BRANCHING ENZYMES

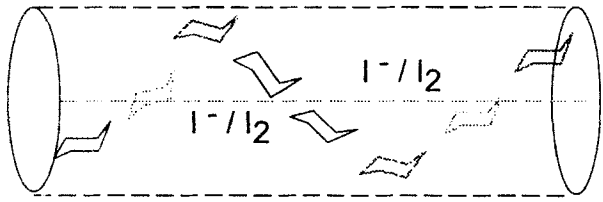
I. INTRODUCTION

The third reaction in the starch biosynthetic pathway is catalyzed by the 1,4-α-D-glucan: 1-4-α-D-glucan 6-glycosyl-transferase (branching enzyme, BE; E.C. 2.4.1.18), responsible for the synthesis of the α-1,6 linkages found in amylopectin and in glycogen. In *E. coli* or other bacteria, only one branching enzyme and one gene are present, whereas in plants, genetic and biochemical evidence indicates there can be two or more forms of branching enzyme. The challenge is to identify the role of each multiform in the synthesis of the complex structure of starch and to understand how differences in enzyme structure determine the specificities in acceptor and branching modes. As usual with plant enzymes, this task is made more difficult by the presence of proteases in crude extracts, and also by the fact that proteolyzed enzymes can still have activity even when their specific properties have been altered in an irreversible manner.

II. ASSAY

Several assays (Fig. 1) are available to measure branching enzyme activity. The iodine assay is based on the decrease in absorbance of the glucan–iodine complex (Krisman, 1962) resulting from the branching of amylose or amylopectin by the enzyme, provided that α-amylases are absent from the enzyme preparation or their activity is greatly reduced by adjusting the assay conditions.

The phosphorylase-stimulation assay (Hawker *et al.*, 1974) is based on the stimulation of the "unprimed" (without added glucan) phosphorylase activity (Fig. 2) of the phosphorylase from a rabbit muscle, as the branching enzyme present in the assay mixture increases the number of nonreducing ends available to the phosphorylase for elongation. It was made more sensitive by using ^{14}C-labeled glucose-1-P and measuring its incorporation into starch, rather than by measuring the formation of P_i.



Helical Iodine / α-D-glucan complex

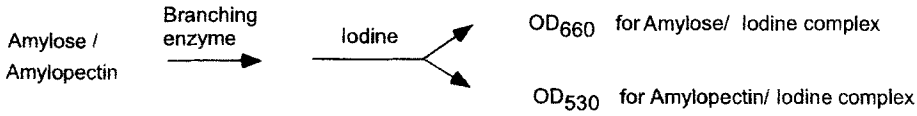


FIG. 1. Iodine staining assay. The substrate, amylose or amylopectin, is branched by the branching enzyme. The assay is terminated by adding iodine/iodide reagent. Amylose and amylopectin form complexes with the iodine/iodide reagent, whose peak of absorbance varies depending on the polymer, and the wavelengths used are 660 nm for amylose/iodine complex and 530 nm for the amylopectin/iodine complex. Branching of the substrate decreases the absorbance of the complex, a fact used to quantify the activity of branching enzyme. Figure reprinted with permission from Binderup (1997).

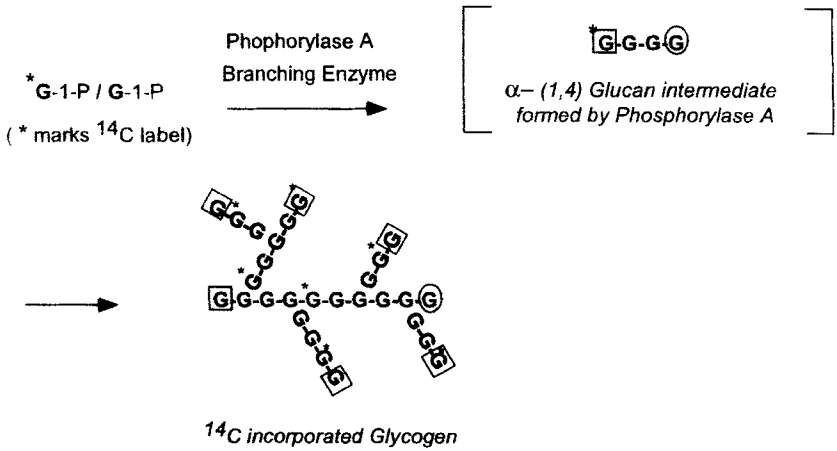


FIG. 2. Phosphorylase stimulation assay of branching-enzyme activity. The phosphorylase a elongates an endogenous primer from the nonreducing end [G]. The branching enzymes originate new branch points, which constitute additional nonreducing terminals, which phosphorylase A can elongate further. The elongated polymer contains ^{14}C-labelled glucose (*G). The only reducing end in the molecule is indicated by the Ⓖ. Figure reprinted with permission from Binderup (1997).

The new branching-linkage assay ("BL assay"; Takeda *et al.*, 1993) is the only one that measures the number of events catalyzed by the branching enzyme (rather than an indirect effect of its action as in the two assays described in the preceding) (Fig. 3). The enzyme fraction is incubated with the substrate, $NaBH_4$-reduced amylose, the reaction is then stopped by boiling, and the product is then incubated with *Pseudomonas* isoamylase for debranching. Finally, the reducing power of the oligosaccharide chains transferred by the enzyme is measured. Reduced amylose is used as the substrate to eliminate or minimize the reducing power of the amylose itself.

