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Direct measurement of ascorbic acid biosynthesis in *Arabidopsis* cell suspension culture using capillary electrophoresis

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Abstract

We describe procedures to directly measure the biosynthesis of vitamin C (L-ascorbic acid, L-AA) in crude extracts of an *Arabidopsis thaliana* cell suspension culture by capillary electrophoresis. Optimal conditions have been established for the quantitation of L-AA formed by the oxidation of three different substrates: L-galactose, L-galactono-1,4-lactone, and L-gulono-1,4-lactone. We also demonstrate that L-galactono-1,4-lactone dehydrogenase activity does not require exogenous cofactor. The minimal sample handling requirements, the high selectivity, and short analysis times represent significant advantages over existing protocols.

Keywords: *Arabidopsis thaliana*; Ascorbic acid; Vitamins; Enzymes

1. Introduction

Ascorbic acid (L-AA), is one of the most abundant hydrophilic antioxidants, occurring in concentrations of up to 50 m*M* in plant chloroplasts [1]. The numerous metabolic functions of L-AA in both animal and plant systems [2–4] has led to great interest in the metabolism of this important component in plants, although the biosynthetic route has for many years been a matter of dispute. Recently, Wheeler et al. [5] reported that the pathway of plant L-AA biosynthesis proceeds via D-mannose and L-galactose (L-Gal) (Eq. (1))

D-glucose → → D-mannose-1-P →

GDP-D-mannose↔GDP-L-galactose →

(L-galactose-1-P) → L-galactose →

L-galactono-1,4-lactone → L-AA (1)

L-Gal is formed by the 3,5-epimerisation of GDP-mannose, and is subsequently oxidized to L-galactono-1,4-lactone (L-GL) by a new, NAD-dependent enzyme, L-galactose dehydrogenase (L-GalDH) [5]. L-GL itself is then oxidized to L-AA by L-galactono-1,4-lactone dehydrogenase (GLDH), a relatively well-characterized enzyme that has been purified from a number of sources [6–11] and that has recently been cloned [9,11]. Others have reported that certain other hexose derivatives are also converted to L-AA [12,13]. Recent work from our laboratory has shown that in addition to L-GL and L-Gal, L-gulono-1,4-lactone (L-GuL), methyl-D-galacturonic acid, and D-glucurono-1,4-lactone, are all precursors of L-AA in *Arabidopsis*, with varying degrees of success (Davey et al., submitted).

In the majority of existing procedures, the L-AA biosynthetic activity of cell-free extracts is measured spectrophotometrically. Such procedures include

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monitoring the increase in absorbance at 265 nm due to L-AA formation [14], the decrease in absorbance at 265 nm due to the oxidation of newly synthesized L-AA following addition of ascorbate oxidase to extracts [15], the decrease in absorbance at 600 nm due to oxidation of 2,6-dichlorophenol-indophenol by L-AA in the assay mixture [16], and the derivatization of L-AA oxidation products with 2,4-dinitrophenylhydrazine [6,10]. GLDH activity, the terminal enzyme of Eq. (1), is typically determined by measuring the increase in absorbance at 550 nm because of the reduction of cytochrome C, which acts as an electron acceptor [6–9,17]. Spectrophotometric assays are convenient, require relatively inexpensive equipment, and allow the processing of multiple samples. However as pointed out [18], these assays are non-specific, and plant extracts in particular may contain many interfering components.

High-performance liquid chromatographic (HPLC) analysis with electrochemical detection [19], with fluorescence detection [20], or with refractive index detection [16], avoids the problems of non-specific interference, but analysis times are long because columns need to be reconditioned between analyses. Further, in most cases the extracts must be partially purified and assays are generally carried out on mitochondrial or microsomal preparations. These additional handling steps are labour intensive and can lead to mitochondrial and membrane degradation so that quantitative comparisons of the biosynthetic activities of different tissues may be difficult and/or unreliable.

