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Research paper

The glycogen of *Galdieria sulphuraria* as alternative to starch for the production of slowly digestible and resistant glucose polymers

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ABSTRACT

Highly branched glucose polymers produced from starch are applied in various products, such as peritoneal dialysis solutions and sports drinks. Due to its insoluble, granular nature, the use of native starch as substrate requires an energy consuming pre-treatment to achieve solubilization at the expense of process costs. Glycogen, like starch, is also a natural glucose polymer that shows more favorable features, since it is readily soluble in cold water and more accessible by enzymes. The extremophilic red microalga *Galdieria sulphuraria* accumulates large amounts of a small, highly branched glycogen that could represent a good alternative to starch as substrate for the production of highly branched glucose polymers. In the present work, we analyzed the structure-properties relationship of this glycogen in its native form and after treatment with amyloglucosidase and compared it to highly branched polymers produced from potato starch. Glycogen showed lower susceptibility to digestive enzymes and significantly decreased viscosity in solution compared to polymers derived from starch, properties conferred by its shorter side chains and higher branch density. The action of amyloglucosidase on native glycogen was somewhat limited due to the high branch density but resulted in the production of a hyperbranched polymer that was virtually resistant to digestive enzymes.

1. Introduction

Starch is the energy storage carbohydrate accumulated by many plants, including dietary relevant crops such as corn, wheat, rice and potato. It is composed of two types of polymers: amylose and amylopectin. Amylose is a virtually linear polysaccharide of glucose residues linked by α-(1 → 4) glycosidic bonds and amylopectin is a branched polysaccharide with a backbone of α-(1 → 4) linked glucose units and side chains attached through α-(1 → 6) bonds. The branching linkages in amylopectin represent around 5% of the total and are confined to clusters, leading to the appearance of crystalline regions in the molecule and the formation of insoluble starch granules (Buléon, Colonna, Planchot, & Ball, 1998). Starch is not only one of the main components of the human diet but it is also an industrially relevant raw material. Starch modified *via* different physical, chemical or enzymatic treatments results in a large range of derivatives that can be used as *e.g* gelling agents in food products or as adhesives and coatings in the paper and textile making (Ellis et al., 1998; Röper, 2002; van der Maarel, van der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002)

One of such starch derivatives are the so-called highly branched glucose polymers, obtained by acid and/or enzymatic treatment of starch in order to increase the branching density through hydrolysis of α-(1 → 4) linkages and/or the creation of new α-(1 → 6) linkages. The higher degree of branching and lower chain length distribution of these polymers make them readily soluble in water, non-retrogradable (Li et al., 2016) and more resistant to degradation by digestive enzymes such as α-amylases and glucosidases, which hydrolyze the α-(1 → 6) linkages at a lower rate than the α-(1 → 4) linkages (Ao et al., 2007). This allows their application in fields where these features are determinant. These highly branched glucose polymers can be used, for example, as osmotic agents for peritoneal dialysis or as slowly digestible carbohydrates in sports drinks (Backer & Saniez, 2005; Fuertes, Roturier, & Petitjean, 2009).

Peritoneal dialysis is the introduction of a solution in the peritoneal cavity of a patient suffering from kidney failure to drain excess water and toxins from the blood. In this case, the branched glucose polymer represents an osmotic agent alternative to glucose that is not easily absorbed into the bloodstream, creating a long-lasting osmotic gradient that moves fluids from the

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blood into the peritoneal cavity through the peritoneum (Mistry & Gokal, 1994). The company Baxter Healthcare commercializes a peritoneal dialysis solution named Extraneal® that contains the so-called icodextrin, a glucose polymer with less than 10% of α-(1 → 6) linkages and a weight-average molecular weight (M_w) of 1.3–1.9 × 10^4 Da (Moberly et al., 2002), and its effectiveness in long dwell peritoneal dialysis treatments has been reviewed (Frampton & Plosker, 2003). A patent has been filed on the enzymatic treatment of starch amylopectin to produce glucose polymers with a very high degree of branching (between 20 and 30%), a feature that would significantly improve their performance as osmotic agents with slow glucose release into the bloodstream (Deremaux, Petitjean, & Wills, 2013).

When carbohydrates are used as an ingredient in sports drinks, they should delay the onset of fatigue by replenishing depleted body reserves and, at the same time, allow fast fluid absorption from the stomach into the small intestine to counteract dehydration (Maughan, 1998). In this regard, branched glucose polymers represent a more optimal energy source than glucose or short linear oligosaccharides due to their slower degradation by digestive enzymes, leading to a more gradual glucose appearance in the bloodstream and a lower insulin response (Takii, Ishihara, Kometani, Okada, & Fushiki, 1999). Because of their high molecular weight, branched glucose polymers have a negligible contribution to the osmotic value of the solution even at high carbohydrate concentration, and can be combined with essential electrolytes to produce hypotonic sports drinks with the optimum osmolality value to achieve fast gastric emptying (Takii et al., 2005). The product Cluster Dextrin®, commercialized by the company Glico, is widely sold as a quickly absorbed but slowly metabolized carbohydrate ingredient for sports meals that provides a constant supply of energy during exercise.

