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An HPLC method for the assay of starch synthase

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Abstract

A method is presented for the assay of starch synthase activity in plant extracts which does not require the use of radioactive substrates. The method is based on the quantification by HPLC of phosphorylated nucleosides generated in the reaction mixture during incorporation of $ADP_{glucose}$ (ADPGlc) into polymeric glucan. Estimation of starch synthase activity based on the rate of ADP accumulation in the medium or on the incorporation of glycosyl residues of ADP[U-^{14}C]Glc into polymeric produced comparable results in extracts of tobacco, spinach or potato leaves. With potato tuber extracts AMP accumulated in the medium instead of ADP. This is ascribed to the activity of apyrase (EC 3.6.1.5), a fluoride-insensitive ATP phosphohydrolase which removes γ-phosphoryl groups of ATP and ADP and it is particularly abundant in potato tubers. In this case, the rate of AMP accumulation in the reaction mixture well correlated with the rate of ADP[U-^{14}C]Glc incorporation into polymeric glucan. Thus, the HPLC method presented for the assay of starch synthase activity is also suitable for tissues containing apyrase activity.

Keywords: Starch synthase; HPLC method; Apyrase

1. Introduction

In eukaryotes and prokaryotes polymeric glucan is stored intracellularly in soluble or insoluble forms. Most bacteria, yeast, fungi and animal cells accumulate glycogen, a soluble molecule based on α-1,4-linked D-glucose residues with numerous α-1,6-glucosidic branch points. Green algae and higher plants accumulate starch: a mixture of unbranched α-1,4-linked D-glucose chains (amylose) and α-1,4 chains with frequent α-1,6 branch points (amylopectin) which results in the formation of compact, highly anhydrous granules. The growth of polymeric glucan complexes is based primarily on the elongation of existing glucan chains via the formation α-1,4 bonds with new glucose units. This reaction is catalysed by α-1,4 glucan-4-glucosyl transferases which use diphosphorylated nucleoside glucose as a substrate for chain elongation. Starch synthesis in plants and green algae is catalysed by ADPGlc: 1,4-α-D-glucan-4-α-D-glucosyltransferase (starch synthase; EC 2.4.1.21) (Preiss, 1988). Early methods for the assay of starch synthase were based on the enzymic quantification of the ADP generated during the course of the reaction (Leloir, De Fekete, & Cardini, 1961; Cardini & Frydman, 1966). More recent protocols are based primarily on the incubation of enzyme and ADP[U-^{14}C]Glc in the presence of a suitable primer. Enzyme activity is deduced from the incorporation of covalently bound radioactivity in the insoluble fraction following treatment of reaction mixtures with methanol–KCl which insolubilise polymeric glucose (Smith, 1990). A variation of this method is based on passage on passage of reaction mixtures through anion exchange resins which retain un-utilised ADP[U-^{14}C]Glc but not labelled glucan or products of its degradation (Jenner, Denyer, & Hawker, 1994). A drawback of these methods is the requirement for expensive radioactive substrates. We present here a method for the assay of starch synthase activity which does not require the use of labelled substrates and is based on the quantification by HPLC of ADP released during the course of the reaction. This method is more sensitive than the enzymic quantification of released ADP. As it provides chromatographic separation of mono- and di-phosphorylated adenosine, the method is also suitable for the assay of starch synthases in extracts of apyrase (EC 3.6.1.5)-containing

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plant tissues (e.g. potato tuber parenchyma, (Traverso-Cori, Chaimovich, & Cori, 1965; Viola & Sommerville, 1998)) where the rapid conversion of ADP into AMP during the course of the reaction is observed.