For these reasons we were interested in developing procedures to quantitate the L-AA biosynthetic capacities of plant extracts with a minimum number of sample preparation steps. The minute (nl) injection volumes, rapid analysis times, and high separation efficiencies made capillary electrophoresis (CE) the analytical technique of choice. With this approach we have been able to quantitate L-AA formation from the oxidation of three substrates. These are L-galactono-1,4-lactone (L-GL), (oxidized directly to L-AA by GLDH), L-Gal, which is oxidized to L-AA by the combined activities of L-GalDH and GLDH, and L-Gul, which is probably oxidized to L-AA by an as yet uncharacterized L-gulono-1,4-lactone oxidase/dehydrogenase (L-GuLO) activity. The presence of L-GuLO activity implies the existence of an alternative route of L-AA biosynthesis to Eq. (1). The possible physiological significance of these results is discussed in more detail elsewhere (Davey et al., submitted).

2. Experimental

2.1. Materials

Boric acid, EDTA·2Na, Tris base and sodium hydroxide were purchased from Aldrich (Brussels, Belgium). L-AA, D-isoascorbic acid (D-isoAA), D/L-GL, D/L-GuL, D-glucuronic acid, D-gulonic acid, D-galacturonic acid and metaphosphoric acid (MPA) were all obtained from Sigma (St Louis, MO, USA). HPLC-grade acetonitrile was obtained from Labscan (Dublin, Ireland). NAD and NADP were obtained from Boehringer (Mannheim, Germany). All water used was purified using a 'Nanopure' system (Millipore, Bedford, MA, USA).

2.2. Growth and incubations

A suspension of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia cells was maintained in 500-ml Erlenmeyer flasks on a rotary shaker at 110 rpm. Growth was in modified Murashige and Skoog medium, supplemented with 20 m*M* 2-(*N*-morpholino)ethanesulfonic acid, under conditions of 16 h light/8 h dark at a constant temperature of 24°C. Cells required for experiments were cultured for seven days to mid-log phase and harvested by filtration onto AP-15 glass-fibre prefilters (Millipore).

2.3. Extraction

For the measurement of L-GL-, L-Gal-, and L-GuL-dependent L-AA biosynthesis, approximately 5 g fresh mass of cells were homogenized at 4°C in 1 ml g^{-1} fresh mass extraction buffer using an Ultraturrex T-25 high speed blender (Van Der Heyden, Brussels, Belgium). The extraction buffer consisted of 100 m*M* Tris/HCl, pH 8.5, containing 2.5 m*M* dithiothreitol (DTT), 0.3 *M* mannitol, and 0.1 m*M* EDTA. After centrifugation at 1500 rcf (3800 rpm), for 12 min at 4°C, samples were passed through a

prepacked Sephadex G-25 gel filtration column (Pharmacia Biotech, Uppsala, Sweden), equilibrated in extraction buffer.

2.4. Assay

L-AA biosynthesis was initiated by the addition of substrate at a final concentration of 5 m*M* (L-GL and L-Gal), or 25 m*M* (L-GuL), in a total volume of 500 μl of gel-filtrated cell extract. Incubations with L-Gal additionally contained NAD cofactor at a final concentration of 0.125 m*M*. Incubations were continued for up to 120 min at room temperature and the reaction was monitored by periodically removing 10 μl of reaction mixture and adding it to an equal volume of 'stop' buffer. The stop buffer comprised 6% MPA-2 m*M* EDTA-2.5 m*M* DTT, containing 0.02 mg ml^{-1} D-isoAA as internal standard. Samples were stored at 4°C or frozen at −20°C until analysis.

2.5. Instrumentation

All CE analyses were carried out on a TSP Ultraphoresis instrument (ThermoSeparations Products, San Jose, CA, USA), fitted with a TSP UV-3000 scanning UV–Vis detector and a cooled autosampler. The entire system was controlled and data acquired and processed using the proprietary Spectra 1000 software.