The production of highly branched glucose polymers can be achieved *via* enzymatic reaction using various substrates such as glucose-1-phosphate (van der Vlist et al., 2008) or sucrose (Buttcher & Quanz, 2004), but starch is still the main substrate used in industrial scale processes. However, glycogen – the energy storage compound of prokaryotes, some lower eukaryotes and animals (Ball & Morell, 2003; Manners, 1991a) – could represent a promising alternative to starch. Like amylopectin, glycogen is also a polymer of α-(1 → 4)-joined glucoses with branches attached through α-(1 → 6) bonds, but its greater proportion of branching linkages- 7–18%, depending on the biological source (Matsui, Kakuta, & Misaki, 1993; Martinez-Garcia, Stuart, & van der Maarel, 2016; Wang & Wise, 2011)- distributed randomly along the molecule and its shorter side chains result in glycogen having a globular, water-soluble conformation that differs markedly from that of starch (Manners, 1991a). This conformation would represent an advantage when using glycogen as substrate in an industrial process because the molecule is readily soluble in cold water and easily accessible by enzymes. This would allow reducing process costs associated with the energy-consuming pre-treatment by which the granular structure of starch is disrupted in order to achieve solubilization (*i.e.* starch gelatinization) (Ellis et al., 1998).

The use of glycogen as industrial substrate is however limited by its lower abundance compared to amylopectin. Microorganisms that accumulate glycogen usually do so under conditions of limiting growth (Preiss, 1984). Nevertheless, there are exceptions to this and certain species are efficient glycogen producers. One of these species is the thermoacidophilic red microalga *Galdieria sulphuraria*. This unicellular alga is an ideal source of glycogen because it accumulates considerable amounts of this polymer (reaching 50% of the dry cell weight (unpublished work)) throughout all growth phases (Martinez-Garcia & van der Maarel, 2016) and it can grow to very high cell densities (Schmidt, Wiebe, & Eriksen, 2005). Moreover, the glycogen of *G. sulphuraria* would represent a good substrate for the production of highly branched glucose polymers because it already contains 18% of α-(1 → 6) linkages, higher than any other glycogen, and it has a smaller weight-average molecular weight (M_w) than glycogen from other organisms (Martinez-Garcia et al., 2016).

In this work we analyze the properties of the highly branched glycogen from *G. sulphuraria* in its native form and after enzymatic treatment with amyloglucosidase to further increase the proportion of branching bonds and decrease the molecular weight. For comparison we also analyze the properties of two other branched glucose polymers prepared from starch. By doing this, we want to assess the feasibility of using *G. sulphuraria* glycogen as alternative substrate to starch for the production of highly branched glucose polymers with applicability in several fields.

2. Materials and methods

2.1. Alga strain, cultivation conditions and glycogen extraction

Galdieria sulphuraria strain SAG 108.79 was obtained from the culture collection of the University of Göttingen (Sammlug von Algenkulturen, Germany). Cells were maintained growing on plates of Allen's mineral medium (Allen, 1959) at pH 4 with 1.5% (w/v) agar at 40 °C and constant illumination of 100 μE/(m^2 s). Colonies were transferred to a fresh plate once a month.

For glycogen extraction, *G. sulphuraria* was grown until late exponential phase in Allen medium at pH 2 supplemented with 1% (w/v) glycerol in complete darkness at 40 °C on a rotary shaker at 150 rpm. Cell growth was monitored by measuring the OD at 800 nm. Glycogen was extracted from *G. sulphuraria* cells as previously described (Martinez-Garcia et al., 2016).

2.2. Carbohydrate samples

Highly Branched Starch (HBS), Eliane C100 (waxy potato starch) and Avebe MD20 (a mixture of maltodextrins with an average DP of 6) were a kind gift from Avebe (Veendam, The Netherlands). HBS was prepared by treating potato starch with the thermostable glycogen branching enzyme from *Rhodotermus obamensis* (Shinoahara et al., 2001) commercialized by Novozymes under the name of Branchzyme™. Glucose and maltose were purchased from Sigma-Aldrich.

2.3. Preparation of glycogen and HBS treated with amyloglucosidase

Both the native glycogen from *G. sulphuraria* and HBS were dissolved in sodium acetate buffer 200 mM pH 4 containing 5 mM $CaCl_2$ to a concentration of 10 mg/mL and treated with amyloglucosidase (AMG) from *Aspergillus niger* (Megazyme) at an enzyme dose of 100 U/g substrate for 180 min at 40 °C with constant mixing by stirring. The reaction was stopped by incubation at 100 °C for 15 min and the AMG-treated polymer was precipitated by adding 1 vol of ethanol 100%, recovered by centrifugation at 10,000 × *g* for 10 min and freeze-dried. The dry sample was resuspended in ultra-pure water, centrifuged at 20,000 × *g* for 10 min to remove the inactivated enzyme and dialyzed against ultra-pure water at 4 °C for 24 h using a membrane with a molecular weight cut-off of 100–500 Da (Spectrum Labs) in order to remove any possible traces of enzymatically-released glucose and of glycerol coming from the solution in which the commercial amyloglucosidase is supplied. The polymer was re-precipitated from the dialyzed solution with 1 vol. of ethanol 100% and freeze-dried.

