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Multiple Forms of the Constitutive Wheat Cinnamyl Alcohol Dehydrogenase

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ABSTRACT

Three cinnamyl alcohol dehydrogenase (CAD) isoenzymes were separated from etiolated wheat seedlings (*Triticum aestivum* L.) and examined by native gel electrophoresis. Two of these enzymes (CAD-1 and CAD-2) were purified to apparent homogeneity. They exhibited a marked difference in substrate affinity. On sodium dodecyl sulphate-acrylamide gel the isolated isoenzymes showed only one protein band each with an M_r 45 000 and 40 000 daltons, respectively, whereas on native gel two bands were identified for each protein. Isoenzymes from a variety of diploid, tetraploid, and hexaploid wheats were compared. The results indicated that the CAD polymorphism could be genetically determined.

Key words: Cinnamyl alcohol dehydrogenase, lignin, *Triticum aestivum* L.

INTRODUCTION

Cinnamyl alcohol dehydrogenase (CAD) catalyses the last step of the synthesis of cinnamyl alcohols and is potentially able to act on three different substrates (coniferaldehyde, sinapaldehyde, and *p*-coumaraldehyde) corresponding to the three monomer units of lignin. The reaction proceeds according to the following equation:

$$\text{Cinnamaldehyde} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{cinnamyl alcohol} + \text{NADP}^+.$$

This enzyme, already isolated from *Forsythia* stems (Mansell, Gross, Stöckigt, Franke, and Zenk, 1974), poplar stems (Sarni, Grand, and Boudet, 1984) and soybean cell suspension culture (Wyrambik and Grisebach, 1975), has an absolute specificity for the cinnamyl aldehydes and is widely distributed in the plant kingdom (Mansell *et al.*, 1974). With the exception of soybean cell suspension culture (Wyrambik and Grisebach, 1975), *Salix*, and a few other plants (Mansell, Babbel, and Zenk, 1976) the majority of plants studied contain only one enzymatic form of CAD. Until now, nothing was known about wheat CAD polymorphism, its origin and the possible involvement of CAD multiforms in the control of lignin formation. This study deals with the variability of CAD extracted from etiolated seedlings of *Triticum aestivum* L. (var. Fidel). The origin of wheat CAD polymorphism is investigated and the putative role of this enzyme in lignification is discussed.

MATERIALS AND METHODS

Plant material

Seeds of *Triticum aestivum* L. (var. Fidel) were purchased from La Quinoleine SA, Oissel, France. The diploid (*T. monococcum* L. and *Aegilops squarrosa* L.), the tetraploid (*T. dicoccoides* Körn, *T. dicoccum* Schrank and *T. durum* Desf.) and the hexaploid wheats (*T. aestivum* L. var. Arina and Probus) were kindly provided by G. Kleyer (Federal Agricultural Research Station, Changins, CH-1260 Nyon). Wheat seedlings were grown on wet filter paper in the dark at 30 °C for 6 d.

Chemicals

Bio-Gel TSK DEAE-5PW, Bio-Sil TSK 250, and hydroxylapatite for column chromatography were purchased from BIO-RAD Laboratories Ltd. $NADP^+$-agarose ($NADP^+$ attached through ribose hydroxyls) and iminodiacetic acid-agarose (iminodiacetic acid attached to epoxy activated cross-linked agarose) were obtained from Sigma (St. Louis, USA). Immobilon transfer membranes were obtained from Millipore (Bedford, USA) and Centricon 10 microconcentrator from Amicon (Danvers, USA). Prepacked PD-10 column containing Sephadex

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G-25 M were purchased from Pharmacia (Uppsala, Sweden). Endoproteinase Glu-C (V8) sequencing grade from *Staphylococcus aureus* V8 was obtained from Boehringer Mannheim (Germany). Coniferaldehyde, *p*-coumaraldehyde, sinapaldehyde, and *p*-coumaryl alcohol were synthesized by Dr Obrecht (Maag Ltd.). Coniferyl alcohol and other chemical compounds were purchased from Fluka (Switzerland), Sigma (St. Louis, USA), and Applied Biosystems Inc. (Foster City, USA).

