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Short communication

Analysis of starch-bound glucose 3-phosphate and glucose 6-phosphate using controlled acid treatment combined with high-performance anion-exchange chromatography

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Abstract

The products after controlled acid hydrolysis of starch, glucose (Glc), glucose 6-phosphate (Glc6P), glucose 3-phosphate (Glc3P), glucose-3 phosphate conversion products and inorganic phosphate (Pi) were separated using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAC–PAD). Pi was derived from partial hydrolytic cleavage of the phospho–monoester bond in glucose 3-phosphate. The identity of the products was verified using ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR). The study demonstrates that Glc3P, despite of its susceptibility to acid catalysed dephosphorylation, can be liberated from starch together with Glc3P conversion products and Glc6P by controlled acid conversion enabling subsequent separation and analysis using HPAC.

Keywords: Glucose phosphate; Starch; Inorganic phosphate

1. Introduction

Numerous phosphorylated sugars serve as key intermediates in metabolic reactions. A phosphorylated sugar that has a less clear function is glucose 3-phosphate (Glc3P). Glc3P has been suggested to be formed as an intermediate as a result of diabetes related complications [1,2]. In addition, Glc3P is found as a rare constituent in phosphorylated starch isolated from roots and tubers of many plants [3–6]. In phosphorylated starch isolated from potato tuber about one out of 300 glucose residues are phosphorylated and one-third of the phosphate groups are located at the O-3 position whereas two-thirds are in the O-6 position of the glucosyl residue [4]. The proportion of phosphate groups positioned at the O-3 and O-6 position has been determined by ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR) of gelatinised starch degraded by α- and iso-amylase [5–7]. Because the Glc3P remains glucan-bound when assayed, this methodology does not offer the possibility to isolate free Glc3P. The glucosidic linkages of starch can be cleaved by acid or by enzymic hydrolysis. Amylolytic enzymes, despite of their superior selec-

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tivity towards specific glucosidic linkages, do not hydrolyse all glucosidic bonds in the vicinity of a phosphate group. This results in Glc and phosphooligosaccharides as the final hydrolytic products [8]. Glc can be released from many starches with good recovery and conveniently analysed using a flow injection system combined with immobilised enzyme reactors [9]. Some starches, e.g., cereal starches, are partly resistant to amylolytic enzyme attack [9] as a result of high lipid and protein content. Upon acid hydrolysis of phosphorylated starches, Glc and Glc6P is released in high yield permitting direct spectrometric determination [7]. Because of the inherent instability of the phosphoester linkage at the O-3 compared to the O-6 position, acid hydrolysis results in some loss of Glc3P.

It has recently been shown that the molecular structure of starch, as measured by the unit chain length distribution profile, is tightly correlated to the degree of phosphorylation [10]. Because the degree of phosphorylation also affects the physico–chemical properties [11,12] and the crystallinity [13] of starch, the possibility to optimise and control the level of starch phosphorylation in vivo constitutes an important parameter in biotechnological approaches to produce starches with tailor-made properties.

In this report we present a procedure by which starch bound Glc3P as well as Glc6P can be determined. The procedure is based on controlled acid hydrolysis and subsequent analysis by high-performance ion-exchange chromatography with pulsed amperometric detection (HPAC–PAD).

2. Experimental

2.1. Chemicals

Glc3P was a kind gift from Dr. Francis Kappler (Department of Nuclear Magnetic Resonance and Medical Spectroscopy, Fox Chase Cancer Center, Philadelphia, PA, USA). All other chemicals used were of highest purity commercially available. $^{33}PO_4^{3-}$ was purchased from Amersham (Buckinghamshire, UK).

2.2. Plant material and preparation of starch

Starch was prepared from freshly harvested potato tubers of *Solanum tuberosum* cv. Dianella and *Solanum phureja* [7].