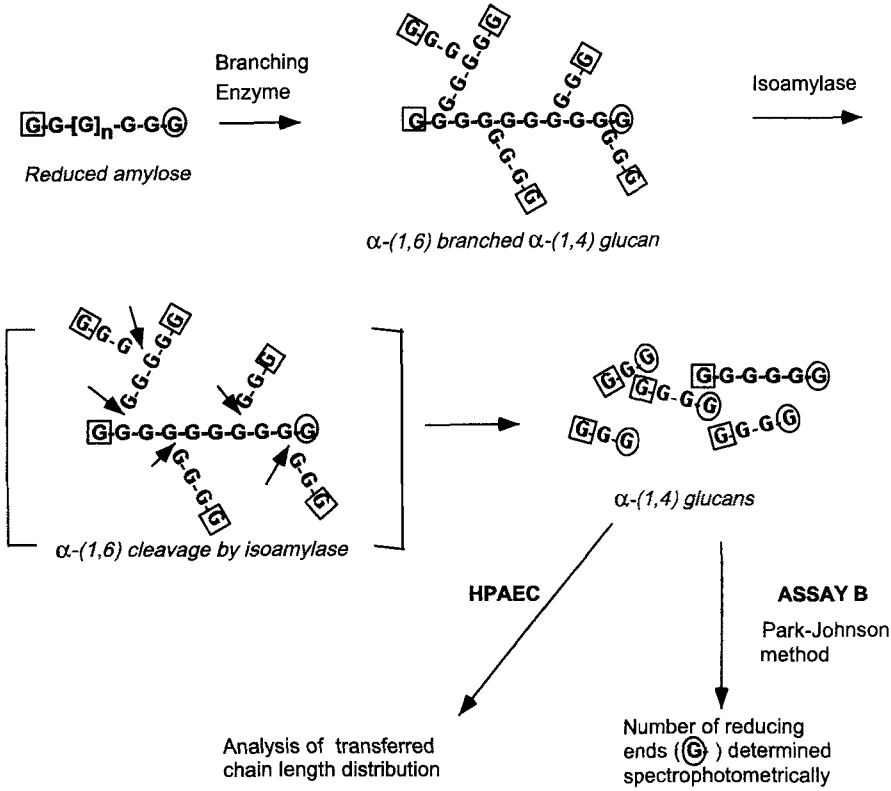


FIG. 3. The mostly linear substrate is branched by branching-enzyme during the assay. After termination of the assay, the now branched polymer is debranched using pure isoamylase, resulting in the liberation of one reducing terminal per chain. In the last step, the reducing terminals are quantified by the Park–Johnson method. Alternatively, the distribution of transferred chains can be determined using HPAEC. Nonreducing terminals are denoted by Ⓖ, reducing ends are denoted by Ⓖ. Figure reprinted with permission from Binderup (1997).

The branching-linkage assay is the most quantitative assay for the branching enzyme, but amylolytic activity interferes most with this assay; the phosphorylase-stimulation assay is the most sensitive and the iodine assay is not very sensitive but allows the testing of branching enzyme specificity with various α-1,4-dextrins, providing information on the possible role of the different branching enzyme isoforms. For complete characterization, it may be best to employ all three assays when studying the properties of the branching enzymes. Above all, however, if reliable information is being sought, the branching enzymes must be purified to the extent that all starch degradative enzymes are eliminated before studying the properties of branching enzymes.

III. PURIFICATION OF BRANCHING ENZYME MULTIFORMS

Multiple forms of branching enzyme have been found in many plants, for example, spinach leaf, maize endosperm, maize leaf (Dang and Boyer, 1988), castor bean (Goldner and Beevers, 1989), developing seeds of pea and sorghum, and teosinte seeds (for reviews see Preiss, 1988). Smyth (1988) resolved two or three peaks of Indica rice starch-branching enzyme on an anion-exchange column. The properties of these fractions are similar to those reported for other plants, except that their molecular weights were determined to be only 40,000.

In maize endosperm, there are three BE isoforms (Singh and Preiss, 1985; Guan and Preiss, 1993), and reports on other tissues are consistent with the presence of more than one isoform. A procedure described by Boyer and Preiss (1978a,b) and by Singh and Preiss (1985), which resulted in enzyme fractions with high specific activity but with traces of amylases, was further improved in our laboratory (Guan and Preiss, 1993). The purification steps involve homogenization, ammonium sulfate precipitation, and chromatography on DEAE-sepharose ("fast-flow"), in which the three isoforms are first separated. BEI is further purified by chromatography on ω-aminodecyl agarose, and by FPLC using MonoQ and Superose 12. Further purification of both BEIIa and BEIIb requires chromatography on ω-aminooctyl agarose and MonoQ. The resulting preparation of BEIIa requires chromatography on BioGel P10 to remove contaminating carbohydrate. Monoclonal antibodies were prepared against BEI and a mixture of BEs IIa and IIb. Singh and Preiss (1985) concluded that although some homology exists among the three starch BEs, there are major differences in the structure of BEI when compared with BEIIa and BEIIb, as shown by its different reactivity with some monoclonal antibodies, and there are differences in amino-acid composition and in proteolytic digest maps. It

was also concluded (Singh and Preiss, 1985) that BEs IIa and IIb are similar and possibly are the products of the same gene. Further evidence (Fisher *et al.*, 1993, 1996a), however, suggests that BEIIa and BEIIb may, after all, be products of two different genes (see the later section, "How Many Genes for Three Maize-Branching Enzymes?").

