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Structural and physical properties of granule stabilized starch obtained by branching enzyme treatment

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ABSTRACT

Chemical cross-linking of starch is an important modification used in the industry for granule stabilization. It has been demonstrated that treatment with branching enzyme (BE) can stabilize the granular structure of starch and such treatment thereby provides a potential clean alternative for chemical modification. This study demonstrates that such BE-assisted stabilization of starch granules led to partial protection from BE catalysis of both amylose (AM) and amylopectin (AP) in their native state as assessed by triiodide complexation, X-ray diffractometry (XRD) and differential scanning calorimetry (DSC). The granule stabilizing effects were inversely linked to hydration of the starch granules, which was increased by the presence of starch-phosphate esters and suppressed by extreme substrate concentration. The data support that the granule stabilization is due to the intermolecular transglycosylation occurring in the initial stages of the reaction prior to AM–AP phase separation. The enzyme activity needed to obtain granule stabilization was therefore dependent on the hydration capability of the starch used.

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1. Introduction

Starch is the primary energy storage of higher plants and is an important part of the human diet. Due to its unique physical properties it is a widely used food ingredient, serving as a gelling agent, thickener or stabilizer (Mason, 2009). In its native state, starch consists of the two glucose polymers AM and AP, which by the plant have been deposited as water insoluble, highly organized, semicrystalline granules (Damager, Engelsen, Blennow, Møller, & Motawia, 2010). When heated in presence of water the starch granules swell due to water uptake of mainly AP, which thereby loses its organization. Also part of the AM leaches out and eventually the granules rupture (Srichuwong & Jane, 2007). Starch gelatinization as a fundamental characteristic makes starch a useful food ingredient. Phosphate groups covalently monoesterified to the C-6 and C-3 positions of starch are tremendously important to support starch granule plasticity, starch hydration and starch paste viscosity (Hansen et al., 2009; Hejazi et al., 2008; Viksø-Nielsen et al., 2001). The functional properties of native starches in most cases are not directly useful as ingredients. Most starches are therefore modified either physically or chemically in order to better suit diverse applications. Covalent cross-linking is a common starch modification used to obtain better granular stability and increased resistance of the starch toward acidity, heating and shearing during food processing (Chiu & Solarek, 2009). Traditionally, cross-linking is done chemically by using agents such as phosphoryl chloride, sodium trimetaphosphate, sodium tripolyphosphate, epichlorohydrin, or adipic–acetic mixed anhydride (Koo, Lee, & Lee, 2010; Mason, 2009). In recent years, there has been intense focus on cleaner technologies for modification of starch. For example, for cross-linking starch films, green alternatives using organic acids such as citric acid and malonic acid as cross-linking agents have been developed (Dastidar & Netravali, 2012; Reddy & Yang, 2012). Another green alternative using enzyme technology to obtain starch with a stabilized granular structure was introduced in a recent study (Jensen, Larsen, Bandsholm, & Blennow, 2013). Here it was shown that granule stabilization of certain starch types could be obtained by enzymatic modification using BE under specific circumstances. Stabilization herein refers to a tightly packed granular structure which was obtained after BE treatment and observed by microscopy in contrast to the control samples, which showed highly swollen "ghost structures". It was shown that potato, maize and a transgenic low-phosphate potato starch (asGWD – antisense

Abbreviations: AM, amylose; AP, amylopectin; asGWD potato starch, antisense glucan water dikinase; BE, branching enzyme; DM, dry matter; DSC, differential scanning calorimetry; GPC, gel-permeation chromatography; LD, limit dextrins; WAXS, wide angle X-ray scattering.

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Glucan Water Dikinase) could all be modified at high substrate concentration by BE, but the remaining granular structure after modification was highly dependent on the content of starch-bound phosphate. High-phosphate starches, such as potato starch, completely lost their granular structure upon treatment with heat and high activity of BE. In contrast, granules from starches with low phosphate content, such as maize, were stabilized and appeared as native granules after high activity BE treatment, while a large part of the granular organization in the control maize samples was lost. When treated at low BE activity, high-phosphate starch, as well as low-phosphate starch, lost granular structure. The observations were explained with a hypothetic model, suggesting that high BE activities were able to connect starch segments through intermolecular transglycosylation prior to gelatinization when treated at high substrate concentration and thereby keeping the main granular structure intact. A more general model for the observed molecular effects remains to be explored.

In this study, starches with different molecular and structural features, were subjected to enzyme treatments at lower starch concentrations. Physical and structural properties of these modified starches were analyzed with diverse techniques. Further evidence for the presence of extensively modified starch granules locked by a network of branch linkages formed by BE treatment is presented.

2. Materials and methods

2.1. Materials

Starches from potato, maize, pea, tapioca and wheat were obtained from KMC (Brande, Denmark). Low phosphate potato starch was isolated from a transgenic glucan water dikinase antisense suppressor line (asGWD) (Viksø-Nielsen et al., 2001).

