Trade Science Inc.

September 2009 Volume 8 Issue 3

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 8(3) 2009 [308-313]

Analysis of the xanthans, produced by *Xanthomonas albilineans*, by capillary electrophoresis: Identification of the product of reaction of an UDP-glucose dehydrogenase

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Received: 24th June, 2009 ; Accepted: 3rd July, 2009

ABSTRACT

Leaf scald, a bacterial-vascular disease of sugar cane, has *Xanthomonas albilineans* as casual organism. The organism may invade the parenchyma between the bundles and produces reddened pockets of gum, identified as a xanthan. This xanthan consists of a basal tetramer composed by two molecules of glucose, one mannose rest and a final glucuronic acid that is highly repeated to form the macromolecule. The occurrence of glucuronate rest in the polysaccharide requires the action of bacterial UDP-glucose dehydrogenase. In order to verify that the obtained protein is a true UDP-glucose dehydrogenase, the identification of the UDP-glucuronic acid like only product of reaction was necessary. The detection of the UDP-glucuronic acid has been carried out by Capillary Electrophoresis.

KEYWORDS

Capillary Electrophoresis;
UDP-glucuronic acid;
UDP-glucose dehydrogenase;
Sugarcane;
Xanthomonas albilineans;
Xanthans.

INTRODUCTION

Leaf scald, a bacterial vascular disease of sugarcane, has *Xanthomonas albilineans* as casual organism.[1,2] The pathogen is confined mainly to the leaf and stalk vascular bundles, which are often partly or completely occluded with a xanthan-like polysaccharide, producing desiccation of leaves.

The xanthan produced by *X. albilineans* consists of a basal tetramer that is repeated to form the macromolecule. This basal tetrasaccharide, composed by two molecules of glucose, one mannose rest and a final glucuronic acid, is highly repeated to form the macromolecule.[3] The occurrence of the glucuronate rest in the polysaccharide requires the action of an UDP-glucose dehydrogenase which catalyses a redox reaction using UDP-glucose as substrate and NADPH as a cofactor, with oxygen dependence for enzymatic oxidation of NADPH.

It belongs to a small group of dehydrogenases that are able to carry out the two-fold oxidation of an alcohol to an acid without the release of an aldehyde as intermediate.[4] This enzyme has a wide range of functions. In plants, UDP-glucose dehydrogenase is the main enzyme in the pathway of synthesis of hemicelluloses and pectins, which are the components of newly formed cell walls.[5]

In plant-pathogenic bacteria like *X. albilineans*, UDP-glucose dehydrogenase is not only absolutely required to the production of the xanthan gum but also it is considered as a determinant factor for virulence.

In order to verify that the previously purified protein

is a true UDP-glucose dehydrogenase, the identification of the UDP-glucuronic acid as the only product of the reaction was absolutely required.

Separation and identification of UDP sugars derivatives, including UDPglucuronic acid and amino sugars, in organic samples[6] or enzymatic reaction mixtures have been currently achieved by RP-HPLC on C18 columns[7], or columns packet (DEAE-2SW) with weak-anion gel.[8] When enzymatic conversion of UDPglucose into UDPglucuronic acid was monitorized by HPLC, a good correlation was observed between the reduction in the area of the substrate peak and the occurrence of product peak(s).[9] This highly reproducible method for enzyme assay is fast since no intermediate reaction mixture is required.

UDP-sugars analysis has been carrying out by techniques other than HPLC methods.[10,11] Capillary electrophoresis (CE) has emerged as a highly promising technique consuming an extremely small amount of sample and capable of rapid, high-resolution separation, characterization and quantitation of analytes. Numerous capillary electrophoresis methods for analysis of intact glycosaminoglycans (GAGs) and glycosaminoglycan(GAG)-derived oligosaccharides have been developed. In the last few years, CE has proved to be a very attractive alternative separation technique for GAGs and GAG-derived oligosaccharides. CE affords high resolving power and great flexibility the separation order.

