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Preparation and characterisation of linear dextrins and their use as substrates in in vitro studies of starch branching enzymes

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Abstract

Essentially linear glucose chains with a relatively narrow molecular weight range were produced by enzymatic degradation of commercially available retrograded starch. The retrograded maize starch was hydrolysed by thermostable α-amylase and amyloglucosidase, which degraded the enzyme-available starch fraction. Gel permeation chromatography (GPC) of the enzyme-resistant dextrins revealed a molecular weight distribution with a peak maximum at a degree of polymerisation (dp) 50–60. Hydrolysis with β-amylase gave a β-amylolysis limit of 92%, suggesting that the dextrins were essentially linear. These linear dextrins were used as a substrate in a study of starch branching enzyme I from potato. The enzyme products were debranched and analysed by HPAEC-PAD revealing two major populations of chains with a dp around 11 and 30, respectively. GPC analysis of the same sample, before debranching, gave a peak with a maximum similar to that of the original substrate. However, hydrolysis of α-(1,6)-linkages by isoamylase clearly shifted the elution peak towards lower molecular weights and showing also that the majority of the glucose chains being longer than 60 glucose units had been used by the branching enzyme.

Keywords: Linear dextrins; Gel permeation chromatography; Potato starch branching enzyme

1. Introduction

Starch is a major storage carbohydrate in plants. It consists of two types of glucose polymers, amylopectin which is a highly branched macromolecule composed of glucose residues linked together with α-(1,4)- and α-(1,6)-linkages and amylose which is composed of mainly linear glucose chains containing α-(1,4)-linkages.

Starch polymers can be hydrolysed enzymatically by α-amylase, which is one of the enzymes responsible for in vivo degradation of starch. Retrograded starches, however, are not completely hydrolysed during digestion by α-amylase, leaving an enzyme-resistant starch fraction that consists mainly of linear amylose-type chains (Cairns, Morris, Botham & Ring, 1996; Russell, Berry & Greenwell, 1989; Siljeström, Eliasson & Björk, 1989). The enzyme-resistant residue is partially crystalline (Cairns, Leloup, Miles, Ring & Morris, 1990; Russell et al., 1989; Siljeström et al., 1989) which makes it resistant to hydrolysis by α-amylase unless the sample has been dissolved in alkali or DMSO (Englyst & Cummings, 1984). The glucose chains in the crystallites are organised as double helices (Wu & Sarko, 1978) where a minimum degree of polymerisation (dp) of 10 is thought to be required for the formation of a double helix (Gidley & Bulpin, 1987). However, chains containing as few as six glucose units can co-crystallise in the presence of longer chains (Gidley & Bulpin, 1987). The average dp of the enzyme-resistant residue has previously been reported to 40–70 glucose units in retrograded starch or amylose obtained from different botanical sources (Jane &Robyt, 1984; Russell et al., 1989; Siljeström et al., 1989).

Starch is synthesised by a number of enzymes including starch synthases, branching enzymes and debranching enzymes (Smith, 1999). For many of these enzymes their precise mechanism and their role in starch synthesis are not yet fully understood. The branching enzymes, which are responsible for the formation of α-1,6-linkages in starch, can be divided into two classes. Potato starch branching enzyme I (SBEI) belongs to class B, according to the division into class A and B by Martin and Smith (1995) based on amino acid sequences and activity characteristics, where class B is relatively more active on longer chains. The branching reaction of this enzyme has been reported to be an inter-molecular chain transfer (Borovsky, Smith & Whelan, 1976; Viksø-Nielsen, Blennow, Hamborg Nielsen