HPLC analyses were carried out using a 600E pump and pump controller (Waters, Milford, MA, USA) after which a Waters 997 photodiode array detector was serially connected. Injections were carried out automatically using a Waters WISP 712 autosampler. Separations were performed on a 250× 4.6 mm, 3-μm spherical particle-size LiChrosorb C_{18} RP-HPLC column, fitted with a 7-mm guard column (Alltech Associates, Deerfield, IL, USA). Spectrophotometric assays were carried out using a SpectraMAX (Molecular Devices, Sunnyvale, CA, USA), running the proprietary SOFTMax Pro software.

2.6. Quantitation of L-AA by CE

L-AA was quantitated by CE, using modifications of previously developed methods [21]. Separations were carried out on a 40.1 cm (34 cm to detector)× 50 μm fused-silica capillary thermostatted in a forced-air oven at 20°C. Before each analysis, the capillary was successively rinsed with 0.2 *M* HCl and 200 m*M* borate pH 9.0–200 m*M* sodium dodecyl sulfate (1:1, v/v), for 0.4 min, followed by background electrolyte (BGE) for 0.6 min at 100 p.s.i. (1 p.s.i. = 6894.76 Pa). The BGE consisted of 200 m*M* borate pH 9.0–10% (v/v) acetonitrile–0.1 m*M* EDTA. Samples were maintained at 8°C in a cooled automatic sampler and injected hydrodyamically for up to 10 s at 0.8 p.s.i. Separations were carried out under an applied voltage of +26 kV (648 $V\,cm^{-1}$), with a 30 s voltage ramp, generating a current of approximately 85 μA. Detection was carried out at three wavelengths simultaneously: 197 nm, 267 nm, and 297 nm; L-AA was quantitated at 267 nm, the absorption maximum for L-AA at this pH. Data were acquired at a rate of 8 Hz. The L-AA concentration of samples was calculated from the mean UV response of L-AA standards prepared in 3% MPA/1 m*M* EDTA, stabilized with 1 m*M* DTT, and run at the beginning of each set of analyses.

2.7. HPLC analysis of L-AA

HPLC analysis of L-AA was essentially as previously described [22]. Separations were carried out isocratically at 0.8 ml min^{-1} using a mobile phase of 400 μl l^{-1} phosphoric acid, 1 m*M* EDTA, and 1 m*M* KCl. The column was regenerated between runs with a 10-min linear gradient of 0% to 20% acetonitrile in the same mobile phase composition given above. The entire system was controlled and data collected and analyzed using the Millenium 2015 (version 3.15) software package (Waters Associates). Data were collected over the range 195–300 nm at a rate of 2 Hz and a spectral resolution of 2.4 nm. Quantitation of L-AA was carried out at 243 nm, the absorption maximum for L-AA at this pH.

2.8. Identity of L-AA peak

The identity of the L-AA peak was confirmed by co-migration with authentic standards in CE and by co-elution with authentic standards in HPLC. L-AA was also identified on the basis of its characteristic UV absorption spectrum (190–300 nm), using the scanning and photodiode array options of the CE and HPLC systems, respectively. Finally, peak identity

was confirmed by its disappearance upon treatment of extracts with ascorbate oxidase, as previously described [22].

2.9. Spectrophotometric assays

The GLDH activity of mitochondrial tissue preparations was determined spectrophotometrically by following the substrate-dependent reduction of cytochrome C at 550 nm, essentially as previously described [6,9]. L-GalDH was measured by following the L-Gal-dependent reduction of NAD at 340 nm in 100 m*M* Tris pH 8.5, containing 5 m*M* glutathione, and 0.125 m*M* NAD cofactor. We were unable to detect L-GuLO activity spectrophotometrically in crude, mitochondrial, or soluble fractions of cell suspension extracts.

