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Characterisation of the in vitro products of potato starch branching enzymes I and II

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Abstract

An enzyme substrate obtained by ethanol fractionation of linear dextrins was branched by potato starch branching enzymes (SBE). During the branching process, samples were withdrawn from the incubation mixture and the chain length distributions were analysed by high performance anion exchange chromatography (HPAEC). The relative composition of chains with a degree of polymerisation of 9–35 was essentially constant throughout the branching process for both SBEI and SBEII. This showed that the change in size and structure of the substrate during the branching reaction did not considerably affect the patterns of chains produced by the enzymes. Chain length patterns as well as branching rates were different for the two SBE isoforms, supporting the theory that they have different roles in amylopectin synthesis. Despite the differences in chain length profiles, the degree of branching was found to be 3.7% for both SBE products when analysed by NMR. Using proton 2D-NMR, the structural differences between intact branching products and their β-limit dextrins were determined. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Branching enzyme; Starch; NMR; Degree of branching; Limit dextrin

1. Introduction

Amylopectin, which is generally the major component of starch (70–80%), is a branched polysaccharide of a high molecular weight (10^7–10^9). It is composed of linear α-(1 → 4)-linked glucose chains connected by α-(1 → 6)-linkages, forming the branch points. Potato starch, which displays a B-type X-ray diffraction pattern, has amylopectin molecules containing fewer short chains and more long chains than amylopectins from A-type starches (e.g. most cereals). Average chain lengths of 23–29 glucose residues has been found for potato amylopectin, whereas most cereal amylopectins have an average chain length of 18–25 (Hizukuri, Kaneko, & Takeda, 1983; Jane et al., 1999). Many factors, including the amylose/amylopectin ratio and the amylopectin fine structure, affect the physical properties of starch. In order to understand starch biosynthesis, knowledge about the precise mechanisms of the enzymes involved in amylopectin synthesis is of great importance. The biosynthesis of starch is governed by at least three types of enzymes: ADP glucose pyrophosphorylases, starch synthases and branching enzymes (for reviews, see Kossmann & Lloyd, 2000; Smith, Denyer, & Martin, 1997).

Starch branching enzymes (SBEs; EC 2.4.1.18) act by cleaving an α-(1 → 4)-linkage and reattaching the chain to an acceptor chain by an α-(1 → 6)-linkage. Multiple forms of SBE have been identified in potato (Blennow & Johansson, 1991; Borovsky, Smith, & Whelan, 1975; Larsson et al., 1996; Vos-Scheperkeuter, de Wit, Ponstein, Feenstra, & Witholt, 1989) and in many other species. Distinct properties for the two isoforms, SBEI and SBEII, have been shown for potato (Rydberg, Andersson, Andersson, Åman, & Larsson, 2001) and maize isoenzymes (Guan & Preiss, 1993; Takeda, Guan, & Preiss, 1993) suggesting different roles for the two enzymes in starch biosynthesis.

The activities on different substrates, such as amylose and amylopectin, as well as the chain length profiles of the branched products were shown to be different for the potato SBE isoforms (Rydberg et al., 2001). Potato SBEI showed a higher rate of branching amylose and a lower activity on amylopectin compared to SBEII, and both enzymes were stimulated by phosphate added to the incubation buffer. The most abundant chains produced by potato SBEI had a degree of polymerisation (dp) of 6 and 11–12, and a broader population of chains with a peak at dp 29–30. The

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branching products of potato SBEII contained large amounts of glucan chains with dp 6 and a peak around dp 13–14.

In this paper, the in vitro products of potato SBE I and II obtained by branching of a linear substrate were further characterised by enzymatic and spectroscopic methods. The substrate was prepared by removing the low molecular weight material of linear dextrins (Andersson, Rydberg, Larsson, Andersson, & Åman, 2002) by ethanol precipitation. SBE branching products obtained after various times of incubation with this substrate were examined by high performance anion exchange chromatography (HPAEC) and some characteristics of the final branching products obtained were determined by NMR and β-amylolysis.

2. Experimental

Starch branching enzymes I and II from potato were expressed in *Escherichia coli* and purified by ammonium sulphate precipitation, starch affinity chromatography and anion exchange chromatography according to Khoshnoodi (1997) and Larsson (1999). The preparations of potato SBEI and II were highly pure as judged from SDS polyacrylamide gel electrophoresis where only one additional, faint band could be seen for SBEII and none for SBEI in Coomassie blue staining (Larsson, 1999). Solutions of 1 μM SBE in 50 mM Tris buffer pH 7.5 with 1 mM DTT and 10% glycerol were stored at −70 °C until use. Isoamylase (EC 3.2.1.68, 59 000 U/ml) was obtained from Hayashibara Biochemical Laboratories Inc., Okayama, Japan. Pullulanase (EC 3.2.1.41, 429 U/ml) was from Megazyme, Ireland and β-amylase (EC 3.2.1.2, 22 880 U/ml) from Sigma Chemical Co., St Louis, USA.

