Characterization of the Reactions of Starch Branching Enzymes from Rice Endosperm

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To our knowledge the present paper shows for the first time the kinetic parameters of all the three starch branching enzyme (BE) isozymes, BEI, BEIIa and BEIIb, from rice with both amylopectin and synthetic amylose as glucan substrate. The activities of these BE isozymes with a linear glucan amylose decreased with a decrease in the molar size of amylose, and no activities of BEIIa and BEIIb were found when the degree of polymerization (DP) of amylose was lower than at least 80, whereas BEI had an activity with amylose of a DP higher than approximately 50. Detailed analyses of debranched products from BE reactions revealed the distinct chain length preferences of the individual BE isozymes. BEIIb almost exclusively transferred chains of DP7 and DP6 while BEIIa formed a wide range of short chains of DP6 to around DP15 from outer chains of synthetic amylopectin and amylose. On the other hand, BEI formed a variety of short chains and intermediate chains of a DP ≤40 by attacking not only outer chains but also inner chains of branched glucan while BEIIa or BEIIb could only scarcely or could not attack inner chains, respectively. The comprehensive in vitro studies revealed different enzymatic characteristics of the three BE isozymes and give a new insight into the distinct roles of individual BE isozymes in amylopectin biosynthesis in the endosperm. Based on these results, the functional distinction and interaction of BE isozymes during amylopectin biosynthesis in cereal endosperm is discussed.

Keywords: Amylopectin • Amylose • Rice • Starch biosynthesis • Starch branching enzyme.

Abbreviations: ae, amylose-extender; APTS, 8-amino-1,3,6-pyrenetrisulfonic acid; BCA, bicinchoninic acid; BE, starch branching enzyme; CE, catalytic efficiency; DP, degree of polymerization; DPn, number-average degree of polymerization; DTT, dithiothreitol; GPC, gel permeation chromatography; HPAEC-PAD, high-performance anion-exchange chromatography equipped with a pulsed amperometric detector; IPTG, isoprpyl-β-D-thiogalactopyranoside; Φ-LD, phosphorylase-limit dextrin; Mw, the number-average molecular weight; MOS, maltooligosaccharides; Mw, the weight-average molecular weight; OD, optical density; SS, starch synthase.

Introduction

α-Glucan is a polysaccharide in which the glucose moiety is linked with α-1,4- and α-1,6-glucosidic linkages. Plant starch is composed of linear or slightly branched amylose and highly branched amylopectin. Amylopectin has a distinct highly ordered structure called a ‘tandem-cluster structure’, in which most of side chains are arranged in parallel and neighboring chains form double helices (Kainuma and French 1972) when linear portions of facing chains reach a length equivalent to a degree of polymerization (DP) ≥10 (Gidley and Bulpin 1987). The formation of double helices in the amylopectin cluster dramatically induces its hydrophobicity and crystallinity. These specific features of amylopectin fine structure are enabled by the rhythmic localization of the branch position in the cluster (Thompson 2002). The starch synthesis system has developed during the process of evolution of plants, and key enzymes involved in the construction of amylopectin tandem-cluster structure have differentiated into multiple isozymes with distinct functions, whereas in glycogen-synthesizing organisms such as bacteria and animals no such functional differentiations in glycogen synthesis enzymes have occurred (Deschamps et al. 2008).

The fine structure of amylopectin is known to differ depending on plant species and various plant tissues, and this variation influences the specific properties of the individual starches. Starch branching enzyme (BE) catalyzes the formation of α-1,6-glucosidic linkages of amylopectin during starch biosynthesis, and therefore BE plays a very important part in the formation of a distinct fine structure of amylopectin. Green plants have two types of BE, BEI and BEII, with distinct structures and enzymatic properties (Martin and Smith 1995, Nakamura 2002). BEI and BEII can be distinguished from each other in terms of...
their reactivities to chemicals such as cyclodextrins (Viksoe-Nielsen 1998) and phosphorylated compounds (Morell et al. 1997a), temperature responses (Takeda et al. 1993), association with starch granules (Mu-Forster et al. 1996) and expression patterns during development of reserve tissues (Mizuno et al. 1993, Burton et al. 1995, Morell et al. 1997a, Ohdan et al. 2005). BEI and BEII also exhibit different kinetic parameters for various α-glucans. In vitro experimental results showed that maize BEI preferentially branches amylose-type glucans with few branches whereas BEII has a higher capacity for branching amylopectin-type highly branched glucans (Guan et al. 1993, Takeda et al. 1993, Guan et al. 1997). Guan et al. (1997) further defined the differences in the size of chains transferred between maize BEI and BEII, and showed that BEI predominantly transfers longer chains with a DP >10, while BEII preferentially transfers shorter chains with DP3–9, with the most abundant chains having DP6 and DP7.

It is known that BEI is further differentiated into BEIIa and BEIIb isoforms in cereals, although BEIIb is usually specifically expressed in endosperm while BEIIa is ubiquitously present in every tissue (Mizuno et al. 1993). Our recent biochemical studies using various mutants of rice in which BEI (Satoh et al. 2003b) or BEIIb (Nishi et al. 2001) is defective and transgenic plants of rice in which the BEIIb expression level is varied (Tanaka et al. 2004) strongly suggest that BEIIa plays a role in transferring longer chains which are eventually linking multiple clusters of amylopectin and medium size chains which are located in the amylopectin amorphous lamellae, whereas BEIIb specifically transfers short chains which represents the border between the amorphous lamellae and the crystalline lamellae of amylopectin (for a review, see Nakamura 2002). The specific function of BEIIb in rice endosperm might be fairly unimportant because no significant changes in the structure of amylopectin and the physicochemical properties of starch granules are found in endosperm of BEIIb-deficient mutants (Nakamura 2002, Satoh et al. 2003a). The physiological role of BEIIb might be to support at least partially the function of BEI and BEIIa, although the activity of BEIIa accounts for about 20% of the total BE activity in rice endosperm (Yamanouche and Nakamura 1992), whereas BEIIa plays the predominant part in leaves in which BEIIa is not expressed. These results apparently contradict the observation that maize BEIIa and BEIIb have the same kinetic parameters for amylose (Guan et al. 1993, Takeda et al. 1993).

The in vivo studies strongly suggest that each BE isozone in monocotyledonous plants contributes to the fine structure of amylopectin, playing a distinct role in the biosynthesis process in the endosperm. To prove the hypothesis in order to help to understand the regulation of starch biosynthesis, it is crucial to characterize the BE isozone through detailed analysis of in vitro experiments. The mechanism of BE enzyme action has been poorly understood, in spite of numerous investigations on BE reaction products (Borovsky et al. 1975a, Borovsky et al. 1975b, Borovsky et al. 1976, Borovsky et al. 1979, Takeda et al. 1993, Guan et al. 1997).

To reveal the characteristics of BE reactions, it is necessary to separate completely and quantify precisely and reproducibly various malto-oligosaccharides (MOS) with different DP values included in the glucan substrate for BE and its reaction products. However, in the pioneering studies on the BE reaction mechanism by Whelan’s group (Borovsky et al. 1975a, Borovsky et al. 1975b, Borovsky et al. 1976, Borovsky et al. 1979) the debranched chains were separated by conventional gel permeation chromatography (GPC), although MOS cannot be separated from each other by this method. In recent investigations (Guan et al. 1997, Mizuno et al. 2001, Rydberg et al. 2001) MOS were mainly quantified by high-performance anion-exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD), but it is known that this method of detection is not fully quantitative for MOS with various DP values (Koizumi et al. 1991). Under these circumstances, there are still many questions unresolved regarding BE reaction mechanisms and properties.