3. Results and discussion

The chemical properties of L-AA are such that it is readily oxidized to dehydroascorbate. As previously discussed [18,22], in L-AA analyses, the choice of extraction buffer is critical to prevent this oxidation and to fix the L-AA-dehydroascorbate redox equilibrium. In our experience, the L-AA content of plant extracts is most stable in 3% MPA-1 m*M* EDTA. Consequently, the CE procedures described here were developed to accommodate the use of this extraction buffer during the determination of L-AA levels of plant tissue and the L-AA biosynthetic capabilities of plant extracts.

3.1. Optimization of CE separation for L-AA analysis

The conditions used for the analysis of L-AA levels are based on methods previously developed for the quantitation of L-AA and glutathione in plant material [21,22]. Here, we found that a high concentration of buffer in the BGE is necessary to counteract the anti-stacking effects that result after injection of samples dissolved in 3% MPA-1 m*M* EDTA (extraction solvent). Good resolution of L-AA was obtained using a BGE of 200 m*M* borate pH 9.0 [22]. However, we occasionally observed co-elution of the L-AA peak with other components, depending on the tissue being analyzed and additives included in the assay mix. Resolution in this system can be easily improved simply by including acetonitrile in the BGE. As shown in Table 1, acetonitrile causes a linear and uniform increase in the actual pH of the BGE. This pH increase in turn augments the proportion of analyte complexed with borate [23], which improves resolution due to the competing influences of the electroosmotic flow-driven bulk liquid transfer to the cathode, and anodic migration of the borate complexes. Therefore, the addition of acetonitrile provides a convenient means of modifying the BGE selectivity in a pH-dependent manner, without increasing the overall ionic strength (and Joule heat production), such as occurs by alkalinization with NaOH.

Good resolution of biological extracts was achieved with a BGE containing 10% acetonitrile, which increased the actual pH from 9.0 to 9.34. There was no advantage to be gained from further increasing acetonitrile concentrations, and above 15% (v/v), peak efficiencies decreased and analysis times nearly doubled. With 200 m*M* borate pH 9.0–10% (v/v) acetonitrile–0.1 EDTA as BGE, L-AA and D-isoAA are well resolved in biological extracts, allowing the use of D-isoAA as an internal standard. Furthermore, the quantitation of L-AA and D-isoAA is unaffected by the presence of either DTT, detergents, FAD, NAD, or NADP. Typical electropherograms for this analysis are shown in Fig. 1.

3.2. Detection limits

For a standard 8-s injection at 0.8 p.s.i., with a

Table 1
Influence of percentage acetonitrile in 200 m*M* borate pH 9.0–0.1 m*M* EDTA on the actual pH of BGE

Acetonitrile %	pH[a]
0	9.00
4	9.14
8	9.27
12	9.41
16	9.52
20	9.68

[a] Actual pH = (0.0336 × % acetonitrile) + 9.0, $r^2 = 0.999$. pH was measured using a HI 8250 pH meter with a glass electrode (Hanna Instruments, Ronchi di Villafranca, Italy).