2.1. Fractionation of linear dextrins

Linear dextrins obtained from retrograded high-amylose maize starch (Andersson et al., 2002) were dissolved in DMSO by heating followed by stirring for 16 h. Water was added to get a concentration of approximately 10 mg dextrins/ml in 80% DMSO. After cooling, ethanol was slowly added under continuous stirring until a concentration of 45% ethanol was reached. The sample was left in an ice-bath for 1 h and the precipitate, containing the high molecular weight material, was recovered by centrifugation. The pellet was then washed twice with ethanol and twice with acetone before drying at 40 °C. The supernatant, containing the shorter glucose polymers, was discarded.

2.2. Changes in chain length distribution during branching

Substrate (fractionated linear dextrins) were incubated with branching enzymes essentially according to Andersson et al., 2002. Approximately 10 mg of substrate was dissolved in 2 M KOH using the following procedure: 150 μl water was added, the sample was heated slightly and after cooling, 150 μl of 4 M KOH was added. The sample was then stirred for 30 min and incubated in a Tris buffer (pH 7.5, 60 mM) together with SBEI or II (200 μl). Portions of the incubation mixture (0.5 ml, 3 mg of substrate/ml) were removed at intervals from 10 min to 21–24 h from the addition of SBE, and immediately placed in boiling water for 5 min to stop the enzyme activity. After the addition of 40 μl sodium acetate buffer (1 M, pH 3.6) and adjustment of the pH to 3.6, each sample was debranched with isoamylase (5 μl) at 38 °C. After 3 h, the debranching activity was terminated in a boiling water bath and to each sample was added 60 μl 4 M KOH prior to analysis by HPAEC.

To confirm that the final branching product had been obtained after 21–24 h of incubation, fresh SBEI or II was added to an aliquot of each final incubation mixture. After another 4 h of incubation, the sample was analysed by HPAEC, which showed no further detectable change in chain length distribution.

2.3. Preparation of β-limit dextrins and intact SBE products

Substrate (fractionated linear dextrins) were incubated with branching enzymes. Typically, 10 mg of substrate was dissolved in 2 M KOH (as described earlier) and incubated for 16 h with SBEI or II (200 μl) in a phosphate buffer (60 mM, pH 7.5) at a final concentration of 3 mg/ml. The sample was heated in boiling water for 5 min to inactivate the enzyme, 500 μl of sodium acetate buffer (0.1 M, pH 4.8) was added and the pH was adjusted to 4.8 with 1 M HCl. β-Amylase (23 U) was added and the samples were incubated at 25 °C for 6 h. β-Limit dextrins were collected on a BioGel P-2 column (100 × 1.6 cm) eluted with water and connected to an RI-detector. The β-limit dextrins were freeze-dried before further characterisation. Prior to β-amylolysis, an aliquot of each incubation mixture was withdrawn and the intact SBE products were collected and freeze-dried as above.

2.4. β-Amylolysis limits

The β-amylolysis limit value for each branching product was determined by injecting 1.1 mg of each β-amylolysate on the BioGel P-2 column (as above). The carbohydrate content in collected fractions was determined 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. At least duplicate samples were analysed.

2.5. Debranching of β-limit dextrins

Approximately 3 mg of β-limit dextrin was dissolved in 90 μl of 90% DMSO by heating the sample in boiling water for 20 min followed by stirring at room temperature for 20 h. Sodium acetate buffer (470 μl, 70 mM, pH 5.0) was added to each sample which was then incubated with