To characterize the BE isozone, it is important first to determine its kinetic parameters precisely, and secondly to select the appropriate glucans used as substrate. The present study has been performed to reveal the kinetic parameters of three rice BE isozymes, BEIIb, BEIIa and BEI, using their recombinant proteins expressed in *Escherichia coli*. In this study, amylopectin prepared from rice endosperm of a BEIIb-deficient mutant line EM10 (Nishi et al. 2001), namely αe-amylopectin, was used since it is a physiologically probable glucan for BE isoforms. In addition to phosphorylase-limit dextrin (Φ-LD) prepared by treatment with phosphorylase of amyloglucan from a rice *waxy* mutant endosperm, synthetic amyloses which are enzymatically synthesized from potato glucan phosphorylase1 and maltohexaose were used since they are not completely linear glucans free from α-1,6-glucosidic linkages and their molecular masses are varied (Kitamura et al. 1982, Kitamura et al. 1989, Nakaniishi et al. 1993). To clarify the chain preference of the BE isozymes, the chain length distribution of the BE reaction product debranching with bacterial isoamylase and pullulanase was analyzed by the capillary electrophoresis method (O’Shea and Morell 1996, Morell et al. 1997b). The method not only can completely separate MOS having different DPs ranging from 3 up to approximately 100, but also can determine the molar ratio of these MOS because every chain is labeled with a fluorescent probe, 8-amino-1,3,6-pyrenetrisulfonic acid (APTS), at its reducing end. This APTS-assisted method is ideal to analyze in detail the fine structure of BE reaction products and hence the mechanism of the BE enzymatic reaction by characterizing the chain length preference of each BE isozone.

This study determined the kinetic parameters of all the three BE isozymes from rice towards both αe-amylopectin and synthetic amylose as glucan substrate. In addition, several in vitro experiments have been designed to distinguish BEIIb from BEIIa because their functional differences, if any, have not been documented.
Results

Purification of three recombinant rice BE isozymes

Since in this study it is necessary to prepare the purified rice BE isozymes free from the other BE isozymes and hydrolytic enzyme activities, each BE isozyme, BEI, BEIIa or BEIIb, was expressed in E. coli cells, and the recombinant protein was purified by using anion-exchange and hydrophobic column chromatography. All the enzyme preparations were purified to near homogeneity as judged by SDS–PAGE (Supplementary Fig. S1), and the purified preparations were free from hydrolytic activities measured by zymogram.

Assay of BE isozymes

To reveal the kinetic parameters and the chain preference of each BE isozyme, it is essential to quantify its enzymatic activity efficiently and reproducibly. We recently established the bichinchoninic acid (BCA) method in which the amount of enzyme protein and the duration of the reaction.

Table 1

<table>
<thead>
<tr>
<th>K_m values</th>
<th>BEI (µM)</th>
<th>(mg ml⁻¹)</th>
<th>BEIIa (µM)</th>
<th>(mg ml⁻¹)</th>
<th>BEIIb (µM)</th>
<th>(mg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-1160 (DPn6,510)</td>
<td>0.18</td>
<td>0.20</td>
<td>1.2</td>
<td>1.4</td>
<td>0.78</td>
<td>0.90</td>
</tr>
<tr>
<td>AS-55 (DPn317)</td>
<td>2.9</td>
<td>0.16</td>
<td>31</td>
<td>1.7</td>
<td>2.6</td>
<td>0.15</td>
</tr>
<tr>
<td>AS-7 (DPn59)</td>
<td>Not determined</td>
<td>Not detected</td>
<td>Not detected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ne-amylopectin</td>
<td>18</td>
<td>1.5</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>V_max and related values [V_max k (kat) [CE⁻¹]]</th>
<th>BEI</th>
<th>BEIIa</th>
<th>BEIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-1160 (DPn6,510)</td>
<td>3.0 (7.4) [42]</td>
<td>2.7 (6.8) [5.8]</td>
<td>0.19 (0.46) [0.59]</td>
</tr>
<tr>
<td>(0.980)</td>
<td>(0.976)</td>
<td>(0.978)</td>
<td></td>
</tr>
<tr>
<td>AS-55 (DPn317)</td>
<td>2.3 (5.6) [2.0]</td>
<td>1.2 (2.9) [0.093]</td>
<td>0.045 (0.11) [0.042]</td>
</tr>
<tr>
<td>(0.981)</td>
<td>(0.841)</td>
<td>(0.925)</td>
<td></td>
</tr>
<tr>
<td>AS-7 (DPn59)</td>
<td>Not determined</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>ae-amylopectin</td>
<td>2.0 (5.0) [0.27]</td>
<td>5.4 (14) [9.3]</td>
<td>0.50 (1.2) [0.98]</td>
</tr>
<tr>
<td>(0.977)</td>
<td>(0.984)</td>
<td>(0.869)</td>
<td></td>
</tr>
</tbody>
</table>

The molar amount of each recombinant BE isozyme protein was calculated by the corresponding Da value: BEI, 148,238.57; BEIIa, 151,248.88; BEIIb, 146,521.18.

Determination of kinetic parameters of rice BE isozymes towards linear α-glucans and branched glucans

The kinetic parameters of the three rice BE isozymes with α-amylopectin as glucan substrate were determined. The K_m value of BEI (18 mg ml⁻¹) was much higher than those of BEIIa and BEIIb although BEIIa and BEIIb exhibited similar K_m values to each other, 1.5 and 1.3 mg ml⁻¹, respectively (Table 1). On the other hand, the maximal velocity (V_max) of BEIIb with α-amylopectin (0.50 µmol min⁻¹ mg⁻¹ protein) was about one order lower than that of BEIIa (5.4 µmol min⁻¹ mg⁻¹ protein), whereas the value for BEI (2.0 µmol min⁻¹ mg⁻¹ protein) was between that of BEIIa and BEIIb (Table 1).

The kinetic parameters of the three BE isozymes for linear α-glucans were compared with each other using synthetic amyloses having a different number-average degree of polymerization (DPn) of approximately 6,510 (AS-1160) and 317 (AS-55), respectively (Kitamura et al. 1982, Kitamura et al. 1989, Nakanishi et al. 1993, Utsumi et al. 2009). The V_max values for BEI and BEIIa were about the same using the long amylose with DPn6,510, while that of BEI was slightly higher than that of BEIIa using the short amylose with DPn317 as the glucan donor (Table 1). On the other hand, the V_max value for BEIIb with both amyloses was more than one order of magnitude lower than those of BEI and BEIIa (Table 1). When AS-1160 (DPn6,510) was the glucan substrate, the K_m value of BEI (0.18 µM) was markedly lower than those of BEIIa (1.2 µM) and BEIIb (0.78 µM).
Chain transfer properties of rice starch branching enzymes

(Table 1). The \( K_m \) values of BEI and BEIIa with the small amylose were remarkably higher than those with the large amylose on a molar concentration basis. In contrast, the \( K_m \) value of BEIIb with the large amylose (0.78 μM) was significantly smaller than that with the small amylose (2.6 μM) and, hence, the \( K_m \) value of BEI towards amylose with DPn317 (2.0 μM) was similar to that of BEIIb. Although AS-7 with DPn59, the smallest available amylose, was used as substrate, no parameters were obtained, possibly due to no activity or the extremely low activity of each BE isozyme towards AS-7.