The degree of starch phosphorylation was determined by enzymatic quantification of glucose and Glc6P after acid hydrolysis in 0.7 *M* HCl at 100°C [7]. Based on the assumption that the phosphate content of Glc3P constitutes 30–40% of the total content of starch phosphate in potato tubers [4,6,7], this screening methodology provides an estimation of the total content of starch phosphate. Inorganic phosphate (Pi) was analysed as described by Ref. [14]. Time course hydrolysis of starch was performed using 50 mg of starch in 1 ml of 0.7 *M* HCl. Aliquots (100 μl) were withdrawn at $t=0.5$, 1.0, 1.5, 2.0 and 4.0 h, neutralised with 2 *M* NaOH and subjected to HPAC analysis (see Section 2.3).

2.3. High-performance anion-exchange chromatography

Separation of sugars was performed using a Dionex DX 500 system equipped with an S-3500 auto sampler, GP40 pump, ED40 PAD system fitted with a CarboPac PA-1 column. Aliquots (20 μl) were injected and the sugars were separated (flow-rate: 1 ml/min) using an eluent containing 10 m*M* NaOH and the following linear NaOAc gradient profile: 0–15 min: 10–30 m*M*, 15–20 min: 30–200 m*M* and 20–40 min: 200–300 m*M*. The elution programme was followed by a 5 min washing period with 980 m*M* NaOAc–10 m*M* NaOH. To determine the content of $^{33}PO_4^{3-}$ in the different HPAC fractions, aliquots were neutralised with HCl and mixed with Ecoscint A scintillation liquid (National Diagnostics, Manville, NJ, USA) and counted in a Wallac WinSpectral 1414 liquid scintillation counter (Wallac, Helsinki, Finland) using WinSpectral version 1.0 software.

2.4. Preparation of Glc3P and Glc6P from starch

Dianella starch (20 g) was hydrolysed (1 h, 100°C, 30 ml of 0.7 *M* HCl) to release starch bound Glc3P and Glc6P. After removal of debris by centrifugation the supernatant was freeze–dried and vigorously mixed with 30 ml of EtOH. After centrifugation (30 min, 10 000 *g*, 4°C) the pellet, including the phosphorylated monosaccharides, was washed with 90% EtOH to remove residual amounts of HCl and then

freeze–dried. After solubilisation in water (10 ml) and removal of insoluble matter (non-hydrolysed starch and oligosaccharides) by centrifugation the supernatant was applied to an Amberlite (OH^-) anion-exchange column (10 ml) which was washed with at least 10 column volumes of water. Phosphorylated saccharides were eluted with 2 *M* HCl and after removal of HCl and water by freeze–drying the sample was solubilised in 100 m*M* NaOH (1.0 ml). Trace of insoluble material was removed by centrifugation, and the content of Glc3P and Glc6P was separated using a semi preparative PA-1 column operated at 5 ml/min flow-rate and the same NaOAc profile described above. Na^+ ions were removed from the fractions (0.5 ml) by cation-exchange chromatography (H^+-Dowex 50W, Fluka). The samples were freeze–dried and stored dry at −20°C.

2.5. ^{31}P Nuclear magnetic resonance (^{31}P-NMR)

Samples obtained from the HPAC–PAD fractions or reference compounds were dissolved in 100 m*M* Na–citrate, 100 m*M* bicine, 2 m*M* EDTA and 3% D_2O (600 μl) and pH adjusted to 4.5 by addition of 2 *M* NaOH or HCl if not otherwise mentioned. ^{31}P-NMR spectra were recorded at 300 K using a Bruker AC250P NMR instrument. The chemical shifts reported are referred to external 85% H_3PO_4 subsequently inserted. Several thousand transients were accumulated using a 90° pulse angle, relaxation delay of 2 s and CPD-decoupling. When the noise level permitted so, Lorentz–Gauss resolution enhancement was applied to the spectra.