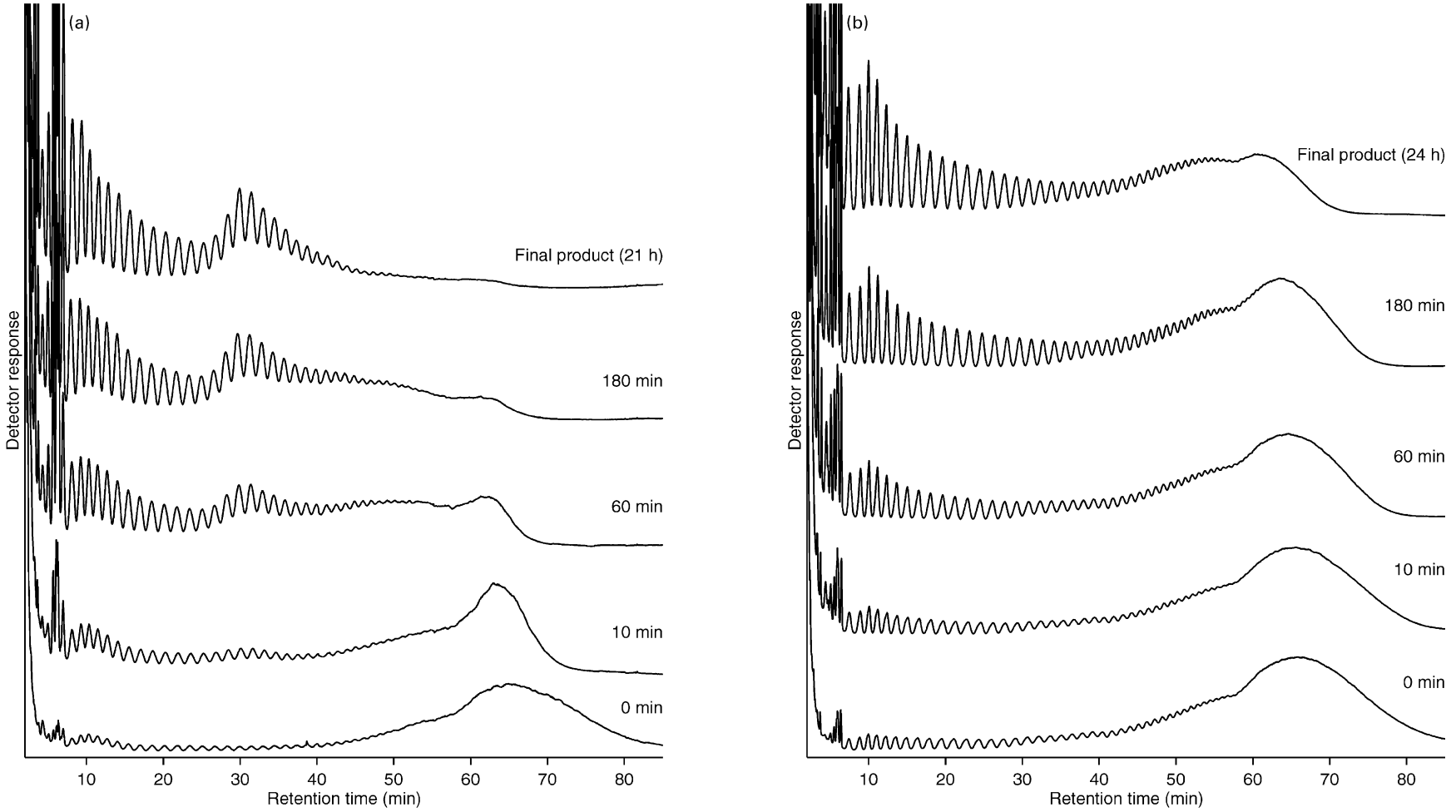

Fig. 1. HPAEC chromatograms of the debranched products of potato SBEI (a) and SBEII (b) obtained after different incubation times.

