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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