Based on these parameters, catalytic efficiency (CE) defined as \( k_{cat} \cdot K_m^{-1} \) was calculated (Table 1). It was shown that the BEI isozyme had higher CE values towards amylose than the BEII isozymes, whereas BEII had higher CE values towards \( \alpha \)-amylopectin than BEI, although the CE values were higher for BEIIa than for BEIIb towards both amylose and \( \alpha \)-amylopectin.

All these kinetic investigations indicate first that BEI prefers linear \( \alpha \)-glucan amylose to branched \( \alpha \)-glucan \( \alpha \text{-amylopectin} \), due to its lower \( K_m \) value for amylose than \( \alpha \text{-amylopectin} \), although \( V_{max} \) values towards amyloses are only slightly larger than those towards \( \alpha \text{-amylopectin} \). In contrast, the \( K_m \) values of BEII isozymes for \( \alpha \text{-amylopectin} \) were much lower than that of BEI because both BEIIa and BEIIb had similar \( K_m \) values towards \( \alpha \text{-amylopectin} \) and long amylose with DPn6,510 on a mg ml \(^{-1}\) basis. Secondly, the present study clearly shows that BEIIa differs greatly from BEIIb in terms of their kinetic parameters towards both \( \alpha \)-amylopectin and amylose. The result contrasts sharply with the previous report with maize BEIIa and BEIIb because they have similar \( K_m \) and \( V_{max} \) values towards amyloses (Guan et al. 1993, Takeda et al. 1993). Thirdly, it is stressed that BE isozymes can react to branched glucan despite a short chain length (the average DP being <20) of its outer chains (Hizukuri 1996, Bertoft and Koch 2000) while their activity towards amylose dramatically diminishes when its chain length becomes <317, especially in the case of BEII isozymes.

Analysis of branched chain products formed after enzymatic reactions by rice BE isozymes

In an attempt to clarify the mechanism underlying the branching characteristics of BE, it is essential to analyze quantitatively in detail the chain length distribution of the debranched BE reaction products. To meet such a requirement, the APTS-assisted capillary electrophoresis method (Morell et al. 1997b) was used in this study.

To analyze clearly the preferences of the three BE isozymes for chain branching, the subtraction of each debranched chain distribution of the products after the BE enzymatic reaction from that of the substrate glucan (\( \alpha \text{-amylopectin} \)) was presented on a molar percentage basis in the left-hand panel of Fig. 1, since it shows to what extent each chain was increased or decreased by the BE enzymatic reaction. In principle no change in the amount of total glucose molecules occurred after the BE enzymatic reaction. According to this principle, the amounts of glucose molar equivalents in individual chains with different DP values were calculated on a glucose molar equivalent percentage basis and presented in the right-hand panel of Fig. 1. Compared with the left-hand panel, the figures in the right-hand panel can more precisely show to what extent chains were used in the BE reaction. For example, if some chains had more glucose molar equivalents after the reaction, it means that the chains were at least the donor segments (transferred chains) or residual chains or residual segments of the donor chains, according to the nomenclature of Borovsky et al. (1976). In addition, since the molar amounts of long chains in the products as well as \( \alpha \text{-amylopectin} \) were lower in the left-hand panel of Fig. 1, the right-hand figures can show more precise information than the left-hand figures as to whether long chains were used as donor chains or produced in or after the BE reactions.

Fig. 1 (left panel) demonstrates that in the BEIIb reaction products with \( \alpha \text{-amylopectin} \), the increase in chains with DP7 and DP6 was remarkable and chains with DP8–10 slightly increased while chains with DP ≥13 decreased. The right-hand figure shows that a significant increase of glucose molar equivalents was found in chains of DP up to 12, and chains of DP13–15 slightly increased. These results suggest that rice BEIIb almost exclusively transfers short chains of DP7 or DP6 by attacking chains of DP ≥13 or 12, and the increase in chains with DP8–12 is due to the formation of residual segments from the donor chains of DP14–19.

The time course experiments show that chains of DP7 and DP6 were predominantly increased from the beginning of the reaction period (1 min) until 10 min, and the ratio of the increase in the DP7 chain to that in the DP6 chain was kept constant during the enzymatic reaction (Fig. 2), supporting the above idea that BEIIb selectively transfers chains having DP7 and DP6.

The pattern of chain distribution of the products after the enzymatic reaction of BEIIa with \( \alpha \text{-amylopectin} \) was apparently different from that of BEIIb (Fig. 1, left panel). The increase in the chain of DP6 was the highest, followed by that in the chain of DP7. The chains of DP8 and DP9 also significantly increased, these increases being slightly lower than that of the chain of DP7. The extent of the increases decreased with chain length up to 11. On the other hand, intermediate and long chains of DP ≥14 decreased. The corresponding right-hand figure shows that an increase in glucose moieties was found until chains up to DP15. These results suggest that although BEIIa most effectively transfers a chain of DP6, the preference for chains transferred is not so strictly selective for the DP6 chain, but the preference gradually decreases with the increase in chain length until around DP13–15. The same pattern of change was observed irrespective of the duration of the enzymatic reaction with BEIIa and \( \alpha \text{-amylopectin} \) (Fig. 2).

The change in chain length pattern of the BEI product was dramatically different from that of either BEIIb or BEIIb (Fig. 1). The extent of the increase in the molar concentration of each short chain was at the same level in the range of DP6–12.
Fig. 1 Chain profiles of glucans produced by BE isozymes using αe-amylopectin as glucan substrate. Top panel: the chain length distribution of αe-amylopectin was expressed as the molar percentage of each chain (left) or glucose molar equivalent percentage (right) in αe-amylopectin. Lower left panels: the products formed by BE enzymatic reactions at 30°C for 30 min were debranched by bacterial debranching enzymes, and the resulting MOS were labeled with APTS at their reducing ends. The APTS-labeled MOS were subjected to capillary electrophoresis, and the molar amounts of separated MOS were measured at 488 nm. The vertical axis shows the subtraction of the relative molar amount of each MOS.
(the figure in the left panel). It is noteworthy that the amount of glucose molar equivalents in the intermediate chains of DP26–39 markedly increased (the figure in the right panel), whereas the increase in those chains was not found in the BEIIa and BEIIb products. Since the chain length profile in free portions from the non-reducing ends until the branch points in B1–B2 chains is likely to be similar to that in A and B1 chains of rice amylopectin through the analysis of the chain length profile of β-limit dextrins of the amylopectin (Bertoft 2000), the result can be explained by assuming that rice BEI can attack the inner α-1,4-glucan chains, and this results in formation of chains with DP26–39, while BEII isoforms have little or no such capacity. The assumption was further examined using Φ-LDs as substrate, as described below.

The chain length distribution of the products was also examined when BE isoforms were incubated with synthetic amylose with DPs of 6,510. Fig. 3 shows that BEIIb activity formed two distinct peaks, namely the first large peak containing chains of DP7 and DP6 and the second small peak including chains of DP ≥8 with a peak chain of DP9. Although the chain profile of the BEIIa reaction products seemingly yielded two peaks, the extent of the second peak of DP ≥8 or DP9 was markedly higher compared with that found in BEIIb. The results are consistent with the idea obtained from the results with ae-amylopectin that BEII almost exclusively transfers short chains with DP7 and DP6 whereas BEIIa transfers short chains in the DP range from 6 up to around DP13–15, with DP6 being the most preferred chain. In contrast, BEI produced two peaks in the lengths of branched chains, namely the first peak with chains of DP10–12 and the second peak with chains of DP29–31, although the DP6 chain was also significantly formed. The result indicates that BEI preferentially forms intermediate and longer chains compared with chains generated by BEIIb and BEIIa.

