Inducible UDP-glucose dehydrogenase from French bean (*Phaseolus vulgaris* L.) locates to vascular tissue and has alcohol dehydrogenase activity

Duncan ROBERTSON,*‡ Colin SMITH† and G. Paul BOLWELL*§

*Department of Biochemistry, Royal Holloway and Bedford New College, University of London, Egham, Surrey TW20 0EX, U.K., and †Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, U.K.

UDP-glucose dehydrogenase is responsible for channelling UDP-glucose into the pool of UDP-sugars utilized in the synthesis of wall matrix polysaccharides and glycoproteins. It has been purified to homogeneity from suspension-cultured cells of French bean by a combination of hydrophobic-interaction chromatography, gel filtration and dye-ligand chromatography. The enzyme had a subunit of M_r 40000. K_m values were measured for UDP-glucose as 5.5 ± 1.4 mM and for NAD^+ as 20 ± 3 μ M. It was subject to inhibition by UDP-xylose. UDP-glucose dehydrogenase activity co-purified with alcohol dehydrogenase activity from suspension-cultured cells, elicitor-treated cells and elongating hypocotyls, even when many additional chromato-

graphic steps were employed subsequently. The protein from each source was resolved into virtually identical patterns of isoforms on two-dimensional isoelectric focusing/PAGE. However, a combination of peptide mapping and sequence analysis, gel analysis using activity staining and kinetic analysis suggests that both activities are a function of the same protein. An antibody was raised and used to immunolocalize UDP-glucose dehydrogenase to developing xylem and phloem of French bean hypocotyl. Together with data published previously, these results are consistent with an important role in the regulation of carbon flux into wall matrix polysaccharides.

INTRODUCTION

UDP-glucose dehydrogenase is responsible for channelling UDP-glucose into the pool of UDP-sugars utilized in the synthesis of wall matrix polysaccharides and glycoproteins. Present evidence suggests that the major controlling factor in the qualitative production of the polysaccharides resides in the complement of synthases found at any one time in the endomembrane system. However, the availability of nucleoside diphosphate sugars appears to be under some fine regulation through a feedback inhibition of UDP-glucose dehydrogenase by the level of UDP-xylose [1,2]. The dehydrogenation of UDP-glucose is therefore potentially an important reaction in the regulation of carbon flux in plant cells. Although much information exists at the biochemical and molecular-biological level for glucose flux into sucrose and starch, only rather speculative understanding of the regulation of fluxes into wall polysaccharides exists.

Some evidence for a regulatory role for UDP-glucose dehydrogenase has been provided by studies in sycamore tissues [1], in cultures of *Catharathus* during the cell cycle [3], in cell suspensions and seedlings of French bean undergoing differentiation [4] and in cell suspension cultures of French bean exposed to biotic stress [5]. In these cases, variation in the level of the dehydrogenase was observed in comparison with the UDP-sugar epimerases, which remained at a fairly constant level under the physiological conditions under study. The key role which UDP-glucose dehydrogenase is thought to play in UDP-sugar metabolism has been further strengthened when considered in relation to enzymes involved in metabolism of starch and sucrose, again for which no comparable changes were observed [4–6].

Following these observations that UDP-glucose dehydrogenase may have a significant role in the supply of UDP-sugars for

cell-wall and glycoprotein biosynthesis, the present work describes the purification and characterization of this enzyme from French bean. After amino acid sequence data were obtained for UDP-glucose dehydrogenase, it was apparent that there was a high degree of similarity to an alcohol dehydrogenase (ADH) which had previously been cloned [7,8]. The present paper describes the co-purification of an UDP-glucose dehydrogenase with ADH activity from cells of French bean, raising of an antibody and immunolocalization to vascular tissue.

MATERIALS AND METHODS

Materials

Cell suspension cultures of French bean were derived as described previously [9]. Elicitor preparations from the fungus *Colletotrichum lindemuthianum* were derived as described previously [9]. Uniformly labelled UDP-[14 C]glucose (11.8 GBq/mmol) was obtained from NEN, Stevenage, Herts., U.K.

Growth of plants and tissue-cultured cells

Cell suspension cultures were subjected to biotic stress between 4 and 6 days after subculture. Elicitor was added to a concentration of 30 μ g/ml glucose equivalents. Seedlings of French bean (*Phaseolus vulgaris* L.) var. The Prince were grown in vermiculite under a 16 h-light/8 h-dark regime at 15 °C.