Because of the difficulties involved in separating the isoenzymes among themselves and from other enzymes such as amylases, it is not yet clear what the best acceptors are and what the products are for each isoenzyme, but some characterization of the enzymes has been done (e.g., K_m for α-glucans such as amylopectin, different animal glycogens, and maltosaccharides). Characterization of the products has been minimal, but some progress has been made in our laboratory (see the next section, "Mode of Action").

In potato tubers, Vos-Scheperkeuter *et al.* (1989) purified a single form of branching activity of molecular mass 79,000, confirming the previous work of Borovsky *et al.* (1975). Antibodies were prepared to the native potato enzyme and it was found they reacted strongly only with maize enzyme I and weakly with IIb. In neutralization tests, they inhibited the activities of both the potato tuber-branching enzyme and of maize-branching enzyme I. Antibodies prepared against denatured potato-branching enzyme reacted with all forms of denatured maize- and potato-branching enzyme in immunoblots, but not with the native enzyme forms. It was concluded that the potato-branching enzyme shows a high degree of similarity to the maize-branching enzyme form I and, to a lesser extent, to the other forms of maize-branching enzyme.

IV. MODE OF ACTION

Some subtle differences among BEI and BEII of maize, in the K_m for several primers, and their response to citrate have been described (see Preiss, 1991). More recently, Takeda *et al.* (1993) have analyzed the branched products made from amylose by each BE isoform. This was done by debranching the products of each isoform using isoamylase, followed by gel filtration.

BEIIa and BEIIb are similar in their affinity for amylose and the properties of the products (Tables I and II). When presented with amyloses of different average chain lengths, all the BEs have higher activity with the longer chain amylose. BEI can still catalyze the branching of an amylose of average chain length (c.l.) of 197 with 89% of the activity shown for the c.l. 405. The activity of BEII drops sharply with a reduction in c.l. The action of BEIIa and BEIIb results in the transfer of chains shorter than

TABLE I

SPECIFIC ACTIVITIES (UNITS/MG OF PROTEIN) OF BRANCHING ENZYME ISOFORMS AS MEASURED USING DIFFERENT ASSAY METHODS[a,b]

BE	BEI	BEIIa	BEIIb
(a) Phosphorylase stimulation	1332	795	927
(b) Branching-linkage assay	2.4	0.32	0.33
(c) Iodine stain assay			
(c_1) Primer: amylose	574	29.5	53
(c_2) Primer: amylopectin	47	59	105
Ratio of activity			
a/b	555	2484	2809
a/c_1	2.3	27	18
a/c_2	49.8	13.5	8.8
c_2/c_1	0.03	2	2

[a] Data from Guan and Preiss (1993).

[b] For this experiment, it is not essential to use enzyme purified to homogeneity, hence the relatively low specific activities. The enzymatic fraction, however, must be free of amylases or contaminating branching activities.

BEI. The action of BEI, BEIIa, and BEIIb on amylopectin has also been studied by Guan and Preiss (1993). Of the three isoforms, BEI had the highest activity in branching amylose and its rate of branching amylopectin

TABLE II

PROPERTIES OF THE PRODUCTS OF THE ACTIVITY OF BRANCHING ENZYME ISOFORMS ON AMYLOSE OR AMYLOPECTIN[a]

α-Dextrin + branching enzyme	λ_{max} (nm)	Decrease in absorbance (%)	β-amylolysis limit (%)	
			Before	After isoamylolysis
Amylose, no BE	639	77	100	—
Amylose + BEI	530	89	44	100
Amylose + BEIIa	593	46	48	99
Amylose + BEIIb	600	47	45	98
Amylopectin, no BE	530	—	60	100
Amylopectin + BEI	495	54	53	100
Amylopectin + BEIIa	491	57	46	98
Amylopectin + BEIIb	489	58	42	99

[a] The substrates, either 1 mg/ml amylose (c.l. 405) or amylopectin (c.l. 21), were incubated with 5 units/ml maize-branching enzyme (measured as by stimulation of phosphorylase *a*; Guan and Preiss, 1993). The decrease in absorbance by the iodine/glucan complex was measured at 660 nm.

was less than 5% of that for amylose. In contrast, the BEIIa and BEIIb isoforms branched amylopectin at twice the rate they branched amylose, and catalyzed branching of amylopectin at six times the rate observed for BEI. The assay used was iodine staining (Boyer and Preiss, 1978a,b). These results are consistent with those of Takeda *et al.* (1993) in suggesting that BEI catalyzes the transfer of longer branched chains and that BEIIa and IIb catalyze the transfer of shorter chains. Thus, it is possible that BEI may produce lightly branched polysaccharides, which then serve as substrates for enzyme complexes of branching enzyme II isoforms and starch synthases to synthesize amylopectin. Branching enzyme II isoforms may play a major role in forming the short chains present in amylopectin (Guan and Preiss, 1993).

The study of the reaction products showed that the action of BEIIa and BEIIb resulted in the transfer of chains shorter than those transferred by BEI. The action of the isoforms on amylopectin has been studied by Guan and Preiss (1993) and, of the three isoforms. BEI had the highest activity (using the iodine assay) on amylose, and its rate of branching amylopectin was less than 5% of that with amylose. In contrast, the BEIIa and BEIIb isoforms branched amylopectin at twice the rate of amylose, and catalyzed the branching of amylopectin at six times the rate observed for BEI. In short, the experimental evidence suggests that BEI may be more involved in producing the more interior (B-) chains of the amylopectin, whereas BEIIa and BEIIb would be involved in forming the exterior (A-) chains.