2. Results and discussion

A method for the rapid chromatographic resolution of ADP, AMP and ADPGlc in starch synthase reaction mixtures was developed. The method was used to quantify the ADP produced during the starch synthase-catalysed incorporation of ADPGlc into glycogen. Figure 1 shows the output of the UV-detector (254 nm; a) and the radiodetector (b) connected in series following the injection of starch synthase assay mixtures containing 25 or 50 μL of potato leaf extracts (or 50 μL of boiled extracts) after a 1-h incubation. The UV chromatogram shows the accumulation of ADP in the reaction mixture, whilst the radiochromatogram shows accumulation of radioactivity in the unbound fraction (glycogen). No accumulation of ADP or radioactivity in the unbound fraction was observed in assay mixtures incubated with boiled extracts. The commercial ADP[U-^{14}C]Glc used for the enzyme assay contained an unidentified radioactive component with a retention time of 6.7 min. The contaminant was found in three different batches from the same manufacturer and contained $22 \pm 3\%$ of the total radioactivity in the commercial ADP[U-^{14}C]Glc stock. The component did not appear to be metabolised by plant extracts within the starch synthase reaction mixture. The presence of the contaminant made it necessary to determine the specific activity of ADP[U-^{14}C]Glc in reaction mixtures by HPLC for each batch used. In Fig. 2 is shown the close correlation between ADP accumulation and incorporation of radioactivity in methanol/KCl insoluble material when mature leaf extracts of three plant sources were assayed for starch synthase activity. When starch synthase activity was assayed in extracts of developing potato tubers, the UV chromatograms showed accumulation of AMP in the reaction mixture instead of ADP (Fig. 3 insert). Potato tubers are known to contain high activity of a soluble ATP phosphohydrolase (apyrase EC 3.6.1.5), which specifically hydrolyses γ-phosphoryl groups of ATP and ADP, the end products being AMP and inorganic phosphate (Traverso-Cori et al., 1965). We have recently reported that this enzyme, which appears relatively insensitive to NaF (our unpublished observation), catalyses the rapid conversion of NTP and NDP into NMP in reaction mixtures during enzyme assays with crude potato tuber extracts (Viola & Sommerville, 1998). When accumulation of AMP was used to calculate starch synthase activity, a good correlation with the radioactive method was found Fig. 3. This indicates stoichiometric conversion to AMP of ADP generated during the course of the reaction and no further hydrolysis of

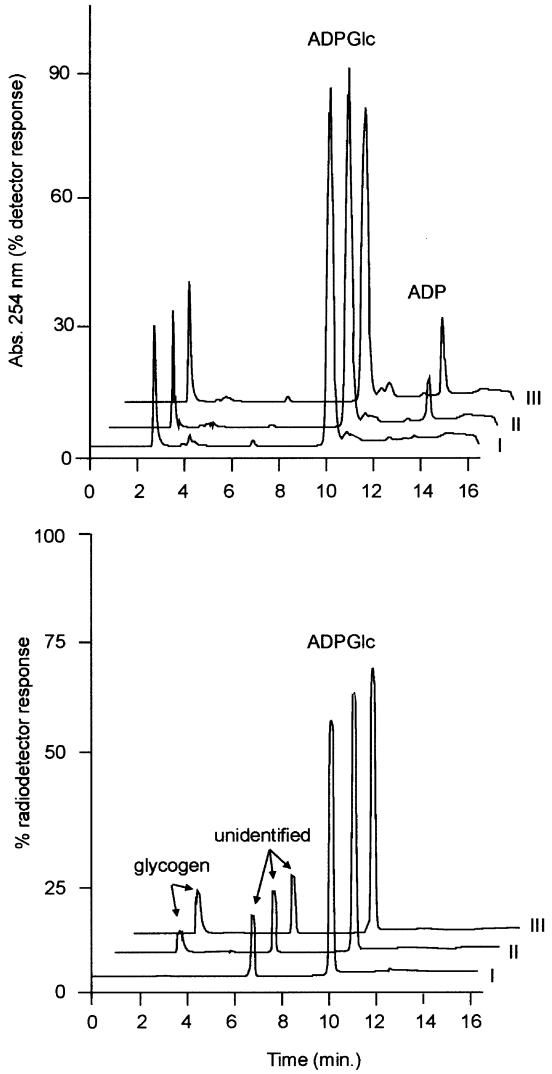

Fig. 1. HPLC analyses of starch synthase assay mixtures containing crude potato leaf extracts after 1 h incubation at 30°C. Samples were boiled and aliquots (40 μL) injected on a Spherisorb SAX column and chromatographic separation monitored using a UV (A_{254}) detector (a) and a flow-through radioactive detector (b) connected in series (for clarity, ^{14}C traces have been adjusted for the detector delay). Chromatograms shown refer to assay mixtures (0.25 mL final volumes) containing (i) boiled extracts (50 μL), (ii) 25 μL crude extract and (iii) 50 μL crude extracts. Components of chromatograms were identified by the injection of authentic compounds. An unidentified radioactive compound was present in the Amersham International stocks of ADP[U-^{14}C]Glc.