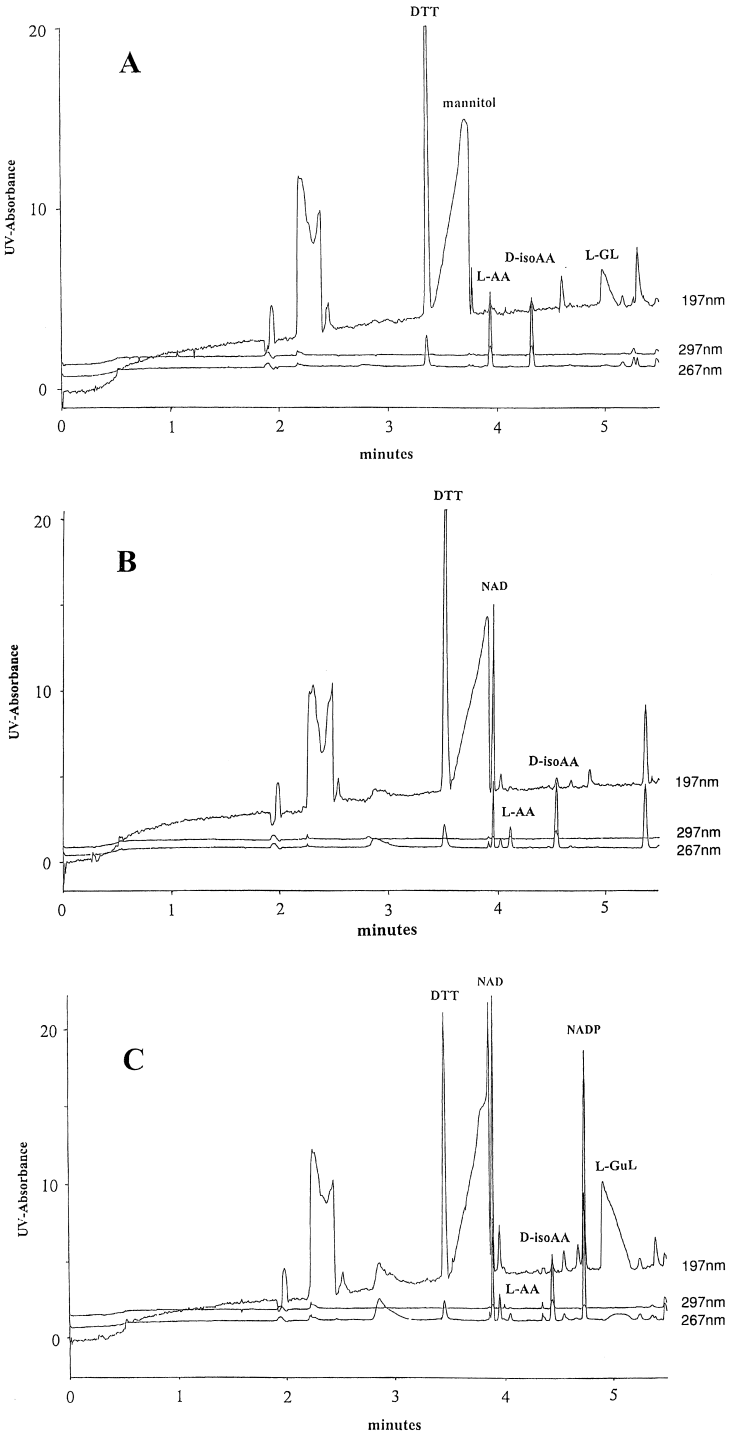

Fig. 1. Typical electropherograms for the quantitation of L-AA formed by crude, Sephadex G-25, gel-filtrated extracts of *A. thaliana* cell suspension culture during incubation with 5 m*M* L-GL, $t = 60$ min (A), 5 m*M* L-Gal + 0.125 m*M* NAD, $t = 60$ min (B), and 25 m*M* L-GuL, $t = 120$ min (C). All analytical conditions were as described (see Experimental) with simultaneous UV monitoring at 197, 267, and 297 nm.

BGE of 200 m*M* borate pH 9.0–10% (v/v) acetonitrile–0.1 m*M* EDTA, the minimum L-AA detection limits, set at approximately three times baseline noise, were in the region of 1 μM. Assuming that the extraction solvent has the same viscosity as water, this corresponds to an injection volume of 16.8 nl, and a mass limit of sensitivity of 0.65 pg/injection. Linearity of detector response was observed between 1 μ*M* and 250 μ*M* ($r^2 = 0.999$), but above this range there was some deviation from linearity as the response factor decreased.