Enzyme assays

UDP-glucose dehydrogenase was routinely assayed spectrophotometrically by monitoring the reduction of NAD^+ as the increase in A_{340} . The reaction mixture contained 5 mM UDP-glucose, 2 mM NAD^+ , 0.1 M glycine (pH 8.5) and enzyme in a

Abbreviations used: ADH, alcohol dehydrogenase; IEF, isoelectric focusing.

‡ Present address: Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, U.K.

§ To whom correspondence should be addressed.

final volume of 1 ml. A control which contained no UDP-glucose was run simultaneously in a Philips twin-beam spectrophotometer model PU 8800. Activity was measured when the absorbance was linear over a 5 min period. ADH activity was measured in the same way as for UDP-glucose dehydrogenase, except that the reaction mixture contained no UDP-glucose and 5 mM ethanol was included.

UDP-glucose dehydrogenase was also assayed radiometrically [1] to check the validity of the product. The final reaction volume was 20 μ l and contained extract, 0.1 M glycine (pH 8.5), 0.4 nmol of UDP-[14 C]glucose (20 μ M), 50 nmol of UDP-glucose (2.5 mM) and 50 nmol of NAD $^{+}$ (2.5 mM). The reactions were carried out for 5 min at 37 °C and stopped by immersion in a boiling-water bath for 3 min. After centrifuging for 10 min at 10000 *g*, the supernatant was hydrolysed with 0.1 M HCl at 100 °C for 15 min. The solution was then dried under vacuum, extracted with 20 μ l of pyridine and subjected to paper chromatography [1]. The radioactivity which co-chromatographed with authentic standards was determined by liquid-scintillation counting.

Extraction and purification of UDP-glucose dehydrogenase

After harvesting by vacuum filtration, 500 g of cells was homogenized in 1 litre of 0.1 M Tris/HCl buffer, pH 7.5, containing 250 mM sucrose, 5 mM 2-mercaptoethanol and 2 mM EDTA. The brei was filtered through three layers of cheesecloth and centrifuged at 15000 *g* for 10 min. The supernatant was made to 25 mM with MgCl $_2$, stirred for 10 min and centrifuged at 15000 *g* for 10 min. The supernatant was made to 50% with solid (NH $_4$) $_2$ SO $_4$, stirred for 20 min, centrifuged for 20 min at 15000 *g* and the pellet discarded. The extract was then subjected to precipitation with 70%-satd. (NH $_4$) $_2$ SO $_4$. The pellet was then resuspended in 30 ml of 0.1 M Tris/HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 2 mM EDTA and dialysed overnight against the same buffer.

The sample was subjected to chromatography on phenyl-Sepharose CL-4B (2.5 cm $\times$ 10 cm) and eluted with a linear gradient of 0–70% ethylene glycol in 0.1 M Tris/HCl buffer, pH 7.5, containing 2 mM EDTA and 5 mM 2-mercaptoethanol. Fractions containing activity were then passed through a Sephadex G-100 column (2.5 cm $\times$ 80 cm) in the same buffer before being subjected to affinity chromatography. The affinity column, composed of Cibacron Blue 3GA (1.5 cm $\times$ 14 cm), was equilibrated in the same Tris buffer, and the sample was loaded and subsequently eluted with a linear gradient of 0–2 mM NAD $^{+}$ in 0.1 M Tris/HCl buffer, pH 7.5, containing 2 mM EDTA and 5 mM 2-mercaptoethanol. After the final purification step, active fractions were pooled and the enzyme was recovered by freeze-drying.

Electrophoretic analysis of protein

One dimensional SDS/PAGE was carried out in a 'mini gel format' [10]. Larger-scale two-dimensional IEF (isoelectric focusing)/SDS/PAGE was also carried out [11]. Protein samples were desalted by methanol/chloroform phase partitioning and methanol precipitation, and reconstituted in urea sample mixture. Once loaded, the rod gels (0.25 cm $\times$ 10 cm) were focused for 16 h at 400 V, followed by 1 h at 800 V. The second dimension was then resolved on a 10%-polyacrylamide/SDS gel (15 cm $\times$ 15 cm). Non-denaturing PAGE was carried out in the mini-gel format on 7.5%-acrylamide gels. Samples were made up to 20% with ethylene glycol and loaded into the sample wells. In adjacent wells Bromophenol Blue (0.01%) and glycerol (10%)