2.4. Determination of the degree of branching, weight-average molecular weight (M_w) and chain length distribution of the polymers

The branching degree of the polymers was determined by ^{1}H NMR analysis as previously described (Martinez-Garcia et al., 2016). The size distribution of the polymers was obtained by size exclusion chromatography (SEC) on an Agilent Technologies 1260 Infinity system (PSS) equipped with a PFG guard-column (PSS) and three columns with porosities of 100 Å (MZ Super-FG, MZ), 300 Å and 4000 Å (PFG, PSSPSS), using DMSO/0.05 M LiBr as eluent at a flow rate of 0.5 mL/min. Data from the multi-angle light scattering (SLD 7000, PSS) and refractive index (G1362A 1260 RID, Agilent Technologies) detectors were processed with WinGPC Unity software (PSS). The M_w from glycogen, glycogen-AMG and HBS was determined from the light scattering signal. A *dn/dc* value of 0.072 (that of pullulan in DMSO, measured and provided by PSS) was applied for M_w calculations. The M_w of HBS-AMG was determined by universal calibration with a standard pullulan series with M_w between 342 and 194000 Da.

For chain length distribution analysis, the polymers were dissolved in sodium acetate buffer 10 mM pH 5 containing 1 mM $CaCl_2$ and debranched by adding 5U of isoamylase from *Pseudomonas* sp., 4.9U of pullulanase M1 from *Klebsiella planticola*, and 6U of oligo-α-1,6-glucosidase (all three enzymes from Megazyme) and incubating at 40 °C for 24 h. Debranched samples were diluted 1:5 in water and analyzed by high pH anion exchange chromatography coupled with pulse amperometric detection (HPAEC-PAD) on a ICS3000 workstation equipped with a CarboPac PA-1 column (2 × 250 mm) and a ICS3000 ED detector (Dionex) using a linear gradient from 30 to 600 mM sodium acetate in 100 mM NaOH in 55 min. A standard series of glucose and malto-oligosaccharides of DP2-DP7 was used for peak assignation.

2.5. In vitro *digestion with pancreatic α-amylase and size distribution of products*

Polymers were dissolved at a concentration of 10 mg/mL in Hepes buffer 100 mM pH7 containing 5 mM $CaCl_2$ and treated with porcine pancreatic α-amylase (type VI-B, Sigma-Aldrich) at an enzyme dose of 300 U/g substrate. The reaction was performed at 37 °C. Aliquots were taken at different times and the enzyme inactivated by incubation at 100 °C for 5 min.

The amount of reducing groups in the reaction aliquots was measured by the Nelson-Somogyi method (Nelson, 1944) and expressed as increase in reducing ends with respect to time 0 min (substrate solution with inactivated enzyme solution added) using maltose as standard. For the size distribution of the products generated after treating the polymers with porcine pancreatic α-amylase for 180 min at 37 °C, an aliquot of the reaction was freeze-dried and dissolved in DMSO containing 0.05 M LiBr and the sample was analyzed by SEC as described in Section 2.4.

2.6. In vitro *digestion with amylase and amyloglucosidase and determination of digestible fractions*

The digestibility of the polymers was measured with a procedure based on the Englyst method (Englyst, Kingman, & Cummings, 1992) with some modifications. Polymers were dissolved at a concentration of 10 mg/mL in sodium acetate 100 mM pH 5.5 containing 5 mM $CaCl_2$ and treated with 100 U/g substrate of porcine pancreatic α-amylase (type VI-B, Sigma-Aldrich) and 100 U/g substrate of amyloglucosidase from *Aspergillus niger* (Megazyme) at 37 °C for 180 min. Aliquots were taken at different times and the enzymes were inactivated by incubation at 100 °C for 5 min. The amount of glucose released by the enzymes after 20 min and 120 min was measured using a glucose oxidase-peroxidase kit (Megazyme). The proportion of undigested sample was calculated by subtracting the glucose released after 120 min from the total sample dry weight.

Table 1
Structural features of glycogen, highly branched starch and the polymers obtained by amyloglucosidase treatment.

	% Branching	Mw (Da)
Glycogen	17.7 ± 0.8[a]	2.3×10^5
Glycogen– AMG	32.0 ± 0.6[a]	1.1×10^5
HBS	8.2	1.2×10^5
HBS – AMG	27.2 ± 3.3[a]	1.8×10^4

[a] Values represent the average of three independent polymer batches ± standard deviation.

2.7. Determination of osmolality

The polymers were dissolved in ultra-pure water and small solutes that might be present in the sample and could influence osmolality values were removed by dialysis and ethanol precipitation. The clean, dry polymers were then dissolved in ultra-pure water at concentrations of 10, 20, 40 and 50% (w/v). Osmolality was determined by measuring freezing point depression in an Osmomat 030 cryoscopic osmometer (Gonotec) using a sample volume of 60 μL. Calibration of the equipment was performed using NaCl solutions of 300, 850 and 2000 mOsm/Kg.

2.8. Determination of viscosity as a function of temperature

The polymers were dissolved in ultra-pure water at concentrations of 25% and 40% (w/v). 2 mL of sample were applied on a Haake MARS III rotational rheometer (ThermoFisher scientific) and the viscosity of the samples was measured in a temperature range from 4 to 40 °C at a constant low shear rate of 5 s^{-1} to avoid any influence of the shear rate on the viscosity of the solution due to shear thinning and to allow polymer chain architecture to be the main determinant of viscosity.

