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

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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. 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*₄ 40000. *Kₘ* values were measured for UDP-glucose as 5.5 ± 1.4 mM and for NAD⁺ as 20 ± 3 µM. It was subject to inhibition by UDP-xyllose. UDP-glucose dehydrogenase activity co-purified with alcohol dehydrogenase activity from suspension-cultured cells, elicitor-treated cells and elongating hypocotyls, even when many additional chromatographic 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.

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UDP-glucose dehydrogenase was routinely assayed spectrophotometrically by monitoring the reduction of NAD⁺ as the increase in *Å₄₅₀*. 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.

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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 µl of 3% BSA/1% (v/v) normal goat serum in 20 mM Tris/Cl 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 µl 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 µl 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 counterstained 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 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 at room temperature in 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 (NH₄)₂SO₄ 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 Mr 40000 (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, 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

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**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.

<table>
<thead>
<tr>
<th>Step</th>
<th>UDP-glucose dehydrogenase</th>
<th>ADH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity (μmol/min)</td>
<td>Protein (mg)</td>
</tr>
<tr>
<td>Crude</td>
<td>52.96</td>
<td>760</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>20.00</td>
<td>17.8</td>
</tr>
<tr>
<td>G-100</td>
<td>8.42</td>
<td>2.31</td>
</tr>
<tr>
<td>Cibacron Blue</td>
<td>6.35</td>
<td>0.234</td>
</tr>
</tbody>
</table>

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![Figure 1 Elution profiles of UDP-glucose dehydrogenase and ADH purified from elicitor-treated French bean cells](image-url)
analysed by two-dimensional PAGE, peptide mapping [13], these appear even more closely related. Amino 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 µM.

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 µM, 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).
In the plant, 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 degradation. As such, it is responsible for entry into physiological states. Purification to homogeneity and analysis on SDS-PAGE showed that the enzyme had an Mr of 40000, and resolved into a number of isoforms on two dimensional IEF/PAGE which showed similar 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 Mr 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
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-xylene, 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.

Figure 7 Immunolocalization in vascular tissue of French bean

(a) Confocal pair showing localization to vascular tissue in hypocotyl 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 xylem (XF) and phloem (PF) fibres. Magnification ×150. Scale bar represents 100 µm. (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) × 30 000; (c) × 30 000; (d) × 15 000. Scale bars represent 0.5 µm. Other abbreviations: CW, cell wall; CYT, cytoplasm; CP, chloroplast; MT, mitochondrion; V, vacuole.
UDP-glucose dehydrogenase from French bean

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 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.

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REFERENCES


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