were loaded to monitor the progress of the electrophoresis. During electrophoresis the apparatus was cooled with water circulated at 7 °C through an internal heat exchanger which features as part of the electrophoresis tank. Non-denaturing IEF/PAGE was carried out in the mini-gel apparatus. The gels consisted of 4% polyacrylamide and 2% ampholines (pH 4–5 and pH 3–10, 1:4, v/v) and the samples were focused for 2 h at 200 V. During electrophoresis the unit was cooled at 7 °C. Samples run under non-denaturing conditions were stained for dehydrogenase activity with either UDP-glucose or ethanol as a substrate. After electrophoresis the gels were immersed in 0.1 M Tris/HCl buffer, pH 8.5, containing 0.4 mg/ml NAD $^{+}$, 0.08 mg/ml phenazine methosulphate, 0.20 mg/ml NitroBlue Tetrazolium and a final concentration of either 5 mM UDP-glucose or 5 mM ethanol. The gels were incubated in the dark for 30 min and the reaction was stopped by replacing the staining solution with distilled water.

Peptide digestion was carried out *in situ* in gel slices from either two- or one-dimensional electrophoretic separations of the most purified fractions which were stained with Coomassie Blue, by using *Staphylococcus* V8 protease (Sigma) [12]. The gel was run at 20 mA until the tracking dye reached the separating gel, then at 30 mA until completion. After electrophoresis, the gel containing the digest was electroeluted on to Problot membrane (ABI Instruments, Foster City, CA, U.S.A.) and stained with Coomassie Blue as directed in the Problot manual. The amino acid sequence for peptides was determined in an ABI model 477 sequencer. Alternatively, depending on which samples were being analysed, the gel containing the digest was directly stained with silver.

Immunostaining for electron microscopy: preparation of tissue for immunomicroscopy

Bean hypocotyls were fixed in freshly prepared 1% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.05 M sodium phosphate buffer (pH 6.8) at 4 °C for 2 h. After incubation overnight in 0.05 M sodium phosphate buffer (pH 6.8), the tissues were dehydrated through graded alcohols and placed in several changes of hydrophilic resin, consisting of 3 parts of LR Gold (London Resin Co., Woking, Surrey, U.K.), 2 parts of GMA (glycol methacrylate – low acid; Polysciences Inc., Warrington, PA, U.S.A.) and 0.1% (v/v) benzoin ethyl ether. The tissue was finally embedded in gelatin capsules or flat-bottomed BEEM (TAAB Laboratories, Aldermaston, Berks., U.K.) capsules, and polymerized for 24 h at room temperature by illumination with UV light at 360 nm. Ultrathin sections of hypocotyl were collected on Formvar (2% in amyl acetate)-coated nickel grids and immunostained. Immunogold labelling was carried out by first incubating sections at room temperature in 20 μ l of 3% BSA/1% (v/v) normal goat serum in 20 mM Tris/HCl buffer (pH 7.6) containing 0.23 M NaCl (TBS) for 20 min. Excess solution was then drained from the section and replaced with 20 μ l of anti-(bean UDP-glucose dehydrogenase) IgG at 1:300 dilution in TBS/BSA/normal goat serum containing 0.05% Tween 20 for 18 h at room temperature. Sections were then washed thoroughly and repeatedly for 5 min in TBS (pH 7.6), and the area around the sections was carefully dried. Sections were immersed in 20 μ l of goat anti-rabbit IgG conjugated to colloidal gold (British Biocell International, Cardiff, U.K.; 5 nm particle size; 1:200 diluted working solution in TBS/ovalbumin/normal goat serum) for 60 min at room temperature. The grids were then washed thoroughly with TBS, followed by distilled water, and dried. Silver enhancement of the colloidal gold was carried out with the Biocell SE kit (British

Biocell International) for 2 min at room temperature. After a thorough washing with distilled water, the grids were counter-stained with 2% aq. uranyl acetate and lead citrate.