3. Results and discussion

3.1. Effect of amyloglucosidase action on the polymer structure

The glycogen of *G. sulphuraria* and HBS were treated with an enzyme in order to generate polymers with an increased proportion of branching linkages combined with a smaller molecular mass and to evaluate their properties.

In this case, the chosen enzyme was amyloglucosidase (EC 3.2.1.3) instead of β-amylase (EC 3.2.1.2), which has been used in previous works to generate highly branched maltodextrins from starch (Lee et al., 2013). Both enzymes are *exo*-acting and hydrolyze linear linkages starting at the non-reducing ends of the side chains. However, amyloglucosidase can also hydrolyze α-(1 → 6) bonds when the next bond in the sequence is α-(1 → 4) (Pazur & Ando, 1959) whereas β-amylase cannot by-pass the branching point. In this way, the trimming of the polymer would not be limited only to the exterior chains and could continue further after the branching linkage has been removed, allowing a greater decrease in the molecular mass of the polymer. This would be especially relevant for the treatment of *G. sulphuraria* glycogen because virtually all of its side chains are equal to or shorter than 10 glucose residues (Martinez-Garcia et al., 2016) and the action of β-amylase would be limited immediately.

The treatment with amyloglucosidase increased significantly the proportion of branching linkages of both glycogen and HBS (Table 1). Glycogen-AMG contained almost double the amount of

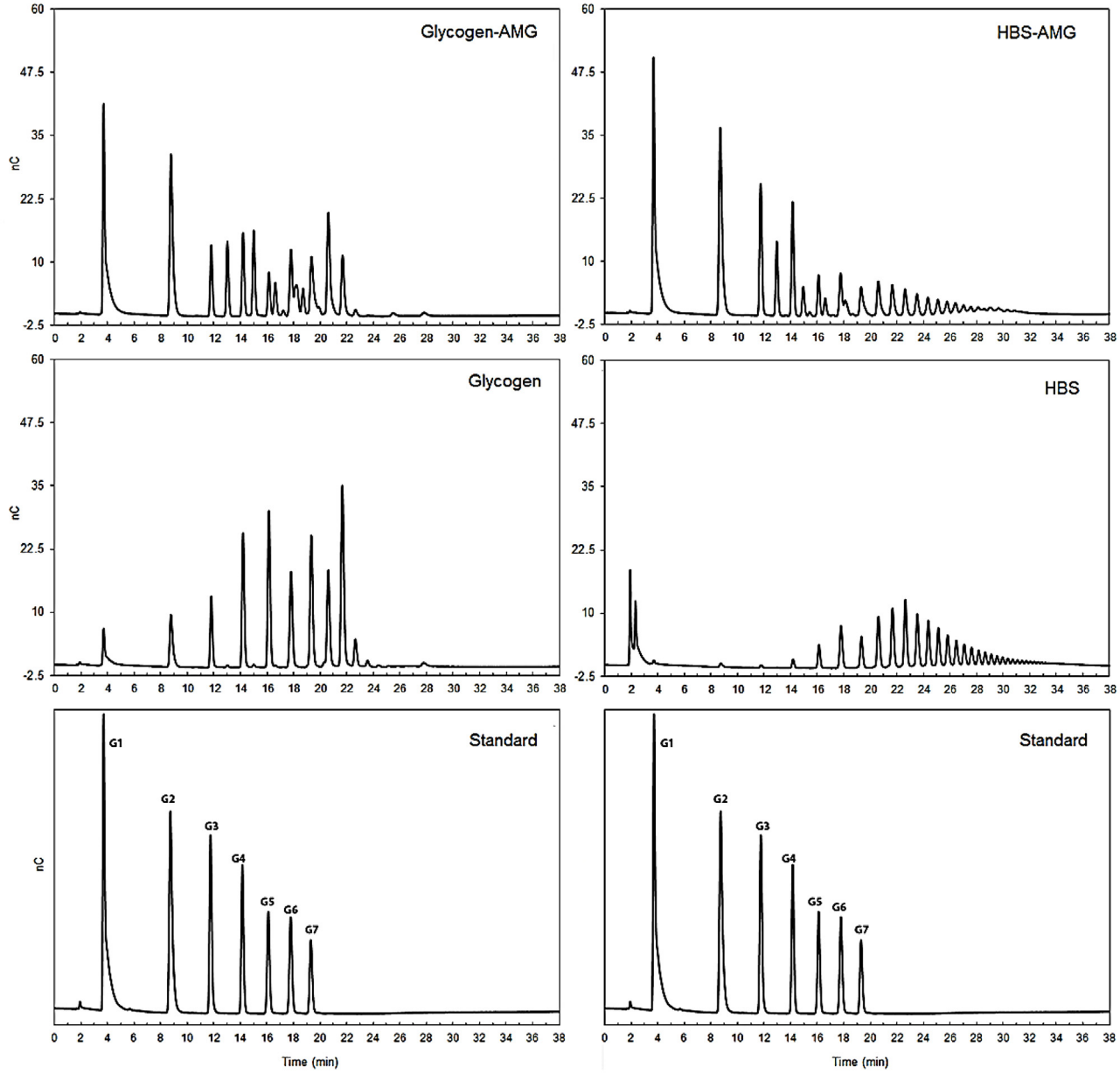

Fig. 1. HPAEC-PAD elution profiles of the polymers after being debranched with a combination of isoamylase, pullulanase and oligo-α-1,6-glucosidase.

