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Extraction, isolation and characterisation of phytoglycogen from su-1 maize leaves and grain

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ABSTRACT

Phytoglycogen is a highly branched soluble α-glucan found in plants, particularly those with decreased activity of isoamylase-type starch debranching enzyme, such as sugary-1 (su-1) maize. An improved technique has been designed to extract and isolate phytoglycogen from the grain and leaves of su-1 maize with minimal degradation for structural characterisation. The structures of extracted phytoglycogen samples were analysed using size-exclusion chromatography (SEC, also termed GPC) and transmission electron microscopy (TEM) and compared with the structure of pig liver glycogen. The SEC weight molecular size distributions indicate that the extraction procedure with protease is most effective in obtaining pure phytoglycogen from grain, whereas that without protease at cold temperature followed by purification using a sucrose gradient is more effective for leaf material. The extracted and purified phytoglycogen samples from both grain and leaf contain wide distributions of molecular sizes (analysed by SEC and TEM), with the smallest being "individual" β particles, which collectively form larger α particles; the latter are dominant in the phytoglycogen samples examined here. The results show that phytoglycogen is similar to liver glycogen in both the range of molecular size distribution and in the presence of α particles.

1. Introduction

Phytoglycogen, a highly branched water-soluble glucan found in plants, has been reported as being structurally similar to glycogen isolated from animal organs (Ball et al., 1996). Phytoglycogen molecules are made up of α-(1→4)-linked glucose units, forming linear chains with average lengths of 11–12 glucose units that are joined together via α-(1→6) glycosidic linkages as branch points. The physiological function of phytoglycogen in plants is, however, still not well understood. Phytoglycogen is accumulated in a large quantity in plants with a mutation in the *sugary-1* gene (*su-1*) encoding an isoamylase-type debranching enzyme (DBE) (Delatte, Trevisan, Parker, & Zeeman, 2005; Kubo et al., 2010; Orzechowski, 2008; Wattebled et al., 2005; Zeeman et al., 1998; Zeeman, Kossmann, & Smith, 2010). Since DBEs are responsible for cleaving excess branch points (α-(1→6) glycosidic linkages) of amylopectin molecules during normal starch biosynthesis, phytoglycogen is thought to be an intermediate that is unable to mature to amylopectin molecules. Another theory has been put forward, stating that phytoglycogen is a form of transient energy storage in leaf material to rapidly remove the excess sucrose synthesised by leaf photosynthesis during daytime in order to reduce osmotic pressure in plant organs (Zeeman et al., 1998), similar to the physiological function of glycogen in animal organs as glucose buffer. With decreased activity of DBEs, the phytoglycogen is not efficiently degraded, leading to its accumulation in the plant. In addition to accumulating in the grain of *su-1* maize, phytoglycogen has also been isolated from the grains of sorghum, rice, and barley as well as the leaves of *Arabidopsis thaliana* (Boyer & Liu, 1983; Burton et al., 2002; Wong, Kubo, Jane, Harada, Satoh, & Nakamura, 2003; Zeeman et al., 1998).

Abbreviations: AUC, area under the curve; C, ice-cold tricine buffer treatment at 0–4 °C; DAP, days after pollination; DBE, debranching enzyme; DMSO/LiBr, dimethyl sulfoxide containing 0.5% (w/w) lithium bromide; SG, sucrose gradient; IUPAC, International Union of Pure and Applied Chemistry; NMR, nuclear magnetic resonance; P, treatment using protease in tricine buffer at 37 °C; R_h, hydrodynamic radius; SEC, size-exclusion chromatography; *sh-2*, *shrunken-2* genetic mutation; *su-1*, *sugary-1* genetic mutation; TEM, transmission electron microscopy; V_h, hydrodynamic volume; W, warm tricine buffer treatment at 37 °C.

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Glycogen is also a soluble highly branched glucan made up of α-(1→4)-linked glucose units, forming linear chains that are joined together via α-(1→6) linkages, with branching structure similar to phytoglycogen (Ball et al., 1996). Glycogen particles range between 20 and 200 nm in diameter and form small β particles (~20 nm), which collectively form the large α particles (50–200 nm). The molecular size distribution of glycogen obtained by size-exclusion chromatography (SEC) does not show a clear distinction between the α and β particles, but rather a continuous distribution where the smallest are "individual" β particles and the larger are α particles with wide ranges of both sizes and numbers of β particles (Sullivan, Vilaplana, Cave, Stapleton, Gray-Weale, & Gilbert, 2010). A greater proportion of larger or smaller particles can be easily observed among the glycogen samples from different tissues and/or at different postprandial periods (Besford, Sullivan, Zheng, Gilbert, Stapleton, & Gray-Weale, 2012; Preiss, 2010; Ryu et al., 2009), e.g. the average molecular size of liver glycogen is generally larger than that of muscle glycogen. Furthermore, the binding between the β particles to form α particles is still not well understood, although there is some evidence that a protein may be involved in this process (Sullivan et al., 2012). In a previous study (Sullivan et al., 2011), the formation of glycogen α particles was reported to be impaired in an animal model of type II diabetes. It is still unknown whether the higher amount of β particles and the lack of large α particles are contributors to the development of type II diabetes or a result of the disease; however, knowledge of the formation of α particles from the β particles might provide new understanding relevant to type II diabetes.

The presence of α and β particles in phytoglycogen has not been reported before, but their existence (particles with ~20 and 50–200 nm in diameter, respectively) can be observed from the transmission electron microscopy (TEM) images in a past study (Putaux, Buleon, Borsali, & Chanzy, 1999). Hence the importance of phytoglycogen α and β particles in plant physiology and the binding between phytoglycogen β particles in forming the larger α particles are virtually unknown. The extraction and purification techniques of phytoglycogen in the past (Delatte et al., 2005; Putaux et al., 1999) have utilised methods that may degrade phytoglycogen α particles to β particles, potentially compromising the structural integrity of the phytoglycogen molecules and producing artefacts in the structural characterisation. One of these techniques utilises perchloric acid to extract phytoglycogen from the leaves of *A. thaliana* (Delatte et al., 2005; Streb et al., 2008). The perchloric acid technique is designed to rapidly inactivate enzymes present in leaf material; however, given the recent finding that α particles extracted from pig liver are rapidly degraded into β particles when exposed to acidic environments (Sullivan et al., 2012), it is likely that the use of perchloric acid cleaves the intra- and inter-molecular bonds of phytoglycogen. Furthermore, the "phytoglycogen" extracted using perchloric acid has a chain length distribution resembling that of amylopectin, but different from that extracted without perchloric acid, suggesting the possibility that the acid hydrolyses amylopectin making it more water-soluble (Delatte et al., 2005).