All these results establish that the three rice BE isoforms have distinct chain preferences of transferred chains in terms of the chain lengths during the branching reactions.

Effects of temperature on the chain preference of BEIIb for the branching reaction

The isoamyloryzates obtained from the reaction of BEIIb with ae-amylopectin at different temperatures were analyzed. The same pattern of chain length distribution was shown when the BEIIb reaction was conducted at 10, 20, 30 or 40°C (Fig. 4), indicating the same chain preference of BEIIb under different reaction temperatures from 10 to 40°C. The fact that the extent of changes in the amounts of chains in the BEIIb reaction products was lower at 40°C than at 20 or 30°C indicates that the BEIIb activity decreases or is inactivated at higher temperature above 30°C, consistent with the result with maize BEIIb (Takeda et al. 1993).

Minimum chain length of amylose required for BE reactions

Classical literature claimed that the minimum chain length of amylose for BE reactions is approximately DP40 when potato BEI is used as the enzyme source (Peat et al. 1952, Borovsky et al. 1976). As described in Table 1, the K_m values for amylose of all rice BE isoforms markedly increased with decreasing DP of the amylose, and their V_{max} values were markedly lower with the smaller amylose than the larger amylose. In fact, when short synthetic amylose with DP35–40 (AS-7) was used as the substrate for rice BEI isoforms, no practical activity was detected as long as the activity was assayed by the BCA method or the iodine staining assay, while substantial activity was found in BEI (data not shown). In an attempt to assay more precisely the BE activity and its reactivity to the short amylose, the chain length analysis of the debranched product obtained after incubation with each BE isoform and AS-7 was conducted by the APTS-assisted capillary electrophoresis method. Although the DP values ranged from 35 to about 80 in AS-7 (Fig. 5A), the chain profile of the BEI incubation product dramatically altered, as shown in the left panel of Fig. 5C, indicating that rice BEI can use AS-7 as the glucan substrate.

The right-hand figure shows that the proportion of chains with DP ≥49 decreased while that with DP ≤47 increased, suggesting that rice BEI can react to the former chains, but not or less to the latter chains, and therefore that the minimum chain length for the branching reaction by BEI is considered to be around DP50. The result is basically consistent with the report by Borovsky et al. (1976) that the minimum chain length for the branching reaction by potato BEI is around 40.

In contrast, no substantial change in the chain profile was found in the product obtained after the enzymatic reaction with the BEIIa isozyme (Fig. 5B) as well as with BEIIb (data not shown). The present observations suggest that both BEIIa and BEIIb were seemingly unable or only slightly able to react with synthetic amylose with DP smaller than about 100, whereas they can attack ae-amylopectin with the most abundant chain of DP around 15 (Table 1, Fig. 1).

BE reactions when Φ-LDs from amylopectin were used as glucan substrate

The above results (Fig. 1) suggest that BEI can attack the inner α-1,4-glucan chain while BEIIa and BEIIb cannot or can be only
Fig. 2 Time course of chain profiles of glucans produced by BE enzymatic reaction at 30°C for 1–10 min. The vertical axis and number on each panel show the same as in the left panel of Fig. 1. Left panel, products of BEIIb; right panel, products of BEIIa. Each value is the mean of duplicate measurements although the standard deviation is too small to detect in the figure. The data shown are used from one representative experiment chosen from at least three independent experiments using different enzyme preparations.
scarcely reactive to the inner chain. To examine this hypothesis, BE isozymes were incubated with Φ-LDs from amylopectin. The chain profile in the intermediate and/or long chains of Φ-LD will alter only when BE attacks the inner chain since all the outer chains of the dextrin have maltotetrasyl units from the branch points after treatment with phosphorylase (Hizukuri 1996) and hence the outer chains are too short for any BE isozyme. Fig. 6 indicates that BEI clearly reacted with the inner chains with a backbone linear chain length with DP ≥about 29 whereas BEIIa could only slightly act on the inner chain and BEIIb was likely to have no such activity.

**Discussion**

BE plays an essential role in starch biosynthesis in forming α-1,6-glucosidic linkages of α-glucan. Three forms of BE isozymes are expressed in cereal endosperm and considered to contribute to the specific fine structure of endosperm amylopectin. To understand how a tandem-cluster structure of amylopectin is constructed, it is essential to resolve several questions related to the mechanism of BE reaction, i.e. what is the minimum chain length of a donor chain for BE reaction, what and where in the glucan chains does BE recognize, what chain lengths are selectively transferred during its chain branching reaction, and how do such enzymatic characters differ for each BE isozyme? To answer these questions, in vitro analysis with purified BE concerning the characterization of BE enzymatic properties and BE reaction mechanism are necessary.

**Comparison of kinetic parameters of the three rice BE isozymes**

For characterization of BE properties, it is fundamental to determine their kinetic parameters; a few reports have shown these parameters of plant BE isozymes towards amylose (Guan et al. 1993, Takeda et al. 1993, Hamada et al. 2001, Nozaki et al. 2001), as summarized in Table 2. To our knowledge only a single report (Hamada et al. 2002) has dealt with these parameters toward amylopectin (Table 2). However, it should be noted that branched maltodextrins, namely amylopectin, pre-amylopectin and/or other types of branched dextrans, are considered to be physiologically much more important glucan donors for BE as compared with amylose during amylopectin biosynthesis because the tandem-cluster units of amylopectin must be synthesized by multiplying the clusters from its precursors and the process can normally be performed even in the absence of amylose, as found in waxy mutant tissues.

In the present investigation, comprehensive analyses of all the three BE isozymes from rice were performed mainly using ae-amylopectin as glucan substrate, and the results were compared with those with synthetic amylose with different chain lengths. It is known that plant BEI and BEII differ from each other with respect to activity towards linear or branched glucan and chain transfer properties. It is generally known that BEI prefers amylose to branched glucan, while BEII has a higher activity towards branched glucan than towards amylose (Guan et al. 1993, Guan et al. 1997). The present investigation indicates that rice BEI had a lower $K_m$ value for amylose than rice BEIIa (Table 1), consistent with the results with maize isozymes (Takeda et al. 1993). The fact that both BEI and BEIIa isozymes from rice exhibited similar $V_{max}$ values (Table 1) is in contrast to a previous report with maize isozymes showing that BEI has a higher $V_{max}$ value than BEIIa (Guan et al. 1993). On the other hand, rice BEI and BEIIb had the same $K_m$ level for short amylose with DPn317, whereas the $V_{max}$ value for the
Fig. 4 Effect of the reaction temperature on chain profiles of glucans produced by BE isozymes. The chain length distribution of the products obtained after enzymatic reaction with rice BEIIb and α-amylopectin for 30 min was determined and shown as in the left panel of Fig. 1, except the reaction temperature (10, 20, 30 or 40°C) as described in the figure.
Fig. 5 Reactivity of rice BE isozyme to short amylose. The BE reactions were conducted using synthetic amylose AS-7 at 30°C for 30 min. (A) The chain length distribution of the substrate amylose AS-7. Note that AS-7 is composed of chains with DP ranging from 35 to 80. (B and C) Left panel: the chain length distribution of the products after the enzymatic reaction of BEIIa (B) or BEI (C) with AS-7 as substrate was determined and is shown on the basis of the molar percentage of each chain, as in the left panel of Fig. 1. (B and C) Right panel: the data were calculated by assuming that the total glucose molar equivalents (by normalizing the total equivalents as 100) were unchanged after the BE enzymatic reactions. The subtraction of glucose molar equivalents of each MOS from the products after BEIIa (B) or BEI (C) enzymatic reaction from that from the substrate AS-7 is shown.