3. Results and discussion

3.1. Time course hydrolysis of starch

Liberation of Glc, Glc6P and Glc3P during acid hydrolysis of starch prepared from potato tubers of cv. ''Dianella'', containing 16.8 nmol Glc6P/mg starch, and from tubers of *Solanum phureja,* containing 25.3 nmol Glc6P/mg starch, is shown in Fig. 1. Glc6P was identified as a single peak appearing at an elution volume of 28 ml after 2–4 h of hydrolysis (Fig. 1). Three components denoted 1, 2 and 3, (Fig. 1, 2 h hydrolysis) with partly overlapping elution profiles are apparent within elution volume 30–31 ml. Authentic Glc3P eluted as a single sharp peak at 31 ml at a position identical to that of component 2 obtained by hydrolysis of phosphorylated starch. Hydrolysis of the Glc3P standard gave rise to a product co-eluting with component 3 obtained by acid hydrolysis of starch. This suggests that components 2 and 3 obtained by acid hydrolysis of phosphorylated starch are Glc3P and a conversion product thereof, respectively.

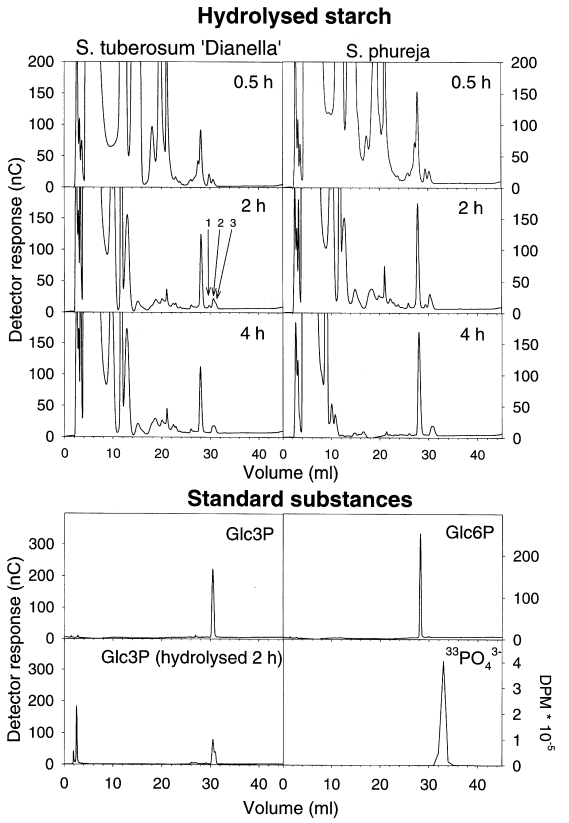

Fig. 1. Products obtained by acid hydrolysis of starch prepared from potato tubers cv. ''Dianella'' and tubers of *S. phureja* as analysed by HPAC–PAD. The elution profile of authentic Glc6P, Glc3P and of Glc3P hydrolysed for 2 h (6 μg each) and of ^{33}Pi are also shown.

The possibility that components 1, 2 and 3 (Fig. 1, 2 h hydrolysis) may be converted into each other was investigated by isolation of components 1 and 2, respectively, after 1 h of hydrolysis as described in Section 2.2. Each of the isolated components was divided into two equal parts. One part was re-analysed on the HPAC to confirm isolation of the target component (Fig. 2A and C). The other part was