This paper reports an improved method for the extraction and purification of phytoglycogen from the grain and leaves of *su-1* maize, modified from a method recently developed to isolate starch molecules from grain flour (Syahariza, Li, & Hasjim, 2010) and that to extract glycogen from animal tissue (Ryu et al., 2009). This method is able to fully dissolve phytoglycogen molecules with minimal degradation, allowing the accurate characterisation of phytoglycogen structure using SEC and TEM. Furthermore, the comparison between the structures of phytoglycogen and glycogen will allow a better understanding in the similarities or differences of their physiological functions in plants and animals, respectively; e.g. whether phytoglycogen is a form of transient glucose storage in plants similar to animal glycogen as a blood glucose buffer or an intermediate of amylopectin biosynthesis. Obtaining a very high yield of phytoglycogen is, however, not the objective of the present study. As long as the loss is not selective, the results from the structural characterisation of extracted phytoglycogen can represent the whole population of phytoglycogen.

2. Materials and methods

2.1. Materials

The kernels of *su-1* and *shrunken-2* (*sh-2*) mutant maize were obtained from Prof. Ian D. Godwin, The University of Queensland, Brisbane, Australia. Some of the kernels were ground and used for phytoglycogen extraction from grain, while others were germinated to provide leaf materials for phytoglycogen extraction. Germination was performed on petri dishes, planted in large pots (in January 2012) and left in the field to grow under sunlight. When the grain started to develop (approx. 86 days after seeding or 14 days after pollination), the topmost five photosynthesising leaves were collected (in April 2012) between 3 pm and 5 pm to maximise the amount of phytoglycogen accumulated during the day. Grain was not harvested from these plants as the amount of phytoglycogen accumulated in the grain may be affected by the removal of leaves during grain development. The harvested leaves were immediately frozen in liquid nitrogen, and then dried overnight using a freeze dryer. The kernels and freeze-dried leaves were ground to a fine powder in a cryo-mill (Freezer/Mill 6870, SPEC CertiPrep, Metuchen, NJ, USA; 1 min precooling followed by 5 min grinding (Syahariza et al., 2010)). This cryogrinding technique has been shown to minimise the mechanical and heat degradation on starch molecules that can occur during dry grinding at ambient temperatures (Syahariza et al., 2010). The *sh-2* maize does not produce phytoglycogen (Holder, Glover, & Shannon, 1974); however, as many plant functions occur in the leaves, the leaf material of the *sh-2* maize was compared with that of the *su-1* maize to check for the presence of contaminants that are purely associated with leaf functions or related to phytoglycogen biosynthesis. Furthermore, the use of a *sh-2* maize mutant, which accumulates small sugars such as sucrose in abundance, allows an investigation into the effectiveness of the ability of the new extraction and purification methods in removing all small sugars, especially in the leaf material.

Glycogen was extracted from pig livers and purified as described in a previous study (Sullivan et al., 2012). The structure of the liver glycogen is used here as a comparison to the structure of phytoglycogen extracted from leaves and grain.

2.2. Extraction of phytoglycogen from leaves and grain

Leaf powder (500 mg) or kernel flour (100 mg) was weighed into a centrifuge tube. Phytoglycogen from each sample was collected as water-soluble extract after the leaf power or kernel flour had been incubated in 2.5 mL aqueous solution for 30 min. Three different incubation treatment conditions were tested: (a) protease (2.5 units/mL; bacterial type XIV, Sigma–Aldrich, Castle Hill, NSW, Australia) in tricine buffer (pH 7.5, 250 mM) at 37 °C; (b) tricine buffer (pH 7.5, 250 mM) without protease at 37 °C as the control for the protease treatment; and (c) ice-cold tricine buffer in ice bath (0–4 °C). These extraction methods are modified from that used to extract starch molecules from grain flour (Syahariza et al., 2010). Although the protease treatment may hydrolyse the binding between β particles, potentially mediated by protein or peptide bonds, in forming the larger α particles of phytoglycogen, the results from a previous study (Sullivan et al., 2012) indicate that

this protease treatment does not significantly affect the molecular size distribution of liver glycogen containing α particles. The fact that α particles are not noticeably degraded by the protease treatment can be clearly observed from the molecular size distributions of phytoglycogen with and without the protease treatment. An additional 2.5 mL ice-cold tricine buffer was added to each sample after incubation followed by centrifugation at 4000 × *g* for 10 min, and the supernatant containing the water-soluble solids was collected. The phytoglycogen in the supernatant was precipitated using approximately four volumes of absolute ethanol then centrifuged at 4000 × g for 10 min, and the precipitate was dissolved in 1.5 mL dimethyl sulfoxide solution containing 0.5% (w/w) lithium bromide (DMSO/LiBr) overnight in a water bath at 80 °C to yield a complete dissolution of the phytoglycogen molecules (Schmitz, Dona, Castignolles, Gilbert, & Gaborieau, 2009). The dissolved sample was centrifuged, and the supernatant was collected. Ethanol precipitation and centrifugation were repeated, and the phytoglycogen precipitate was re-dissolved in 0.5 mL DMSO/LiBr solution in a thermomixer (Eppendorf, Hamburg, Germany) at 80 °C for 2 h and occasionally inverted by hand. The sample was then centrifuged once more, and the supernatant was collected and stored in a vial for SEC analysis. Samples were dissolved in DMSO/LiBr solution as this solvent, coupled with heating at 80 °C and shaking, has been shown to completely dissolve starch molecules with minimal degradation (Schmitz et al., 2009; Syahariza et al., 2010).

2.3. Purification of leaf phytoglycogen

To accurately characterise the structure of phytoglycogen, three purification techniques were tested to remove water-soluble contaminants from leaf phytoglycogen.

2.3.1. Heating in a boiling water bath

The water-soluble extract collected from initial extraction of leaf material followed by dilution with an additional 2.5 mL ice-cold tricine buffer was heated in a boiling water bath (~100 °C) for 15 min before it was centrifuged at 4000 × *g* for 10 min and precipitated with ethanol. The additional heating step is regularly used in the extraction of phytoglycogen from grain endosperms, and is hypothesised to denature water-soluble protein contaminants, causing them to become water-insoluble, which then can be easily removed from the water-soluble phytoglycogen by centrifugation (Inouchi, Glover, Takaya, & Fuwa, 1983). The rest of the treatment is the same as described in the initial extraction method (Section 2.2).