AMP. Much of our knowledge of plant apyrases is restricted to potato (Kettlun et al., 1982, 1992) and it is not clear how widespread is the enzyme in the rest of the plant kingdom. Although its presence in plant extracts could result in total or partial hydrolysis of the ADP generated during the course of the reaction, this should not affect the estimation of enzyme activity by the method presented here which provides chromatographic separation of AMP and ADP. We also have found no evidence of nucleotide pyrophosphatase activity which can

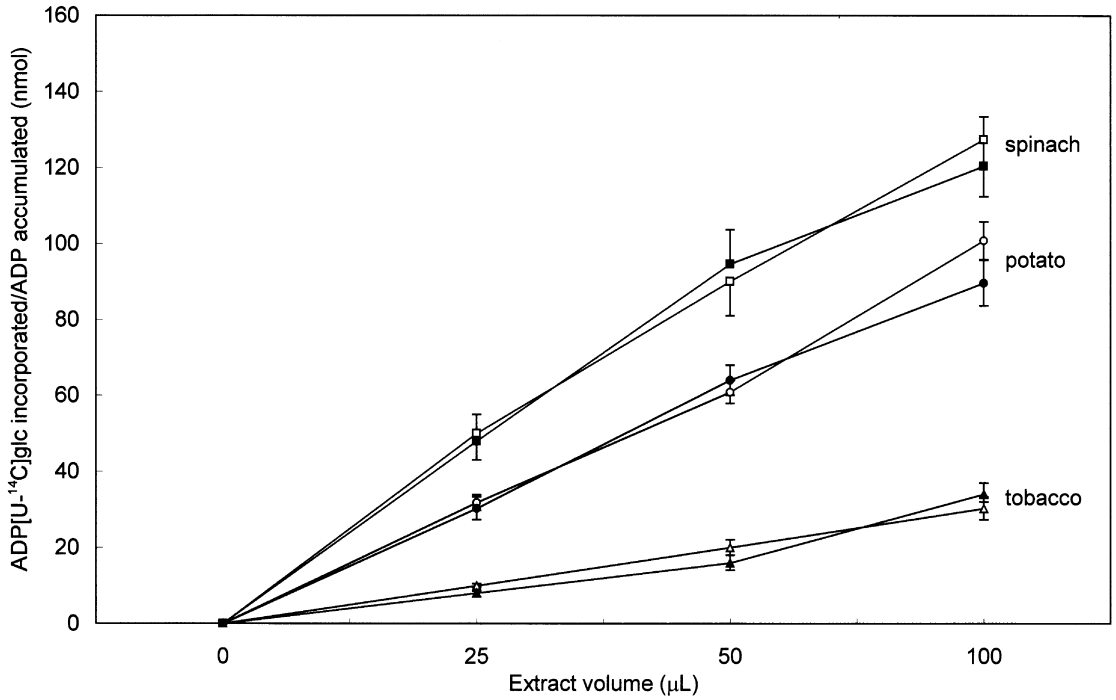

Fig. 2. Correlation between ADP accumulation (open symbols) and ADP[U-^{14}C]Glc incorporation in the methanol/KCl insoluble fraction (closed symbols) during assay of starch synthase activity with leaf extracts. Following incubation, 50 and 150 μL, respectively of the reaction mixtures were used for HPLC quantification of ADP accumulation and for precipitation of the methanol/KCl insoluble fraction. Data are expressed as nmol ADPGlc incorporated or ADP accumulated in the assay mixture (final volume 0.25 mL). Datapoints represent means ± S.D. of four independent determinations.