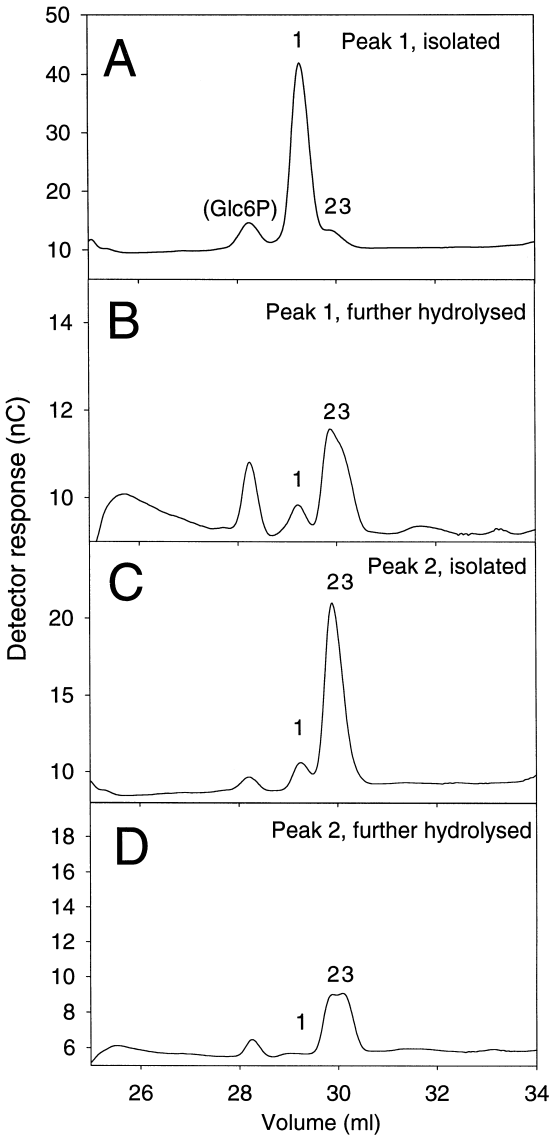

Fig. 2. Inter conversion of Glc3P hydrolysis products. Fractions from preparative HPAC collected at position 1 (A) and 2 (C), respectively, and re-analysed. (B) Peak at position 1, further hydrolysed for 2 h and then re-injected. (D) Peak at position 2, further hydrolysed for 2 h and re-injected. Trace of Glc6P in the samples is indicated.

hydrolysed for an additional 2 h and re-analysed (Fig. 2B and D). The profiles obtained indicate conversion of component 1 to components 2 and 3. Component 2 is partly converted to component 3 giving rise to a double peak superimposable with that observed after 4 h hydrolysis of phosphorylated starch and after hydrolysis of the Glc3P standard (Fig. 1). Because acid treatment of component 1 gives rise to products co-eluting with component 2 (Glc3P) as well as its conversion product (component 3), the data suggest that component 1 represents a partial starch hydrolysis product e.g., maltose 3-P. The fact that a phospho-oligosaccharide can co-elute or elute earlier than a phospho-monosaccharide has been indicated elsewhere [8]. We cannot exclude the presence of trace amounts of O-6 phosphorylated saccharides e.g., maltose-6 P in the isolated samples, or liberation of small amounts of Glc or G6P during further hydrolysis as Glc6P and low-molecular-mass neutral saccharides were not completely removed from the preparations.

Glc had a very weak affinity to the column and eluted at approximately 2.5 min whereas Pi eluted at 33 min as detected by injection of ^{33}Pi. Consequently, Pi was separated from Glc6P and Glc3 as well as from its conversion products.

The components identified as Glc, Glc6P and Glc3P including its conversion products, were quantified at starch hydrolysis periods of 30, 60, 90, 120 and 240 min (Fig. 3). Glc and Glc6P were completely liberated after hydrolysis for 120 min. The total amount of Glc3P and its conversion product rapidly increased within the first 30 min of hydrolysis and then decreased with time, reflecting cleavage of the phospho–monoester bond [4,7]. Because the content of Glc and Glc6P reached their maximal levels after 120 min of hydrolysis and remained constant for additional 120 min, the approximately 20% rise in the Pi content between 120 and 240 min of hydrolysis most likely arises from hydrolysis of the phospho–monoester linkage in Glc3P or its conversion product. We therefore conclude that about 80% of the phosphate bound to Glc3P remained detectable by HPAC–PAD after 120 min of hydrolysis.

3.2. ^{31}P-NMR

The structures of the components isolated by semi-preparative HPAC were verified using ^{31}P-NMR. To obtain optimal separation of the signals related to Glc6P, Pi, Glc3P and its conversion products, the pH dependence of the chemical shifts were determined for these substances and for Glc1P (Fig. 4). The best