Amylose was much higher for BEI than BEIIb, these results being different from maize isozymes in that compared with BEII isozymes, BEI isozyme not only has a higher $V_{\text{max}}$ value towards amylose of DP405 (Takeda et al. 1993), but also exhibits a lower $K_m$ value for the amylose (Guan et al. 1993).

It is particularly interesting in the present results that rice BE isozymes have distinct parameters towards branched glucan ae-amylopectin compared with those towards amyloses. It should be noted that the minimum chain lengths of donor chains required for BE reactions are quite different between amylose and amylopectin because the reactive amylose to rice BE isozymes has a chain length of DPN317 or larger whereas the donor chains of ae-amylopectin have a chain length in the range of DP approximately 12–30 (Fig. 1). Despite much shorter chain lengths of the donor chains in amylopectin molecules as compared with those of amylose, both BEI and BEII type isozymes from rice were found to be highly reactive to ae-amylopectin although the $K_m$ value for ae-amylopectin (BEI, 5,500–7,400 µM; BEIIa, 460–610 µM; BEIIb, 400–530 µM) was much higher than that for amylose (BEI, 0.18–2.9 µM; BEIIa, 1.2–31 µM; BEIIb, 0.78–2.6 µM) on a molar concentration basis of donor chains (Table 1), if we calculate these values by assuming that the average chain length of ae-amylopectin is 15–20. The $V_{\text{max}}$ values for ae-amylopectin of BEI isozymes...
were apparently higher than those for amylose, whereas that of BEI was at the same level or slightly smaller than those for amylose (Table 1). These results indicate that all plant BE isoforms react to branched glucans in different manners from how they react to linear glucans.

The observations strongly suggest that BE can act on branched glucan much better than amylose in which the chain length is short in terms of DP ≤ 100 or a little bit larger, especially in the case of BEII isoforms. The higher $K_m$ value of BE for the branched dextrin cannot be a disadvantage in amylpectin biosynthesis, since amylpectin precursors are considered to be localized on the surface of the developing starch granule and hence the actual concentration of the donor chains of branched precursor must be very high under physiological conditions. There is no direct evidence for the mechanism of how BEs can react to short chains (DP ≤ 20) of branched dextrins while they are unable to attack the amylose (DP approximately ≤ 50). However, the fact that amylpectin cluster chains are present in parallel by forming double helices when their linear portion reaches DP > 10 (Gidley and Bulpin 1987) inclines us to think that the actual substrate for BE might be the double-helical parallel chains, as proposed previously by Borovsky et al. (1976). In addition, the higher solubility and/or ordered arrangement of chains of the branched dextrins in the semi-aqueous circumstance in the stromal compartment as compared with amylose might be properties favorable for them as substrates for BE isoforms.

**Comparison of chain transfer properties among the three rice BE isoforms**

To resolve the basic questions as to how each BE isozyme recognizes the glucan donor chains and differently attacks chains with varied chain lengths and where it forms the branch linkages in the acceptor chains, analysis of the fine structure of BE products is particularly important. Pioneering studies have been conducted by Preiss and colleagues. They found that BEII isoforms form shorter chains of DP3–12 whereas the BEI isoform produces preferentially short to intermediate chains ranging from DP6 to DP > 30 (Takeda et al. 1993, Guan et al. 1997). Later several groups reported basically similar results using BEI and BEII isoforms from rice endosperm (Mizuno et al. 1993), kidney bean embryo (Hamada et al. 2001, Nozaki et al. 2001) and potato tuber (Rydberg et al. 2001), although the minimum length of the transferred chain is considered to be six glucose units.

![Fig. 6 Chain profiles for phosphorylase-limit dextrins (Φ-LDs) in the rice BE reaction products.](https://academic.oup.com/pcp/article-abstract/51/5/776/1821103/786)

The chain length distribution of the products after the enzymatic reaction of BEI, BEIIa, or BEIIb with Φ-LDs of rice endosperm amylpectin at 30°C for 30 min was determined and is shown as a molar percentage of total MOS with the omission of the malto-tetraose chains. Top panel: the chain length distribution of the substrate Φ-LDs. Lower panels: the chain length distribution of the products after the enzymatic reaction of BEI, BEIIa or BEIIb with Φ-LD AS-7 was determined. Note that the amount of the second peak with a maximum DP around 33–35 found in Φ-LD was significantly reduced when Φ-LD was incubated with BEI, whereas the profile of Φ-LD chain length was unchanged when the dextrin was incubated with BEIIb, and the second peak was only slightly distorted by BEIIa. The other conditions are the same as in Fig. 1.
### Table 2: Comparison of kinetic parameters for plant BE

<table>
<thead>
<tr>
<th>Plant</th>
<th>Chain Length</th>
<th>BE with Amylose</th>
<th>BE with Amylose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Maize</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEI</td>
<td>(cl = 405)</td>
<td>2.0 µM (0.13 mg ml⁻¹)</td>
<td>1.04 (µmol min⁻¹ mg⁻¹)</td>
<td>Takeda et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>(cl = 197)</td>
<td>4.1 µM (0.13 mg ml⁻¹)</td>
<td>2.6 (µmol min⁻¹ mg⁻¹)</td>
<td>Guan et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>(cl = 56)</td>
<td>50 µM (0.45 mg ml⁻¹)</td>
<td>3.2 (µmol min⁻¹ mg⁻¹)</td>
<td>Guan et al. (1997)</td>
</tr>
<tr>
<td>BEIIa</td>
<td>(cl = 405)</td>
<td>10 µM (0.66 mg ml⁻¹)</td>
<td>0.32 (µmol min⁻¹ mg⁻¹)</td>
<td>Takeda et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>(cl = 197)</td>
<td>50 µM (1.6 mg ml⁻¹)</td>
<td></td>
<td>Guan et al. (1993)</td>
</tr>
<tr>
<td>BEIIb</td>
<td>(cl = 405)</td>
<td>11 µM (0.72 mg ml⁻¹)</td>
<td>0.14 (µmol min⁻¹ mg⁻¹)</td>
<td>Takeda et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>(cl = 197)</td>
<td>50 µM (1.6 mg ml⁻¹)</td>
<td>0.2 (µmol min⁻¹ mg⁻¹)</td>
<td>Guan et al. (1997)</td>
</tr>
<tr>
<td><em>Wheat</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEI</td>
<td></td>
<td>0.30 mg ml⁻¹ (WBE-IAD)</td>
<td>251 U a mg⁻¹</td>
<td>Morell et al. (1997a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.11 mg ml⁻¹ (WBE-IB)</td>
<td></td>
<td>Morell et al. (1997a)</td>
</tr>
<tr>
<td>BEII</td>
<td></td>
<td>0.65 mg ml⁻¹</td>
<td></td>
<td>Morell et al. (1997a)</td>
</tr>
<tr>
<td><em>Potato</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEI</td>
<td>(cl = 260)</td>
<td>15 (mol s⁻¹ mol⁻¹ enzyme)</td>
<td></td>
<td>Borovsky et al. (1975a)</td>
</tr>
<tr>
<td><em>Kidney bean</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEI</td>
<td>(PvSBE2)</td>
<td>1.27 mg ml⁻¹</td>
<td>251 U a mg⁻¹</td>
<td>Nozaki et al. (2002)</td>
</tr>
<tr>
<td>BEII</td>
<td>(PvSBE2)</td>
<td>0.74 mg ml⁻¹</td>
<td>234 U a mg⁻¹</td>
<td>Nozaki et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>(rPvSBE2)</td>
<td>1.27 mg ml⁻¹</td>
<td>242 U a mg⁻¹</td>
<td>Hamada et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>(rLF-PvSBE2)</td>
<td>4.80 mg ml⁻¹</td>
<td>396 U a mg⁻¹</td>
<td>Hamada et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>rPvSBE2</td>
<td>18.4 mg ml⁻¹</td>
<td>561 U b mg⁻¹</td>
<td>Hamada et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>rLF-PvSBE2</td>
<td>4.4 mg ml⁻¹</td>
<td>135 U b mg⁻¹</td>
<td>Hamada et al. (2002)</td>
</tr>
</tbody>
</table>