CAD-purification

All the experiments were carried out at 4 °C. Five g fresh weight of 6-d-old etiolated wheat seedlings (*T. aestivum*, var. Fidel) were ground in a mortar with liquid nitrogen. The homogenate was extracted in 20 cm^3 of 50 mM sodium phosphate pH 7·5, containing 10% (v/v) ethylene glycol and 2·0 mM reduced glutathione. The crude extract was filtered through a nylon net (pore size 50 μm) and centrifuged at 13 000 *g* for 20 min. The supernatant was fractionated by ammonium sulphate precipitation in the range of 30–70% saturation. The CAD-containing precipitate was dissolved in 2·5 cm^3 of extraction buffer and passed successively through a Sephadex G-25 PD-10 and a hydroxylapatite column (50 × 10 mm). Both columns were equilibrated with the extraction buffer. The next step of purification required a fast protein liquid chromatography (FPLC) method: The protein mixture was adsorbed on a DEAE TSK column (150 × 21·5 mm) equilibrated with 20 mM sodium phosphate pH 8·0 containing 2·0 mM reduced glutathione. Elution of CAD was performed with a linear gradient by increasing the concentration of sodium acetate up to 0·6 M at a flow rate of 4·0 cm^3 min^{-1}. The volume of each fraction was 4·0 cm^3. Fractions 52 to 54 were applied to a column (50 × 15 mm) of $NADP^+$-agarose equilibrated with 20 mM sodium phosphate pH 6·75 containing 10% ethylene glycol and 2·0 mM glutathione and eluted as described by Wyrambik and Grisebach (1979) with 2·0 mM NADP. The eluate was adsorbed on a 1·0 cm^3 DEAE-cellulose column (10 × 5 mm) equilibrated with 20 mM Tris-acetate pH 8·0 and 10% ethylene glycol and CAD was eluted with 1·0 cm^3 of the same buffer containing 0·5 M NaCl. The concentrated enzyme was then fractionated using a zinc-loaded column essentially as described by Kato, Nakamura, and Hashimoto (1986) except that TSK gel Chelate-5PW was replaced by the iminodiacetic acid column (50 × 15 mm). After elution of the first CAD-active fraction (CAD-1) in the void volume, a second active fraction (CAD-2) was recovered by elution with 20 mM Tris-acetate, pH 8·0, containing 0·5 M NaCl, 10% (v/v) ethylene glycol and 0·2 M glycine. The CAD fractions were concentrated using Centricon microconcentrator according to the manufacturers recommendations. The samples were concentrated 20-fold for the electrophoretic analyses, whereas an 80-fold enrichment was required for the chemical analyses.

Enzyme assay and protein determination

Reduction of cinnamaldehydes was performed according to the procedure described by Wyrambik and Grisebach (1975). Protein concentrations were determined by the Biorad assay (Bradford, 1976).

Enzyme molecular weight

The apparent molecular weight of CAD was determined on a Bio-Sil TSK 250 (300 × 7·5 mm) column equilibrated with 0·05 M Na_2SO_4, 0.02 M $NaH_2PO_4.2H_2O$, pH 6·8 and calibrated with thyroglobulin (670 000 daltons), IgG (158 000), ovalbumin (44 000), myoglobin (17 000) and vitamin B12 (1350). The subunit molecular weight was determined by SDS-acrylamide gel electrophoresis.

SDS-acrylamide gel electrophoresis

The method of Laemmli (1970) was used and the concentration of acrylamide was 12·5%. A 50–100 mm^3 protein solution (2–10 μg) was applied to the gel. Calibrating proteins were lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase B. Proteins were detected using the silver staining procedure (Merril, Goldman, and Van Keuren, 1982) and the glycoprotein staining procedure (Zacharius, Zell, Morrison, and Woodlock, 1969).

Native gel electrophoresis

The method of Laemmli (1970) for SDS-acrylamide gel electrophoresis was used except that 10% (v/v) ethylene glycol replaced SDS. The acrylamide concentration was 6% and the protein separation was carried out at 4 °C. A 50–100 mm^3 protein solution (2–10 μg) was applied to the gel. CAD bands detection was performed by incubating the gel with the CAD or ethanol dehydrogenase staining mixtures as described by Mansell *et al.* (1976).