CE also has several advantages over a variety of other analytical methods, including an extremely high separation efficiency, on-line detection, simple operation, short analysis time, automated and reproducible analysis, and very low consumption of samples and buffers.[12]

CE is available in a number of modes including capillary zone electrophoresis (CZE), isoelectric CE, and micellar electrokinetic CE. In all of these modes it is possible to apply normal and reversed polarity. Normal polarity mode is the most common mode of separation by CE. In normal polarity, the sample is injected at the anode and detected at the cathode, and basic or neutral buffer are required.[13] In reversed-polarity mode, the sample is applied at the cathode and detected at the anode, and an acidic buffer is required. At very low pH (<3), the silanol residues on the capillary wall lose their negative charge, thus the electro-osmotic flow is decreased. At acidic pH values, the electro-osmotic flow is too weak to overcome the electrophoretic mobility. The major force in the separation is the mobility of ions under electrophoresis. The resolution achieved by CE under a given set of conditions is dependent mainly on the charge, mass and molecular mobility of the analytes present. Detection is typically by ultraviolet (UV) absorbance or fluorescence emission.[14,15]

In this report, the detection of the UDP-glucuronate acid as the unique product of the reaction catalysed by an UDPglucose dehydrogenase from *Xanthomonas albilineans* has been carried out by CE.

EXPERIMENTAL

Purification of UDPG dehydrogenase

Xanthomonas albilineans were cultured in Willbrink medium, collected by centrifugation and a cell-free extract was prepared as above. Protein was precipitated with 40% (w/v) saturation ammonium sulphate; the mixture was stored at 4 ºC for 1 h and centrifuged at 12,000 × *g* for 15 min at 2 ºC. The pellet was discarded and the supernatant was precipitated again with 60% (w/v) saturation ammonium sulphate. The supernatant was discarded; the pellet was dissolved in 5mL of 10mM phosphate buffer, pH 6.8 and dialysed at 4 ºC against the same buffer until ammonium was completely removed. Solution containing UDP-glucose dehydrogenase was chromatographed through a Dowex column (8% cross-linked, dry mesh, 200–400) positively charged by treatment including a washing with 100 mL 1 N hydrochloric acid and 10 mL 1.7mM sodium chloride in 10mM phosphate buffer pH 6.8. Fractions of 3.0 mL were collected and analysed for protein and UDPG dehydrogenase activity

UDPG dehydrogenase activity was measured in reaction mixtures containing 0.5 mL of bacterial protein solutions, 0.5 mL of 10mM UDP-glucose in 10mM phosphate buffer, pH 6.8, 0.1 mL of 0.3mM NADPH and 1.9 mL of phosphate buffer, pH 6.8. Controls were prepared in absence of the substrate, UDP-glucose. Mixtures were maintained for 30 min at 37 1C, measuring the oxidation of NADPH by the decrease of absorbance at 340 nm. Alternatively, reactions were developed by bubbling air in reaction mixtures to supply oxygen during reaction.[16]

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Capillary Electrophoresis technique with indirect detection and reversion of the polarity

A P/ACE MDQ Instrument from Beckman Coulter (Fullerton, CA, USA) was used to separate UDP-glucuronic acid from UDP-glucose in the reaction mixtures, using UDP-glucose and UDP-glucuronic acid from Sigma Chemical Co., as standards. The system had a diode array detector with a 6 nm bandwidth.

Data were acquired by using 32 Karat ™ (v 7.0) software. Microbore fused-silica tubing coated with polyimide (Beckman Coultier, U.S.A.) of 75 μm i.d. and 190 μm outer diameter (o.d.) with a total length of 61 cm and a separation length of 50 cm were used. The buffers used as electrolyte were 5mM of β-resorcylic acid (dissolved in 0.5% v/v of methanol by volume), pH 3 and 1.0 mM of TTAOH (tetradecyltrimethilammonium hydroxide).

Reaction mixtures as well as their control without UDP-glucose were dried in air flow and the residues were dissolved in 200 μL of 5mM of β-resorcylic acid (dissolved in 0.5% v/v of methanol), pH 3 and 1mM of TTAOH to be analysed. Standard solutions (0.5 mg mL^{-1}) or concentrated standard solutions (1.0 mg mL^{-1}) were injected under pressure (0.5 psi for 5 s, about 40 nL) and the separation voltage was 20 kV. Detection was monitored at 200 nm and 214 nm measuring absorbance decrease.

Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography (MEKC) is a mode of electrokinetic chromatography (EKC) in which surfactants (micelles) are added to the buffer solution. Surfactants are molecules which exhibit both hydrophobic and hydrophilic character. Separation was carried out according to Legaz *et al.*[17] A P/ACE MDQ Instrument from Beckman Coulter (Fullerton, CA) was used for this capillary electrophoresis analysis. In this technique the buffer used as electrolyte were 5mM of β-resorcylic acid (dissolved in 0.5% v/v of methanol) pH 3, and 1mM of TTAOH and 15 mM of SDS (sodium dodecyl sulphate) as anionic surfactant agent.

The samples were prepared in the same conditions as previous as described and dissolved in the same buffer but without SDS. The separation voltage was 20 kV. Detection was monitored at 200 nm and 214 nm measuring absorbance decrease.

Capillary Electrophoresis with anode-cathode polarity

A P/ACE MDQ Instrument from Beckman Coulter (Fullerton, CA, USA) was used to separate UDP-glucuronic acid from UDP-glucose in the reaction mixtures, using UDP-glucose and UDP-glucuronic acid from Sigma Chemical Co., as standards.

Reaction mixtures as well as their control without UDP-glucose were dried in air flow and the residues were dissolved in 10mM sodium borate buffer, pH 9.2, to be analysed. Standard solutions (0.5 mg mL^{-1}) or concentrated standard solutions (1.0 mg mL^{-1}) were injected under pressure (0.5 psi for 5 s, about 40 nL) and separated at 20 kV using 25mM borate buffer, pH 9.2, as electrolyte. The separation voltage was 20 kV. Detection was monitored at 200 nm and 214 nm measuring absorbance decrease.

RESULTS

Capillary electrophoresis with indirect detection and reversion of the polarity and micellar electrokinectic chromatography

The separation of standards, UDP-glucose (as substrate of the enzyme) and UDP-glucuronic acid (as product), both prepared at 1.0 mg mL^{-1}, was resolved in only one peak with migration time values of 4.067 min, identified as UDP-glucose (Figure 1A) and 4.125 min identified as UDP-glucuronic acid (Figure 1B), respectively.

The separation of diluted standards, UDP-glucose and UDP-glucuronic acid, both prepared at 0.5 mg mL^{-1}, was resolved in two peaks with migration time values of 4.05 min, identified as UDP-glucose (Figure 2A) and 4.133 min, identified as UDP-glucuronic acid (Figure 2B), respectively, and both UDP-glucose and UDP-glucuronic acid at 0.5 mg mL^{-1} was resolved as only one peak with a migration time value of 4.15 min (Figure 3).

Capillary electrophoresis technique with indirect detection and reversion of the polarity did not produce then positive results and for this reason, it was come to analyze the samples by micellar electrokinetic chromatography. The separation of standards, both prepared at 1.0 mg mL^{-1}, was resolved in two important peaks for UDP-glucose with migration times value of 6.125 min and 8.6 min, respectively. The first peak could be interpreted as a combination of UDP-glucose and

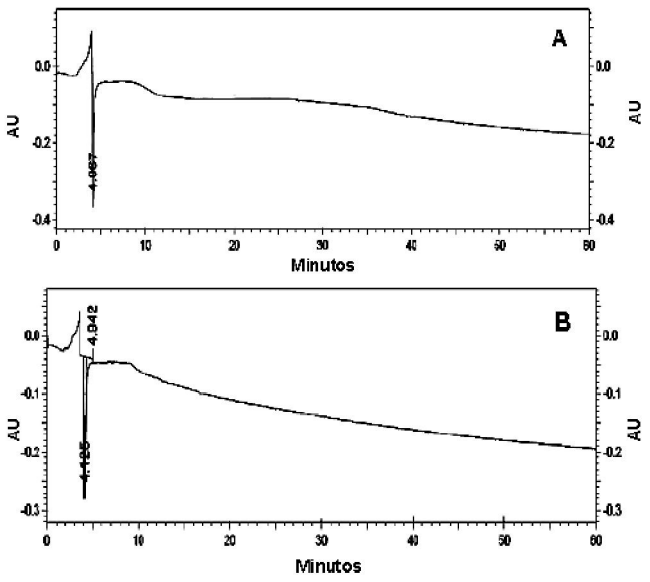

Figure 1 : (A) Electropherogram obtained by capillary electrophoresis with indirect detection and reversion of the polarity for UDP-glucose (1mg mL^{-1}) used as standard. (B) Electropherogram obtained by capillary electrophoresis with indirect detection and reversion of the polarity for UDP-glucuronic acid (1mg mL^{-1}) used as standard. Detection was monitored at 214 nm.