3.3. Optimizing assays for L-AA biosynthetic activity

It was our aim to quantitate the rates of L-AA biosynthesis in crude extracts of plant tissues and so to minimize sample handling and preparation. However, we were unable to use existing spectrophotometric assays to measure L-GL-, L-Gal-, or L-GuL-dependent L-AA formation in either crude or gel-filtrated crude extracts, due to the high, non-specific background reduction of the associated cofactors. These spectrophotometric assays could only be applied after partial purification of the extracts. We therefore concentrated on optimizing conditions for the direct analysis of the L-AA synthesized by CE. To maximize the sensitivity of the CE assay though, it was necessary to determine the optimal conditions for the different enzyme activities involved. Specifically, the extraction conditions, substrate specificities, and the pH and substrate concentration dependence of the conversion of L-GL, L-Gal, and L-GuL to L-AA.

3.3.1. Extraction buffer

Previous work in our laboratory with GLDH purified from cauliflower meristem had shown that the pH optimum for GLDH activity is pH 8–8.5 [9]. Our first choice of extraction buffer was therefore 100 m*M* Tris pH 8.5. Since GLDH is a mitochondrially-associated enzyme [9,10], 0.3 *M* mannitol was included to help preserve mitochondrial integrity, and EDTA to inhibit protease activities [24]. Ideally, the extraction buffer is also the assay buffer, but to prevent the autooxidation of L-AA synthesized during the assay, it was necessary to add 2.5 m*M* DTT. DTT was subsequently also found to stabilise L-GalDH activity during extraction. Detergents have regularly been used in the purification of L-AA biosynthetic enzymes [10,14,15,25,26]. However, we found that the inclusion of ionic detergents such as sodium dodecyl sulfate abolished GLDH activities, and non-ionic detergents such as 0.4% Brij 35, Triton X-100, Tween 20, and *n*-octyl-glucopyranoside, severely depressed L-GL-dependent L-AA formation.

3.3.2. Sample processing

With 100 m*M* Tris, pH 8.5, 0.3 *M* mannitol, 2.5 m*M* DTT, 1 m*M* EDTA as the extraction buffer, we were able to measure L-GL-dependent L-AA formation by CE in crude cellular extracts. However, the high endogenous L-AA levels and autooxidation of L-AA at this buffer pH complicated analyses. Once low-molecular-mass compounds had been removed by Sephadex G-25 gel filtration, we observed a linear, substrate-dependent formation of L-AA with all three substrates using the CE assay. The inclusion of DTT in the assay buffer was necessary to prevent autooxidation of newly synthesized L-AA, and, under these conditions, the rate of L-AA formation remained essentially linear for up to 180 min (Fig. 2). Recoveries of activities were equally high whether cells were extracted by grinding in liquid nitrogen, or by blending on ice with a high-speed mixer.

3.3.3. Cofactor requirements

Surprisingly, GLDH activity did not require a supply of exogenous cytochrome *c*, which is the cofactor normally used as electron acceptor in GLDH assays. This observation suggests that either endogenous, mitochondrial cytochrome *c* levels are sufficient to support GLDH activity, or that another (high-molecular mass) electron acceptor for GLDH is naturally present, possibly as part of the mitochondrial electron transport system. L-Gal-dependent L-AA formation, which represents the combination of GalDH and GLDH activities, was by contrast strictly dependent on the presence of exogenous NAD cofactor. An equimolar mixture of NAD and NADP decreased the rate of L-Gal oxidation by about 12% (data not shown). The relatively small amount of L-GuLO activity present did not require either NAD, NADP, or FAD as cofactor for activity.