In situ *V8 digestion and western blotting*

Material isolated from SDS-acrylamide gels was digested *in situ* with the endoproteinase Glu-C as described by Sweatt, Kennedy, Wager-Smith, Gawinowicz, Barzilai, Karl, and Kandel (1989). Electroblotting of peptides on to Immobilon polyvinylidene difluoride (PVDF) membrane was carried out essentially as described for protein blotting on Polybrene glass by Vandekerckhove, Bauw, Puype, Van Damme, and Van Montagu (1985), except that 0·1% thioglycolic acid replaced mercaptoethanol. Transferred peptides were stained with Ponceau S as described by Eckerskorn, Mewes, Goretski, and Lottspeich (1988).

Amino acid sequence analysis

Amino acid sequence analysis of electroblotted peptides was carried out on a pulsed-liquid phase sequencer model 477A equipped with an on-line PTH-analyser model 120A (Applied Biosystems Inc., Foster City, USA) according to the manufacturers recommendations. All chemicals were purchased from Applied Biosystems Inc. Sequence alignments were made using the algorithm of Lipman/Pearson (Lipman and Pearson, 1985).

RESULTS

Separation of cinnamyl alcohol dehydrogenase isoenzymes

Etiolated wheat seedlings have been shown to be a good source for the purification of CAD. Comparable yields were not obtained with mature leaves. Despite stability optimization by the addition of 10% (v/v) ethylene glycol and 2·0 mM glutathione in the extraction medium, a fast isolation procedure is required for sufficient recovery. Ammonium sulphate precipitation, hydroxylapatite-, ion exchange- and NADP-agarose chromatography are commonly proposed to purify CAD from various sources (Lüderitz and Grisebach, 1981; Sarni, Grand, and Boudet, 1984) and were used in this study (Table 1). When stored at −80 °C in the presence of 2·0 mM NADP, 2·0 mM

TABLE 1. *Fractionation of cinnamyl alcohol dehydrogenase isoenzymes extracted from wheat coleoptiles and first leaves*

One enzyme unit is defined as the amount of activity which converts one nanomol coniferaldehyde per second (1 nKat)

Purification step	Protein (mg)	Specific activity (nkat mg^{-1} protein)	Purification (−fold)	Recovery (%)
Ammonium sulphate	52	1·03	1·0	100
Hydroxylapatite	22	2·5	2·4	104
DEAE-TSK	2·4	8·44	8·2	38
NADP-agarose	0·05	214	208	21
Zn-chelate:				
Fraction CAD-1[a]	0·01	531	515	11
Fraction CAD-2[b]	0·03	66	64	4

[a] Without affinity for the column.
[b] Adsorbed on the column and eluted with glycine.

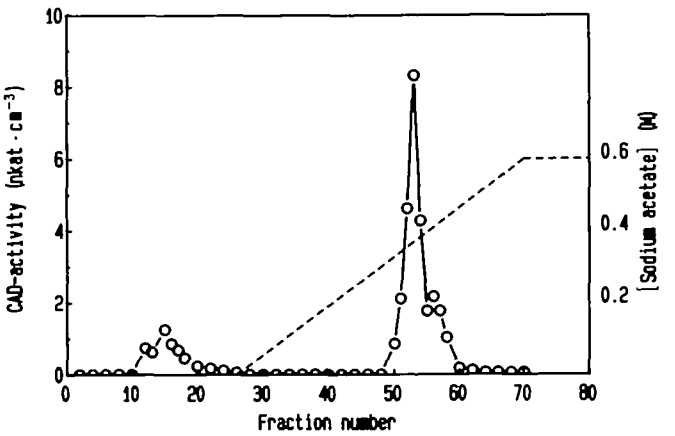

FIG. 1. Separation of wheat cinnamyl alcohol dehydrogenase on DEAE-TSK: (O—O) enzyme activity with coniferaldehyde as substrate; (---) buffer gradient.