α-(1 → 6) bonds than glycogen (32.0% compared to 17.7%) and HBS-AMG showed around 3 times more α-(1 → 6) bonds than HBS (27.2% compared to 8.2%). Both amyloglucosidase-treated polymers had a smaller M_w compared to the original polymers (Table 1). However, the decrease in M_w after enzyme treatment was less dramatic for glycogen (from 2.3×10^5 Da of the original glycogen to 1.1×10^5 Da of glycogen-AMG) than for HBS (from 1.2×10^5 Da of the original HBS to 1.8×10^4 Da for HBS-AMG). This lower extent of hydrolysis of glycogen compared to HBS might originate from the fact that α-(1 → 6) bonds are cleaved by amyloglucosidase more slowly than α-(1 → 4) (Ao et al., 2007; Kerr, Cleveland, & Katzbeck, 1951). Thus, *G. sulphuraria* glycogen, with more than double the amount of α-(1 → 6) linkages than HBS, would have less linkages hydrolyzed by the enzyme over the same period of time. Additionally, due to the short length of the side chains in glycogen and high% of α-(1 → 6) bonds, amyloglucosidase might have encountered steric hindrance, being limited to only hydrolyze the bonds on the most exterior tiers of the glycogen molecule and not being able to continue the trimming further towards the interior part. On the contrary, the longer side chains in HBS would give the enzyme more space to perform extensive hydrolysis and, in consequence, to decrease the molecular weight significantly.

The polymers were debranched with a combination of isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41) and oligo-α-1,6-glucosidase (EC 3.2.1.10) in an attempt to completely hydrolyze the branching bonds, even on very short chains (as those expected to be present in the amyloglucosidase-treated polymers), and the chain length distribution (CLD) of the linear fragments was determined by comparing the HPAEC-PAD elution profiles with a standard series of glucose and malto-oligosaccharides (DP1-DP7). As previously reported (Martinez-Garcia et al., 2016), the CLD of *G. sulphuraria* glycogen showed only short chains, up to DP 11, with chains of DP 4–9 being the most abundant ones (Fig. 1). Some of the branches in glycogen were already very short even before the trimming of the molecule by amyloglucosidase, since considerable peaks corresponding to chains of DP 1–3 could be observed. On the contrary,

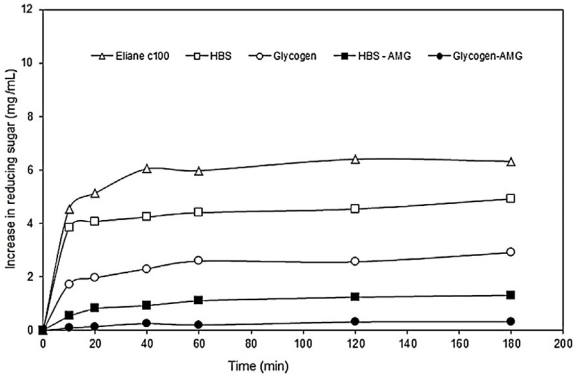

Fig. 2. Increase of reducing sugar over time during *in vitro* digestion of the polymers with pancreatic α-amylase.

these very short branches were practically absent in HBS and the majority of the chains had DP > 10. In both glycogen and HBS, the treatment with amyloglucosidase caused a significant increase in chains of DP1-3 as a result of an extensive hydrolysis of some longer chains by the enzyme. The CLD of both glycogen-AMG and HBS-AMG showed some extra peaks in between those corresponding to linear chains from DP3-DP7, most probably originating from oligosaccharides still containing an α-(1 → 6) bond, which elute earlier in HPAEC than their linear counterparts (van Leeuwen et al., 2008). This suggests that in some parts of the amyloglucosidase-treated polymers, the density of branching points was too high to allow the debranching enzymes to accommodate the chain in its active site and cleave the bonds.

3.2. Digestion properties of the polymers

The native and amyloglucosidase-treated glycogen and HBS were incubated with pancreatic α-amylase (EC 3.2.1.1) in order to describe their resistance to this enzyme. α-amylase is an *endo*-acting enzyme in charge of initiating carbohydrate digestion in the human body by hydrolyzing internal α-(1 → 4) bonds of glucans (Butterworth et al., 2011). The action of pancreatic α-amylase on the polymers was measured as increase in reducing ends over time. All four polymers were less degraded by α-amylase than the waxy potato starch (Eliane C100) used as standard. This is in accordance with their higher content of branching bonds compared to waxy potato starch. The native glycogen of *G. sulphuraria* was significantly more resistant to α-amylase degradation than HBS since the increase in reducing ends over time was around half that of HBS (Fig. 2). Both glycogen-AMG and HBS-AMG showed a much higher resistance to α-amylase degradation than the original polymers from which they had been derived (Fig. 2). Although both polymers seemed to be barely hydrolyzed by the enzyme, the increase in reducing ends over time for glycogen-AMG was even lower than for HBS-AMG. The four branched polymers were also incubated with human salivary α-amylase and the results were identical results to those obtained with porcine pancreatic α-amylase (data not shown).