2.3.2. Chloroform/methanol solution

After the second ethanol precipitation of the initial extraction method (before the final dissolution in DMSO/LiBr solution for SEC analysis), the phytoglycogen extracted from leaf powder was mixed with 0.2 mL (2% anhydrous w/w) $NaSO_4$ solution, and subsequently with 1 mL chloroform/methanol solution (1:1 ratio) to dissolve lipids and small sugars in the extracted phytoglycogen, respectively (Benedict, 2011). The phytoglycogen pellet was collected by centrifugation at 4000 × *g* for 10 min, washed with ethanol, and then dissolved in 0.5 mL DMSO/LiBr solution overnight using the thermomixer at 80 °C, while being occasionally inverted by hand to ensure a homogenous mixture. The sample was centrifuged at 4000 × *g* for 10 min and the supernatant was collected in a vial for SEC analysis.

2.3.3. Sucrose gradient

Similar to the chloroform/methanol purification method, after the second ethanol precipitation of the initial extraction method (before the final dissolution in DMSO/LiBr solution for SEC analysis), the phytoglycogen extracted from leaf powder was dissolved in warm deionised water until the pellet was completely dispersed, and then it was rapidly frozen in liquid nitrogen and freeze-dried overnight. The dried phytoglycogen was resuspended in 0.4 mL glycogen isolation buffer (containing 50 mM tris(hydroxymethyl)aminomethane, pH 8, 150 mM sodium chloride, 2 mM ethylenediaminetetraacetic acid, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, and 1 mM protease-inhibiting phenylmethanesulfonylfluoride) and layered over a modified 3 mL stepwise sucrose gradient (37.5% and 75% in glycogen isolation buffer, a slight modification to the method described elsewhere (Ryu et al., 2009; Sullivan et al., 2012)). The sample was then centrifuged at 488,300 × *g* for 1.5 h at 4 °C. The pellet obtained in the 75% sucrose gradient layer was then resuspended in 1 mL cold glycogen isolation buffer and centrifuged at 600,000 × *g* for 45 min at 4 °C. The supernatant was discarded, and the pellet was dissolved in 0.2 mL deionised water and then precipitated with 1 mL ethanol to remove excess sucrose. The sample was centrifuged at 4000 × *g* for 10 min at 10 °C, and the supernatant was discarded. The pellet was resuspended in 1 mL warm deionised water and freeze-dried overnight. The dried and purified phytoglycogen sample was dissolved in 0.2 mL DMSO/LiBr solution using the thermomixer at 80 °C for 2 h and occasionally inverted by hand. The sample was then centrifuged at 4000 × *g* for 10 min, and the supernatant was collected in a vial for further SEC analysis.

2.4. Chain length distribution of phytoglycogen from su-1 grain and pig liver glycogen

After the second ethanol precipitation of the extraction method (before final dissolution in DMSO/LiBr solution for SEC analysis), the phytoglycogen extracted from leaf powder was dispersed in 0.9 mL of warm deionised water and heated in a boiling water bath until completely dispersed. The dispersion was cooled to room temperature where 5 μL of sodium azide solution (0.04 g/mL) and 0.1 mL acetate buffer (0.1 M, pH ~ 3.5) were added. Isoamylase solution (2.5 μL; Megazyme Interdations, Bray, Co. Wicklow, Ireland) was added to the solution which was then vortexed and incubated in a 37 °C water bath for 3 h. The debranched starch was neutralised to pH ~ 7 by adding sodium hydroxide solution (0.1 M) dropwise to the solution. The sample was then transferred to a preweighed 2 mL microcentrifuge tube and freeze-dried overnight. The dried sample was subsequently dissolved in 1 mL DMSO solution containing LiBr (0.5%, w/w) and heated at 80 °C for 24 h in a thermomixer to ensure complete dissolution. The solution was then transferred to a SEC vial and stored at room temperature until SEC analysis.

2.5. Size-exclusion chromatography

SEC, also known as gel-permeation chromatography (GPC), separates polymers based on their hydrodynamic volume (V_h), which is defined by the International Union of Pure and Applied Chemistry (IUPAC) as the volume of a hydrodynamically equivalent sphere (Jones, Stepto, & Wilks, 2009); equivalent meaning is dependent on the particular technique used (thus, for example, *V*h is a different quantity between dynamic light scattering and SEC). The result is presented as the SEC weight distribution, w ($\log V_h$), of the phytoglycogen molecules as a function of hydrodynamic size. The assumption that SEC separates any polymer only by its V_h regardless of its structure is termed the 'universal calibration' principle (Gilbert, 2011; Kostanski, Keller, & Hamielec, 2004). For convenience, the SEC weight distributions are presented in terms of hydrodynamic radius (R_h) with $V_h = (4/3)\pi R_h^3$.

The molecular structures of branched and debranched phytoglycogen and glycogen were analysed using an Agilent 1100 Series SEC system (Agilent Technologies, Waldbronn, Germany) equipped with a refractive index (RI) detector (RID-10A, Shimadzu Corp., Kyoto, Japan). GRAM preColumn and GRAM 30 and 3000 analytical columns (Polymer Standards Service (PSS) GmbH, Mainz, Germany) were used in series and placed in a column oven at 80 °C, and DMSO/LiBr solution was used as the mobile phase. The flow rate was set at 0.3 mL/min, which results in minimal shear scission while maintaining adequate size separation resolution (Cave, Seabrook, Gidley, & Gilbert, 2009). Shear scission is an SEC problem that may occur during the size separation of large polymers.

Pullulan standards (PSS) with a range of peak molecular weights of 342–2.35 × 10^6 were directly dissolved in DMSO/LiBr solution and run through the system to generate a universal calibration curve, thus allowing the determination of V_h from the elution volume (see, e.g. Gilbert, 2011).

2.6. Transmission electron microscopy

TEM imaging was conducted using the method of Scheffler, Huang, Bi, and Yao (2010) with some modifications. A 400-mesh grid, coated in 1.1% polyvinyl formal in chloroform, was glow-discharged before use. Droplets of ~0.01% phytoglycogen dispersed in Tris buffer (0.5 mL, 50 mM Tris, pH 7, 150 mM NaCl) were placed on the grid and allowed to dry (approx. 5 min). The phytoglycogen sample and grid were then stained with 2% aqueous uranyl acetate. Samples were imaged on a JEM 1010 TEM (JEOL, Tokyo, Japan) at the Centre for Microscopy and Microanalysis, The University of Queensland, Brisbane, Australia, operating at 100 kV. Images were recorded digitally with a SIS Veleta CCD camera (Olympus, Münster, Germany), and measurements were prepared using AnalySiS image management software. This technique is commonly used to view glycogen samples from animals and has been used in the past to view phytoglycogen samples (Putaux et al., 1999). Although, in principle, the size distribution of particles can be obtained using TEM, the results are biased by the relatively small number of particles in the images and by how well the images represent the whole population. To overcome this problem, the size distribution of particles in a heterogeneous system, such as phytoglycogen and glycogen, requires thousands of particles in a large number of images chosen randomly from the TEM grid, which is labour and time intensive. Thus this is not performed in the present study as SEC is the preferred method to provide the same information with greater efficiency.