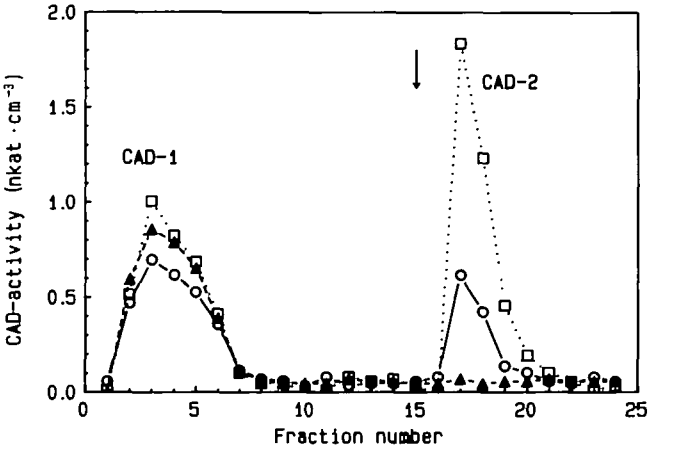

FIG. 2. Separation of wheat cinnamyl alcohol dehydrogenase on Zn-chelate affinity chromatography. CAD-activity was measured with coniferaldehyde (O), sinapaldehyde (▲) and coumaraldehyde (□) as substrate. Adsorbed CAD was eluted with 0·2 M glycine as indicated by the arrow.

glutathione and 10% (v/v) ethylene glycol, enzyme activity was stable for several months.

As shown in Fig. 1, two active fractions can be separated by ion exchange chromatography (DEAE-TSK). The main activity peak (Fractions 52–54, about 90% of the total) was further purified on NADP-agarose. After Zn-chelate affinity chromatography, this CAD-activity was again separated into two fractions (CAD-1 and CAD-2), which exhibited marked differences in substrate specificity (Fig. 2). CAD-1 acted on the three natural substrates whereas CAD-2 had no affinity for sinapaldehyde. Both purified enzymes were apparently homogeneous after SDS-gel electrophoresis and silver staining (Fig. 3). The isolated proteins did not stain for glycoprotein (data not shown).

Heterogeneity of the isolated CAD-isoenzymes

Native gel electrophoresis of the crude extract revealed the presence of six isoforms (designated a, b, c, d, e, f) by using coniferyl alcohol as substrate (Fig. 4). Occasionally, a fast migrating seventh band (designated g) was observed (Fig. 6). An analogous banding pattern was observed with coumaryl alcohol (data not shown). With ethanol and NAD, three strong bands were found and the R_m values for these bands differed from those obtained with coniferyl alcohol and coumaryl alcohol (data not shown). Analysis of the fractions separated on DEAE-TSK indicated that isoforms a and b were eluted in fraction no. 15 whereas isoforms c, d, e, and f were concentrated in the main peak (data not shown). In native gel electrophoresis the two fractions CAD-1 and CAD-2, separated by Zn-chelate affinity chromatography, showed two bands each. CAD-1 gave rise to bands d and e and CAD-2 to bands c and f (Fig. 4). The two active species of each CAD fraction were visible by using either coniferyl alcohol or coumaryl alcohol as substrate.

Molecular weight of enzymes and subunits

The apparent molecular weight of the CAD isoenzymes was determined by gel filtration on a Biosil-TSK column (300 × 7·5 mm). The fraction corresponding to approximately 70 000 daltons contained the total CAD activity

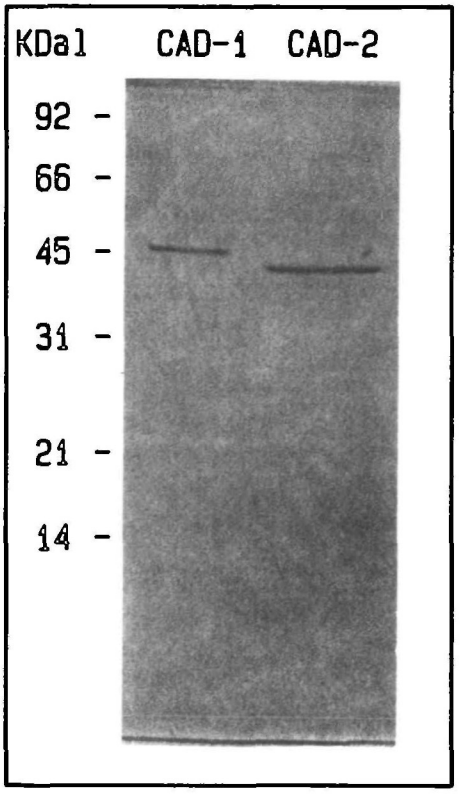

FIG. 3. SDS-polacrylamide gel patterns of CAD isoenzymes separated by Zn-chelate affinity chromatography.