Immunostaining for light microscopy

Sections (1 μm) of hypocotyl, prepared as for electron microscopy, were cut and transferred to drops of water on clean dry microscope slides and were allowed to dry overnight at 37 °C. Immunogold labelling was carried out by first incubating sections at room temperature in 20 μl of TBS/BSA/normal goat serum for 30 min. Excess solution was then drained from the sections and replaced with 20 μl of anti-(bean UDP-glucose dehydrogenase) IgG at 1:300 dilution in TBS/BSA/normal goat serum containing 0.05% Tween 20 for 1 h at 37 °C. Sections were then washed thoroughly and repeatedly for 5 min in TBS (pH 7.6), and excess buffer was removed without allowing the sections to dry out. Sections were immersed in 20 μl of goat anti-rabbit IgG conjugated to colloidal gold (5 nm particle size; 1:200 working solution in TBS/BSA/normal goat serum) for 30 min at room temperature and then washed thoroughly in TBS, followed by a final rinse in distilled water. Silver enhancement was carried out with a Biocell SE kit for 3 min at room temperature in accordance with the manufacturer's instructions. Slides were rinsed in distilled water and allowed to dry before mounting and examination in a confocal microscope (model MRC 500; Bio-Rad Laboratories, Watford, Herts., U.K.).

RESULTS

Co-purification of UDP-glucose dehydrogenase and ADH

The UDP-glucose dehydrogenase was purified to a high specific activity from elicitor-treated suspension-cultured French bean cells (Table 1). The initial homogenate was subjected sequentially to $(\text{NH}_4)_2\text{SO}_4$ precipitation, hydrophobic-interaction chromatography, gel filtration and blue-dye affinity chromatography (Figure 1). On one-dimensional SDS/PAGE the purified preparation gave a polypeptide of M_r 40 000 (Figure 2a), which could be separated into multiple forms on two-dimensional gels (Figure 2b). Extensive protein sequencing revealed high sequence similarity to PSADH1, an ADH from pea [8]. Further analysis of the UDP-glucose dehydrogenase from this source and two other sources (Figures 2c and 2d), unelicited cells containing highest specific activity of the enzyme and similarly from elongating hypocotyls [4], showed co-purification of both UDP-glucose and ADH activities and similar patterns on two-dimensional IEF/SDS/PAGE. It was found that the retention times, fold purification and recovery for both activities, and their ratios,

Figure 1 Elution profiles of UDP-glucose dehydrogenase and ADH purified from elicitor-treated French bean cells

Soluble extract preparation, precipitated between 50–70% satn. with $(\text{NH}_4)_2\text{SO}_4$ and dialysed overnight, was subjected to a three-step chromatographic purification. The first step (a) was phenyl-Sepharose eluted with a 0–70% gradient of ethylene glycol (—), followed by (b) Sephadex G-100 and finally (c) Cibacron Blue 3GA eluted with a gradient of NAD^+ . Since NAD^+ absorbs strongly at 280 nm, there was no need to include an additional line representing the gradient over and above the UV trace. (●) UDP-glucose dehydrogenase activity ($\Delta A_{280}/\text{min}$ per aliquot of fraction) relative to (○) ADH activity ($\Delta A_{340}/\text{min}$ per aliquot of fraction). — — —, A_{280} of fraction.

were the same throughout the purification scheme from all three sources. The final ratios were similar for preparations from the three distinct physiological conditions, being for cells in log phase growth 0.32, for elicited cells 0.35 and for elongating hypocotyls 0.47. These purified preparations were also subjected to native PAGE or IEF followed by activity staining, indicating that both UDP-glucose dehydrogenase and ADH activities shared the same mobilities and banding pattern on IEF (Figure 3). The product of the final stage of purification could not be further differentiated into separate activities by chromatofocusing, cation and anion exchange, liquid-phase IEF, various

Table 1 Purification of UDP-glucose and ADH activities from suspension-cultured French bean cells after 8 h exposure to elicitor

For the extraction, 500 g of tissue was used and UDP-glucose dehydrogenase was purified. Throughout each chromatogram ADH activity was also found to match the UDP-glucose dehydrogenase profiles.