V. HOW MANY GENES FOR THREE MAIZE-BRANCHING ENZYMES?

In neutralization tests, antibody raised against form I inhibits BEI but not BEIIa or BEIIb, and antibody prepared against IIa and IIb inhibits IIa and IIb but not BEI, confirming earlier reports by Fisher and Boyer (1983). However, when an enzyme-linked immunoadsorbent assay (ELISA) was used, some reaction of BEIIa and BEIIb was detected when using antiserum raised against BEI (Singh and Preiss, 1985). The antisera prepared with IIa and IIb, however, reacted only to a very small extent with BEI. This was also observed in using purified monoclonal antibodies raised against BEIIa and BEIIb (Singh and Preiss, 1985). Three monoclonal antibodies could react with all three branching enzymes in the ELISA assay, whereas three other lines produced monoclonal antibodies specific for forms IIa or IIb (Fig. 4). Neutralization of enzyme activity of all three branching enzymes was seen with the antibody that reacted with all three in the ELISA test, whereas the monoclonal antibody that reacted with only

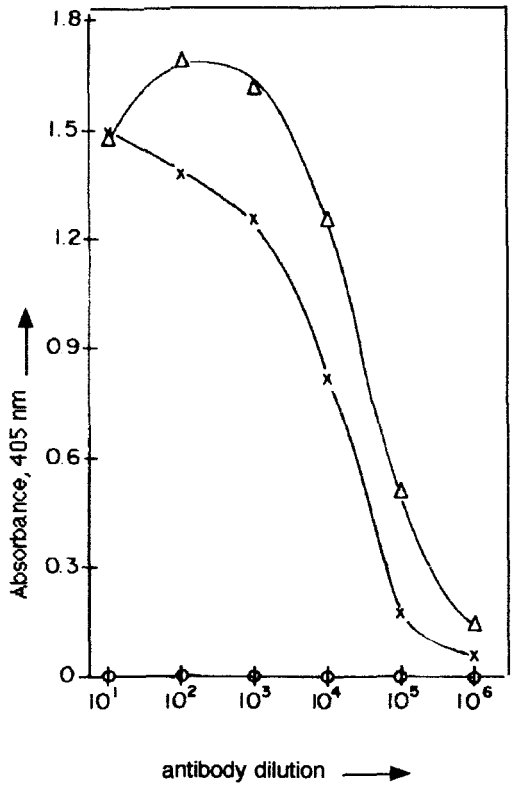


FIG. 4. Monoclonal antibodies obtained from different cell lines (only one cell line is illustrated as an example) reacted with both BEIIa and BEIIb, but did not react with BEI. ○, BEI; x, BEIIa; △, BEIIb. Figure reprinted with permission from Singh and Preiss (1985).

IIa and IIb inhibited their activities in neutralization tests but did not inhibit enzyme I activity.

Amino-acid composition studies also indicate major differences between BEI and the other two forms, whereas only minor differences are observed between IIa and IIb (Singh and Preiss, 1985). High-performance liquid chromatography (HPLC) patterns of a BEI peptide digest (whether trypsin or chymotrypsin were used) are very different in both the number of peptides and their retention times than those obtained with BEIIa or BEIIb digests. The digest patterns produced by trypsin are shown in Fig. 5. In contrast, no differences in HPLC are observed between the peptide maps of BEIIa and BEIIb forms using either trypsin (Fig. 5) or chymotrypsin (Singh and Preiss, 1985). It was concluded that some homology exists among the three starch-branching enzymes, but major differences exist between

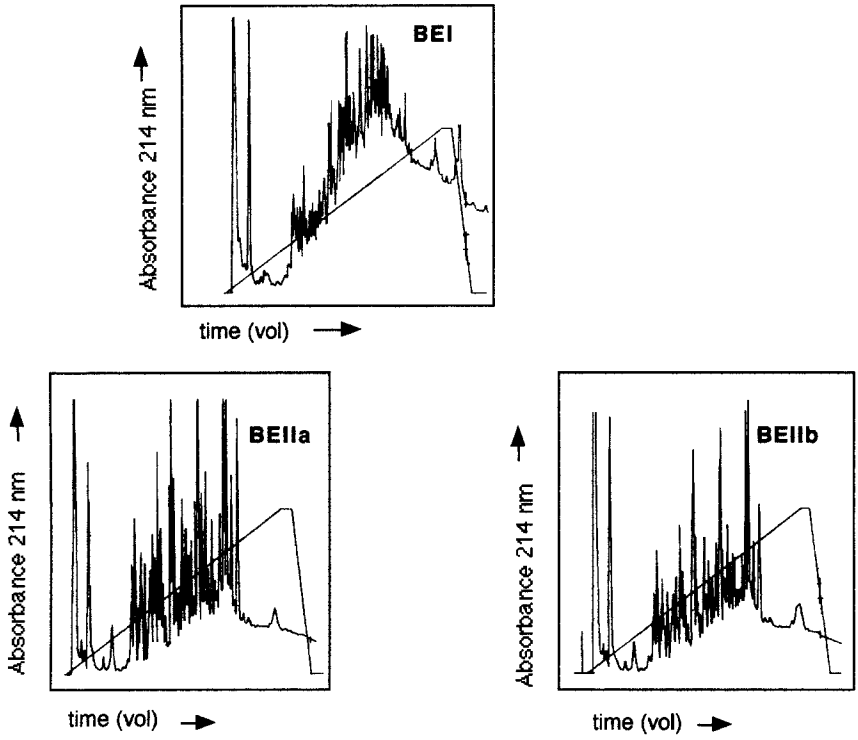


FIG. 5. Tryptic peptide maps of the maize-branching enzymes showing the similarity between the pair BEIIa/BEIIb and differences between the pair and BEI. Approximately 1.25 nmol of peptide digest was chromatographed on a reverse-phase column. Figure reprinted with permission from Singh and Preiss (1985).