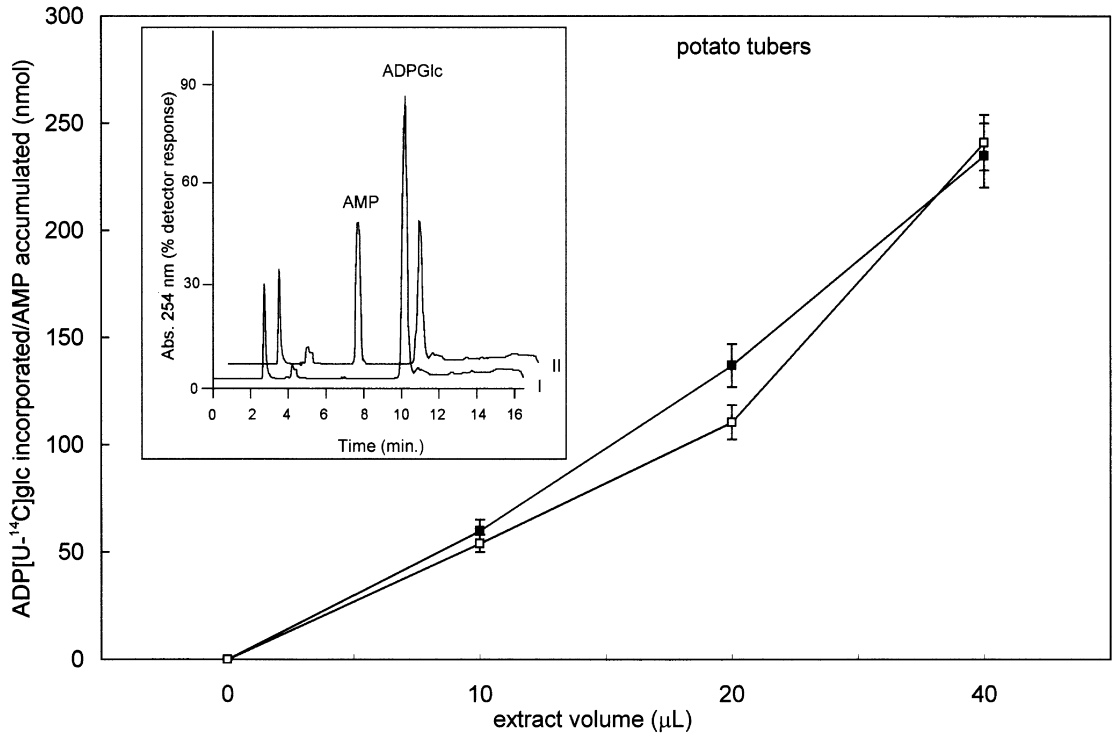

Fig. 3. Correlation between AMP accumulation and ADP[U-^{14}C]Glc incorporation in the methanol/KCl insoluble fraction during assay of starch synthase activity with potato tuber extracts (symbols as in Fig. 2). In the insert are shown UV (A_{254}) chromatograms of starch synthase assay mixtures containing 20 μL of boiled (i) or crude (ii) potato extract. Data are expressed as in Fig. 2 and represent means ± S.D. of three independent determinations.

convert NDPGlc into Glc-1P and NMP and could generate artefacts in the starch synthase assay. Nucleotide pyrophosphatase activity has been reported in a variety of plant tissues (Balakrishnan, Vaidyanathan, & Rao, 1977; Thom & Maretzki, 1989; Salvucci & Crafts-Brandner, 1995) including potato tubers (Bartkiewicz, Sierakowska, & Shugar, 1984). Under our assay conditions no AMP was detected during assay of starch synthase with potato, tobacco, and spinach leaf extracts Fig. 1. In the assay with potato tuber extracts the rate of AMP production showed a good correlation with the rate of ADP[U-^{14}C]Glc incorporation into soluble glucan indicating that the AMP is generated from the ADP produced by starch synthase. The inclusion of commercial apyrase from potato tubers (Sigma type A 9149) during starch synthase assays with leaf extracts resulted in the accumulation of AMP instead of ADP, whilst no AMP was formed in the absence of the plant extracts (data not shown). It is worth pointing out that nucleotide pyrophosphatase activity during radioactive starch synthase assays may originate artefactual results as the [U-^{14}C]Glc-1P generated may be incorporated into α-glucan via starch phosphorylase. The HPLC method proposed here allows the detection of AMP accumulation during starch synthase assay and enables further investigations on the eventual activity of nucleotide pyrophosphatases or phosphodiesterases.

In conclusion, the HPLC method presented here for the assay of starch synthase provides a valid alternative to the conventional method based on the use of radiolabelled ADP[U-^{14}C]Glc. The present method is specific for starch synthase and more sensitive than spectrophotometric determinations with a lower detection limit 0.7–1 nmol ADP/AMP (ca. 5-fold more senstitive than the enzymic method). Moreover, it eliminates the risk of under-estimation of enzyme activity associated with the use of ADP[U-^{14}C]Glc due to the presence of contaminants in commercial batches of isotope or to the de-polymerisation of radioalabelled glucan during the course of the assay.

3. Experimental

3.1. Chemicals

All chemicals used for enzyme assays were obtained from BDH Chemical Co., Poole, Dorset, UK or from Sigma Chemical Co., St Louis, USA. Chemicals for HPLC analyses (HPLC-grade) were from Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK. ADP[U-^{14}C]Glc (NH_4 salt; 10.5 GBq/mmol) was from Amersham International (Amersham, UK).

3.2. Plant material

Developing tubers (ca. 10 cm diameter, cv Record) and mature leaves from spinach, tobacco and potato were collected from plants grown in UC compost (Thompson & Taylor, 1979) in unheated glasshouses.