Step	UDP-glucose dehydrogenase					ADH			
	Total activity ($\mu\text{mol}/\text{min}$)	Protein (mg)	Specific activity (nmol/min per mg)	Recovery (%)	Purification (fold)	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity (nmol/min per mg)	Recovery (%)	Purification (fold)
Crude	52.96	760	69.68	100	1	175.49	230.91	100	1
Phenyl-Sepharose	20.00	17.8	1120	38	16	64.93	3648	37	16
G-100	8.42	2.31	3643	16	52	31.59	13670	18	59
Cibacron Blue	6.355	0.234	27170	12	390	19.30	78745	10	341

Figure 2 Gel analysis of purified UDP-glucose dehydrogenase from French bean cells

(a) One-dimensional SDS/PAGE on 12.5% acrylamide and silver staining of a preparation from elicitor-treated French bean cells. Track 1, M_r markers; track 2, UDP-glucose dehydrogenase. (b)–(d) Two-dimensional IEF/SDS/PAGE of preparations from (b) elicitor-treated cells, (c) suspension-cultured cells at peak of activity [4] and (d) elongating hypocotyls at peak of activity [4]. Spots of UDP-glucose dehydrogenase marked 1–8 and purified from elicited cells are in the order in which they were subjected to peptide mapping as shown in Figure 4.

Figure 3 Gel analysis of purified UDP-glucose dehydrogenase, followed by activity staining

Track 1, non-denaturing PAGE, followed by activity staining for UDP-glucose dehydrogenase. Track 2, non-denaturing PAGE, followed by activity staining for alcohol dehydrogenase. Samples were run on the same 8% acrylamide gel under non-denaturing conditions, and separate lanes were cut out and stained for the requisite activity. Tracks 3 and 4: flat-bed IEF stained for (3) UDP-glucose dehydrogenase or (4) ADH; experimental design was as for tracks 1 and 2.

dye columns, and affinity chromatography on AMP-Sepharose and immobilized UDP-glucuronate (results not shown).

In order to estimate the degree of relatedness between individual spots obtained when highly purified preparations were analysed by two-dimensional PAGE, peptide mapping *in situ* was carried out with V8 protease [12]. The resulting peptide map for each protein when characterized by SDS/PAGE indicated that there was a high degree of similarity between all eight spots (Figure 4). However, due to the relative abundance of Coomassie-Blue-stained material present in each spot, it was difficult to load the same amount of protein per lane in the peptide separating gel. Even so, the possibility that there are distinctions between each species is not high. Compared with the relatedness of the isoforms of phenylalanine ammonia-lyase from French bean on peptide mapping [13], these appear even more closely related. Amino

Figure 4 Peptide mapping of purified dehydrogenase isoforms

Cleveland [12] map of separated isoforms from a purification of UDP-glucose dehydrogenase from elicitor-treated cells on a 15% acrylamide gel. Spots separated on a large-scale (20 cm × 20 cm) IEF/SDS/PAGE system were excised and subjected to proteolytic mapping using V8 protease. Spots 1–8 were loaded in order of separation from the anode end (see Figure 2). The gel was silver-stained.

acid sequences obtained from two peptides generated from the two most abundant individual spots also showed a high degree of similarity to pea ADH, and complements sequence obtained from peptides generated from the initial purified preparations [8]. One peptide, VNAEMTNGRN, corresponds to PSADH1 amino acid sequence positions 257–266 (VIAEMTNGGV) and the other, LTRINVDAG, corresponds to amino acids 116–124 (LLRINTDRG).

Kinetic properties

UDP-glucose activity was measured both spectroscopically and radiometrically. The latter assay confirmed UDP-glucuronate as the only product, and the enzyme activity has similar parameters in both assay systems. The spectroscopic assay was used routinely. Kinetic properties were determined for both UDP-glucose dehydrogenase and ADH on the most highly purified fraction. By keeping the concentration of NAD^+ constant and using a range of either UDP-glucose or ethanol concentrations, the K_m for each substrate was estimated by use of double-reciprocal plots. The K_m for UDP-glucose was 5.5 ± 1.4 mM and the K_m for ethanol was 1.8 ± 0.5 mM. Similarly, by using a fixed concentration of either UDP-glucose or ethanol and varying the concentration of NAD^+ , the K_m for NAD^+ was estimated. When either substrate was held at a fixed concentration, the K_m for NAD^+ was found to be the same in each case: 20 ± 3 μ M.

The same purified preparations were also tested with free glucose and xylose as substrates in NAD^+ -dependent spectrophotometric assays, where no detectable activity could be measured. When butan-1-ol or propan-1-ol was used as substrate over a range of concentrations up to 30 μ M, the activity detected for each compound was approx. 30% of that found at the equivalent concentration of ethanol. Substituting propan-2-ol in place of ethanol gave approx. 10% of the activity, whereas substituting butan-2-ol for ethanol gave no detectable activity (results not shown). Inhibitors such as pyrazole, an NAD^+ analogue used at relatively low concentrations, and trifluoro-ethanol, an inhibitor of ADH used at relatively higher concentrations, inhibited both ADH and UDP-glucose dehydrogenase activities to the same extent. However, when UDP-xylose was tested as an inhibitor only the UDP-glucose dehydrogenase activity was inhibited (Figure 5).