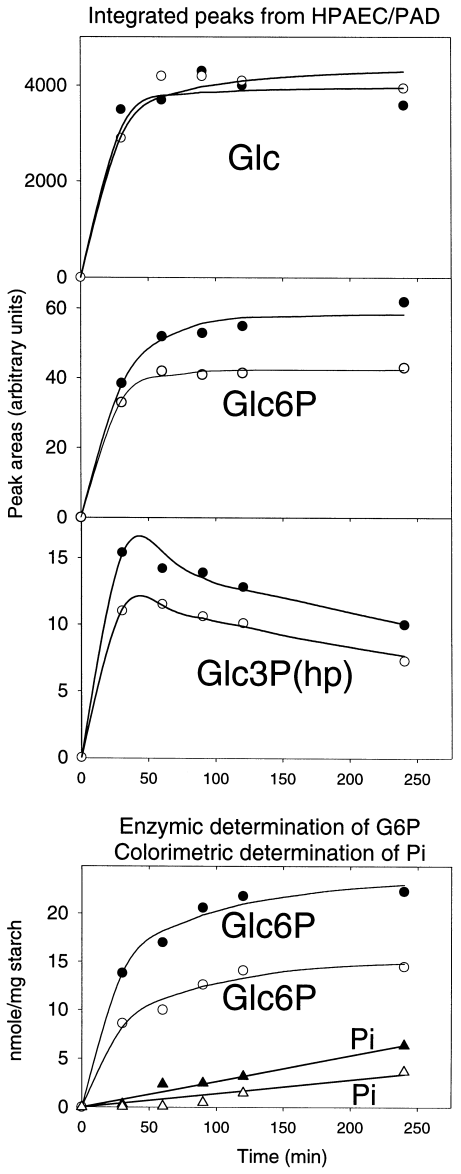

Fig. 3. The effect of hydrolysis time on the accumulation of Glc, Glc6P, Glc3P and its conversion products [Glc3P(cp) as studied using potato tuber starch isolated from. *S. tuberosum*, ''Dianella'' (– ○ –) and *S. phureja* (– ● –). The content of Glc6P determined enzymatically [7], and Pi released from *S. tuberosum*, ''Dianella'' starch (open symbols) and from *S. phureja* starch (filled symbols) are also shown.

separation and stability of signals were observed at pH≤4.5 (Fig. 4). At pH≥7.0 the signals derived from Glc3P and Glc6P were poorly resolved. At pH

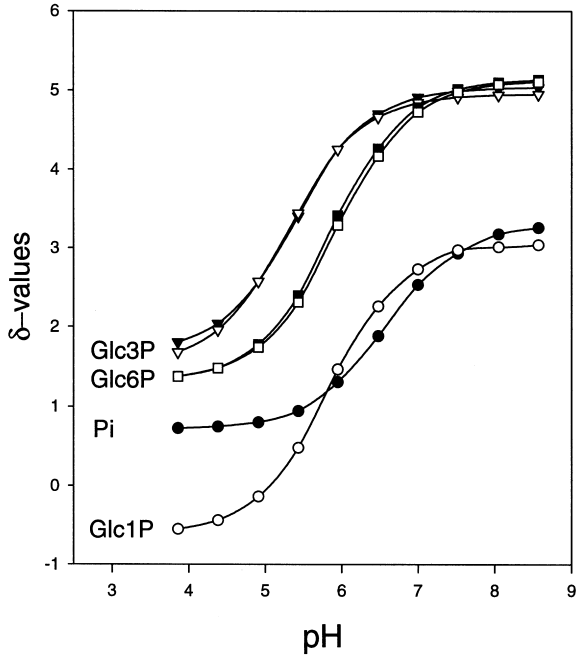

Fig. 4. Effect of pH on the ^{31}P chemical shifts for Glc6P, Glc3P, Glc1P and Pi.

5.0–7.0 the high pH dependence of the chemical shifts made this area a poor choice for distinguishing between Glc6P and Glc3P.