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*a* One unit of activity was defined as the decrease in absorbance of 0.1 at 660 nm per min when measured by the iodine staining assay.

*b* One unit of activity was defined as the decrease in absorbance of 0.1 at 540 nm per min when measured by the iodine staining assay.

c-l, chain-length.
However, the properties of the BE chain transfer reaction have been inadequately characterized. In most previous investigations the reaction products were analyzed using gel filtration chromatography and recently by HPAEC-PAD. As pointed out by Thiemann et al. (2006), however, the HPAEC-PAD method has two major limitations. First, the response of the detector PAD depends on the size of the MOS, and no data on such calibration for large MOS with a high DP value have been available (Koizumi et al. 1991). Thus the amount of each MOS liberated from dextrin after treatment of isoamylase is not quantitative and it cannot be compared with each other. Secondly, MOS are completely separated by HPAEC only when their DP values are smaller than about 30. To overcome these disadvantages, the capillary electrophoresis method is used in the present study because this method is applicable for the quantitative analysis of long MOS with DP30–100 (Morell et al. 1997b, Nakamura et al. 2002).

In principle, before and after the BE enzymatic reaction, first, no change occurs in the amount of total glucose molecules although the number of chains and therefore the number of non-reducing ends doubles on every reaction. Secondly, the chain lengths of the reaction products, namely the transferred chains and the residual segments, decrease. Thirdly, when the BE reaction proceeds, on a molar basis the proportion of the transferred chains is the same as that of the residual chains which remain after the cleavage of the substrate chains followed immediately by transfer of the chains residing in the non-reducing side of the substrate chains, although it is impossible to distinguish the transferred chains from the residual chains with this methodology. Fourthly, the increase in the number of chains can be calculated.

Based on the first criterion, changes in the glucose molar equivalents were quantitatively analyzed, as shown in the right-hand panel of Fig. 1. Based on figures from the right-hand panel, we can calculate and compare the numbers of individual chains which were increased or decreased after the BE reaction. When BEIIb was reacted with α-amylopectin for 30 min, the increase in the number of chains with DP6 and D7 accounted for approximately 76% of the total number of increased chains (DP6–15). The most probable explanation of these results is that almost all of the donor segments (the transferred chains) of the donor chains produced by BEIIb have lengths corresponding to DP7 and DP6, while the lengths of most residual segments of the donor chains (in the case of the interchain BE reactions) and/or the residual chains (in case of the intrachain BE reactions) ranged from DP6 up to a DP of at least about 15 (nomenclature after Borovsky et al. 1976), because the molar ratio of the transferred chains to the residual segment should be 1. On the other hand, after the BEIIa reaction the increase in the number of chains of DP7 was comparable with those of DP9 and D9, and hence the extent of increases in number of chains of DP8–15 reached up to about 47% of the total amount of increased chains (DP6–15). The result suggests that BEIIa transfers short chains with a more broad DP ranging from DP6 to about DP15, although the DP6 chain is most effectively transferred while the residual segment chain has a DP from 6 to about 15. In summary we assume that the chain preference of BEIIb is highly specific because the BE isozyme almost exclusively transfers branched chains with DP7 and DP6 by recognizing and attacking the seventh and sixth α-1,4-linkages, respectively, from the non-reducing end of the outer chain. On the other hand, BEIIa transfers a wide range of short outer chains, the lengths of DP ranging from 6 to about 15. The combined results shown in Figs. 1 and 6 also show that BEIIa can efficiently use both intermediate chains of DP17–26 and long chains of DP ≥40 as donor substrate while BEIIb can act on the intermediate chains at a high rate, but much less on the long chains of DP ≥40. The results suggest that BEIIb predominantly attacks the outer chains derived from cluster chains, but BEIIa can attack outer chains emerging from both the cluster chains and the cluster-linking chains.

In contrast, the profile of chains which BEI generated markedly differs from that produced by BEIIa and BEIIb. BEI formed not only short chains of DP6–15 with the double peaks of DP11 and DP6, but also middle size chains of DP26–39, indicating that BEI can attack the inner region of long chains of DP ≥40 to produce those middle chains while the inner chain cannot be used at all as a substrate by BEIIb although BEIIa seemingly can only partly act on the inner chain (Fig. 6). It is also noted that the chain profiles of the reaction products of BEI with synthetic amylose showed a bimodal pattern while that of BEIIa or BEIIb did not exhibit such a pattern, consistent with the previous studies with BEI-type isozymes from maize (Takeda et al. 1993) and potato (Borovsky et al. 1976, Rydberg et al. 2001, Andersson et al. 2002, Hanashiro et al. 2003).

Minimum chain lengths of linear α-glucans or branched α-glucans required for branching reactions by rice BE isozymes

Whelan and co-workers reported in their pioneering investigations on the mechanism of amylose branching action by potato BEI that the BEI requires an amylose substrate with at least 40 glucose units (Peat et al. 1952, Borovsky et al. 1975b, Borovsky et al. 1976). However, their conclusion was based on indirect evidence, but did not show directly the capacity of the BE isozyme using pure amylose with known DP values. In addition, they only conducted tests using potato BEI isoyme, but not any BEII isozymes. On the other hand, Nussenbaum and Hassid (1953) reported that the minimum chain length of amylose for potato BEI must lie between 42 and 116. Later Rydberg et al. (2001) measured the isoamylolysisates of the potato BE reaction products with linear dextrins having a peak DP value of about 60. For BEI, the majority of the linear dextrins with DP >60 were missing, while for BEII those with DP >70 apparently decreased, suggesting that BEI and BEII can attack amylose with DP >60 and DP >70, respectively. The DP values reported so far for various MOS must be questionable because all the studies used gel filtration chromatography to separate MOS.

We precisely measured the effects of BE activity on the shift of the isoamylolysate profile using synthetic amylose AS-7...
having a DP ranging from 35 to 80 with a maximum DP value of approximately 59 by the APTS-assisted capillary electrophoresis method (Fig. 4), indicating that the minimum chain length of amylose for BEI branching activity was a DP of approximately 50, while both BEIIa and BEIIb were unable to react with amylose having DP ≤80 and possibly no substantial activity can be shown towards amylose of DP <80 or DP <317 (Table 1). The results led us to the possibility that linear dextrans cannot be donor chains for BE isozymes under ordinary physiological conditions because our preliminary analysis failed to detect a substantial amount of linear MOS with DP >45 in the stromal compartment in developing rice endosperm.