A preparation of BE (α-1,4 → α-1,6 glycosyltransferase, E.C. 2.4.1.18) was obtained from Novozymes, Bagsværd, Denmark. The enzyme was produced by heterologous expression of a synthetic gene coding for glycogen BE from *Rhodothermus obamensis* in a genetically modified strain of *Bacillus subtilis*. The preparation was tested for absence of amylolytic activity (<0.2 CU/mL by Ceralpha method (Megazyme, Wicklow, Ireland).

α-Amylase from *Bacillus amyloliquefaciens* (EC 3.2.1.1) was obtained from Megazyme (Wicklow, Ireland). The activity of α-amylase was determined at pH 6.5 and 25 as previously described °C (Bertoft, Manelius, & Qin, 1993). β-Amylase of barley (EC 3.2.1.2, specific activity 705 U/mg) was obtained from Megazyme.

2.2. Enzymatic modification

Reaction mixtures were prepared in microfuge vials with 30% and 40% (w/v) dry matter (DM) starch, respectively. Samples of 1.00 g were thus prepared by mixing 300 mg or 400 mg DM starch, respectively, with 50 mM sodium phosphate buffer, pH 6.5, and BE at 25 °C. BE was added in volumes equalling 0, 750, 1500 and 2250 BE units per gram sample, respectively, and are here denoted control samples and samples with low, medium and high BE activity respectively. We defined one BE unit as the quantity of the enzyme that causes a decrease in absorbance at 660 nm of an AM–triiodide complex of 1%/min (pH 7.2; 60 °C). The samples were incubated in a thermomixer (Eppendorf, Hamburg, Germany) with continuous mixing (1400 rpm) for 1 h at 70 °C. Following incubation, samples were cooled to 25 °C and the enzyme was inactivated by lowering pH to 1 using 4 M HCl, incubated for 15 min at 25 °C, and neutralized to pH 6.5 using 4 M NaOH and dried at 30 °C *in vacuo*. Low substrate controls comprised 3% DM and 0, 75 or 225 BE units per sample, respectively.

Time course studies were conducted with 1.00 g samples as described above. Individual samples were prepared for each time point and the reaction was stopped at the given time as described above.

Control and high BE activity samples of maize, potato and asGWD potato starch were prepared on a larger scale (250 g reaction mixture). The reactions were carried out in beakers in a water bath and were stirred by a steel propeller on a shaft. Stirring was stopped once the reaction mixture had reached 70 °C.

2.3. Starch phosphate monoester content

Starch phosphate monoester content was determined as content of glucose 6-phosphate and glucose 3-phosphate after hydrolysis of starch and analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex BioLC system; Dionex Corp., Sunnyvale, CA, USA). Starch hydrolysates with verified glucose-6-phosphate and glucose-3-phosphate contents were used as standards and injected repeatedly along with the samples (Blennow, Bay-Smidt, Wischmann, Olsen, & Møller, 1998).

2.4. Microscopy

The granular state of the starch samples was visualized by bright field light microscopy as described by Jensen et al. (2013). Fluorescence images for visualization of enzyme penetration into the starch granules were obtained after staining the samples (5 mg) with 30 μL SYPRO Ruby Protein Gel Stain (Invitrogen, California, USA) for 2 h using a fluorescence Leitz, SM-Lux-Pol, Leica Microsystems (Leitz, Wetzlar, Germany) microscope with a fluorescent filter (450 nm). Images were taken using a Leica DC300F camera and IM50 software.

2.5. Average DP of debranched starch determined by HPAEC-PAD

Samples were gelatinized and enzymatically debranched at 40 °C by 0.3 unit isoamylase (Megazyme) per 1 mg of sample. The obtained linear glucan fragments were analyzed by high-pressure anion-exchange chromatography with pulsed amperometric detection. Samples of 20 μL (100 μg of linear α-glucan) were injected on a CarboPac PA-200 column using 0.4 mL/min flow rate, 150 mM isocratic NaOH and the following NaOAc gradient profile: 0–5 min: 0–110 mM linear gradient, 5–130 min: 110–350 mM convex gradient. Single peaks between the degree of polymerization (DP) 2–80 were integrated and corrected for detector response (Blennow et al., 1998), and the average DP was calculated from the corrected values of the relative content of each chain.

2.6. Amylose (AM) content

AM content was determined by triiodide colorimetry (Wickramasinghe, Blennow, & Noda, 2009).

2.7. Iodine vapor exposure

The ability of iodine to complex starch chains in granules was determined by equilibrating starch samples at room temperature in a desiccator (25 cm diameter and 2.0 cm deep) containing saturated K_2SO_4 solution (300 mL). Samples were weighed and equilibrated to 0.97 water activity. Sodium azide (1%, w/w) was added to the K_2SO_4 saturated salt solution to prevent microbial growth. To determine iodine binding, a thin layer of the equilibrated starch sample (0.2 g) was spread in a plastic dish and exposed for 24 h at room temperature to iodine vapour generated from 2 g of iodine crystals placed in the desiccator containing the starch samples. The

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