3. Results and discussion

All SEC analyses were performed in duplicate. The results from the duplicate measurements show similar trends in regards to the different extraction and purification treatments. However, the SEC weight distributions from the same extraction or purification treatment are not superimposed, and hence only one of the duplicate measurements is used as the representative in this paper. The duplicate SEC weight distributions for each extraction or purification treatment are shown in the Supplementary Data.

3.1. Determining optimal extraction conditions

Three different extraction techniques were utilised to test which extraction condition is the most effective to extract phytoglycogen from the grain and leaves of *su-1* maize. The protease treatment (extraction using protease in tricine buffer solution at 37 °C) was applied to break up any protein matrix that may surround the phytoglycogen in the grain or leaves, as protein matrices have been reported to envelop starch granules in sorghum (Taylor, 2005). The warm treatment (extraction using tricine buffer solution at 37 °C without protease) was applied to test if endogenous enzymes, potentially degrading the phytoglycogen, were activated at 37 °C during the extraction process. The cold treatment (extraction using ice-cold tricine buffer solution without protease after equilibrating the sample in an ice bath) was used to prevent the activation of endogenous degrading enzymes during the extraction of phytoglycogen and to observe if the protein matrix can hinder the leaching of phytoglycogen. Water has been used in similar ice-cold extraction conditions in the past to extract phytoglycogen from grains, as phytoglycogen is highly water-soluble and cold temperatures can reduce the solubility of other components in grain, including starch (Burton et al., 2002; Delatte et al., 2005).

All three extraction conditions yielded three populations from the *su-1* maize leaf sample, as observed in the SEC weight distributions (Fig. 1a). The first population (denoted by i) has a peak (or shoulder) at R_h ~ 1.5 nm. The peak of the second population (denoted by ii) is at R_h ~ 4–6 nm. The peak of the third population (denoted by iii) is at R_h ~ 25–30 nm. The proportions of these three populations vary with the extraction conditions. Only the third population (peak iii) is present in the extracts from the *su-1* maize grain sample regardless of the extraction conditions (Fig. 1b). The SEC weight distributions of the extracts from the *sh-2* maize leaf sample (Fig. 1c), which does not accumulate phytoglycogen (Holder et al., 1974), displays two populations similar in the molecular size to the first and second populations (peaks i and ii, respectively) of the extracts from the *su-1* maize leaf sample (Fig. 1a). These small molecular-sized populations appear to be uniquely associated with leaf material as there is no evidence of these populations in the extracts from the *su-1* maize grain; however, the third population (peak iii) is present in both the *su-1* maize grain and leaf materials, indicating that this population is most probably phytoglycogen, which the *su-1* maize plant is known to accumulate (Boyer & Liu, 1983; Rahman, Wong, Jane, Myers, & James, 1998). Furthermore, the molecules in the first two populations (peaks i and ii) extracted from the leaf materials are too small to be phytoglycogen molecules, which are similar in size to glycogen molecules (20–200 nm in diameter). Hence they are most likely to be contaminants or residual substances not removed by the initial extraction techniques. These small molecular-sized residual substances may also be degraded molecules arising from the activation of degrading enzymes during the extraction treatments, especially at 37 °C and in the presence of protease (i.e. warm and protease treatments), such as amino acids or peptides resulting from the degradation of proteins as well as phenolic compounds from the degradation of lignins; degraded molecules are more soluble in the tricine buffer solution because of their smaller molecular size. In addition, the water-soluble extracts from leaf materials have a yellow/brown tint regardless of the extraction treatments, whereas this colouration is not observed in the extracts from grain, indicating that the small molecules in peaks i and ii are probably degraded proteins and lignins, which are normally coloured. The small molecular-sized populations (peaks i and ii) may also be partially degraded phytoglycogen arising from the fast reaction of endogenous enzymes when the leaf materials were incubated in tricine buffer during extraction. It is unlikely that the small molecules in the leaf materials are from the degradation of phytoglycogen as a result of subsequent dissolution steps after the initial extraction, as the DMSO/LiBr solvent has been shown to have minimal degradative effects on starch molecules at 80 °C with shaking (Schmitz et al., 2009; Syahariza et al., 2010). Additionally, these small molecular-sized populations are not observed in the extracts from the *su-1* maize grain treated with the same subsequent dissolution steps. As the objective of the present study is to extract and purify phytoglycogen from grain and leaves for structural characterisation, the characterisation of small molecular-sized contaminants (peaks