the structure of branching enzyme I when compared to the pair IIa and IIb, as shown by the absence of reaction with some monoclonal antibodies, differences in amino acid composition, and differences in their proteolytic digest maps. There were significant differences between BEI and the pair BEIIa and BEIIb, but these two are similar in their reactions to polyclonal and monoclonal antibodies, molecular weight, K_m for substrates, and so on. In maize endosperm (Fisher *et al.*, 1993; Guan *et al.*, 1994a,b) and in rice seed (Mizuno *et al.*, 1992, 1993; Nakamura and Yamanouchi, 1992), two different cDNAs were isolated encoding two branching enzyme isozymes, BEI and BEII.

Results obtained with the maize endosperm mutant, amylose extender (*ae*), suggested that *Ae* was the structural gene for either BEII or BEIIb (Boyer and Preiss, 1978b, 1981; Preiss and Boyer, 1980). BEI levels were

not affected. In gene dosage experiments, Hedman and Boyer (1982) showed that there is a nearly linear relationship between increasing the dosage of the dominant *Ae* allele and BEIIb activity. Since the separation of form IIa from IIb was not optimal, it was still possible that the *Ae* locus was also affecting the level of IIa.

In short, the evidence accumulated by 1982 suggested that only two genes were coding for branching enzymes, and that the slight differences between BEIIa and BEIIb could be attributed to posttranscriptional processing. However, on the basis of the analysis of 16 isogenic lines that have independent alleles of the maize *amylose extender* (*ae*) locus, Fisher *et al.* (1996a) suggested that BEIIa and BEIIb are encoded by separate genes; the BEIIb enzyme would be encoded by the *Ae* gene. They isolated a cDNA clone labeled *Sbe* 2b with a predicted amino-acid sequence between residues 58 and 65 as the N-terminal sequence of the maize BEIIb that they had purified (Fisher *et al.*, 1996a,b). Moreover, they could not detect any mRNA in *ae* endosperm extracts using the *Sbe* 2b cDNA clone. Some BE activity that chromatographed as BEIIa was detected in the *ae* extracts. Although the results of Fisher *et al.* are suggestive, it remains to be shown that the activity they labeled as BEIIa in the *ae* mutant is the same as what has been termed as BEIIa in wild-type maize. It is possible that the residual enzyme activity seen in the *ae* mutant is another BE isozyme, additional to the BEII isozymes studied previously, especially because the maize lines Fisher and his collaborators used are of different genetic background from those used in the classic studies.

VI. OTHER SPECIES

In potato tubers, Vos-Scheperkeuter *et al.* (1989) purified a single form of branching activity of molecular mass 79,000. Antibodies to the native potato enzyme were prepared and the enzyme was found to react strongly only with maize BEI and very weakly with BEIIb. In neutralization tests, the enzyme inhibited the activities of both the potato tuber BE and of maize BEI. It was concluded that the potato BE shows a high degree of similarity to the maize BEI and, to a lesser extent, to the other maize BE.

However, whether potato tubers have two isoforms of BE had not been resolved. Borovsky *et al.* (1975) isolated from potato tubers a BE of molecular mass 85,000. This is close to the mass of 79,000 found by Vos-Scheperkeuter *et al.* (1989). It has been claimed that a BE of molecular mass 97,000 and 103,000 can be isolated (Blennow and Johansson, 1991; Khoshnoodi *et al.*, 1993). It is still not resolved whether the previous lower

molecular mass values of 79,000 and 85,000 are the results of proteolysis during purification of the 103,000 BE or whether these proteins could be the products of different allelic forms of the BE gene or different BE genes. It is of interest to note that BEs isolated from other plants, bacteria, or mammals have molecular masses ranging from 75,000 to approximately 85,000. These molecular masses have been consistent with the molecular weights obtained from deduced amino-acid sequences obtained from isolated genes or cDNA clones.

In potato and cassava, there is no clear evidence yet for the existence of more than one isoform. Antisense experiments in potato tuber did not eliminate BE activity completely. If there were two genes coding for enzymes, antisense neutralization if one of them would not affect the expression of the other. However, this result is inconclusive because antisense experiments usually decrease the amounts of the protein encoded but do not reduce it to zero. Purification and characterization of the remaining activity in antisense experiments could resolve the matter, but was not done in this particular case.

Smyth (1988), using Indica rice, resolved two or three peaks of BE on an anion-exchange chromatography. Although the molecular weight of the BE seemed to be only about 40,000, activity was determined using the iodine assay and a crude extract, and amylase contamination or possible proteolysis apparently were not taken into account.