3.3. Enzyme extraction and assay

Slices of potato tubers or whole leaves were ground to a fine powder with mortar and pestle under liquid N_2. The tissues were resuspended in 3 vol of 0.1 M Bicine-KOH, pH 3.0, containing 1 mM EDTA, 5 mM DTT, 1 mM PMSF and 10 mM sodium sulphite. The extracts were centrifuged (30,000*g*; 10 min) and the supernatants de-salted by passage through PD 10 columns (Pharmacia) equilibrated with the extraction buffer. For standard assays, aliquots of extracts (10-40 μL) were incubated with 0.1 M Bicine-KOH (pH 8.0), 0.5 M sodium citrate, 5 mM NaF, 2 mM ADPGlc and 1.5 mg glycogen in a final volume of 0.25 mL. Radioactive assays also contained ADP[U-^{14}C]Glc at a specific activity of 1.35 Kbq mmol^{-1}. Control samples contained boiled extracts. Assays were incubated for 60 min at 30°C and the reaction was stopped by heating at 100°C for 2 min.

3.4. Methanol/KCl assay of ADP[U-^{14}C]glc incorporation into glycogen

The glycogen in the reaction mixture was precipitated by the addition of 3 mL of chilled MeOH (70 vol%) containing 1% (w/v) KCl. After incubation for 5–10 min in the cold, insoluble material was pelleted by centrifugation (14,000*g*; 5 min). The insoluble pellets were washed twice by resuspension and precipitation in MeOH/KCl. The final glycogen pellets were resuspended in 1 mL distilled water and transferred to scintillation vials containing 10 mL scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, GA). The radioactivity incorporated in the glycogen was determined in a Packard scintillation counter.

3.5. HPLC assay of ADP production

Prior to HPLC analyses, boiled reaction mixtures were centrifuged (14.000*g*; 10 mm). Aliquots (40 μL) of assay mixture were injected onto a Spherisorb-SAX (25 × 1 cm; HPLC Technology, Macclesfield, UK). Mobile phases were (A) 10 mM ammonium phosphate, pH 3, and (B) 450 mM ammonium phosphate, pH 4.5. Flow rate was 1 mL/min. The gradient employed was: isocratic 20% B for 2 min, linear increase to 82% B over 11 min, linear decrease to 20% B over 1 min, isocratic 20% B over 5 min. The detection system consisted of a Shimadzu SPD-6A UV detector (Shimadzu, Kyoto, Japan) set at 254 nm connected in series with a Reeve 9701 radioactive flow-detector (Reeve Analytical, Glasgow, UK). Under these conditions, glycogen was not retained by the column and eluted in the unbound fraction, whereas AMP, ADPGlc

and ADP eluted at 6.9 min, 10.2 min and 13.6 min, respectively.

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References

Balakrishnan, C. V., Vaidyanathan, C. S., & Rao, N. A. (1977). *Eur. J. Biochem.*, *78*, 95.

Bartkiewicz, M., Sierakowska, H., & Shugar, D. (1984). *Eur. J. Biochem.*, *143*, 419.

Cardini, C. E., & Frydman, R. (1966). *Methods Enzymol.*, *8*, 387.

Jenner, C. F., Denyer, K., & Hawker, J. S. (1994). *Aust. J. Plant. Phys.*, *20*, 329.

Kettlun, A. M., Uribe, L., Calvo, V., Silva, S., Rivera, J., Mancilla, M., Valenzueka, M. A., & Traverso-Cori, A. (1982). *Phytochemistry*, *21*, 551.

Kettlun, A. M., Urra, R., Leyton, M., Valenzuela, M. A., Mancilla, M., & Traverso-Cori, A. (1992). *Phytochemistry*, *31*, 3691.

Leloir, L. F., De Fekete, M. A. R., & Cardini, C. E. (1961). *J. Biol. Chem.*, *236*, 636.

Preiss, J. (1988). In J. Preiss (Ed.), *The Biochemistry of plants* (Vol. 14, pp. 181–249). San Diego: Academic Press.

Salvucci, M. E., & Crafts-Brandner, S. J. (1995). *Plant Physiol.*, *108*, 1269.

Smith, A. M. (1990). In P. J. Lea (Ed.), *Methods in plant biochemistry* (Vol. 3, pp. 93–102). San Diego: Academic Press.

Thom, M., & Maretzki, A. (1989). *Plant Physiol. Biochem.*, *27*, 87.

Thompson, R., & Taylor, H. (1979). *Sci. Hortic.*, *10*, 309.

Traverso-Cori, A., Chaimovich, H., & Cori, O. (1965). *Arch. Biochem. Biophys.*, *109*, 173.

Viola, R., & Sommerville, L. B. (1998). *Plant Sci.*, *132*, 127.