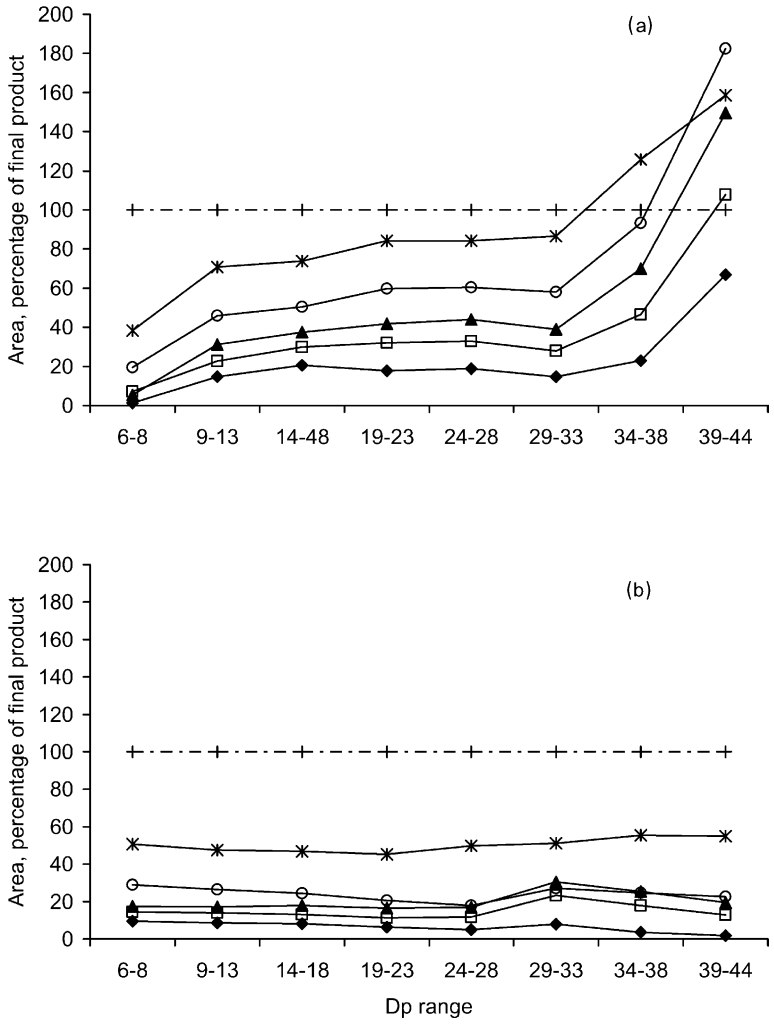

Fig. 2. Changes in the relative amounts of glucose chains of various lengths after different times of incubation with potato SBEI (a) and SBEII (b). The amount of the individual chain length (determined by HPAEC) is expressed as a percentage of the amount in the final product (dotted line). Averages were calculated for groups of chains as indicated in the figure. Lines marked –◆–, –□–, –▲–, –○–, and – ∗ – represent the relative amounts of different chains after 10, 20, 30, 60 and 180 min of incubation, respectively. Samples representing the final products were incubated for 21 h (SBEI) and 24 h (SBEII).

pullulanase (3 U) for 16 h at 38 °C. The debranching activity was stopped in a boiling water bath and the samples were diluted to ~0.9 mg/ml before analysis by HPAEC. Duplicate samples were analysed.

2.6. High performance anion exchange chromatography (HPAEC)

Analyses were performed on a Dionex DX500 instrument (Sunnyvale, USA) with a CarboPac PA-100 anion exchange column (250 × 4 mm) connected to a pulsed amperometric detector (ED40). The eluents were: 150 mM NaOH (eluent A), and 150 mM NaOH containing 500 mM NaOAc (eluent B). The gradient was run with a flow rate of 1 ml/min and the following eluent mixtures: 0–5 min, linear gradient from 34.3 to 45% of eluent B; 5–55 min, linear gradient from 45 to 67% and at 55–91 min, linear gradient up to 100% eluent B. Finally, eluent B decreased linearly, reaching 34.3% at 97 min. The column was equilibrated with the initial eluent composition for 20 min before each sample injection.

2.7. ^{1}H NMR spectroscopy

SBE products for NMR studies were prepared by incubating 10 mg of substrate with SBEI or II in a phosphate buffer as described earlier. The samples were then desalted on a BioGel P-2 column, freeze-dried, dissolved in D_2O and freeze-dried again. The lyophilisation in D_2O was repeated twice and the samples were finally dissolved in D_2O (~10 mg/ml) prior to analysis. β-Limit dextrin and maltoheptaose (Boehringer Mannheim, Germany) samples were treated in a similar way. ^{1}H NMR spectra were obtained on a DRX400 Bruker Avance spectrometer operating at 400.13 MHz and 30 °C. A pulse-angle of 30° was used in all experiments, pulse repetition time was 5.1 s and the number of scans was ≥64. ^{1}H–^{1}H COSY using gradients was recorded with 8 scans over 512 increments (zerofilled to