Mizuno *et al.* (1992) has reported four forms of BE from immature rice seeds that were separated by chromatography on DEAE-cellulose chromatography. It seems that two of the forms, BE1 and BE2 (composed of BE2a and BE2b) were the major forms, whereas BE3 and BE4 were minor forms comprising less than 10% of the total BE activity. The molecular weight of the BEs were: BE1, 82,000; BE2a, 85,000; BE2b, 82,000; BE3, 87,000; BE4a, 93,000; and BE4b, 83,000. However, BE1, 2a, and b seem to be immunologically similar in their reaction to maize endosperm BEI antibody. Moreover, the rice seed BE1, BE2a, and BE2b had similar *N*-terminal amino acid sequences. All three BEs had two N-terminal sequences, TMVXVVEEVDHLPIT and VXVVEEVDHLPITDL. The latter sequence is similar to the first sequence, lacking just the first two N-terminal amino acids. Thus, although these activities came out in separate fractions from the DEAE-cellulose column, they seem to be the same protein on the basis of immunology and N-terminal sequences. BE2a, however, was 3 kDa larger. The antibody against BE3 reacted strongly against BE3 but not toward BE1 and 2a, 2b. Thus, rice endosperm, as noted for maize endosperm, had essentially two different isoforms of BE.

Because of the many isoforms existing for the rice seed branching enzymes. Yamanouchi and Nakamura (1992) studied and compared the BEs

from rice endosperm, leaf blade, leaf sheath, culm, and root. The BE activity could be resolved into two fractions, BE1 and BE2, and both fractions were found in all tissues studied in different ratios of activity. The specific activity of the endosperm activity, either on the basis of fresh weight or protein, was 100- to 1000-fold greater than other tissues studied. On native gel electrophoresis, rice endosperm BE2 could be resolved into two fractions: BE2a and BE2b. Of interest was the fact that for electrophoresis of the other tissue BE2 forms, only BE2b was found. BE2a was only detected in the endosperm tissue. It appears that in rice there could be tissue-specific isoforms of BE.

Fisher *et al.* (1996b) were able to isolate two cDNAs from an *Arabidopsis thaliana* hypocotyl library that seemed to be those coding for BEIIa and BEIIb. The two cDNAs had diverged 5′ and 3′ ends, but the amino-acid sequences encoded by them were 90% identical. The two cDNAs hybridized to transcripts that showed similar expression patterns in the vegetative and reproductive tissues.

A pea variety with wrinkled seeds has a reduced starch level, about 66–75% of that seen in the round seed; also, the amylose content is about 33% in the round form, but is much higher (60–70%) in the wrinkled pea seed. Edwards *et al.* (1988) measured the activities of several enzymes involved in starch metabolism in wrinkled peas at four different developmental stages and found that BE activity was, at its highest, only 14% of that seen for the round seed. The activities of other starch biosynthetic enzymes and phosphorylase were similar in the wrinkled and round seeds. These results were confirmed by Smith (1988), who also showed that the *r*(*rugosus*) lesion (as found in the wrinkled pea of genotype *rr*) was associated with the absence of one isoform of branching enzyme. Edwards *et al.* (1988) proposed that the reduction in starch content observed in the mutant seeds is caused indirectly by the reduction in BE activity through an effect on the starch synthase, suggesting that, in the absence of branching enzyme activity, the starch synthase activity is not optimal in α-1$\rightarrow$4-glucan synthesis. Indeed, in a study of rabbit muscle glycogen synthase (Carter and Smith, 1978) it was found that prolonged elongation of the outer chains of glycogen caused it to become an ineffective primer, thus decreasing the apparent affinity of the glycogen synthase for the primer. It was also found that the concentration of ADPglucose was higher in the wrinkled pea than in the normal pea, suggesting that activity of the starch synthase was restricted in vivo. Under optimal in vitro conditions, in which a suitable primer such as amylopectin or glycogen is added, starch synthase activity in the wrinkled pea was equivalent to that found in the wild type.

The *r* locus of the pea seed was cloned using an antibody toward one of the pea-branching enzyme isoforms and screening a cDNA library (Bhatta-

charyya *et al.*, 1990). It appears that the branching enzyme gene in the wrinkled pea contains an 800-bp insertion, causing it to express an inactive branching enzyme. The sequence of the 2.7-kb clone showed more than a 50% homology to the glycogen-branching enzyme of *E. coli* (Baecker *et al.*, 1986), and the authors concluded that the cDNA that they had cloned corresponded to the starch-branching enzyme gene of the pea seed.

VII. RELATIONSHIP BETWEEN STRUCTURE AND FUNCTION

After purifying and characterizing the enzymes and cloning the genes, the next step is to understand the relationship between the structure of the branching enzymes and catalysis—in other words, the way in which variations in protein structure affect the substrate preference, the length of the branches formed, and the pattern of branching. This knowledge would obviously facilitate the manipulation of the branching enzyme gene to obtain the desired change in enzyme function. Subsequent transformation of the altered gene into plants would result in starch granules with altered structure and physical properties that could be used by the starch industry with positive economic effects.

In addition to the studies discussed in the preceding (e.g., the size of the chains transferred by the maize endosperm BE to form the α-1,6 linkages), no studies have been conducted with respect to identifying amino-acid residues involved in catalysis. However, amino-acid sequences deduced for the branching enzymes from several bacterial genes, and for many plant BE isozymes (from the cDNA clones), show high identity, leading to some information on possible amino-acid sequences that may be important for catalysis. It is of interest that the glycogen-branching enzyme from the photosynthetic cyanobacterium, *Synechococcus* sp. PCC7942, has been cloned (Kiel *et al.*, 1989) by using the *glgB* gene from *E. coli* (Baecker *et al.*, 1986) as a hybridization probe. The sequence for the cyanobacterial *glgB* gene was determined (Kiel *et al.*, 1990) and its deduced amino-acid sequence has extensive similarity to the amino-acid sequence (62% identical amino acids) in the middle area of the *E. coli* protein. It appears, therefore, that branching enzymes in nature have extensive homology, whether their specificity is for high branching, as observed in glycogen (~10% α-1,6 linkages), or a lower degree of branching, as that seen in amylopectin (~5% α-1,6 linkages).