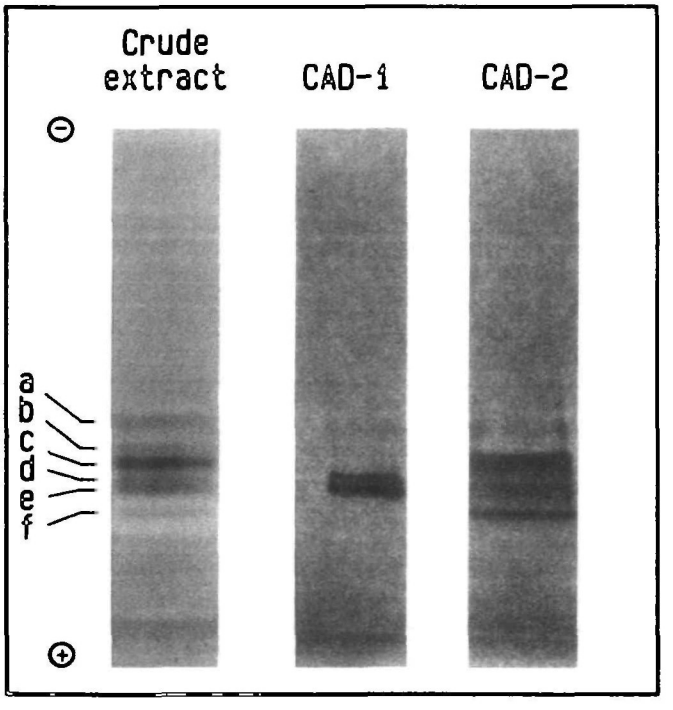

FIG. 4. Native polyacrylamide gel pattern of CAD-isoforms contained in the crude extract and separated by Zn-chelate affinity chromatography. Activity bands due to CAD are designated a, b, c, d, e, f and detected with coniferyl alcohol as substrate.

applied on the column. Analysis of the samples by native gel electrophoresis indicated that both isoforms of each CAD are present in the same fraction and have the same molecular weight (data not shown). The subunit molecular weight, determined by SDS-gel electrophoresis, was 45 000 and 40 000 daltons for CAD-1 and CAD-2, respectively (Fig. 3).

V8 digestion and amino acid sequence analysis

Direct N-terminal amino acid sequence analysis of CAD-1 and CAD-2 subunits, isolated from SDS-gel and immobilized on PVDF membrane by electroblotting, was unsuccessful (data not shown). Therefore, both proteins, isolated from preparative SDS-acrylamide gels, were digested *in situ* using the endoproteinase Glu-C. After separation by SDS-gel electrophoresis and western blotting on PVDF membrane the resulting peptide bands were cut out and sequenced directly. The typical Ponceau S staining pattern obtained from the subunits of CAD-1 and CAD-2 is illustrated in Fig. 5. The amino acid sequence of each analysed peptide was compared to the sequence obtained from the nucleotide sequence of CAD4 cDNA encoding CAD mRNA induced in *Phaseolus vulgaris* by a fungal elicitor (Walter, Grima-Pettenati, Grand, Boudet, and Lamb, 1988). No structural homology was detected.

Because of the difficulty in concentrating up to 80-fold CAD under native form, the procedure described here was not appropriate for the chemical analysis of each isoform of CAD-1 and CAD-2.

Genetical dependence of CAD polymorphism

The CAD polymorphism was screened in seedlings of different diploid, tetraploid and hexaploid wheat lines of *Triticum*: Two diploids (*T. monococcum* and *Aegilops squarrosa*), three tetraploids (*T. dicoccoides*, *T. dicoccum* and *T. durum*) and three different hexaploids (*T. aestivum* var. Arina, Probus, and Fidel) produced by natural hybridization between *T. dicoccoides* and *Aegilops squarrosa*. The isoenzyme pattern, from the crude extracts of each wheat line, tested by native gel electrophoresis is represented in Fig. 6. In both diploid and tetraploid ancestors of *T. aestivum* only three isoforms of CAD were detected, whereas seven activity bands were observed in the three hexaploid wheats. The other tetraploid types do not have the same banding pattern as *T. dicoccoides*. The diploid *T. monococcum* contains only one CAD isoenzyme, which is present in all the tested wheats. The slow migrating band in panel B (also present in the gel incubated without substrate) was not identified.