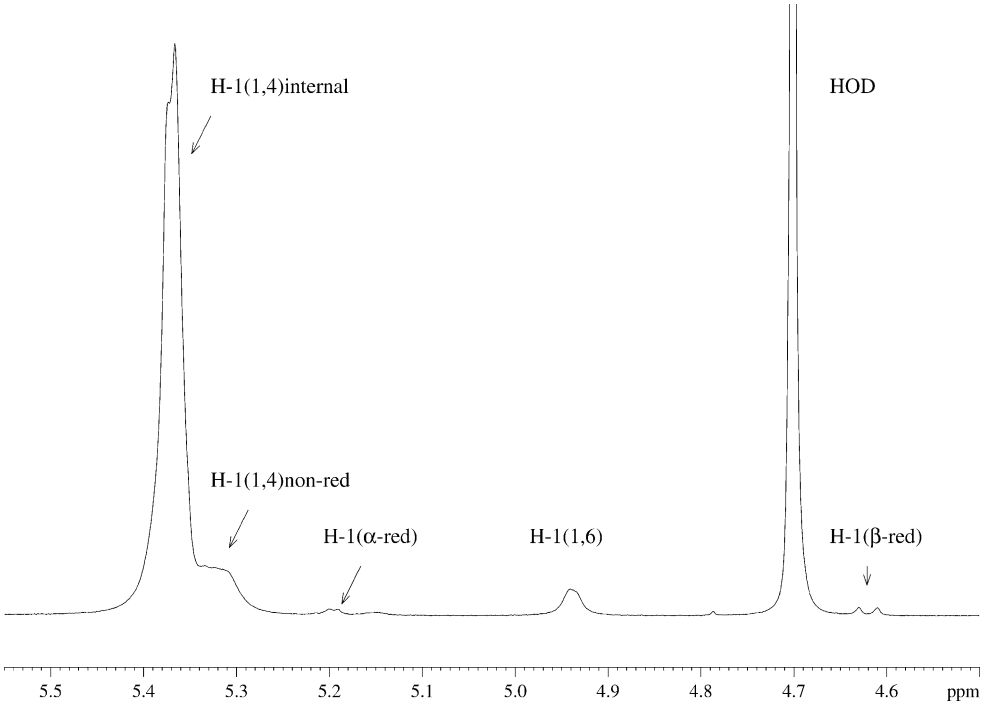

Fig. 3. Anomeric region of a 400 MHz ^{1}H NMR spectrum of an SBE product in D_2O.

1k) and 2k data points with spectral widths of 1035 Hz in both dimensions.

3. Results and discussion

3.1. Fractionation of the enzyme substrate

Linear dextrins, prepared from retrograded high-amylose starch (Andersson et al., 2002), were fractionated by ethanol precipitation. Selective precipitation of the high molecular weight material was obtained by a slow addition of ethanol, under continuous stirring of the sample. Most of the low molecular weight material was removed with the supernatant. The resulting enzyme substrate, with only minor amounts of short glucose chains (dp < 40), was estimated by gel filtration to have a chain length range of dp 50–250 (not shown). An amount of 850 mg of the enzyme substrate was obtained by batch-wise fractionation of 2.5 g of linear dextrins, giving an average yield of 34% (SD $= 3.6$, $n = 9$). The precipitate obtained from each fractionation was examined by HPAEC analysis before being pooled. The similarities in elution profile between the individual precipitates (not shown), as well as the yield from each fractionation indicated a relatively high reproducibility for the fractionation method.

A removal of essentially all low molecular weight material (dp < 50) could be achieved by repeating the ethanol precipitation step. However, this gave a yield of only 15%, making it unsuitable for our purpose of producing large amounts of substrate. The procedure involving only one precipitation step was therefore chosen for the preparation of the enzyme substrate used in this study.

3.2. Changes in chain length distribution during branching

The pattern of chains produced during the branching process was examined by taking out samples from 10 min to around 20 h of incubation. The samples withdrawn were debranched with isoamylase and analysed by HPAEC. In the final SBEI product (Fig. 1a), there was an accumulation in chains of dp 6 and two populations of chains with peaks around dp 11–12 and 29–30, as previously described (Andersson et al., 2002; Rydberg et al., 2001). The chromatograms of the SBEI products also show that all unit chains present in the final product were formed already after short incubation times. A general increase in short chains, with peaks at dp 6, 9–11 and a smaller population around dp 13–17, was observed in the products of SBEII (Fig. 1b). Chains of dp 6 have been reported to be donor chains remaining after SBE transfer (Drummond, Smith, & Whelan, 1972).

From the chromatograms in Fig. 1a and b, we could also see how SBEI reduced the number of the longest chains more drastically than SBEII. SBEI initially (0–60 min) produced large amounts of chains with a dp around 35–75, but those chains later decreased as they were used by the branching enzyme again. The fast reduction in the longest chains (dp > 80) in the SBEI incubation mixture does not necessarily mean that the enzyme has a preference for those chains. It could also be the result of a continuous mass transfer from longer to shorter chains, with no new formation of the longest chains. In comparison with SBEI, the