Sequence comparisons done by a number of groups, most notably by Svensson and her colleagues (Svensson, 1994), indicate that the various starch- and glycogen-branching enzymes contain consensus sequences to the four regions that are postulated to be the catalytic regions of the α-

amylase family of enzymes. This family includes pullulanase, isoamylase, glucosyl transferase, and cyclodextrin glucanotransferase (Fig. 6). The four regions are in the central portion of the amino-acid sequences of these enzymes.

The conservation of the putative catalytic sites of the α-amylase family in the starch- and glycogen-branching enzymes should be no surprise, as BE catalyzes two consecutive reactions for the synthesis of α-1,6 glucosidic linkages: cleavage of α-1,4 glucosidic linkages and then transfer to C-6 hydroxyl groups of a glucose residue in the growing polysaccharide. These reactions are probably similar to those catalyzed by the other α-amylase family enzymes, namely cleavage and transfer to another glucose residue or to water.

Some of the eight highly conserved amino-acid residues of the α-amylase family may also be functional in branching enzyme catalysis. Research using

	Region 1	Region 2
B. subtilis α-amylase	97 **DAVINH**	171 **GFRFDAAKH**
B. sphaericus cyclodextrinase	238 **DAVFNH**	323 **GWRLDVANE**
P. amyloderamosa isoamylase	291 **DVVYNH**	370 **GFRFDLASV**
K. pneumonia pullulanase	602 **DVVYNH**	673 **GFRFDLMGY**
Maize endosperm BE I	277 **DVVHSH**	347 **GFRFDGVTS**
Maize endosperm BE II	315 **DVVHSH**	382 **GFRFDGVTS**
Potato tuber BE	355 **DVVHSH**	424 **GFRFDGITS**
Rice seed BE 1	271 **DVVHSH**	341 **GFRFDGVTS**
Rice seed BE 3	337 **DVVHSH**	404 **GFRFDGVTS**
E. coli glycogen BE	335 **DWVPGH**	400 **ALRVDAVAS**

	Region 3	Region 4
B. subtilis α-amylase	204 **FQYGEILQ**	261 **LVTWVESHD**
B. sphaericus cyclodextrinase	350 **IIVGEVWH**	414 **SFNLLGSHD**
P. amyloderamosa isoamylase	412 **RILREFTV**	499 **SINFIDVHD**
K. pneumonia pullulanase	702 **YFFGEGWD**	826 **VVNYVSKHD**
Maize endosperm BE I	402 **TVVAEDVS**	470 **CIAYAESHD**
Maize endosperm BE II	437 **VTIGEDVS**	501 **CVTYAESHD**
Potato tuber BE	453 **VTMAEEST**	545 **CVTYAESHD**
Rice seed BE 1	396 **TIVAEDVS**	461 **CVTYAESHD**
Rice seed BE 3	459 **ITIGEDVS**	524 **CVTYAESHD**
E. coli glycogen BE	453 **VTMAEEST**	517 **NVFLPLNHD**

FIG. 6. Primary structures of several branching enzymes compared with the four conserved regions of the α-amylase family. The sequences have been derived from references cited in this text and in Svensson (1994). Four types of enzymes from the amylase family are compared with the branching enzymes. Svensson (1994) compared more than 40 enzymes ranging from amylases, glucosidases, several α-1,6-debranching enzymes, and four branching enzymes. The conserved amino-acid residues are in bold letters.

site-directed mutagenesis (Kuriki *et al.*, 1996) suggests that the conserved Asp residues of regions II and IV and the Glu residue of region III are important for BE activity. Their exact functions are unknown, and further experiments, such as chemical modification and analysis of the three-dimensional structure of BE, are required for determination of their role as catalytic residues and for detailing the mechanism of reaction. Other studies with phenylglyoxal, a reagent for modification of Arg residues, have shown that the maize endosperm BEs can be inactivated (Cao and Preiss, 1996). The phenylglyoxal inactivation can be prevented by the addition of amylose to the modification reaction mixture. Thus, arginine residues in BE may be involved in the binding of the substrate or in catalysis. Similarly, diethyl pyrocarbonate, a reagent specific for histidine residues, also inactivates maize BE activity, and this inactivation can also be prevented by the presence of amylose (Funane and Preiss, unpublished experiments, 1996).

It should also be noted that the amino-acid sequences at the N- and C-termini of the various BEs are dissimilar; these regions may be important with respect to substrate specificity as well as to the size of chain transferred and to the extent of branching.

Kuriki *et al.* (1997) described the construction of chimeric enzymes made from the maize branching-enzymes. The maize branching-enzymes differ in specificity of substrate and pattern of branching of the substrate. Comparing the amino-acid sequences BEI and mBEII of maize, the identity is 58%, with the identity higher (67%) in the central portion of the enzymes, which contains the regions I–IV highly conserved in the α-amylase family (Baba *et al.*, 1991; Takata *et al.*, 1992; Jespersen *et al.*, 1993; Guan *et al.*, 1994b). When amino-acid residues with similar functional side chains are taken into consideration, the two enzymes are 75% similar, with similarity 94% for the central region. Conversely, the amino and carboxy-terminal sides are quite dissimilar, suggesting that these structural differences may be responsible for the functional differences between the isozimes. To test this hypothesis, several different chimeric enzymes were constructed (Fig. 7).