The starch hydrolysis product identified as Glc6P as well as component 1 and components 2+3 were isolated by HPAC (Fig. 1) and prepared for ^{31}P-NMR as described in Section 2.5. The chemical shifts and relative intensities of signals of spectra recorded at pH 4.50 document that the component co-eluting with Glc6P is indeed Glc6P (Fig. 5). Component 1 and the mixture of components 2 and 3 produced NMR spectra with signals close to that of the Glc3P standard. The slight differences in chemical shifts between the Glc3P spectra is probably caused by the presence of salt in the hydrolysed sample. Up to 0.2 ppm variation in the δ values for Glc3P under the conditions employed has been observed (data not shown). Component 1 gave a double signal centered at δ 1.91 at pH 4.5 and one major single resonance at δ 5.01 at pH 7.9 (not shown), which could not unambiguously be identified as derived from either Glc3P or Glc6P. Trace of components 2+3 is also identified in this fraction

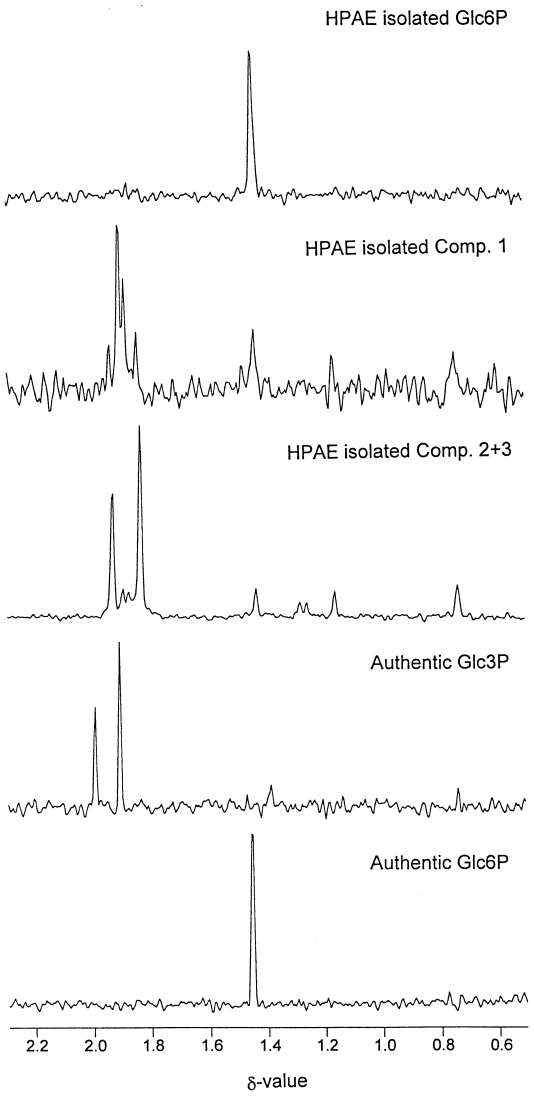

Fig. 5. ^{31}P-NMR recorded at pH 4.50 of components isolated by preparative HPAC. Authentic standard compounds are also shown.

(see below). The double resonance most likely reflects the presence of α/β anomeric forms. In agreement with the inter conversions observed by acid hydrolysis we conclude that component 1 contains O-3 bound phosphate most likely in the form of the partial starch hydrolysis product maltose-3 P. The ^{31}P-NMR spectrum of the mixture of component 2 and 3 is very similar to that of Glc3P strongly supporting the assignment of component 2 as Glc3P. Since component 3 appears to be derived from component 2 by acid hydrolysis it could be a product of oxidation, phosphate migration or isomerization of the glucose ring resulting in a furanose form. The latter situation seems most likely since its rate of acid catalysed dephosphorylation was the same as for Glc3P and no alterations in the chemical shifts were found after 120 min of hydrolysis (results not shown).

The strategy to combine controlled acid hydrolysis with HPAC–PAD does not allow exact quantification of starch-bound Glc3P because of the multitude of reactions taking place during acid hydrolysis. However, assuming that the decomposition rate of the phospho–monoester bond at the O-3 position is independent of adjacent glucosidic bonds, hydrolysis rates of Glc3P at defined conditions i.e., type of acid, concentration of acid, temperature and time, can be used to estimate the amounts of starch bound Glc3P. As the detector response for glucose phosphates is not suspected to vary significantly in relation to position on the glucose unit [15] peak areas for Glc3P and Glc6P, respectively, can be used for calculations of Glc3P/Glc6P ratios in starch.

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