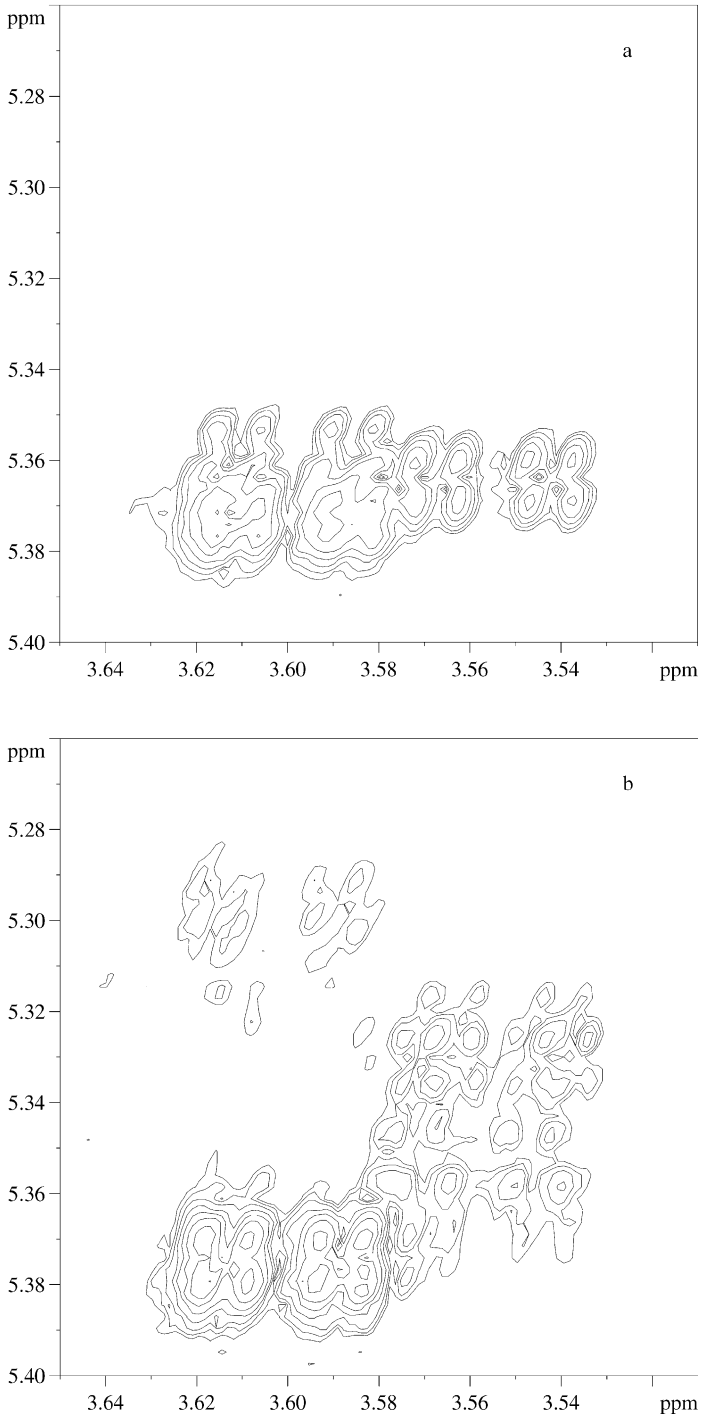

Fig. 4. Four hundred megahertz ^{1}H COSY spectra of H-1/H-2 cross peaks of (a) the SBEII product and (b) the SBEII β-limit dextrin. Chemical shifts for H-1 and H-2 are shown on left and bottom axis, respectively.

SBEII isoform worked more slowly on this substrate and seemed not to be very active on chains with a dp less than ~55. These results illustrate that the two SBE isoforms acted differently on this substrate. Similar results have previously been reported for the branching enzymes from maize (Takeda et al., 1993).

Changes in the relative amounts of individual glucose chains of dp 6–44, after different incubation times, are shown in Fig. 2. The amount of the individual chain length is expressed as a percentage of the amount in the final product. For SBEI, there was a slower increase for the shortest chains, dp 6–8, in the beginning (0–180 min) of the branching process, whereas chains within the dp range 9–33 followed a similar pattern of chain transfer throughout the branching reaction (Fig. 2a). Longer chains (dp > 33) increased more rapidly at the beginning, but the numbers of

these chains later decreased, as also seen in the HPAEC chromatograms. This means that even if chains that are more abundant in the final product increase more rapidly than less abundant chains, the relative composition of chains of dp 9–33 remained the same during incubation with SBEI. For SBEII, the relative composition of all chains examined (dp 6–44) was similar throughout the whole branching process (Fig. 2b). This shows that the two enzymes produce chains of certain lengths, almost independent of how the chain length and degree of branching of the substrate varies during the branching process.