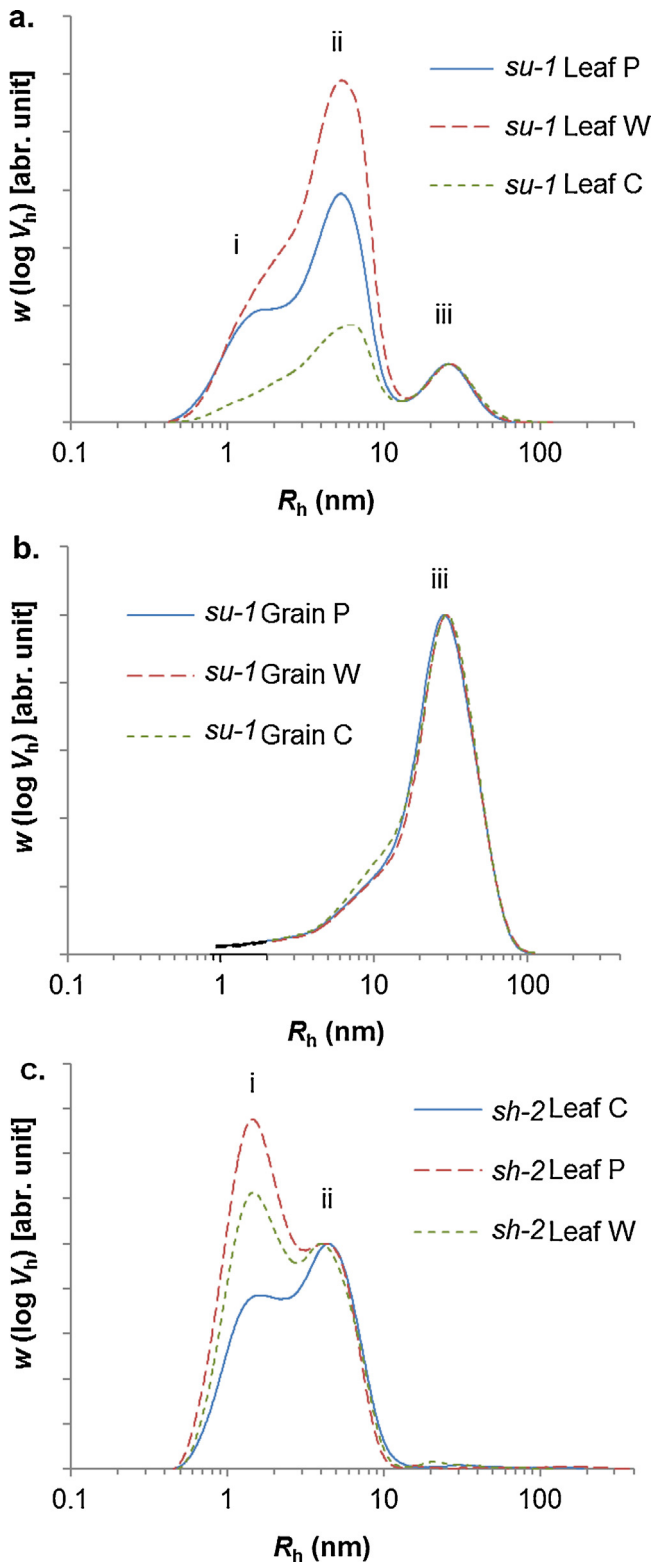

Fig. 1. SEC weight distributions of water-soluble extracts obtained using protease (37 °C, P), warm buffer (37 °C, W), and ice-cold buffer (~0 °C, C) treatments from *su-1* maize (a) leaves and (b) grain normalised to yield the same height of peak iii (R_h between 10 and 100 nm), as well as (c) *sh-2* maize leaves normalised to peak ii (R_h between 3 and 10 nm) as this mutant maize does not have peak iii.

i and ii) in the water-soluble extracts, which were dependent on the extraction conditions, was deemed to be less scientifically important and not within the scope of the present study; hence it was not carried out.

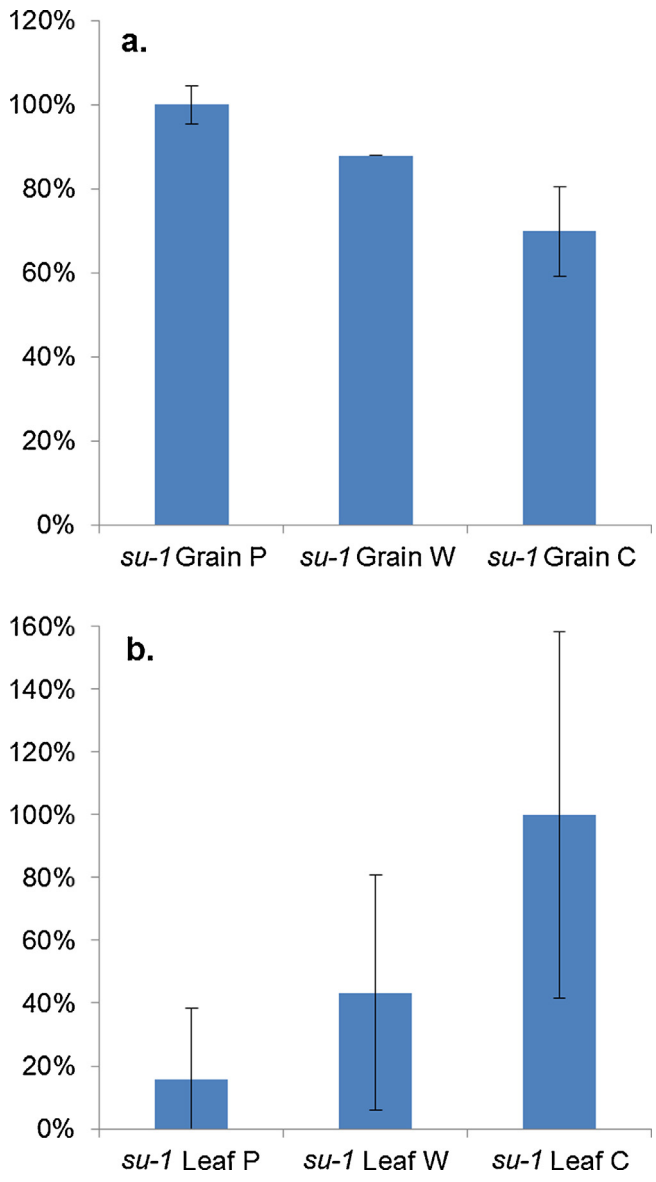

Fig. 2. Relative AUC of phytoglycogen peak (or peak iii) in the SEC weight distributions of water-soluble extracts from (a) grain and (b) leaves of *su-1* maize plants obtained using protease (37 °C, P), warm buffer (37 °C, W), and ice-cold buffer (~0 °C, C) treatments.

The comparisons among the three different treatments to extract phytoglycogen from the *su-1* maize grain demonstrate that the protease extraction treatment results in the greatest area under the curve (AUC) of phytoglycogen (population iii) in the un-normalised SEC weight distributions, whereas the warm and cold extraction treatments only yield 88% and 70%, based on the AUC, of the amount of phytoglycogen (peak iii) extracted by the protease treatment (Fig. 2a). The results suggest that the protease treatment is the optimal treatment to extract phytoglycogen from grain. The cold extraction, however, results in the greatest AUC of phytoglycogen (population iii) from the *su-1* leaf material and the smallest AUCs of the small molecular-sized contaminants (populations i and ii) in the un-normalised SEC weight distributions. The yields of the phytoglycogen (peak iii) extracted from the *su-1* leaf material using the protease and warm treatments are 16 and 43%, respectively, based on the AUC, of that extracted using the cold treatment (Fig. 2b), suggesting that protein matrix does not hinder the phytoglycogen extraction from leaf material. Furthermore, the molecular size distributions of peak iii are superimposable,

regardless of the extraction conditions (Fig. 1a and b for *su-1* maize leaf and grain, respectively), indicating that the incomplete extraction of phytoglycogen is not selective and hence the extracted phytoglycogen can represent the whole population of phytoglycogen.