DISCUSSION

The results of this study are in agreement with earlier works, which demonstrate that cinnamyl alcohol dehydrogenase exists in multiple forms (Wyrambik and Grisebach,

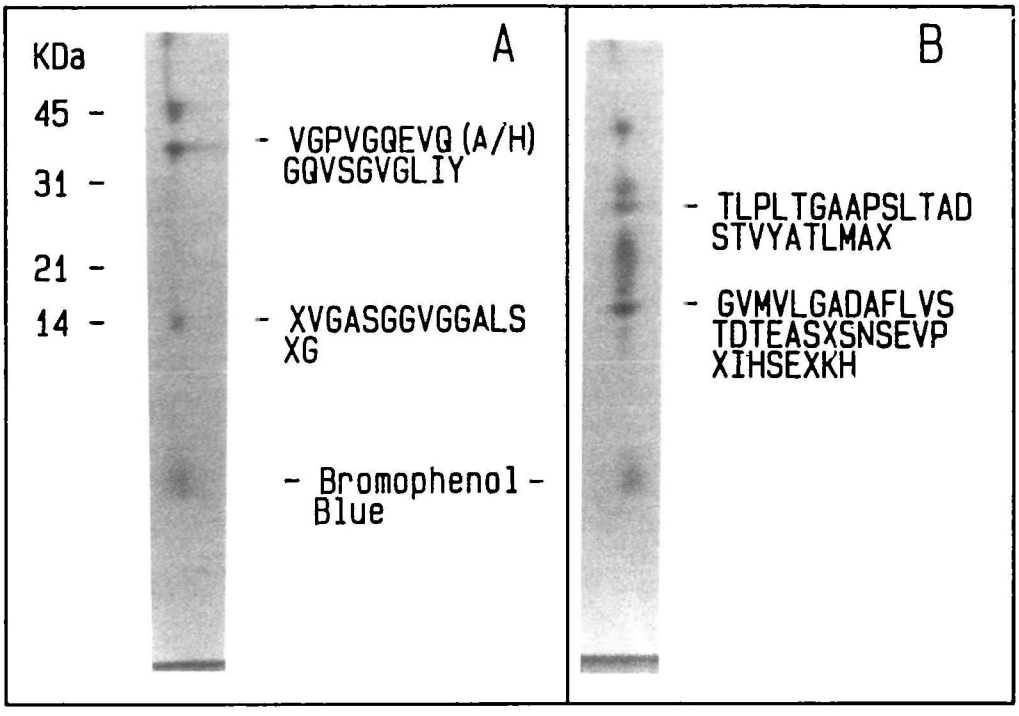

FIG. 5. Ponceau S-staining pattern obtained after *in situ* V8 digestion and electroblotting of the subunits of the CAD-1 (A) and CAD-2 (B) isoenzymes. The amino acid sequence of each peptide is indicated.

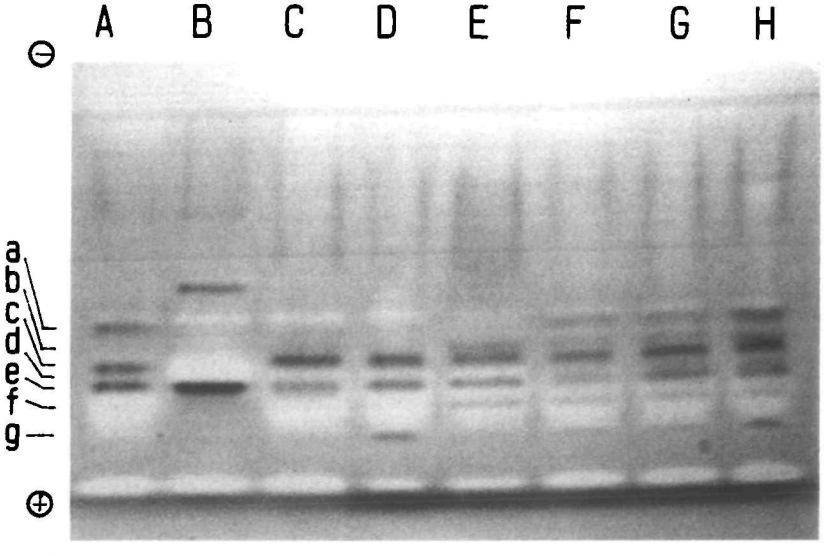

FIG. 6. Native polyacrylamide gel pattern of CAD isoenzymes extracted from various etiolated 6-d-old seedlings (coleoptile + 1st leaf): *Aegilops squarrosa* (A), *T. monococcum* (B), *T. dicoccoides* (C), *T. dicoccum* (D), *T. durum* (E), *T. aestivum* var. Probus (F), var. Arina (G) and var. Fidel (H). Activity bands due to CAD were detected with coniferyl alcohol as substrate and are designated by a, b, c, d, e, f, and g.