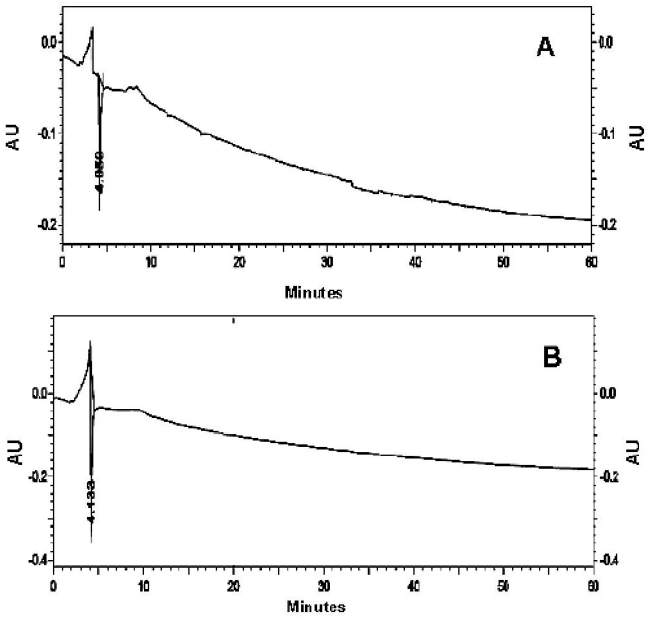

Figure 2 : (A) Electropherogram obtained by capillary electrophoresis with indirect detection and reversion of the polarity for UDP-glucose (0.5 mg mL^{-1}) used as standard. (B): Electropherogram obtained by capillary electrophoresis technique with indirect detection and reversion of the polarity for UDP-glucuronic acid (0.5 mg mL^{-1}) used as standard. Detection was monitored at 214 nm.

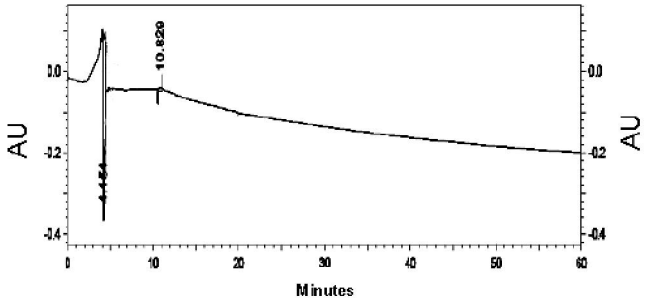

Figure 3 : Electropherogram obtained by capillary electrophoresis with indirect detection and reversion of the polarity of a mixture of UDP-glucose and UDP-glucuronic acid (0.5 mg mL^{-1} each one) used as standards. Detection was monitored at 214 nm.

SDS micelle, whereas the second peak could be identified as UDP-glucose (Figure 4A). The analysis of UDP-glucuronic acid was also resolved in two peaks.