The contaminants, especially those falling within the range of the second population (peak ii), have a large AUC and appear to overlap with the phytoglycogen peak (peak iii). To further understand the biosynthesis of phytoglycogen, including branching structure, and to better understand the similarities between phytoglycogen and glycogen in relation to size and the presence of the two particle (α and β) populations, the small molecular-sized contaminants need to be removed completely or largely reduced. The purification of the extracted phytoglycogen is therefore essential for characterisation of its fine structure in future work. The fact that the second population (peak ii) is present in leaf samples (see Supplementary Data), even from the *sh-2* maize plants that do not yield phytoglycogen, indicates that this population of molecules is not associated with phytoglycogen biosynthesis. As the cold extraction method seems to be the optimal way to extract the phytoglycogen from leaf material, further purification to remove the small molecular-sized contaminants and to obtain pure phytoglycogen was performed only on the water-soluble extracts of *su-1* maize leaf material obtained from the cold extraction method.

3.2. Purification of phytoglycogen by heating

An additional heating step has been used to aid the purification of the phytoglycogen extracted from maize grain (Inouchi et al., 1983). It has been hypothesised that the additional heating denatures any water-soluble proteins present in the extract, making the proteins insoluble and facilitating their removal. This technique, however, increases the AUC and the molecular size range of the small molecular-sized contaminants in the extracts from the su-1 leaf material, as seen in the SEC weight distribution (Fig. 3a). In addition, peak ii of the contaminants remains overlapping with the phytoglycogen peak (peak iii) in the SEC weight distribution. The results suggests that the additional small molecular-sized contaminants are the small compounds that are trapped in cell-wall and/or protein matrices and are released when these matrices are disrupted by additional heating in a boiling water bath. These additional small molecular-sized contaminants could also be the products from the molecular degradation of heat-labile substances in the su-1 leaf samples.

3.3. Purification of phytoglycogen by chloroform/methanol solution

One technique that has been used to purify glycogen utilises a mixture of chloroform and methanol to remove lipids and soluble sugars, respectively, while precipitating glycogen from homogenised mosquitoes (Benedict, 2011). This technique was applied to the water-soluble extract (containing phytoglycogen) from the *su-1* leaf material, and almost completely removed peak i of the small molecular-sized contaminants (Fig. 3b), indicating that these smallest molecules are mainly soluble sugars (e.g. sucrose soluble in methanol) and/or small hydrophobic compounds (soluble in chloroform). Phytoglycogen polymers, similar to glycogen, are precipitated by methanol because they can form insoluble single helical inclusion complex with methanol. Peak ii of the small molecular-sized contaminants, while appearing to be substantially reduced, is still present and overlapping with the phytoglycogen peak (peak iii), suggesting that the molecules in peak ii are soluble in neither chloroform nor methanol.

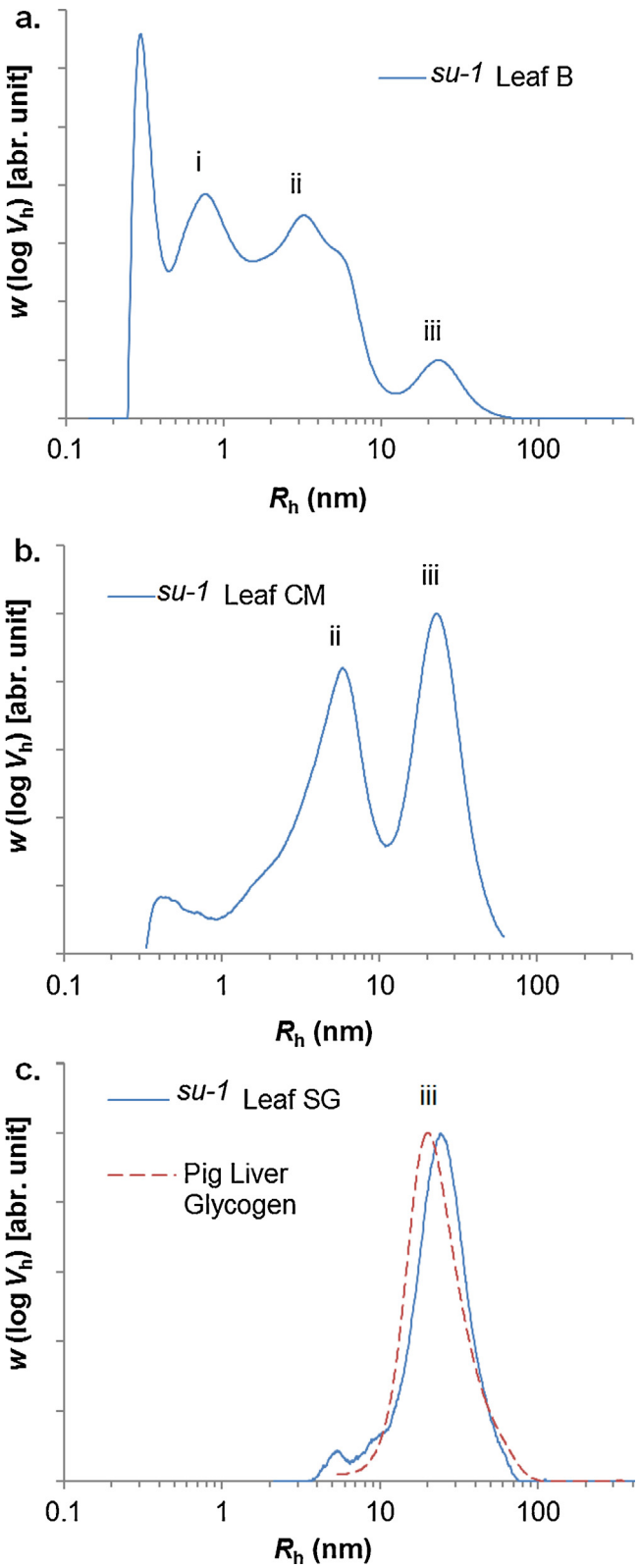

Fig. 3. SEC weight distributions of water-soluble extracts obtained using ice-cold buffer treatment (~0 °C) from *su-1* maize leaves after purification by (a) boiling treatment (100 °C, 15 min; B), (b) chloroform/methanol treatment (CM), and (c) sucrose gradient method (SG). Glycogen isolated using the same SG method from pig liver is included for comparison.

