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A mass spectrometric method for quantifying C3 and C6 phosphorylation of starch

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ABSTRACT

The glucosyl residues comprising starch can be phosphorylated at either the C3 or the C6 position of the molecule because of the activities of two distinct dikinase enzymes. After hydrolysis of the starch, the C6 phosphorylation is easy to measure using a routine enzyme assay for glucose 6-phosphate, but the C3 phosphorylation is more difficult to assay. A mass spectrometric (MS) method has been developed that, in a single run, can distinguish and quantify the glucose 3-phosphate and glucose 6-phosphate produced by hydrolysis of starch and can also measure the glucose content to give an accurate estimate of the starting material. The MS method involves quantification by LC/MS with external standards, using normal-phase hydrophilic interaction liquid chromatography and selective reaction monitoring. The MS method has been used to determine degrees of starch phosphorylation in a diverse group of potato lines, revealing threefold differences in phosphorylation between high- and low-phosphate lines. The method was also used to show that cold storage of potato tubers for up to 24 weeks had little substantive effect on the levels of starch phosphorylation. MS provided an effective and efficient means of determining both the C6 and the C3 phosphorylation of starch.

The degree of phosphorylation of starch determines its physicochemical properties and is therefore relevant for industrial uses of starch. Levels of starch phosphorylation have been manipulated by downregulating the genes involved, resulting in changes to properties such as gelatinization temperature, viscosity, pasting, and gel hardness [1–3]. Starch phosphorylation is also an important aspect of plant metabolism because of its role in starch degradation. Reducing the extent of starch phosphorylation has been shown to impair the degradation of starch [4,5], whereas an increase in starch phosphorylation was shown to stimulate the activity of the starch-degrading enzyme β-amylase [6].

The glucosyl residues that comprise starch can be phosphorylated at either the C6 or the C3 position [7]. In potato tuber starch, C6 phosphorylation predominates, making up 70–80% of the phosphorylation, whereas C3 makes up 20–30% [8,9]. There are also negligible amounts (1%) of C2 phosphorylation [7]. Phosphorylation at the C6 and C3 positions is achieved by different enzymes: C6 phosphorylation by glucan/water dikinase (GWD)[1] and C3 phosphorylation by phosphoglucan/water dikinase (PWD). The activity of PWD is dependent on prior C6 phosphorylation by GWD [10].

The C6 phosphorylation can be easily measured via acid hydrolysis of the starch and a standard enzymatic assay for glucose 6-phosphate (Glc6P) [8,11]. C3 phosphorylation is more difficult to determine, and a variety of methods have been explored. One approach is to measure the total phosphate content of the starch by ashing the organic matter and measuring inorganic phosphate by a colorimetric assay [12] and then estimating C3 as the difference between total phosphate and C6 phosphorylation. However, this method is indirect and does not take into account minor components such as C2 or the presence of any inorganic phosphate. C3 phosphorylation of starch has also been measured by ^{31}P NMR, which is a robust method but requires 200–300 mg of starch and is too slow to be suitable for high-throughput experiments [8,13]. Glc6P and glucose 3-phosphate (Glc3P) from hydrolyzed starch have also been measured by high-performance anion-exchange chromatography with pulsed amperometric detection [14]. However, this method was later shown to be compromised by the coelution of Glc3P and maltose-6-P derived from partial hydrolysis of starch [10].

A mass spectrometric method was developed by Haebel et al. [15] that measured relative levels of Glc6P and Glc3P in hydrolyzed starch using their unique differing abundances of product ion fragments produced from their common precursor mass in negative-ion electrospray tandem mass spectrometry (MS). The method was fast and required less than 1 mg of starch, but involved a complex calculation to determine the proportions of Glc3P and Glc6P based on ratio abundances of product ions relative to the change in individual concentrations. This technique repeated in our

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[1] *Abbreviations used:* GWD, glucan/water dikinase; PWD, phosphoglucan/water dikinase; Glc6P, glucose 6-phosphate; Glc3P, glucose 3-phosphate; TFA, trifluoroacetic acid; HILIC, hydrophilic interaction liquid chromatography; SRM, selective reaction monitoring; REML, restricted maximum likelihood; LOD, limit of detection; LOQ, limit of quantitation.

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laboratory highlighted a discrepancy between calculated and actual concentrations (a fraction G6P/G3P of 0.7/0.3 would calculate as 0.8/0.2). However, Haebel et al. [15] were successful in optimizing a method for hydrolyzing starch using trifluoroacetic acid (TFA), ensuring that the Glc3P is not dephosphorylated, as occurs during hydrolysis with HCl. Another MS method used hydrophilic interaction liquid chromatography (HILIC) to separate carbohydrate-related metabolites from plants and was effective for quantifying glucose and Glc6P [16] but did not include Glc3P.

We have combined the optimized TFA starch hydrolysis [15] with a modified HILIC MS method, to separate and quantify the glucose, Glc6P, and Glc3P from hydrolyzed starch. The glucose content provides an accurate measurement of the amount of starch hydrolyzed, and thus our method enables determination of the absolute quantities of C6 and C3 phosphorylation in a starch sample.