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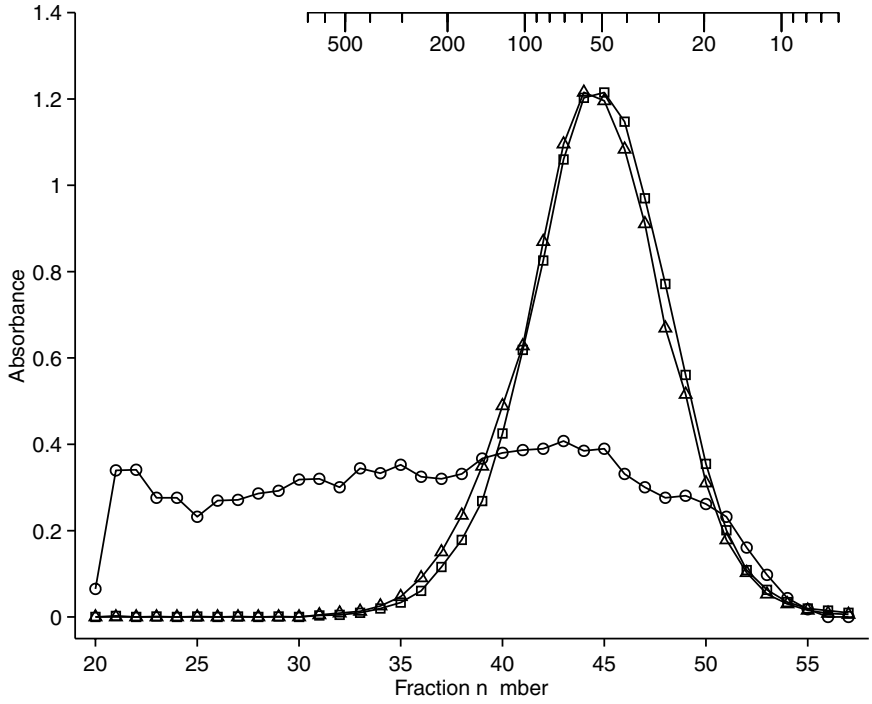

Fig. 1. GPC elution profile of essentially linear dextrins prior to (-△-) and after (-□-) hydrolysis by isoamylase. Retrograded starch prior to hydrolysis by α-amylase and amyloglucosidase is also shown (-○-). Dp values obtained after column calibration with pullulans are shown on the upper axis.

& Lindberg-Møller, 1998), although intra-molecular transfer of chains could not be excluded from the results in those experiments.

For studies of the action of branching enzymes and their effects on starch structure, methods such as iodine staining, phosphorylase stimulation assay and branch-linkage assay, have commonly been employed (Boyer and Preiss, 1978; Krisman, 1962; Peat, Whelan & Bailey, 1953; Takeda, Guan & Preiss, 1993). Today, high performance anion exchange chromatography (HPAEC) is a frequently used method of analysis of the resulting enzyme products. As an enzyme substrate, amylose or amylopectin could be used. However, a well-characterised linear substrate with a narrow molecular weight range would be preferred.

The purpose of this study was to prepare and characterise linear dextrins and to use these as substrates in a study of starch branching enzyme action.

2. Materials and methods

2.1. Material

Retrograded high-amylose maize starch (Novelose®) was obtained from National Starch and Chemical Company, Bridgewater, USA. A non-commercial sample of retrograded high-amylose maize starch produced by Cerestar, Vilvoorde, Belgium was used in some analyses. Thermostable *Bacillus licheniformis* α-amylase (EC 3.2.1.1) and *Aspergillus niger* amyloglucosidase (EC 3.2.1.3) were obtained from Megazyme, Bray, Wicklow, Ireland. Isoamylase (EC 3.2.1.68, 59 000 U/mg protein) was purchased from Hayashibara Biochemical Laboratories, Okayama, Japan and β-amylase (EC 3.2.1.2, 880 U/mg protein) from Sigma Chemical, St Louis, MO, USA. Potato branching enzyme I (103 kDa form) was over-expressed in *E. coli* and purified by starch affinity chromatography and ion-exchange chromatography as described by Khoshnoodi (1997). Pullulan standards were from Shodex, Tokyo, Japan.

2.2. Preparation of linear dextrins

Retrograded starch (approx. 500 mg) was dispersed in 15 ml of 0.1 M acetate buffer pH 5.0 and treated with 40 μl α-amylase (300 U/ml) for 1 h in a boiling water bath. After cooling, 500 μl of amyloglucosidase, diluted to 140 U/ml in the acetate buffer, was added and the sample was incubated overnight at 60°C. Finally, the sample was centrifuged, washed twice with the acetate buffer, twice with acetone and then dried overnight at 40°C.

2.3. β-Amylolysis

β-Amylolysis was performed by dissolving 5 mg of the dextrins in 200 μl DMSO in a boiling water bath. The sample was then diluted with 1 ml of 0.1 M sodium acetate, pH 4.8 and 1 μl of β-amylase (22 880 U/ml) was added. The incubation (at 25°C) was stopped after 5 h by boiling the sample for 5 min. The sample was diluted 1:1 with water prior to injection on a Biogel P-2 column (1,6 × 90 cm)