Figure 5 Inhibition of UDP-glucose dehydrogenase and ADH activities

Inhibition curves are shown for the effect of pyrazole (-----), trifluoroethanol (---) and UDP-xylose (---) on UDP-glucose dehydrogenase (○) or ADH (●) activities in a highly purified enzyme preparation from elicitor-treated French bean cells.

Figure 6 Specificity of antibodies to UDP-glucose dehydrogenase

Western blots are shown for (a) track 1, preimmune serum, and track 2, immune serum, on a purified preparation of UDP-glucose dehydrogenase from elicited French bean cells, and for (b) the levels of enzyme present in soluble cell extracts from cells harvested at intervals after addition of elicitor. Tracks: 1, 0 h; 2, 2 h; 3, 4 h; 4, 6 h; 5, 8 h; 6, 10 h. The second-antibody detection was with goat anti-rabbit IgG conjugated to alkaline phosphatase.

Immunological studies using anti-(UDP-glucose dehydrogenase) serum

Antibodies were raised against the most highly purified preparation of the dehydrogenase. These proved not to be particularly inhibitory, and at a titre of 1:10 inhibited both UDP-glucose dehydrogenase by 53% and ADH activity by 60%. The anti-serum recognized a single band of M_r 40000 when compared with preimmune serum (Figure 6). A Western blot of soluble extracts from cells harvested at different time points after addition of elicitor showed increased abundance of the enzyme subunit, reaching a peak at 8 h, and confirming that the induction of enzyme activity described previously [5,8] is accounted for by increased appearance of enzyme protein.

Control immunolocalization studies

A number of controls employing preimmune rabbit sera, rabbit antisera to the M_r -42000 chitin-binding wall-bound protein [14], phenylalanine ammonia-lyase and peroxidase [15], secondary cell-wall-specific glycoproteins [16] and chloroplastic acyl-carrier protein [17] were used. No binding of gold particles was observed when tissue was incubated with preimmune serum and second-antibody-gold complexes, or with second-antibody-gold complex alone. Most of the control rabbit antisera employed bound to cell components other than those to which the anti-(UDP-glucose dehydrogenase) IgG bound. Anti-(acyl-carrier protein) serum bound to chloroplasts only, whereas anti-(M_r -42000 chitin-binding protein), anti-(secondary cell-wall-specific glycoprotein) and anti-(cationic peroxidase) sera bind to the cell wall and associated compartments (results not shown). Only anti-(phenylalanine ammonia-lyase) serum bound to vascular cytoplasm as well. Overall, there was no indication of non-specific interactions with the methodologies employed.

Immunogold localization of UDP-glucose dehydrogenase to vascular tissues in French bean hypocotyls

Antibodies raised against proteins purified from cell-suspension cultures of French bean have proved suitable for localization studies in the plant many times [2]. The antibody raised in this study was used to immunolocalize UDP-glucose dehydrogenase in French bean hypocotyls. The labelling was strong enough to produce reflection images observed with confocal optics showing localization of silver-enhanced gold particles in cells undergoing secondary cell-wall thickening (Figure 7a). Labelling was observed most strongly in sections taken from the younger part of hypocotyls, where secondary cell walls were developing below the cotyledons and first leaves. These results show a different timing of induction comparable with those obtained for enzymes of lignin synthesis [15] or secondary cell-wall-specific glycoproteins [16]. Labelling was present in late cambial cells and developing xylem tracheary elements and developing xylary and phloem fibres. The absence in mature xylem is marked, suggesting that the enzyme is expressed during earlier stages of vascular differentiation when secondary walls are starting to be laid down, giving structural evidence for a key role in the provision of intermediates for cell-wall polysaccharide biosynthesis. When observed by electron microscopy, deposition of gold particles was found at high density in the cytoplasm of developing vascular cells (Figures 7b–7d).