3.3. Degree of branching

The final products of both SBEI and SBEII were analysed by NMR spectroscopy. Most proton and carbon chemical shifts for starch, starch-related products and glycogen have earlier been assigned (McIntyre & Vogel, 1990; Zang, Howseman, & Schulman, 1991). Previous studies have also shown that reliable values for the degrees of branching of various starches and amylopectins could be achieved by NMR spectroscopy (Gidley, 1985; Nilsson, Bergquist, Nilsson, & Gorton, 1996). The spectra of the two branching products were very similar and contained the signals characteristic for a starch spectrum. The degree of branching was calculated as the intensity of the anomeric signal for α-(1 → 6)-linked glucose residues, divided by the intensities of all anomeric signals representing the total amount of glucose residues (Fig. 3). The reducing ends were included in the calculation due to the relatively small molecular size of the branched products.

The samples branched by SBEI and SBEII both had a degree of branching of 3.7%. This means that a product with a branching density similar to that of amylopectin (4–5%) (Manners, 1989) could, at least in vitro, be obtained with each of the SBE isoforms, and in the absence of other starch biosynthetic enzymes. That similar degrees of branching were obtained for the two enzyme products was rather interesting bearing in mind the differences in their chain length profiles. The combination of many short chains and some relatively long chains, as in the SBEII product, could thus give a branching density similar to that of the SBEI product, with its populations of 'medium-sized' chains.

3.4. 2D NMR experiments

The final branching products and their β-limit dextrins were examined by 2D ^{1}H homonuclear NMR spectroscopy (COSY). In the one-dimensional spectra it was difficult to separate the H-1 resonances for the internal α-(1 → 4)-linked glucose residues from the non-reducing ends since they have similar chemical shifts, but more information could be gained from the H-1/H-2 cross peaks in the 2D-spectra. The COSY-spectrum of the SBEII product contained two main H-1/H-2 cross peaks, at 3.60 and 3.56 ppm, of α-(1 → 4)-linked glucose residues (Fig. 4a). The signals were identified as H-1/H-2 of internal and non-reducing end residues, respectively, by comparison with a spectrum of maltoheptaose, to which chemical shifts were previously assigned (Sugiyama et al., 2000).

In β-limit dextrins, the B-chains have a short stub of 1 or 2 glucose units external to the branch point while the hydrolysed A-chains contain 2 or 3 glucose units. In the COSY spectrum of an SBEII product hydrolysed by β-amylase, an additional set of H-1/H-2 cross peaks appeared (Fig. 4b). The additional cross peaks at 3.56 ppm presumably represented the non-reducing ends of both A- and B-chains, whereas the cross peaks at 3.6/5.3 ppm were derived from 1,4,6-linked glucose residues (Jodelet, Rigby, & Colquhoun, 1998). The COSY spectra of the SBEI product and its β-limit dextrin were very similar to those presented in Fig. 4.

If α-(1 → 6)-linkages were present on the second or third glucose residue from the non-reducing end of the acceptor chain in the SBE product, this would give a spectrum containing some additional H-1/H-2 cross peaks, as seen in the β-limit dextrin spectrum. By comparing the two spectra (Fig. 4), it was apparent that the presence of α-(1 → 6)-linkages that close to the non-reducing end of the SBE product was negligible. In our previous studies of potato branching enzymes (Andersson et al., 2002; Rydberg et al., 2001), both SBEI and SBEII made branched products with a size similar to that of the original linear substrate. This could be explained by intra-molecular branching, where donor and acceptor chains belong to the same molecule. If present, such intra-molecular branch points could possibly appear near the non-reducing end of the acceptor chain, unless the enzymes move the cleaved chain along the acceptor chain before reattachment. The results presented here indicate that branch points were not formed close to the non-reducing ends. Whether or not intra-molecular branch points were formed at other positions on the B-chain, however, could not be determined by these methods.

3.5. β-Amylolysis limit values and characterisation of β-limit dextrins

Analysis of the enzyme products from SBEI and II gave limits of β-amylolysis of 66 and 74%, respectively. β-Amylolysis values have previously been reported for the products of maize branching enzymes and were found to be in the range of 45–50% on an amylose substrate (Guan & Preiss, 1993). The relatively high values obtained here could be affected by the relatively small substrate used in this study, which gives small, branched products with a relatively high proportion of external chains. Small amounts of long linear chains, which could be present in the SBEII product as indicated in Fig. 1b, would also give a higher limit of β-amylase degradation.