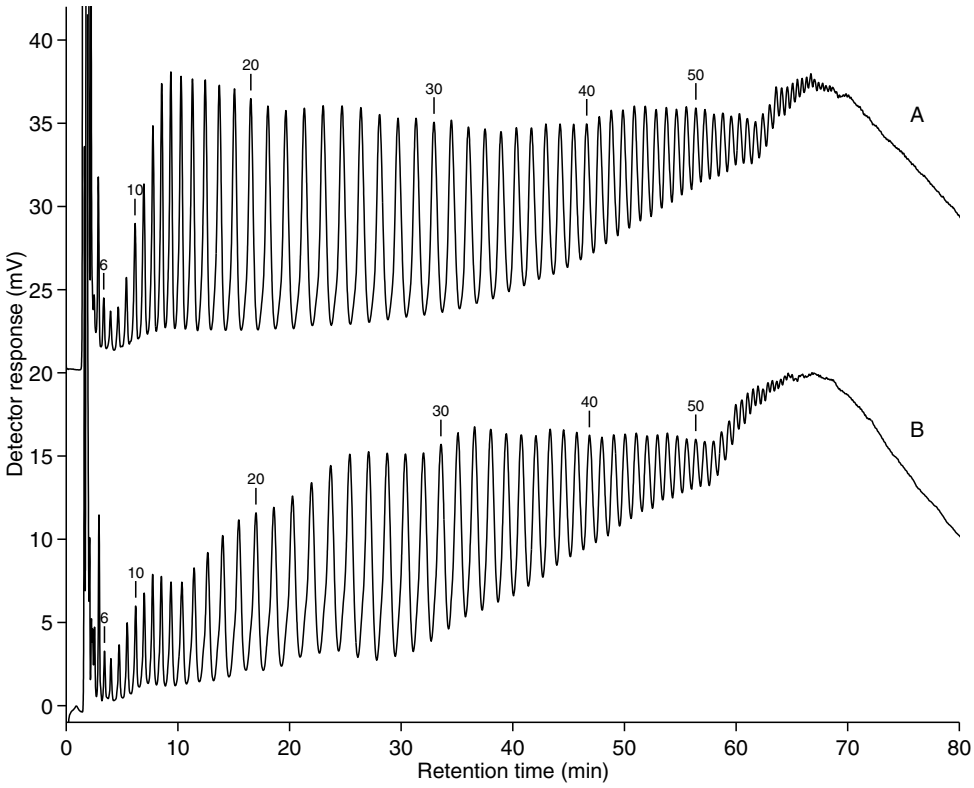

Fig. 2. High performance anion exchange chromatography of linear dextrins prepared from Novelose (A) and Cerestar starch (B). Dp values are indicated above the peaks.

eluted with water. Carbohydrates in collected fractions (2 ml) were detected by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The β-amylolysis limit was calculated as the proportion of carbohydrates with a dp <4.

2.4. Chromatographic methods

Gel permeation chromatography (GPC) on a Sepharose CL-6B column eluted with 0.25 M KOH was conducted as previously described (Fredriksson, Silverio, Andersson, Eliasson & Åman, 1998). The relative amounts of carbohydrate in the collected fractions were measured by the phenol–sulphuric acid method (Dubois et al., 1956). High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and a CarboPac PA-100 column was used as described by Koch, Andersson & Åman (1998). The samples for HPAEC were either dissolved in 150 mM NaOH or in the branching enzyme incubation mixture described below.

2.5. Assay for potato starch branching enzyme I

Samples for analysis by HPAEC or GPC were prepared by dissolving linear dextrins (6–8 mg) in 100 μl of 2 M KOH. 1 ml of water and 200 μl of 0.5 M Tris buffer pH 7.6 was added, the pH was adjusted to 7.6 with HCl and the sample was diluted with water to 2 ml. To 900 μl of the solution was added 100 μl of a 1 μM solution of starch branching enzyme I or 100 μl of water (control sample). The samples were incubated for 16 h at room temperature and the reaction was then terminated by heating in a boiling water bath for 5 min. 1 M acetate buffer pH 3.6 (~150 μl) and 5 μl of isoamylase (295 U) were added and the samples were incubated at 38°C for 5 h. Prior to injection the enzymatic reaction was stopped by boiling for 5 min and the pH of the samples was adjusted to pH > 10 using M NaOH.

3. Results and discussion

3.1. Preparation and characterisation of linear dextrins

The starting material, commercially available retrograded maize starch, was hydrolysed with α-amylase and amyloglucosidase producing α-amylase-resistant fractions. About 2/3 of the original material was hydrolysed during this enzyme treatment.