Materials and methods

Plant material

Thirty tetraploid potato lines (*Solanum tuberosum*) representing a diverse phenotypic range were field-grown near Lincoln, New Zealand, in the summer of 2008–2009. The lines were as follows: 119-52, 2000A, 2770.6, 3090.2, 4164A.3, 4164B.16, 4164C.3, 4164D.1, 4164E.1, 4164F.2, A233-1, "Allura," "Brodick," Crop 20, "Fraser," "Gladiator," "Golden Miracle," "Kaimai," "Karaka," "Kufri Joyti," "Ladies Fingers," "Pink Fir Apple," "Purple Heart," "Purple Passion," "Rima," V116, V170-1, V201, V394, and VT^n 62-33-3. For each line, six typical tubers were harvested from each of 3 plants from a single 12-plant plot, and cylindrical samples were taken through the middle of each tuber using an 11-mm cork borer. The six cores from each plant were pooled and freeze-dried giving three pooled samples per line. During sampling and subsequent procedures, the 90 samples were processed in batches of 6. The processing order was derived from a Latinized resolvable incomplete block design, such that 6 samples were processed together in the order given and a complete replicate of lines (five sets of 6) was processed sequentially. The design was created with CycDesigN [17].

Six lines were chosen from the 30 listed above to represent a range of levels of starch phosphorylation: VT^n 62-33-3, V201, "Brodick," 4164A3, "Fraser," and "Kaimai." For each line, all tubers were harvested from 12 plants in a single field plot during the summer of 2009–2010. The tubers were pooled and kept in cold storage (4 °C) for 0, 3, 6, 12, or 24 weeks. For each time point and line, a single sample was taken from each of nine randomly selected tubers using a cork borer. The nine samples were pooled into three samples and then freeze-dried. At each time point, the lines were processed in the order given above. For subsequent procedures, each replicate was processed as a group of 30 (6 lines × 5 time points).

Starch extraction

Starch was prepared from freeze-dried potato tuber samples as follows. Samples were crushed and 3 mg of the resulting powder was suspended in 100 ml water with vigorous shaking and then filtered with a coarse cloth filter to remove fibrous plant material. The filtrate was left to stand for 1 h allowing the starch granules to settle. The liquid was poured off and replaced by 50 ml water, and the sample was shaken vigorously to resuspend the starch. This was repeated four more times. After the final wash was removed, the starch was freeze-dried. Deionized water was used throughout to maintain consistency.

Hydrolysis

Hydrolysis of starch followed the method of Haebel et al. [15]. Starch (1 mg) was hydrolyzed in 100 μl of 2 M TFA for 3 h at 95 °C. A 20-μl aliquot was then removed and added to 200 μl of sterile water in a microplate. The sample was dried overnight in a Speedvac (Savant Instruments, Farmingdale, NY, USA) at room temperature. Another 200 μl of water was added, and the sample was dried again by Speedvac for 7 h at room temperature. The dried hydrolysate was resuspended in 200 μl of 80% ethanol with 0.1% formic acid with the aid of vortex mixing.

Chromatography

The LC/MS system consisted of a Thermo Electron Corp. (San Jose, CA, USA) Finnigan Surveyor MS pump, a Thermo Accela Open autosampler (PAL HTC-xt with DLW), and a ThermaSphere TS-130 column heater (Phenomenex, Torrance, CA, USA).

A 2-μl aliquot of each prepared extract was separated with a mobile phase consisting of (A) 0.1% formic acid with 5 mM ammonium acetate in water and (B) 0.1% formic acid in acetonitrile, by normal-phase chromatography (zwitterionic ZIC-HILIC stationary phase: 3.5 μm, 150 × 2.1 mm i.d.; Merck SeQuant, Umea, Sweden, guard HILIC GRACE) maintained at 25 °C with a flow rate of 300 μl/min [16]. A modified gradient was applied (time/%A): 0 min/5% A, 2 min/5% A, 3 min/30% A, 10 min/38% A, 10.1 min/95% A, 12 min/95% A, 12.1 min/5% A, and 16 min/5% A.

The eluent was analyzed by electrospray ionization/MS (LTQ, 2D linear ion-trap; Thermo Finnigan, San Jose, CA, USA). Quantification was performed with an external standards mix in the range of 0.5–50 μg/ml for phosphates and 1000–10,000 μg/ml for glucose using selective reaction monitoring (SRM) of the analyte precursor and product ions. Glucose *m/z* 225 $[M-H+46]^-$ (product, *m/z* 179, 157), Glc3P *m/z* 259 $[M-H]^-$ (product, *m/z* 241), and Glc6P *m/z* 259 $[M-H]^-$ (product, *m/z* 97) were recorded for SRM at respective retention times of 6.3, 7.7, and 9 min. Glucose (Cat. No. G-8270) and Glc6P (G-7879) used as standards were purchased from Sigma. Glc3P was prepared in 30% overall yield from D-(1,2)(4,6)-diacetone glucose (Aldrich) as described by Ritte et al. [18]. The data were consistent with those reported.

Instrument tuning based on Glc6P gave a good balance of sensitivity, diminishing that for the high glucose content and increasing it for the low glucose phosphate concentrations, thereby maintaining signal responses for all species within the linear dynamic range of the detector. External standards were used to measure the amounts of glucose, Glc6P, and Glc3P based on areas under the curves. For each LC/MS plate, full sets of standards were analyzed at the start and finish of each plate and the 5 μg/ml (Glc6P and Glc3P) and 3000 μg/ml (glucose) standards were analyzed every 12th run. Values were calculated with the aid of Xcalibur 2.1 software (Thermo Electron Corp.). Standard curves for Glc6P and Glc3P were fitted using the quadratic curve function and for glucose using the cubic spline forced through zero function.

Statistical analyses

For the trial using 30 lines, initial analyses using mixed models fitted with restricted maximum likelihood (REML) [19] were carried out to assess the importance of the sets of six and the processing order. Since neither was found to be important, the data were analyzed with analysis of variance.

For the storage trial involving six lines, to allow for the repeated measuring of tubers from the same plants, the analysis potentially needed to be adjusted by allowing for varying correlations between times. Various models (each fitted with REML) were assessed and an unstructured correlation model was found to