β-Limit dextrins were collected, debranched and analysed by HPAEC. The chain length profiles of the β-limit dextrins, and those of the intact products of SBEI

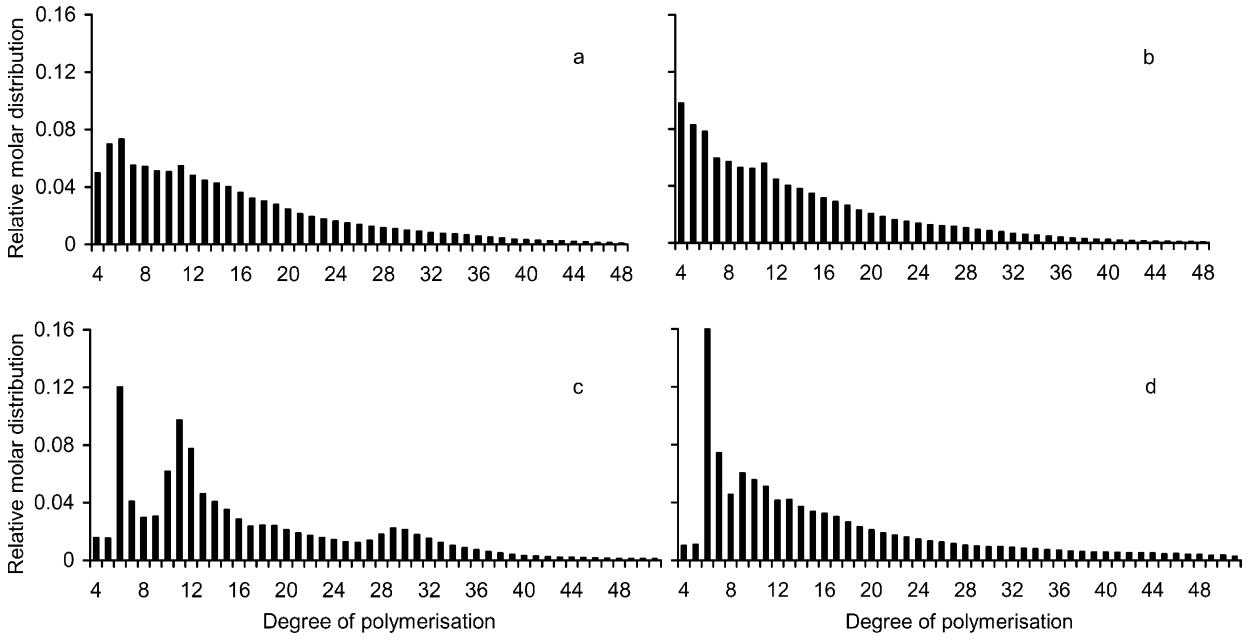

Fig. 5. Relative molar chain length distributions of debranched SBEI (a) and SBEII (b) β-limit dextrins and debranched products of SBEI (c) and SBEII (d).

and II, are shown in Fig. 5. The populations observed in the intact branching product of SBEI (Fig. 5c) were absent in the β-limit dextrin (Fig. 5a). This indicates that these chains were at least partly available to β-amylase degradation. The chain length distribution for the SBEII β-limit dextrin showed a pattern similar to that of the SBEI β-limit dextrin but with an increased proportion of very short chains (Fig. 5b). The abundance of short B-chains could be interpreted as branch points being formed either close to each other in a multiply branched molecule or close to a reducing end. The SBEII β-limit dextrin profile was not considerably different from that of the SBEII product apart from a reduction in the proportion of the most abundant chains of dp 6 and 9–10 (Fig. 5d).

3.6. Concluding remarks

Distinct chain length profiles, as previously reported by Rydberg et al. (2001) were produced by the two potato SBE isoforms on branching a linear substrate. In the present study, the substrate was fractionated to remove the shortest chains and thereby reducing the risk of short substrate chains affecting the chain length profiles of the branched products. During the branching process, samples were withdrawn from the incubation mixture and the chain length distributions were analysed by HPAEC. The relative composition of chains with a degree of polymerisation of 9–35 was essentially constant throughout the branching process for both SBEI and SBEII. This showed that structural changes in the substrate did not considerably affect the patterns of chains produced by the enzymes. Using ^{1}H NMR it was shown that the final products of both SBEI and SBEII had a degree of branching of 3.7%.

SBEI was more effective than SBEII in reducing the number of long chains. Whether this is due to differences in substrate specificity between the two enzyme isoforms, or because of differences in the structure of the branched products is not known. The longest chains in the final products of SBEII could be branched in a way that makes them inaccessible to further branching, or the enzyme could have a low affinity for these long chains. However, the high β-amylolysis value (74%) together with the relatively small molecular size reported for similar branched molecules (Rydberg et al., 2001), argue in favour of the latter explanation.

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