Chromatographic and enzymatic methods were used to determine molecular size distribution and branching of the dextrins. GPC elution profiles of retrograded starch (Novelose®) prior to and after hydrolysis with α-amylase and amyloglucosidase are shown in Fig. 1. The retrograded maize starch exhibited a broad molecular weight distribution while the elution profiles of the dextrins revealed a peak with a maximum in the range of dp 50–60 based on column calibrations with pullulan standards. This was in line with

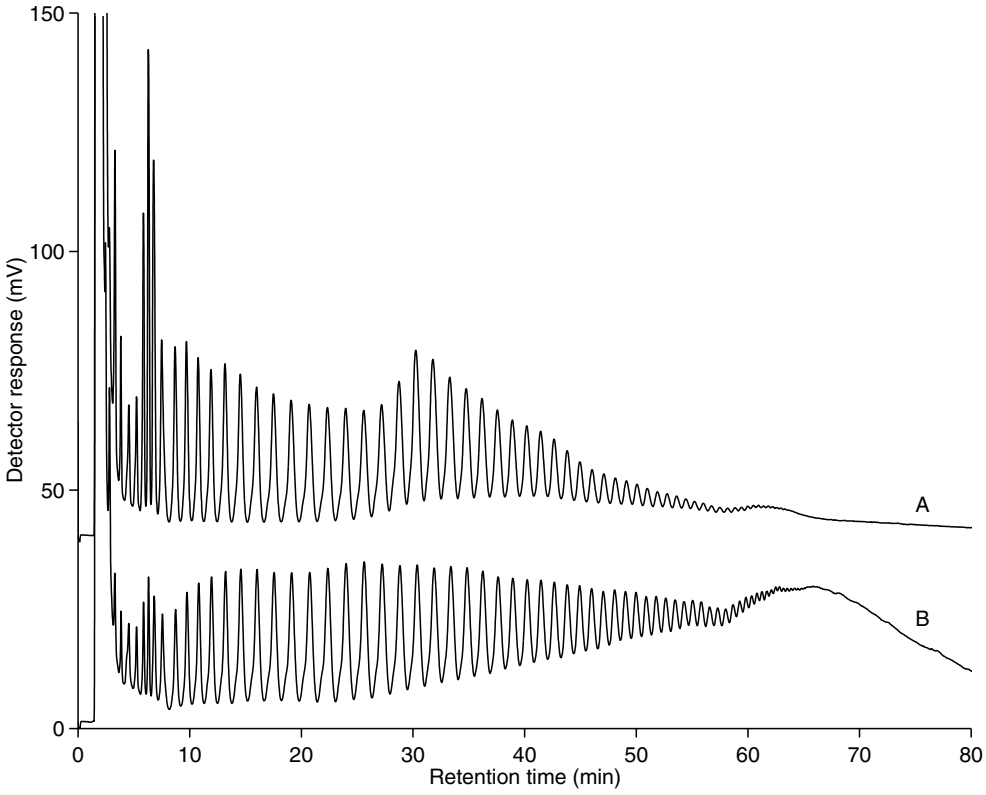

Fig. 3. Chromatogram of linear dextrins incubated with (A) or without (B) SBEI and debranched with isoamylase prior to analysis by HPAEC.

results reported from other studies (Jane et al., 1984; Russell et al., 1989; Siljeström et al., 1989). After debranching with isoamylase the elution profiles did not change indicating that the dextrins contained little or no branched material. A retrograded starch obtained from Cerestar was also enzymatically digested and analysed by GPC giving similar elution profiles (not shown).

With HPAEC, individual chains up to DP 72 could be separated (Fig. 2A). The majority of the chains observed were longer than eight glucose residues but minor amounts of shorter chains could also be detected. This is in line with previous studies on enzyme-resistant starch (Gidley, Cooke, Darke, Hoffmann, Russell & Greenwell, 1995). The individual peaks were sharp and well resolved, strongly suggesting that the dextrins were linear. Furthermore, debranching of the samples did not change the elution pattern (not shown). A considerable portion of the glucose chains had a dp above 70, as confirmed by GPC. These chains were not separated into individual peaks under these chromatographic conditions but were still visible in the chromatogram.

A comparison of dextrins obtained from Novelose and the Cerestar starch revealed a clear difference in chain length profiles (Fig. 2A and B). Linear dextrins obtained from Novelose contained a higher proportion of chains shorter than ~22 glucose units whereas linear dextrins obtained from the Cerestar starch contained a relatively larger amount of chains with a dp of 22–50.

β-Amylase hydrolyses alternate α-(1,4)-linkages in amylose and amylopectin, producing mainly maltose and β-limit dextrins. After digestion with β-amylase, 92% of the dextrins had been hydrolysed by the enzyme. This value further supported the assumption that the glucose chains were essentially linear.