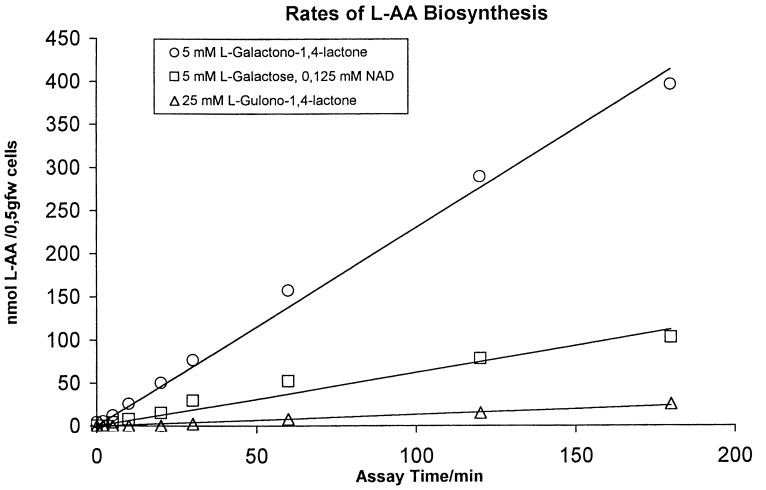

Fig. 2. Rates of L-AA formation by crude Sephadex G-25 gel-filtrated extracts of *A. thaliana* cell suspension culture, expressed in nmoles formed/min/0.5 g fresh masses (gfw) cells. Incubations carried out as described (see Experimental) with 5 m*M* L-GL, 5 m*M* L-Gal + 0.125 m*M* NAD, or 25 m*M* L-GuL. The relative rates of L-AA formation are $y = 2.30x$ ($r^2 = 0.994$), $y = 0.62x$ ($r^2 = 0.960$), and $y = 0.13x$ ($r^2 = 0.977$), for L-GL, L-Gal + NAD, and L-GuL, respectively.

3.3.4. Substrate specificity

In accordance with previous work in our laboratory [9], crude, gel-filtrated cell extracts from *A. thaliana* cell suspension appeared to be absolutely specific for L-GL. L-AA biosynthesis could not be measured using either D-GL, D-GuL, D-glucuronic acid, D-galacturonic acid, L-xylonic acid-1,4-lactone, or D-mannono-1,4-lactone as substrate, even after overnight incubation. We interpret the L-GuL-dependent L-AA biosynthesis that is present as being due to a separate protein activity, because partially purified GLDH from *A. thaliana* cell suspension culture had no detectable L-GuLO activity (data not shown). L-GalDH was also strictly specific for L-Gal, with no cross-reactivity towards D-galactose, D/L-glucose, D/L-GL, D/L-GuL, D-glucuronic acid, D-galacturonic D-gulonic acid, or D/L-glyceraldehyde. L-GalDH is also absolutely dependent on NAD as cofactor.

3.3.5. pH dependence

The pH dependence of the rate of L-AA biosynthesis from L-GL, L-Gal and L-GuL was determined over the pH range 5 to 10.5 (Fig. 3). In all cases, maximum activity was found between pH 8 and 9. In the case of L-Gal oxidation, this represents the pH optimum for the combined activities of L-GalDH and GLDH. Since there was a rapid loss of GLDH and L-GalDH activity above pH 9, optimal conditions were set as pH 8.5 for both extraction and assays. At this pH the relative rates of biosynthesis of L-AA were 1, 4.7, and 17.4 with L-GuL, L-Gal, and L-GL, as substrates, respectively.

3.3.6. Substrate concentration

The substrate concentration dependence for L-Gal- and L-GL-dependent L-AA biosynthesis was determined over the range 0.1–25 m*M*. In both cases, maximal rates of L-AA biosynthesis were observed at concentrations between 1 and 5 m*M*. Above 5 m*M*, rates decreased slowly. Previous work from our laboratory on the purified enzyme from cauliflower indicated that substrate inhibition of GLDH was observed at concentrations above 32 m*M* [9]. By comparison, in spinach [10] and white potato tubers [6], substrate inhibition was observed at concentrations greater than 8.4 m*M*. Maximal L-GuLO activity was observed at concentrations of L-GuL around 25 m*M*. This high value suggests that L-GuLO activity will not be as physiologically important as L-GL- and L-Gal-dependent L-AA biosynthesis.