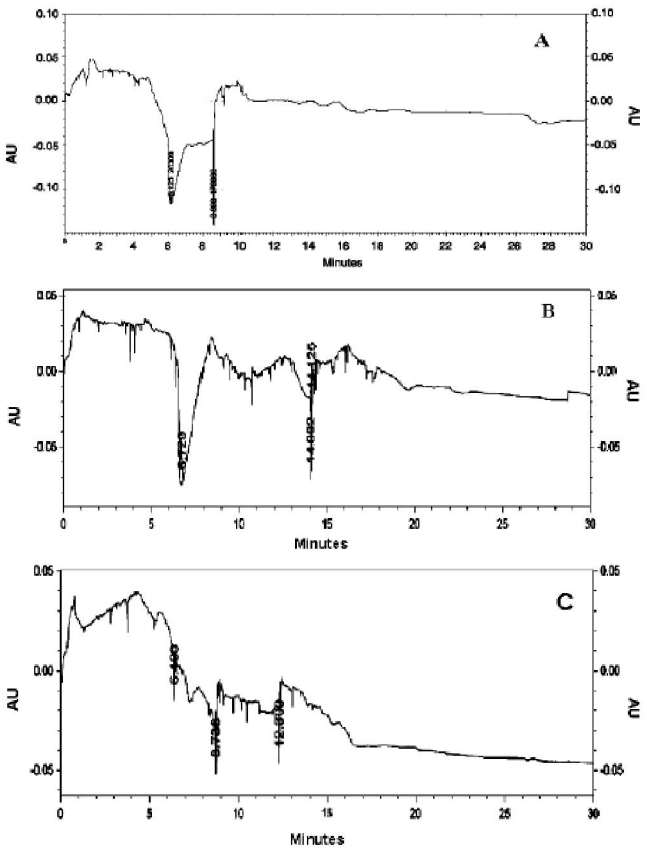

Figure 4 : (A) Electropherogram obtained by micellar electrokinetic chromatography for UDP-glucose (1.0 mg mL^{-1}) used as standard. (B): Electropherogram obtained by micellar electrokinetic chromatography for UDP-glucuronic acid (1.0 mg mL^{-1}) used as standard. (C) Electropherogram obtained by micellar electrokinetic chromatography of a mixture of UDP-glucose and UDP-glucuronic acid (1.0 mg mL^{-1}) used as standards. Detection was monitored at 214 nm.

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The first one, with migration time value of 6.72 min, could be identified as UDP-glucuronic acid and SDS micelle. The second peak with migration time value of 14.092 min could be identified as free UDP-glucuronic acid (Figure 4B). The mixture of UDP-glucose and UDP-glucuronic acid at 1.0 mg mL^{-1} was resolved in three main peaks with migration times values of 6.4 min, 8.73 min and 12.3 min, respectively. The two first detected peaks could be identified as UDP-glucose if they are compared to the individual pattern analysis. The third peak could be identified as UDP-glucuronic acid, equivalent to the obtained peak from the individual pattern analysis (Figure 4C).

The control reaction (without UDP-glucose) did not produce any significant peak (Figure 5A) whereas the reaction mixture analysis (with substrate and presumably product formed during reaction (Figure 5B) produced a great peak with a migration time value of 15.97 min.

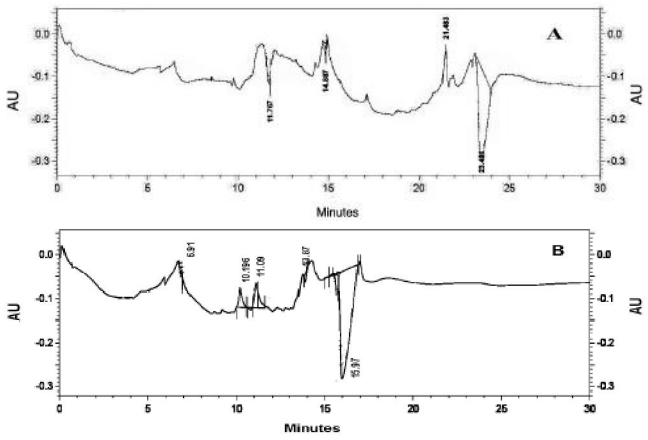

Figure 5 : (A) Electropherogram obtained by micellar electrokinetic chromatography of a control reaction mixture (without UDP-glucose). Detection was monitored at 214 nm. (B) Electropherogram obtained by micellar electrokinetic chromatography of the reaction mixture containing substrate, enzyme and cofactor, after 30 min. Detection was monitored at 214 nm.

Capillary electrophoresis with anode-cathode polarity

UDP-glucose (0.5 mg mL^{-1}) moved in CE as a main peak with a migration time value of 4.26 min, whereas UDP-glucuronic acid, at the same concentration, separated as a single peak with a migration time value of 5.25 min. Both compounds were conveniently separated from a mixture with identical migration times (Figure 6A) whereas the same sample loaded with supplementary UDP-glucose and UDPglucuronic acid (1.0 mg mL^{-1}) maintained well separated peaks with migration time values of 4.70 min for UDP-glucose and 5.76 min for UDP-glucuronic acid (Figure 6B).

Control of UDP-glucose dehydrogenase reaction without substrate did not produce any identified peak in CE (Figure 6C) whereas the separation of the component of a true reaction mixture, containing the enzyme and its substrate, revealed a main peak with a migration time value of 6.27 min (Figure 6D) identified as UDP-glucuronic acid. Untransformed UDP-glucose migrated at 4.88 min. Thus, the catalytic conversion of UDP-glucose by UDP-glucose dehydrogenase in the presence of both oxygen and NAPDH really produced UDP-glucuronic acid.