The minimum chain length of amylopectin for BE cannot be determined only from the chain length preference studies because it is impossible to distinguish the transferred chains from residual chains. However, the fact that we failed to detect chains having a chain length of ≤5 after any BE enzymatic reactions (Figs. 1–4) indicates that the minimum chain length for BE reaction must be 12 glucose units or longer, although the precise length for each BE isozyme remains to be elucidated.

The present study demonstrates that all rice BE isozymes can react with both branched glucan ae-amylopectin and linear glucan synthetic amylose, and their distinct chain length preferences in their BE reactions are not changed by any glucan substrates (Figs. 1, 3). However, it is noted that every BE reacts poorly with the synthetic amylose with the decrease in its DP value due to the increase in $K_m$ value and the decrease in $V_{max}$ value, and it is actually difficult to detect the activity when $DnP$ values are <317 (AS-55, DPN317; AS-7, DPN59) (Table 1), basically consistent with the results with maize BE isozymes (Takeda et al. 1993). It is noted that during the course of the BE reaction with amylose, the branched product might be the donor substrate for the subsequent BE reaction, and this might be, at least to some extent, the reason for the same chain profile of the product irrespective of the type of donor substrate: branched glucan or synthetic amylose.

**Possible roles of rice BE isozymes in amylopectin biosynthesis**

Based on results obtained from the present investigations, Table 3 summarizes the differences in chain length preference among BEI, BEIIa and BEIIb activities towards amylopectin.

Previous observations (Nishi et al. 2001, Satoh et al. 2003b) support the idea that there are two types of branches in the amylopectin cluster; namely the branches in the amorphous lamellae and the branches residing on the border between the amorphous lamellae and the crystalline lamellae, and the former branches are mainly catalyzed by BEI while the latter branches are almost specifically produced by BEIIb (Nakamura 2002). Although the idea has not yet been proven, the present in vitro study with three recombinant rice BE isozymes indicates that individual BE isozymes play distinct roles in amylopectin biosynthesis, and the results are consistent with the above idea leading from the in vivo studies, as illustrated in Fig. 7. The specific role of BEIIb in the synthesis of short A and B1 chains in the crystalline lamellae influences the fine structure of amylopectin stored in cereal endosperm of which starch has an A-type X-ray diffraction pattern whereas starch in the absence of BEIIb activity shows a B-type pattern (Nishi et al. 2001). Our previous reports strongly suggest that rice starch synthase I (SSI) plays a specific role in elongating chains of DP6 and DP7 to short

**Table 3** Summary of chain transfer properties of rice BE isozymes towards amylopectin

<table>
<thead>
<tr>
<th>Chains Type</th>
<th>BEI</th>
<th>BEIIa</th>
<th>BEIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Outer chains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferred chains</td>
<td>DP6–15 or longer</td>
<td>DP6–15</td>
<td>DP7 and DP6</td>
</tr>
<tr>
<td>Residual chains</td>
<td>(DP ≥6)</td>
<td>DP6–15 or longer</td>
<td>DP6–15 or longer</td>
</tr>
<tr>
<td>Donor chains</td>
<td>(DP ≥12)</td>
<td>DP12–30 or longer</td>
<td>DP12–22 or longer</td>
</tr>
<tr>
<td><strong>Inner chains</strong></td>
<td>Active</td>
<td>Poorly active</td>
<td>Inactive</td>
</tr>
</tbody>
</table>
chains of DP8–12 (Fujita et al. 2006) while rice SSIIa is involved in the synthesis of cluster chains of DP \leq 24 from chains of DP \leq 10 (Nakamura et al. 2005). We hypothesize that in maturing rice endosperm where starch is synthesized at a high rate, the uniformity of the length of branched chains of DP7 and DP6 formed by BEIIb might be important in the formation of the same fine structure of clusters in amylopectin molecules and in the efficiency of amylopectin synthesis by facilitating synchronized and concerted action of each SS isozyme because the capacities of rice SSI (Fujita et al. 2006), SSIIa (Nakamura et al. 2005) and SSIIa (Fujita et al. 2007) are highly dependent on the chain lengths of substrate chains for their elongation reactions. Although the mechanism of how the synthesis of the new cluster is initiated from the preceding cluster during amylopectin synthesis is unknown, the properties of BEI which preferentially transfers chains with a wide range of DP values with a peak around 11 from intermediate and long chains including the inner chains of amylopectin might have more advantage of initiation of chains in the amorphous lamellae in the new cluster as compared with BEII isozymes.

Materials and Methods

Materials

Three amylose samples, AS-7, AS-55 and AS-1160, were enzymatically synthesized by a previously reported method using potato phosphorylase (EC 2.4.1.1) (Kitamura et al. 1982, Kitamura et al. 1989, Nakanishi et al. 1993). The number- and weight-average molecular weights (\(M_n\) and \(M_w\)) and molecular weight distributions were evaluated by high-performance GPC using a low-angle laser-light-scattering photometer (LALLS; LS8000; Tosoh Co., Ltd., Japan) and a differential refractometer (RI; RI-8011; Tosoh Co., Ltd.) as detectors. Based on the \(M_n\) values, the DPn can be calculated as 6,510 (AS-1160), 317 (AS-55) or 59 (AS-7), respectively, (Fig. 5A). The amylose was dissolved in 0.25 M aqueous CH\(_3\)COOK at a concentration of endosperm is demonstrated in six stages. (1) After the synthesis of a cluster is completed, one of the chains is elongated by SSIIa. (2) BEI transfers the inner chains and/or the long outer chains to the elongated chain, at the positions as presented by filled circles. (3) The newly synthesized chains are elongated mainly by SSIIa and/or to some extent by SSIIa. (4) When the lengths of the non-branched region of the elongated chains reach \geq 12, BEIIb attacks them to form the second branches, as shown by open circles. Due to the specific function of BEIIb, the newly formed chains have the similar length of DP7 and DP6. (5) These chains are elongated by coordinate actions by SSI (Nakamura 2002, Fujita et al. 2006) and SSIIa (Nakamura 2002, Fujita et al. 2007). (6) Through the enzymatic actions from (1) to (5), the new cluster is synthesized. It should be noted that starch debranching enzymes are considered also to be involved in the amylopectin by clearing ill-positioned branches which are accidentally formed during the BE reactions illustrated in (2) and (4), although the possible function is not described in the figures (see Nakamura 2002).
ether, and stored at 80°C. The procedure was repeated four times. The final resulting precipitate was washed with acetone, followed by diethyl ether, and the supernatant was added to 3 vols. of ethanol. The precipitate was washed with acetone, followed by diethyl ether, and stored at −30°C until use.

Φ-LD was prepared from starch seeds of a BEIIb-deficient amylose-extend (ae) mutant line EM10 (Nishi et al. 2001) was removed from amylose which was precipitated with 1-butanol and isoamylalcohol according to the method of Takeda et al. (1986). To remove the lower molecular weight glucans in the AS-1160 and AS-820 preparations, 20 mg of these preparations were dissolved in 1 ml of 100% dimethylsulfoxide (DMSO) at 80°C and added to 8 ml of distilled water. The dissolved amylose was precipitated by adding 1 ml of 100% 1-butanol and 100 µl of 5 M NaCl, and centrifuged at 10,000 × g for 20 min. The procedure was repeated four times. The final resulting precipitate was dried by washing with acetone and diethyl ether, and stored at −30°C until use.