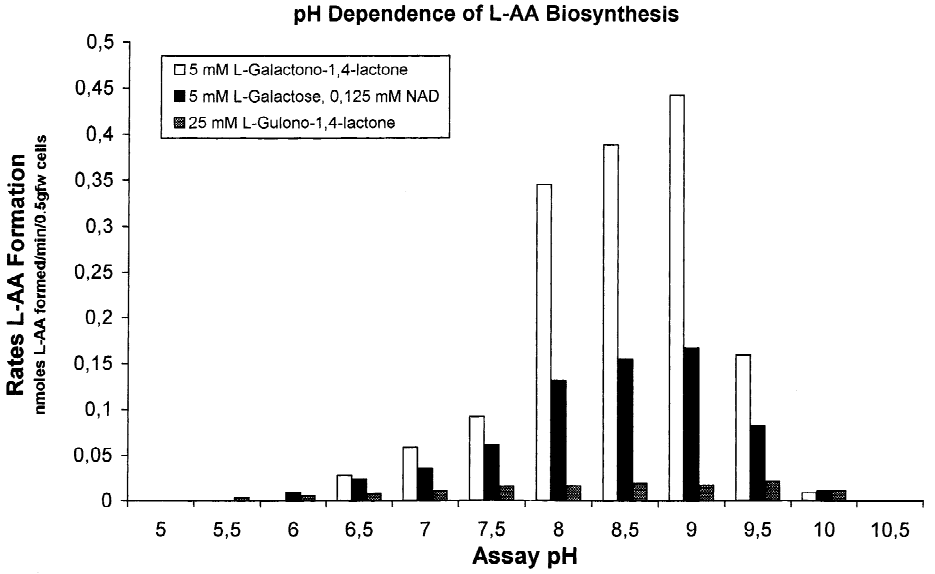

Fig. 3. pH dependence of rates of L-AA formation of crude, Sephadex G-25 gel-filtrated extracts of *A. thaliana* cell suspension culture, from L-GL, L-Gal, and L-GuL. The buffers used were as follows: pH 5.0 and 5.5, 100 m*M* sodium acetate; pH 6.0–pH 7.5, 100 m*M* potassium phosphate; pH 8.0–pH 9.0, 100 m*M* Tris/HCl; and pH 9.5–10.5, 100 m*M* sodium bicarbonate. All other conditions were as described in the text.

3.3.7. Stability of GLDH activity

Crude *A. thaliana* cell suspension extracts lost GLDH activity with a half life of about 2.5 days at 4°C. This loss of activity was not improved by the inclusion of 10% ethylene glycol, 1 to 5 m*M* DTT, or 10 m*M* EDTA/EGTA in the extraction buffer. Sephadex G-25 gel-filtrated extracts appeared to be slightly more stable, but there was rapid and irreversible loss of activity upon aeration of extracts. In the absence of DTT in the extraction buffer, L-GalDH activity disappeared within about 24 h.

4. Conclusions

In summary, we describe methods to determine the rate of L-AA biosynthesis from L-GL, L-Gal, and L-GuL in extracts of an *A. thaliana* cell suspension culture by CE. The analysis is complete within 5 min, and directly measures substrate-dependent L-AA formation in crude, Sephadex G-25 gel-filtrated extracts. Optimal conditions regarding substrate concentration and assay pH have been determined and we demonstrate for the first time that exogenous cytochrome *c* is not required for GLDH activity. There is further an L-GuL-dependent L-AA biosynthetic activity simultaneously present in vitro. The high optimal substrate concentrations of this L-GuLO activity however, suggest that it is physiologically a much less important substrate.

While it is also possible to monitor the rate of L-AA biosynthesis by HPLC, baseline resolution of L-AA and D-isoAA is incomplete, and DTT present in the assay buffer does not elute until after approximately 18 min under the conditions described here. Indeed, the need to regenerate the column between injections means that the turnaround time between injections is 35 min. The CE assay therefore has significant advantages in terms of speed, resolution, and is insensitive to the presence of detergents, organic solvents, and other additives in the assay buffer.

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