3.4. Purification of phytoglycogen by sucrose gradient

The sucrose gradient technique separates particles/molecules based on their densities. Phytoglycogen particles can penetrate both 37.5% and 75% sucrose layers of the gradient resulting in a white coloured pellet, the same as that observed from liver glycogen (Ryu et al., 2009; Sullivan et al., 2012). On the other hand, the coloured components, which are likely to be part of the small molecular-sized contaminants not removed by the initial extraction process, remained in the upper (37.5%) sucrose fraction. The SEC weight distribution of the pellet obtained from the lower sucrose fraction (75%) shows only the phytoglycogen peak (peak $R_h \sim 25$ nm with R_h range of 4–100 nm), verifying that the phytoglycogen molecules have been purified and that the small molecular-sized contaminants have been mostly, if not completely, removed (Fig. 3c). The molecular size of phytoglycogen from the *su-1* leaf material after purification by sucrose gradient is within the same range as that of phytoglycogen from the *su-1* grain from initial extraction method (Fig. 1b) and that of glycogen from pig livers also purified using the same sucrose gradient method (Fig. 3c). The phytoglycogen samples from the *su-1* grain and leaf materials have peaks at a slightly larger R_h (~25–30 nm) than liver glycogen ($R_h \sim 20$ nm), and the phytoglycogen isolated from *su-1* maize leaf has a slightly smaller size distribution than that isolated from *su-1* maize grain. As liver glycogen has been reported to contain mainly α particles (Preiss, 2010), it can be inferred that phytoglycogen also consists of mainly α particles. Furthermore, since phytoglycogen has been described in the past as having a similar size range to glycogen, it can also be inferred that the extraction and (sucrose gradient) purification techniques result in minimal degradation of phytoglycogen. If degradation had occurred, it would be expected that the molecular size range of phytoglycogen would be smaller than that of glycogen.

3.5. Chain length distributions and degree of branching of phytoglycogen from grain and pig liver glycogen

The SEC number distributions of debranched grain phytoglycogen and glycogen, $N_{de}(X)$, are shown in Fig. 4. Unlike the chain-length distributions (CLDs) of non-waxy starches, there is only one major peak for phytoglycogen. The weight CLDs of the phytoglycogen and glycogen are very similar. The number-average chain lengths of the phytoglycogen and glycogen, respectively, are approximately 11 and 10 DP, corresponding to a degree of branching (DB) for phytoglycogen and glycogen, respectively, of 9.2% and 9.6%. The average DP and DB of phytoglycogen from the grain and rat liver glycogen obtained from debranched SEC corresponds with those reported in the past (Huang & Yao, 2011; Putaux et al., 1999; Ryu et al., 2009; Zeeman et al., 2010). There is potential error converting the SEC weight distribution to $Nde(X)$, which involves using the Mark-Houwink relation. The DB from ^{1}H nuclear magnetic resonance (NMR) spectroscopy of phytoglycogen from grain and leaf material in attrition to pig liver glycogen is given in the Supplementary Data.

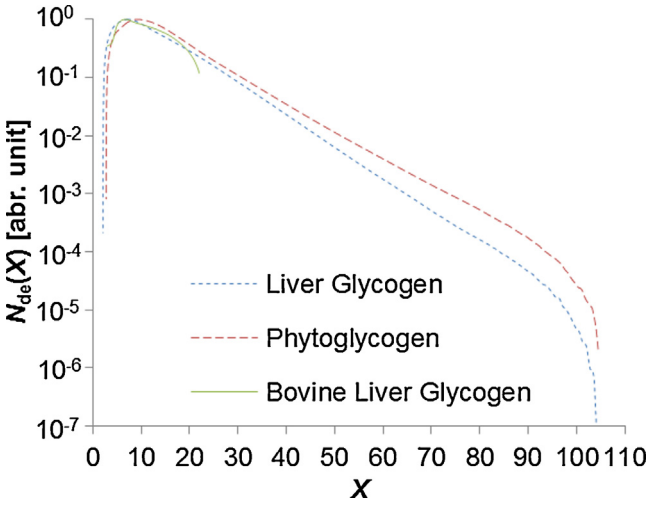

Fig. 4. SEC chain length number distributions from debranched, protease extracted *su-1* grain phytoglycogen and sucrose gradient purified pig liver glycogen. FACE chain length number distribution results from debranched commercial bovine liver glycogen (Sigma, St. Louis, MO) are overlayed for comparison.

Fluorophore assisted carbohydrate electrophoresis (FACE) would also be a useful technique to use to determine average chain-length and DB as it has increased sensitivity for small branches and does not have the problems associated with SEC, such as band broadening. There are, however, some shortcomings associated with the use of FACE, in particular it has difficulty detecting longer branch chains (Morell, Samuel, & O'Shea, 1998). FACE results for commercial bovine liver glycogen (Sigma, St. Louis, MO) were taken from the literature (Castro, Dumas, Chiou, Fitzgerald, & Gilbert, 2005) and replotted for comparison to phytoglycogen from *su-1* grain and rat liver glycogen SEC debranched results (Fig. 4). The bovine liver glycogen DB obtained from FACE was 8.7%, very similar to that obtained by SEC (9.6%). Although these samples are similar, commercial bovine liver glycogen has been shown to consist of very small β particles (<5 nm) rather than the combination of α and β particles found in rat liver glycogen (20–200 nm) (Ryu et al., 2009). The structural differences between the two samples may account for the differences found for DB. Because of the limited amount of phytoglycogen that can be extracted from the *su-1* maize leaf material, the characterisation of fine structure of the phytoglycogen from leaf material was unable to be obtained from the present study.

3.6. TEM of phytoglycogen from su-1 leaf and grain material in addition to pig liver glycogen

Pig liver glycogen has a wide size distribution of α and β particles (Preiss, 2010). The β particles are approximately 20 nm in diameter, while the diameter of α particles can range between 50 and 200 nm. The SEC weight distributions show that the extracted and purified phytoglycogen from *su-1* grain and leaves has a range of R_h of ~4–100 nm (Figs. 1b and 3c, respectively). Thus phytoglycogen, similar to glycogen, contains a distribution from simple β particles through to large α particles. TEM was used in the present study to qualitatively confirm the findings observed from the SEC weight distributions, as TEM has been used previously to identify the α and β particles of glycogen (Huang & Yao, 2011; Putaux et al., 1999; Sullivan et al., 2010). The presence of α and β particles in the TEM image of the extracted phytoglycogen from the *su-1* maize leaf material (Fig. 5) was similar to those seen in grain phytoglycogen and animal glycogen (Putaux et al., 1999; Sullivan et al., 2010). Furthermore, the molecular size distributions of phytoglycogen peak (peak iii) are superimposed regardless of the extraction conditions (Fig. 1a and b for *su-1* maize leaf and grain, respectively), indicating that the protease treatment did not degrade the phytoglycogen α particles. This is similar to the results from a past study (Sullivan et al., 2012), showing that treating liver glycogen with the same protease did not result in a significant change in its apparent molecular size distribution. It is suspected that the protease is unable to diffuse into α particles because of steric hindrance. Further study is needed to understand how β particles are joined together to form α particles in phytoglycogen and glycogen.