3.2. Studies on potato starch branching enzyme I

The linear dextrins were used as substrate for studies on the mode of action of starch branching enzyme I from potato. The substrate is well defined with little or no branching and with a comparatively narrow chain length distribution. The fact that it could be prepared in fairly large quantities and that it has a molecular weight range suitable for analysis by HPAEC made it well suited for use in in vitro enzyme assays.

Chain length distributions were determined by HPAEC, giving a picture of how the proportions of individual chains had changed during incubation with potato starch branching enzyme I (Fig. 3). In the control sample (Fig. 3B), incubated without enzyme, peaks with chains up to dp ~ 70 were resolved while polysaccharide chains longer than 70 glucose units were detected as unresolved peaks. For the sample incubated with SBEI, much of this high molecular weight material had disappeared (Fig. 3A). Two new populations were clearly visible; one with a peak maximum around dp 11 and a second with a maximum around dp 30. A similar pattern of chain distribution is shown in a

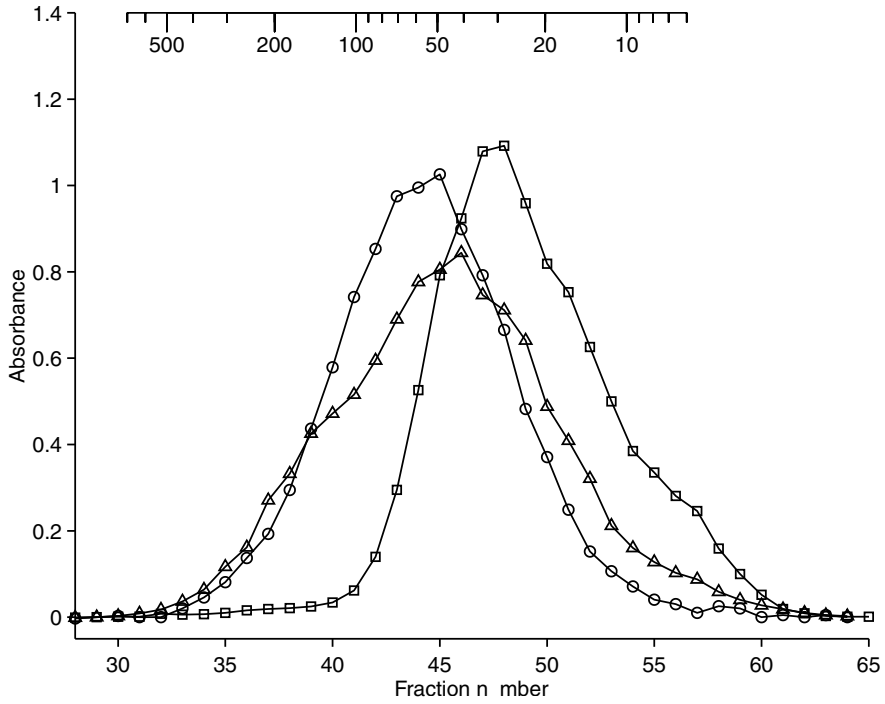

Fig. 4. GPC elution profiles of the products formed by incubation of potato starch branching enzyme I (SBEI) with linear dextrins. Control sample without SBEI added (-○-), SBEI-products before (-△-) and after (-□-) debranching with isoamylase. Dp values obtained after column calibration with pullulan standards are shown on the upper axis.

study of starch branching enzyme I from maize (Takeda et al., 1993).

Linear dextrins incubated with SBEI were also analysed on a Sepharose CL-6B column before and after debranching with isoamylase (Fig. 4). Before debranching, the samples showed an elution profile with a peak maximum at a dp of 40–50. After debranching, the peak maximum shifted to dp 30. The peak was well defined in the high molecular weight area where the majority of chains above dp 60 were missing. At the same time, the proportion of short chains was increased and new chains shorter than in the original substrate had been formed. This showed that most chains above dp 60 served as substrates for the branching enzyme. Furthermore, the molecular weight distributions for the control sample (without branching enzyme added) and the branched sample prior to debranching by isoamylase are similar. This could be interpreted as the transfer of chains by potato SBEI being intra-molecular rather than inter-molecular. However, inter-molecular transfer cannot be excluded and has also been reported in earlier studies (Borovsky et al., 1976; Peat et al., 1953; Viksø-Nielsen et al., 1998).

In this paper, linear dextrins have been prepared by enzymatic hydrolysis of retrograded starch. The use of these linear dextrins as an enzyme substrate in in vitro studies of the action of a branching enzyme has been demonstrated.

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