DISCUSSION

UDP-glucose dehydrogenase may act as a key enzyme in the control of carbon flux into the pool of UDP-sugars required for cell-wall polysaccharide biosynthesis and oligosaccharide decoration of glycoproteins. As such, it is responsible for entry into the pool of UDP-sugars which form the precursors of cell-wall pectins and hemicelluloses. The enzyme has been purified and characterized from French bean cells and tissues in various physiological states. Purification to homogeneity and analysis on one-dimensional SDS/PAGE showed that the enzyme had an M_r of 40000, and resolved into a number of isoforms on two dimensional IEF/SDS/PAGE which showed similar abundance whether the source material was actively growing suspension-cultured cells or cells from elongating hypocotyls [4] or elicitor-treated suspension cultured cells [5]. This multiplicity is not unusual for plant cytosolic enzymes, and is seen for phenylalanine ammonia-lyase from French bean, for example [18]. Kinetic

Figure 7 Immunolocalization in vascular tissue of French bean

(a) Confocal pair showing localization to vascular tissue in hypocotyls of French bean. Sections were incubated with anti-(UDP-glucose dehydrogenase) IgG followed by gold-antibody complex. Deposited gold particles were silver-enhanced and observed with confocal optics creating pairs of phase-contrast and reflection images. Labelling with anti-(UDP-glucose dehydrogenase) was specific for cells with developed secondary cell walls: cambium (C) and xylary (XF) and phloem (PF) fibres. Magnification $\times 150$. Scale bar represents $100\ \mu\text{m}$. (b), (c) Subcellular localization by immunogold electron microscopy. The antigen is localized to the cytoplasm of developing xylem vessels (b and c) and phloem (d). Arrows show examples of gold labelling, which is profuse. Magnifications: (b) $\times 30\,000$; (c) $\times 30\,000$; (d) $\times 15\,000$. Scale bars represent $0.5\ \mu\text{m}$. Other abbreviations: CW, cell wall; CYT, cytoplasm; CP, chloroplast; MT, mitochondrion; V, vacuole.

parameters have been determined for the enzyme. Furthermore, it was also determined that when ADH was purified from elicited suspension-cultured cells [8], the activities always co-purified. When this co-resolution was studied in depth, this was found to be true for the preparations from all three distinct sources. Despite extensive investigation, the activities could not be separated by a wide series of methodologies.

This equivalence of the two activities suggests they may be a function of the same enzyme system. This conclusion is based on the co-purification, non-denaturing gel electrophoresis followed by specific activity staining, identical behaviour with inhibitors (except UDP-xylose, which may act at a different site), the fact that all spots separated by two-dimensional IEF/SDS/PAGE were found to be blocked when N-terminal protein sequencing was carried out, that peptide maps of individual spots resolved by two-dimensional IEF/SDS/PAGE were identical, and peptide

sequences of peptides derived from the two most abundant spots were identical. Co-purification is not surprising, since dye ligand chromatography has been used for the purification of ADH [19–21]. However, the inhibitors of ADH, trifluoroethanol and pyrazole, were equally effective against UDP-glucose dehydrogenase. There is some contradictory evidence, e.g. the lack of effect of UDP-xylose on the ADH activity of the enzyme preparation, but, weighed against the other evidence, the conclusion that UDP-glucose dehydrogenase has intrinsic ADH activity is compelling.

There has been a considerable amount of knowledge accumulated about the regulation of ADH expression in response to oxygen deficits [22]. However, there are instances where ADH is expressed under circumstances unlikely to have been triggered in response to anaerobic conditions. This has been observed in ripening tomato fruit [23,24] and enhanced root growth in barley

[25]. It is noteworthy that, for the extensively studied maize ADH isoforms, one form, ADH2, has intrinsically lower specific activity (10-fold) than ADH1 [26]. The *adh2* gene promoter confers expression in roots and vascular tissue of maize [27], and it was concluded that *adh* expression in the leaves of tomato [28], potato [29] and petunia [30] is predominantly associated with the vascular tissue. ADH2 has been detected in stem nodes [31]. In the ADH field, it is a problem to explain why it is expressed in vascular tissue [22]. One possibility underlying the expression of ADH in vascular tissue may be indicated by the present study. The UDP-glucose dehydrogenase immunolocalized to vascular tissue in hypocotyls of French bean. From the data presented suggesting its dual specificity, it may well be that these are equivalent and that the UDP-glucose dehydrogenase/ADH functions in the provision of UDP-sugars required for the formation of secondary wall in vascular tissues. In this context, using the *Zinnia* model system for studying differentiation, a cDNA was cloned that showed sequence similarity to ADH and was expressed in immature xylem and phloem, and was most pronounced in larger phloem cells [32,33]. The antiserum described here has been used in this system and localizes to developing tracheids at an early stage (G. Peter and G. P. Bolwell, unpublished work). All these data may indicate a family of ADH-like dehydrogenases that are expressed during vascular differentiation, of which UDP-glucose dehydrogenase is an important example.