The amylopectin from rice seeds from a BEIIb-deficient amylose-extend (ae) mutant line EM10 (Nishi et al. 2001) was removed from amylose which was precipitated with 1-butanol and isoamylalcohol according to the method of Takeda et al. (1986). To remove the lower molecular weight glucans in the AS-1160 and AS-820 preparations, 20 mg of these preparations were dissolved in 1 ml of 100% dimethylsulfoxide (DMSO) at 80°C and added to 8 ml of distilled water. The dissolved amylose was precipitated by adding 1 ml of 100% 1-butanol and 100 µl of 5 M NaCl, and centrifuged at 10,000 × g for 20 min. The procedure was repeated four times. The final resulting precipitate was dried by washing with acetone and diethyl ether, and stored at −30°C until use.

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Construction and induction of recombinant rice BE

The cDNA used as template for the following PCRs was prepared as follows. Total RNA of a japonica rice cv. Nipponbare was extracted from developing seeds at the early to mid-milking stage with the RNAeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. The RNA (2 µg) was used for first-strand cDNA synthesis with the iScript cDNA Synthesis Kit (Bio-Rad).

The DNA fragment encoding rice BEI mature protein was generated by PCR with forward primer 5′-tagtgaaccatattgagcttgagttggaggaggtcgaccactctcc-3′ and reverse primer 5′-agaggcaagttggaggtctcgctggagcatcctgcagctgagtacc-3′ and reverse primer 5′-aagggccctcgagttagtcctct and reverse primer 5′-aagggccctcgagttagtcctct, and cDNA as template. The PCR product was digested with Hpal–PstI and was assembled with the PshA1–PstI fragment of pET44b (Novagen). The DNA fragment encoding rice BEIIa mature protein was generated by PCR with forward primer 5′-tagtgaaccatattgagcttgagttggaggaggtcgaccactctcc-3′ and reverse primer 5′-aagggccctcgagttagtcctct, and cDNA as template. The PCR product was digested with Hpal–Xhol and was assembled with the PshA1–Xhol fragment of pET44b (Novagen). The DNA fragment encoding rice BEIIb mature protein was generated by PCR with forward primer 5′-atttaaatggcgcgggccggccgggctagtcc-3′ and reverse primer 5′-caagggccctcgagttagtcctct, and cDNA as template. The PCR product was digested with Nael–Sall and was assembled with the PshA1–Sall fragment of pET44b (Novagen). The OsBEIIb fragment containing all of the tag sequence of pET44b was transferred to pColdIII (TAKARA), because the OsBEIIb protein level in E. coli was low when its gene was inserted into pET44b. All of these plasmids were incorporated into the E. coli AD494 (DE3) pLySts strain (Novagen). The transformed cells were incubated in 10 ml of Luria broth containing 100 µg ml⁻¹ chloramphenicol and 15 µg ml⁻¹ kanamycin overnight at 37°C. A 10 µl aliquot of the pre-culture medium was incubated in 1 liter of Luria broth containing 100 µg ml⁻¹ ampicillin, 34 µg ml⁻¹ chloramphenicol and 15 µg ml⁻¹ kanamycin at 37°C. When the optical density at 600 nm (OD₆₀₀) of the BEI culture medium reached 0.6, BEI protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by incubation at 37°C for 3 h. When the OD₆₀₀ of the BEIIa culture medium reached 0.6, both proteins were induced by the addition of 1 mM IPTG followed by incubation at 25°C for 20 h. When the OD₆₀₀ of the BEIIb culture medium reached 0.6, the culture was incubated at 15°C for 30 min, and the BEIIb protein was induced by the addition of 1 mM IPTG followed by incubation at 15°C for 20 h. The cells were collected by centrifugation at 7,000 × g for 10 min. The collected cells were stored at −80°C until use.

Purification of BE

All the procedure was performed at 0°C. The frozen cells per liter of the above culture were suspended in 50 ml of Medium A including 50 mM imidazole·HCl (pH 7.4), 8 mM MgCl₂, 1 mM dithiothreitol (DTT) and 12.5% glycerol. The cells were lysed by sonication and lysed cells were centrifuged twice at 10,000 × g for 30 min. The supernatant was added to 3 vols. of ethanol. The precipitate was washed with acetone, followed by diethyl ether, and dried. The resulting Φ-LD preparation (~1.2 g) was used in the present study.
to HPLC using a TSKgel DEAE-SPW column (7.5 mm in diameter×75 mm in length, Tosoh Corporation, Tokyo, Japan), which had been equilibrated with Medium A. The proteins were eluted with a linear gradient of NaCl concentration (0–0.5 M) in Medium A for 60 min at a flow rate of 1 ml min⁻¹.

The BE activity in each fraction was detected with a modification of the phosphorylase a stimulation assay (Hawker et al. 1974). The reaction mixture contained 50 mM HEPES-HCl buffer (pH 7.0), 50 mM glucose 1-phosphate, 2.5 mM AMP, rabbit muscle phosphorylase a (0.1 unit, SIGMA) and an aliquot (1–5 µl) of each fraction in a total volume of 20 µl. The reaction was run for 30 min at 30°C and terminated at 100°C for 5 min. The activity was visualized by treatment of the reaction mixture with iodine solution. The peak fraction of BE activity was desalted and concentrated as described above and stored at −80°C until use.

**BE enzymatic reaction**

The BE reaction was conducted at 30°C for 30 min in 0.2 ml of the mixture containing 100 mM Na-phosphate buffer (pH 7.5), 1 mM DTT, 128 µg of ae-amylopectin and the recombinant BE from the rice BEIIb, BEIIa or BEI isozyme. The enzymatic reaction was terminated by boiling the reaction mixture for 2 min, and the BE activity was measured by the BCA method as described below.

**Assay of BE**

Assay of BE for determining kinetic parameters was conducted according to the method of Utsumi et al. (2009). Solution A consisted of 97.1 mg of disodium 2,2′-bicinchoninin (BCA), 3.2 g of sodium carbonate monohydrate and 1.2 g of sodium bicarbonate in a total volume of 50 ml. Solution B consisted of 62 mg of copper sulfate pentahydrate and 63 mg of l-serine in a total volume of 50 ml. The working reagent was freshly prepared by mixing equal volumes of Solutions A and B (Fox and Robyt 1991). A 225µl aliquot of the carbohydrate sample was added to 225 µl of the working reagent in an Eppendorf tube, and the mixture was incubated at 80°C for 40 min in a water bath. After the incubation, the assay mixture was then cooled to room temperature and left to stand for 10 min. An aliquot (150 µl) of the BCA-treated sample was taken and its absorbance at 560 nm was measured by using a microplate spectrophotometer (Bio-Rad). The absorbance at 560 nm was found to be proportional to the concentration of maltose or glucose in the range of 0 to at least 25 µM of the assay mixture.

**Analysis of chain length distribution of glucans**

The glucans in the rest of the reaction mixture were debranched by incubating in a containing 40 mM Na-acetate buffer (pH 4.4), *Pseudomonas amylofera* isomaltase (2 U, Hayashibara Biochem. Laboratory, Okayama, Japan) and *Klebsiella pneumonia* pullulanase (0.9 U; Waka, Tokyo, Japan) at 37°C overnight in a total volume of 0.3 ml. The glucan chain length distribution analysis of the resulting mixture was conducted by capillary electrophoresis after labeling of the glucans with the fluorescent probe APTS at their reducing ends according to the method of Morell et al. (1997b).

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


Chain transfer properties of rice starch branching enzymes