The different resistance of the four polymers to pancreatic α-amylase action could also be seen in the size distribution of the polymers before and after digestion with the enzyme. The α-amylase hydrolysate of glycogen showed a bimodal distribution, with one peak corresponding to small molecular weight compounds (eluting at the same volume as maltose) and another peak corresponding to high molecular weight compounds showing a size distribution that partially matched that of the intact polymer (Fig. 3). This bimodal distribution of the products points to a multiple attack action of the enzyme on the glycogen molecule, as previously reported for pancreatic α-amylase (Bijttebier, Goessaert, & Delcour, 2008). In this mode of action, the enzyme hydrolyzes successively several linkages before dissociating from the substrate chain, generating as products maltose and a limit dextrin with a reduced M_w compared to the initial polymer (Atichokudomchai, Jane, & Hazlewood, 2005). The fact that the limit dextrins in the α-amylase hydrolysate of glycogen still eluted within the same volume range as the intact polymer suggests that, due to the high branch density in the glycogen molecule, the enzyme was limited to act solely on the chains localized in the most external tiers. This caused only a small reduction of the M_w of the polymer, in a similar way as what happened when glycogen was incubated with amyloglucosidase (Table 1).

A bimodal size distribution could also be observed for the α-amylase hydrolysate of HBS (Fig. 3). However, the limit dextrins generated as products from HBS showed a size distribution shifted towards smaller M_w compared to the intact polymer. This suggests that the lower% of branching points of HBS compared to glycogen (Table 1) provided more space for α-amylase to attack the internal chains, reducing the M_w of the resulting limit dextrin more drastically.

The high resistance to pancreatic α-amylase of glycogen-AMG depicted in Fig. 2 was corroborated by the size distribution of the products obtained after incubation with the enzyme. In this case, only a small proportion of the hydrolysate consisted of small molecular weight compounds (mainly maltose) and most of the products still had a polymeric nature with a size distribution almost matching completely that of the undigested polymer (Fig. 3). In glycogen-AMG, the chains localized in the most external tiers of the molecule had already been hydrolyzed by amyloglucosidase prior to the incubation with pancreatic α-amylase, leaving no substrate for this enzyme to act upon. In consequence, glycogen-AMG remained virtually intact after incubation with pancreatic α-amylase.

Despite the high resistance to α-amylase also displayed by HBS-AMG, the size distribution of the products in the α-amylase hydrolysate was more heterogeneous than for glycogen-AMG, with a big peak corresponding to limit dextrins of intermediate M_w (Fig. 3). Even though the proportion of α-(1 → 6) linkages in both glycogen-AMG and HBS-AMG is similar (Table 1), HBS-AMG still contains some longer chains (DP > 10) in its structure which are completely absent in glycogen-AMG (Fig. 1). This reduces the branch density in some parts of the HBS-AMG molecule, providing α-amylase with more space to attach to and hydrolyze some internal chains and generate as products smaller branched limit dextrins along with maltose.

In a previous work using a chemically synthesized branched malto-decaose containing two α-(1 → 6) linkages, it was hypothesized that a distance of only three glucose units between two branch points should be sufficient to enable porcine pancreatic α-amylase binding and bond cleavage (Damager et al., 2005). However, the short length of the branches in this malto-decaose, consisting of only 2 glucose units, might have facilitated the accommodation of the substrate in the active site of the enzyme, something that might be impossible when using a highly branched polymer with longer side chains such as glycogen.

The four polymers were also incubated with a combination of pancreatic α-amylase and amyloglucosidase to simulate their digestion in the human body and to assess their impact on the glycemic index (GI) by determining the proportion of sample converted to glucose after 20 and 120 min, as described in the Englyst method (Englyst et al., 1992). Glycogen showed a higher overall resistance to digestion than HBS and the waxy potato starch (Eliane C100) used as a control, since 64% of the sample was still not converted into glucose after 120 min (Table 2). Both glycogen-AMG and HBS-AMG were highly resistant to the digestive enzymes, with