G.P.B. thanks the BBSRC for funding.

REFERENCES

- Dalessandro, G. and Northcote, D. H. (1977) *Biochem. J.* **162**, 267–279
- Bolwell, G. P. (1993) *Int. Rev. Cytol.* **146**, 261–324
- Amino, S., Takeuchi, Y. and Komamine, A. (1985) *Physiol. Plant.* **64**, 111–117
- Robertson, D., Beech, I. and Bolwell, G. P. (1995) *Phytochemistry* **39**, 21–28
- Robertson, D., McCormack, B. A. and Bolwell, G. P. (1995) *Biochem. J.* **306**, 745–750
- Botha, F. C., O'Kennedy, M. M. and du Plessis, S. (1992) *Plant Cell Physiol.* **33**, 477–483
- Llewellyn, D. J., Finnegan, E. J., Ellis, J. G., Dennis, E. S. and Peacock, W. J. (1987) *J. Mol. Biol.* **195**, 115–123
- Robertson, D., Davies, D. R., Gerrish, C., Jupe, S. C. and Bolwell, G. P. (1995) *Plant Mol. Biol.* **27**, 59–67
- Dixon, R. A. and Lamb, C. J. (1979) *Biochim. Biophys. Acta* **586**, 453–463
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U.K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Bolwell, G. P. and Rodgers, M. W. (1991) *Biochem. J.* **279**, 231–236
- Millar, D. J., Slabas, A. R., Sidebottom, C., Smith, C. G., Allen, A. K. and Bolwell, G. P. (1992) *Planta* **187**, 176–184
- Smith, C. G., Rodgers, M. W., Zimmerlin, A., Ferdinando, D. and Bolwell, G. P. (1994) *Planta* **192**, 155–164
- Wojtaszek, P. and Bolwell, G. P. (1995) *Plant Physiol.* **108**, 1001–1012
- Slabas, A. R. and Smith, C. G. (1989) *Planta* **175**, 145–152
- Bolwell, G. P., Bell, J. N., Cramer, C. L., Schuch, W., Lamb, C. J. and Dixon, R. A. (1985) *Eur. J. Biochem.* **149**, 411–419
- Bicsak, T. A., Lane, R. K., Reiter, A. and Chase, T. (1982) *Arch. Biochem. Biophys.* **216**, 605–615
- Nicols, M. and Crouzet, J. (1980) *Phytochemistry* **19**, 15–18
- Pryor, A. and Huppatz, J. L. (1982) *Biochem. Int.* **4**, 431–438
- Dennis, E. S., Olive, M., Dolferus, R., Millar, A., Peacock, W. J. and Setter, T. L. (1992) in *Inducible Plant Proteins* (Wray, J. L., ed.), pp. 231–245, Cambridge University Press, Cambridge
- Longhurst, T. T., Tung, H. F. and Brady, C. J. (1990) *J. Food Biochem.* **14**, 421–433
- Chen, A. S. and Chase, T. (1993) *Plant Physiol. Biochem.* **31**, 875–885
- Tomoda, Y., Umemura, K. and Adachi, T. (1994) *Biosci. Biotechnol. Biochem.* **58**, 202–203
- Freeling, M. (1974) *Biochem. Genet.* **12**, 407–417
- Paul, A.-L. and Ferl, R. J. (1994) *Plant J.* **5**, 523–533
- Tanksley, S. D. and Jones, R. A. (1981) *Biochem. Genet.* **19**, 397–409
- Matton, D. P., Constabel, P. and Brisson, N. (1990) *Plant Mol. Biol.* **14**, 775–783
- Gregerson, R., McLean, M., Beld, M., Gerats, A. G. M. and Strommer, J. (1991) *Plant Mol. Biol.* **17**, 37–48
- Dlouhy, R. (1980) Ph.D. Thesis, Indiana University
- Demura, T. and Fukuda, H. (1993) *Plant Physiol.* **103**, 815–821
- Demura, T. and Fukuda, H. (1994) *Plant Cell* **6**, 967–981