1975; Mansell *et al.*, 1976). Combination of ion exchange and metal chelate affinity chromatography separated three active CAD fractions from wheat seedlings, two of which (CAD-1 and CAD-2) were purified up to apparent homogeneity and compared.

Although the two isolated CAD-1 and CAD-2 showed up in SDS-acrylamide gel electrophoresis as only one band each, native gel electrophoresis depicted double bands (c + f and d + e). On the basis of the determination of the molecular weight for the subunits and the native

enzymes, it can be concluded that both isoforms of each CAD are dimeric proteins of about 70 kDa. On the other hand, both subunits of each CAD-isoform have the same molecular weight (Fig. 3). The heterogeneity of the isolated CAD-1 and CAD-2 could be due to the following reasons. Each pair of isoforms represents either two different proteins with the same molecular weight or two different conformational states of the same protein. Artefacts, generated during isolation, are also possible. The direct N-terminal amino acid sequence analysis of the isolated isoenzymes was unsuccessful. The isolated proteins have probably blocked amino termini. Therefore, no conclusion can be drawn about the purity of CAD-1 and CAD-2.

The main objective of the present study was to investigate the variability of the wheat cinnamyl alcohol dehydrogenase. The implications of arguments in favour of the hypothesis that CAD-1 and CAD-2 are different proteins, are listed below. (i) Both enzymes with a quite different substrate specificity (Fig. 2) have a different molecular weight in SDS-gel electrophoresis (Fig. 3). (ii) Due to the high formation constants of histidine and cysteine complexes with Zn in aqueous solution, it is assumed that the metal chelate affinity chromatography is a probe for the localization of histidine and cysteine on the protein surface (Hemdan, Zhao, Sulkowski, and Porath, 1989). Our present experiment suggests that peripheral imidazol or thiol groups are present only on the surface of CAD-2 (Fig. 2). (iii) Ultimately, more information about the variability of CAD is provided by the internal amino acid sequence of the peptide fragments generated by cleavage of the two isolated CAD isoenzymes. The digestion of each subunit *in situ* with the Glu-C protease and the resulting peptide mapping provide good evidence that CAD-1 and CAD-2 are different proteins (Fig. 5). The partial amino acid sequence of the present constitutive wheat CAD isoenzymes has no homology with the stress-induced CAD of *Phaseolus vulgaris*, reported by Walter *et al.* (1988). Homology between the bean CAD and other sequences of the wheat CAD-isoenzymes, not found in our samples, cannot be excluded. Yet, extensive sequence similarity to a maize malic enzyme leaves doubt to the true identity of the induced bean CAD (Walter, Grima-Pettenati, Grand, Boudet, and Lamb, 1990). Therefore, no conclusion can be drawn about the divergence between the induced CAD of *Phaseolus vulgaris* and the constitutive one of wheat seedlings.

Isoenzymes are believed to have functional significance. Thus, different CAD isoenzymes, which exhibit different substrate specificities could, potentially, regulate the composition of lignin (Lüderitz and Grisebach, 1981). Because of the extremely low amount of lignin present in the young wheat tissues, a qualitative analysis of lignin in wheat coleoptiles is extremely difficult with the present methods (Whitmore, 1971). On the other hand, it can be argued that other enzymes could also potentially regulate the composition of lignin, i.e. *o*-methyl transferase, hydroxycinnamate-CoA ligase, cinnamoyl-CoA reductase. Therefore, the possible role of CAD in the control of lignin composition remains unclear. A ploidy dependence of the CAD polymorphism is possible as suggested by the different CAD isoenzymes composition of diploid (14 chromosomes), tetraploid (28 chromosomes) and hexaploid wheat lines (42 chromosomes) (Fig. 6). However, the number of bands does not correlate with the increase of ploidy. Especially, the different pattern of isoforms, observed in the different tetraploid types, suggests that the CAD encoding gene duplication is allopolyploidism-independent. Although the origin of the CAD-isoforms cannot yet be determined, it seems likely that the CAD polymorphism is genetically determined.

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