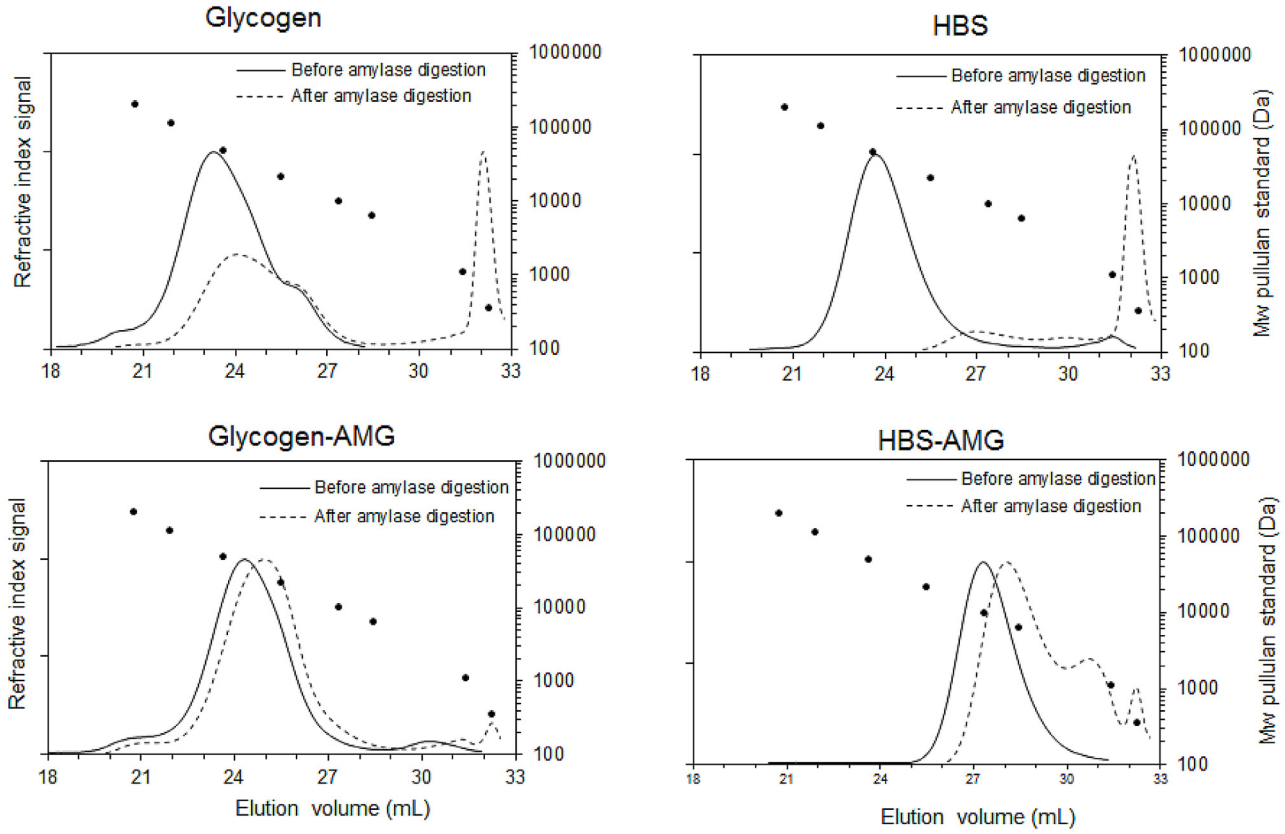

Fig. 3. Molecular size distribution of the polymers before and after digestion with pancreatic α-amylase. Black dots represent the elution volume of pullulan standards.

Table 2
Proportion of sample (% of total dry weight) that was released as glucose or remained undigested during incubation of the polymers with pancreatic α-amylase and amyloglucosidase at 37 °C.

	Sample digested to glucose (%)		Undigested sample (%)
	After 20 min	Between 20–120 min	After 120 min
Eliane c100	34.2	52.1	13.7
HBS	29.4	26.6	44.0
Glycogen	17.8	18.2	64.0
HBS-AMG	2.3	9.3	88.3
Glycogen-AMG	1.5	3.8	94.7

close to or more than 90% of the initial sample not hydrolyzed to glucose at the end of the incubation time. The amount of glucose released during the first 20 min, denominated as rapidly available glucose (RAG) in the Englyst method, has the largest impact on the GI (Englyst, Veenstra, & Hudson, 1996). In this regard, glycogen would have a significantly lower influence on the GI than HBS or amylopectin since the amount of glucose released within 20 min was around half the amount released from the other two polymers. The levels of RAG from glycogen-AMG and HBS-AMG were comparable, 1.5 and 2.3%, respectively, and show that these two polymers would have a negligible contribution to the GI. In view of the high proportion of undigested sample measured *in vitro* for glycogen and glycogen-AMG, it would be of interest to assess potential prebiotic properties of these polymers *in vivo*.

The digestion results presented here, although obtained by preliminary tests under *in vitro* conditions, show the high resistance of the glycogen-type polymers to degradation by α-amylase alone or in combination with amyloglucosidase. This characteristic could be a potential drawback for their application as osmotic agents in peritoneal dialysis solutions. During the dialysis procedure, the polymer is not significantly degraded while being in the peritoneal cavity of the patient (De Waart, Zweers, Struijk, & Krediet, 2001). However, it can be assimilated *via* the lymphatic system and reach the blood plasma, where it is hydrolysed by circulating α-amylase into maltose and other high M_w fractions (Mistry & Gokal, 1993). These products are not further metabolized by the patient due to renal failure and therefore accumulate in the system. In the case of glycogen-type polymers displaying high resistance to enzymatic degradation, most accumulated products would be of high M_w, which could lead to potentially adverse effects. Additional studies mimicking more *in vivo* conditions are necessary to confirm the high resistance of glycogen-type polymers to enzymatic degradation and to test their safety when applied into peritoneal dialysis solutions.

3.3. Osmolality and viscosity properties of the polymers

The osmolality of glycogen, HBS and the amyloglucosidase-treated polymers was determined by measuring freezing point depression in solutions of different concentration and compared to the osmolality of low molecular weight compounds such as glucose, maltose and Avebe MD20.

Table 3
Viscosity values as a function of temperature of the polymers in solution at two different concentrations.

