiency, simplicity, sensitivity and reproducibility are all advantages of HPLC for the separation of extremely low quantities of complex mixtures. The current research

Abbreviations: CAD, cinnamyl alcohol dehydrogenase; HPLC, high performance liquid chromatography.

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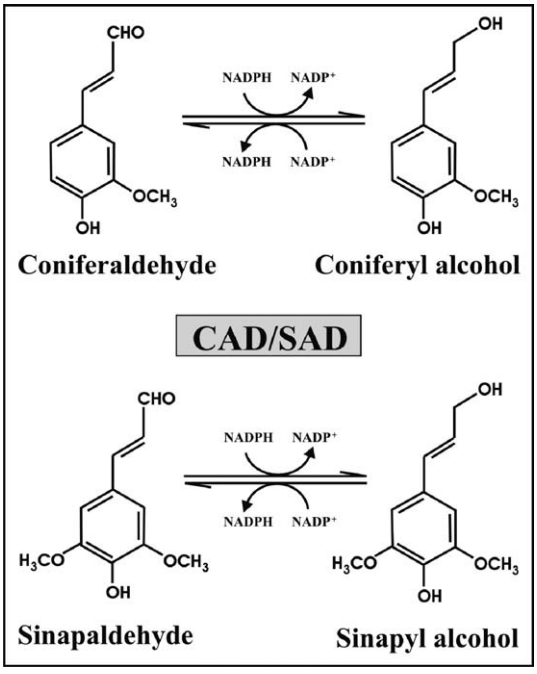

Fig. 1. Last reaction of monolignol biosynthesis in angiosperms.

proposes a simple and sensitive HPLC technique to determine CAD activity. The method is based on a single enzyme extraction of soybean roots. The enzyme activity is assayed chromatographically by following the consumption of coniferyl and sinapyl aldehydes, the substrates of the CAD reaction.

2. Results and discussion

As reported earlier, CAD activity has been assayed spectrophotometrically [5,6,8,12] by the oxidation of hydroxycinnamyl alcohols (at 400 nm, reverse reaction) or by the oxidation of NADPH (at 340 nm, forward reaction). In the present study, CAD was assayed chromatographically by the reduction of the corresponding aldehydes (coniferaldehyde and sinapaldehyde) in the forward reaction. In our previous experiments (data not shown), CAD activity was analyzed chromatographically by the reverse reaction (alcohols to aldehydes). Irregular resolution of the chromatographic profiles proceeded variable concentrations of coniferyl and sinapyl aldehydes were verified. By consequence, no linearity in the concentrations of aldehydes produced from alcohols was obtained. It is known that, contrary to aldehydes, coniferyl and sinapyl alcohols are not stable (air and light sensitive) and require strict storage conditions (under nitrogen and vacuum). Thus, unreliable changes during the enzymatic reaction cannot be discarded, by interfering in the aldehyde formation. In agreement, this technical protocol did not give reliable results in more than fifty independent samples. So, in a broader sense, the measure of disappearance of aldehyde substrate (in forward reaction) was more feasible.

To standardize this technical protocol, two aspects have been considered. First, the possibility of unspecific endogenous consumption of the aldehyde substrate in the crude enzyme extract inasmuch its formation (reverse reaction) was not linear. For that, controls with the enzyme extract plus the substrate (coniferaldehyde or sinapaldehyde), without NADPH were undertaken. Both aldehyde substrates were partially consumed. So, in all subsequent enzyme reactions, these values were subtracted of the aldehyde substrates (50 nmol) and considered as initial concentrations. Second, the confirmation of alcohols formation from aldehyde substrates proceeded by linear behavior of the enzymatic reaction. Fig. 2A,C show the typical HPLC results of CAD reactions with coniferaldehyde and sinapaldehyde substrates, respectively. The HPLC chromatogram (Fig. 2A) shows the CAD reduction of coniferaldehyde (retention time, Rt = 12.52 min) into coniferyl alcohol (Rt = 7.15 min). Similarly, the HPLC chromatogram (Fig. 2C) shows the CAD-mediated sinapaldehyde (Rt = 16.58 min) reduction into sinapyl alcohol (Rt = 8.28 min). The HPLC-profiles reveal that aldehyde substrates and alcohol products were unambiguously identified with their respective authentic standards (Fig. 2B,D). The insets show the coniferyl (Fig. 2a) and sinapyl (Fig. 2b) alcohols produced in reactions containing different quantities of crude protein extract. Results confirm the linearity in the alcohol formation from aldehyde substrates, indicating the specificity of the enzyme reaction.

Fig. 3 shows the chromatographic profiles of coniferaldehyde (left column) and sinapaldehyde (right column). Coniferaldehyde and sinapaldehyde standards (50 nmol) eluted at 12.49 min (Fig. 3A) and 16.56 min (Fig. 3B), respectively. Fig. 3C,D show that the initial concentrations of aldehyde substrates eluted in the same retention times as standards (Fig. 3A,B). For validation of the proposed method, two different protocols were undertaken as indicated in the Methods. Fig. 3E–H shows the chromatographic profiles of aldehyde substrates consumed after the CAD reaction. By comparing retention times of the samples (Fig. 3E–H) and standards (Fig. 3A,B), aldehyde substrates consumed in the enzymatic reactions were identified. In 4-day-old seedling roots (protocol 1), the peak areas of coniferaldehyde (Fig. 3E) and sinapaldehyde (Fig. 3F) were smaller than those of the initial concentration of aldehyde substrates (Fig. 3C,D). Similarly, in 3-day-seedling roots incubated in nutrient solution for 24 h (protocol 2), the peak areas of coniferaldehyde (Fig. 3G) and sinapaldehyde (Fig. 3H) were also smaller than aldehyde substrates. In both cases, this confirms the disappearance of aldehyde substrate and, consequently, the CAD action with further alcohol formation (Fig. 2A,C). CAD activities were determined in 4-day-old seedlings roots (protocol 1) and in incubated seedlings roots (protocol 2). In the first condition, the enzyme activities were 7.24 nmol of coniferaldehyde consumed min^{-1} mg^{-1} protein (Fig. 4A) and 10.32 nmol of sinapaldehyde consumed min^{-1} mg^{-1} protein (Fig. 4B). In the second condition, the enzyme activities were 12.27 nmol of coniferaldehyde consumed min^{-1} mg^{-1} protein (Fig. 4A) and 24.10 nmol of sinapaldehyde consumed min^{-1} mg^{-1} protein (Fig. 4B). In brief, data presented in this communication indicate that the metho-