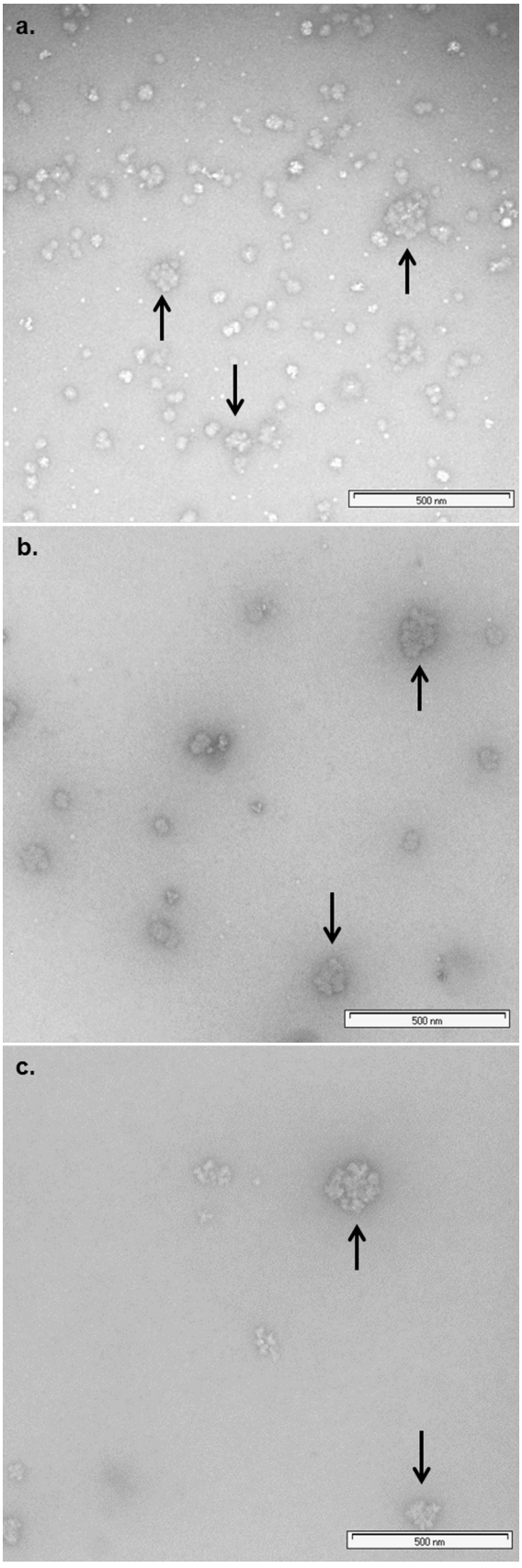

Fig. 5. TEM images of (a) phytoglycogen, purified with the use of a sucrose gradient, from *su-1* maize leaves, (b) phytoglycogen extracted from *su-1* maize grain and (c) pig liver glycogen, purified with the use of a sucrose gradient, showing the presence of α particles (examples as indicated by arrows). Scale bars 500 nm.

4. Conclusions

Phytoglycogen has been successfully isolated (and purified) from both *su-1* maize grain and, for the first time, *su-1* maize leaf material with complete dissolution and minimal degradation for accurate structural characterisation. The protease extraction technique gives the highest yield for the extraction and isolation of phytoglycogen from the *su-1* maize grain, while the extraction using ice-cold tricine buffer in an ice bath without protease appears to be the optimal extraction condition for the phytoglycogen in *su-1* maize leaf material. This may be attributed to a more dominant effect of a protein matrix enveloping, or a constitutional part of, the phytoglycogen in grain, while leaf material is likely to have a significantly greater amount of endogenous degrading enzymes, which may break down phytoglycogen during incubation at higher temperatures. Furthermore, the water-soluble extracts from the *su-1* leaf material contain substantial amounts of small molecular-sized contaminants, whereas those from the *su-1* grain material contain mainly phytoglycogen. This is probably because grain, being a storage organ, has a lower amount of highly soluble small molecular-sized components than leaf, which is the organ for photosynthesis and is filled with small transient nutrients (such as soluble sugars) for transport throughout the plant. It is also probable that degrading enzymes are always active in leaf to break down the transient nutrients, while those in grains are mainly active during grain development and later during germination. Two of the three purification techniques tested in the present study are unable to completely remove the small molecular-sized contaminants from the water-soluble extract of *su-1* maize leaf material. Only the use of a sucrose gradient removes most molecular-sized contaminants, to obtain phytoglycogen from *su-1* maize leaf material with relatively high purity.

SEC size distributions and TEM image demonstrates that a wide size distribution of particles ranging from single small β particles to large multiple-cluster α particles are present in phytoglycogen samples from the *su-1* maize leaves and grain, similar to pig liver glycogen. In both phytoglycogen and glycogen, it is difficult to make a clear distinction between the two populations in the SEC weight distribution; rather there is a continuous size distribution. On the other hand, the phytoglycogen samples from the *su-1* maize leaf and grain seem to consist of a larger amount of α particles than pig liver glycogen. While characterisation techniques, such as ^{1}H NMR spectroscopy and chain length analysis, are needed to confirm if the phytoglycogen extracted from leaf has fine structure similar to the grain phytoglycogen and liver glycogen, obtaining the necessary amount of phytoglycogen from leaves is quite difficult and beyond the scope of this study. The leaf phytoglycogen behaves similarly to the grain phytoglycogen and liver glycogen during extraction (soluble in water, but not ethanol) and subsequent purification (insoluble in methanol/chloroform and able to penetrate through 75% sucrose gradient). In addition, the leaf phytoglycogen has similar molecular size ranges and chain length distributions (from SEC weight distributions) to grain phytoglycogen and liver glycogen, and (α and β) particle structure (from TEM images). Thus it can be confidently inferred that the obtained leaf phytoglycogen has similar molecular structure to grain phytoglycogen and liver glycogen.

The results from the present study have demonstrated that phytoglycogen isolated from the *su-1* maize leaf has SEC weight distribution with a slightly smaller peak R_h than that isolated from the *su-1* maize grain, lending credence to the theory that the phytoglycogen in different organelles can have different molecular or particle sizes, similar to glycogen (larger glycogen particle or molecular size in liver than that in muscle). As phytoglycogen has now been extracted and purified from leaf and grain materials of

su-1 maize, with a complete molecular dissolution and minimal molecular aggregation and degradation, allowing accurate molecular structural characterisation, the foundation has been laid for future investigation into the bonding mechanisms between β particles to form α particles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.09.061.

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