T(°C)	Viscosity (mPa-s)							
	Glycogen		Glycogen-AMG		HBS		HBS-AMG	
	25%	40%	25%	40%	25%	40%	25%	40%
4	22	52	15	42	59	246	45	58
20	13	27	15	22	40	209	27	49
38	8	15	11	12	24	109	9	29

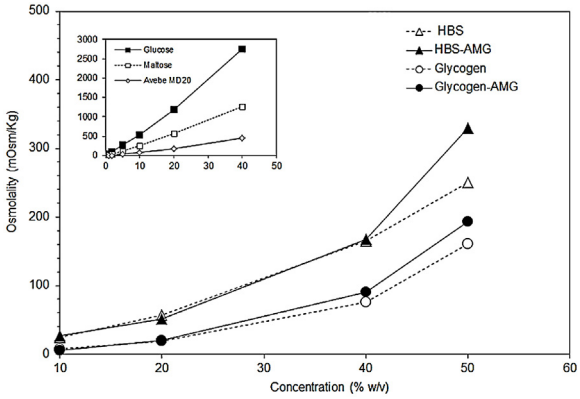

Fig. 4. Osmolality values of the glucose polymers in solution at different concentrations. Inset shows osmolality values of glucose, maltose and Avebe MD20 for comparison.

As expected for high M_w compounds, the osmolality values of the different polymer solutions at every concentration were more than one order of magnitude lower than that of the low molecular weight compounds (Fig. 4). Even at concentrations as high as 50% (w/v), solutions of glycogen, glycogen-AMG and HBS showed osmolality values below human plasma osmolality, reported to be 285–305 mOsm/Kg (Khajuria & Krahn, 2005). The amyloglucosidase-treated polymers showed higher osmolality values than the original polymers, in accordance with the fact that their M_w is smaller and thus, for a same dry sample weight, the number of molecules in solution is higher. The difference in osmolality between the two glycogen polymers was smaller than between the two HBS polymers, reflecting the smaller difference in M_w already discussed in Section 3.1.

A low contribution to osmolality is an advantage when using glucose polymers in applications such as formulation of sports drinks and peritoneal dialysis solutions. In sports drinks, low osmolality is correlated with a faster gastric emptying, allowing the sport drink to be quickly assimilated to provide fast rehydration and energy supply (Takii et al., 2005). In peritoneal dialysis, solutions of glucose polymers with an osmolality below that of blood plasma can still induce effective ultrafiltration (movement of excess body water into the peritoneal cavity through the peritoneum) by a mechanism known as colloid osmosis (Mistry & Gokal, 1993).

The viscosity of the four polymers in solution was measured at two different concentrations, 25 and 40% (w/v), using a fixed shear rate in a temperature range from 4 to 40 °C. The results presented in Table 3 show viscosity values at the three temperatures we considered more relevant in view of the potential applications for these polymers because they are similar to fridge, room and body temperature.

At a concentration of 25%, the viscosity values of all four polymers were of the same order, although HBS and HBS-AMG showed somewhat higher viscosity. However, at a concentration of 40%, a dramatic difference between the viscosity of the glycogen-type polymers and HBS was observed. This result reflects the differences in side chain length between the two types of polymers. In a more concentrated solution, the chance of chain entanglement between polymer molecules is higher than in a less concentrated solution (Guo, Zhang, Hu, Du, & Cui, 2016) and thus, differences in the length of the side chains have a bigger effect on the viscosity of the solution. When longer chains (DP > 10), present in the structure of HBS but absent in glycogen and glycogen-AMG (Fig. 1), get entangled in each other, they slow down the movement of the polymer molecules in the solvent more drastically than shorter chains, leading to the significant increase in viscosity observed. At 40%, the difference in viscosity between HBS and HBS-AMG was more significant than the difference between glycogen and glycogen-AMG, as a consequence of a more remarkable change on the chain length distribution profiles (with a decrease in longer chains in HBS) after the amyloglucosidase treatment.

4. Conclusions

The highly branched glycogen from *G. sulphuraria* in its native form showed improved digestive and rheological properties compared to HBS, a moderately branched glucose polymer produced by glycogen branching enzyme treatment of potato starch. Due to the short length of its side chains and the greater proportion of branching bonds, *G. sulphuraria* glycogen displays a high branch density that is responsible for its decreased susceptibility to digestive enzymes and its markedly lower viscosity in solution compared to HBS. Incubation of this glycogen with amyloglucosidase resulted in a hyperbranched glycogen that did not differ significantly from the native glycogen in its chain length distribution, M_w and rheological properties. This supported the theory that the already high branch density in the native glycogen of *G. sulphuraria* limits the action of hydrolytic enzymes, leaving only a little margin for structural modification by glycoside hydrolases. Nevertheless, the hyper branched glycogen obtained by amyloglucosidase treatment of the native glycogen was virtually resistant to digestive enzymes, a characteristic that might be advantageous for certain applications where polymer digestion is undesirable. The results of the present study show that the native glycogen extracted from the extremophilic red microalga *G.sulphuraria* could be readily used as highly branched glucose polymer with slowly digestible properties or as substrate to produce resistant polymers, thus representing an alternative to starch-derived polymers.

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