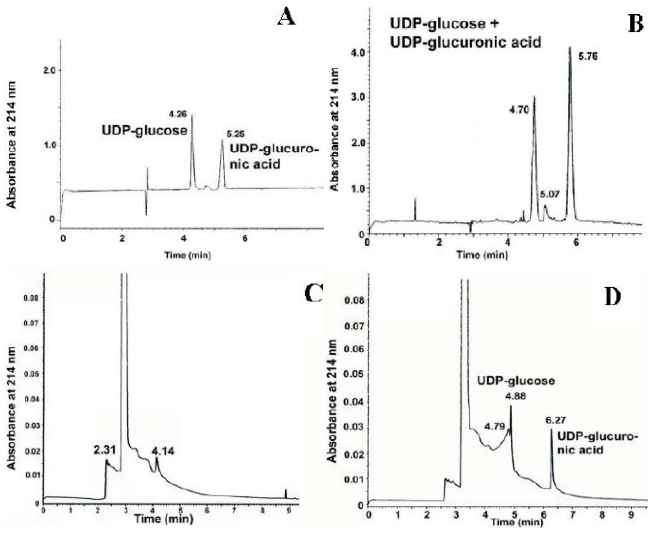

Figure 6 : Electropherograms obtained by capillary electrophoresis with anode-cathode polarity of (A) a mixture of UDP-glucose and UDP-glucuronic acid (0.5 mg mL^{-1} each one). (B) The same mixture containing double amount of UDP-glucose and UDP-glucuronic acid (1.0 mg mL^{-1}). Detection was monitored at 200 nm. (C) Control of enzymatic reaction mixture without substrate added (UDP-glucose). (D) The reaction mixture after 30 min of contact of the enzyme with the substrates (UDP-glucose and oxygen) and coenzyme (NADPH). Detection was monitored at 214 nm.

We tried to separate UDP-glucuronic acid from reaction mixtures for UDPG dehydrogenase assay using CE technique with indirect detection and reversion of the polarity,[14] but the separation of the used standards, UDP-glucose (as substrate) and UDP-glucuronic acid (as product), did not produce positive results (Figures 1-3). For that reason, it was come to analyze the

samples by micellar electrokinetic chromatography,[18,19] when an anionic surfactant such as sodium dodecyl sulfate (SDS) was included in the electrolyte solution (Figure 4). In this case, more clarifying results were obtained in that referred to the possible separation of the used standards (UDP-glucose and UDP-glucuronic acid), but the separation from reaction mixture did not produce convincing results (Figure 5).

Finally, the best UDP-glucuronic acid separation was obtained using the conventional CE with anode-cathode polarity.[20] The product of enzymatic reaction has been identified as UDP-glucuronic acid, with a migration time value of 6.27 min, whereas unreacted UDP-glucose produces a peak at 4.88 min.

Capillary electrophoresis is increasingly recognized as an important analytical separation technique because of its speed, efficiency, reproducibility, ultra-small sample volume, low consumption of solvent, and ease of removal of contaminants. These advantages suggest that capillary electrophoresis is an ideal technique for the determination of UDP-sugars. In addition, separation and identification of UDPGlucuronic acid as the unique product of reaction confirms that the enzyme isolated from *X. albilineans* is a true UDPG dehydrogenase instead of the high functional differences with other UDPG dehydrogenases that do not require molecular oxygen for functioning.[21]

CONCLUSION

The results given in this study demonstrate that UDP-glucuronic acid conveniently separates from UDP-glucose by using the conventional CE with anode-cathode polarity procedure. Attempts to separate those compounds by CE with indirect detection and reversion of the polarity or micellar electrokinetic chromatography do not produce convincing results. Identification of UDP-glucuronic acid from reaction mixtures containing UDP-glucose dehydrogenase from *X. albilineans* and UDPG as a substrate is absolutely required for the identification of the enzyme.

ACKNOWLEDGEMENTS

We are indebted to Miss Raquel Alonso for her excellent technical assistance. This paper has been supported by a Grant from the Dirección General de Investigación Científica y Tecnológica (Ministerio de Ciencia y Tecnología, Spain) BFU2006-14263.

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