Several of the chimeric enzymes constructed and expressed in *E. coli* were inactive and some had little activity, but the purified mBEII-I BspH1, in which the carboxy-terminal part of mBEII was exchanged for that of mBEI at the *Bsp*H1 restriction site (Fig. 7) was active. The resulting enzyme had properties different from both mBEI and mBEII (Table III). Activity was higher when assayed with the phosphorylase stimulation assay. In its preference for amylose (rather than amylopectin), the chimera was similar to mBEI, suggesting that the carboxy-terminal end is the region involved in determining substrate specificity and catalytic capacity. Conversely, the chimeric enzyme transferred shorter chains (d.p. around 6) than mBEI, mimicking mBEII and suggesting that the amino terminal of maize

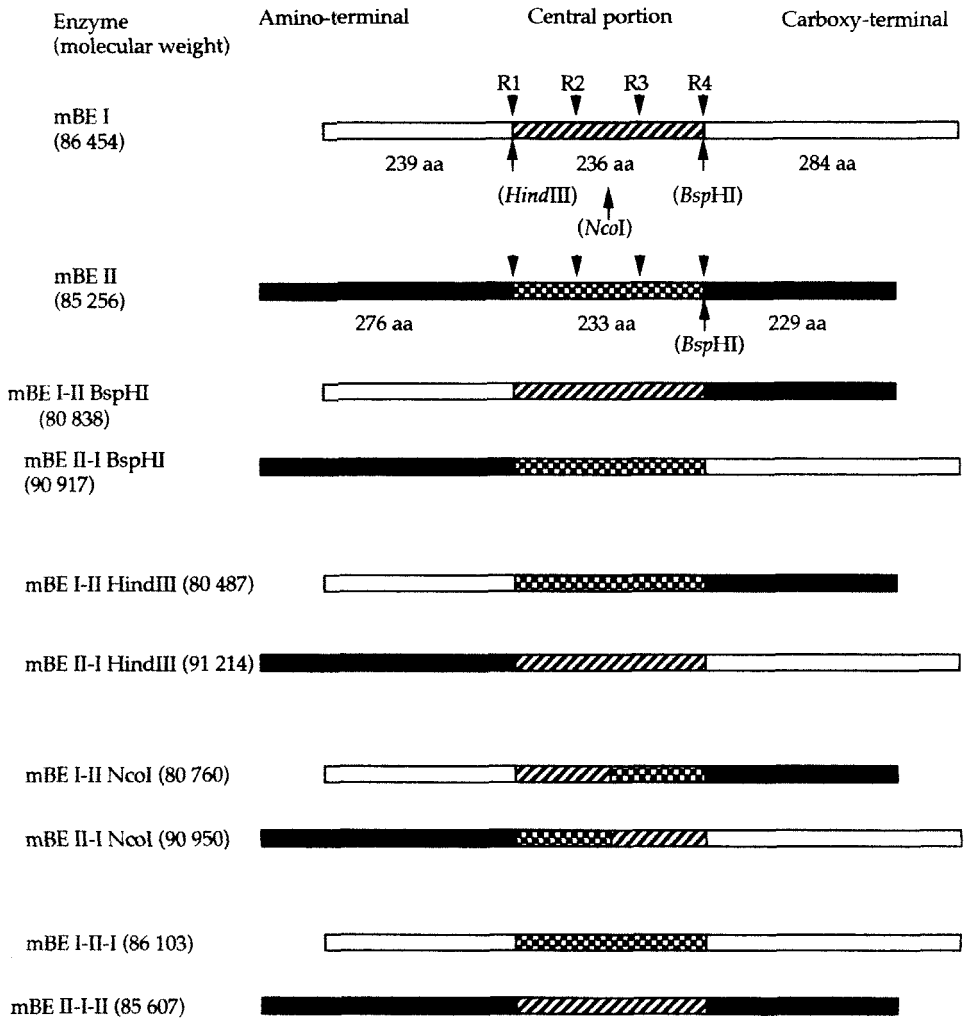


FIG. 7. Schematic diagram representing the structure of the branching enzymes from wild-type maize, mBE I, and mBE II, and of the chimeric enzymes built by genetic manipulation. The figures show amino- and carboxy-terminuses and the four conserved regions (R1 to R4) in the central portion. Some of the restriction sites used to build the chimeras were already present in the cDNAs of the branching enzymes (e.g. *Hind*III, *Nco*I and *Bsp*HI), but one restriction site had to be introduced in the sequence of mBE I by site-directed mutagenesis. The mutation introduced with the purpose of facilitating genetic manipulation has no effect on the amino-acid sequence of the wild-type enzyme and is called a "silent" mutation. The portions of the chimeric enzyme from the N- and/or C-terminal of mBE I are shown in white, whereas those from mBE II are shown in black. The central portion from mBE I is striped and from mBE II has squares. Molecular weights are in Daltons.

TABLE III

SPECIFIC ACTIVITIES OF THE TWO MAIZE ENDOSPERM BRANCHING-ENZYME ISOFORMS, MBEI AND MBEII AS COMPARED TO THAT OF THE CHIMERIC ENZYME MBE II-I BSPHI[a]

Branching-enzyme	mBEI	mBEII	mBE II-I BspHI
Assay a, phosphorylase stimulation	1196	1040	3880
Assay b, branching linkage			
AS320 (100 μM)	2.1	0.4	1.3
AS110 (100 μM)	1.3	0.2	0.89
AS70 (100 μM)	0.32	0.03	0.48
Assay c, iodine stain			
Amylose (c_1)	90	6.4	69
Amylopectin (c_2)	2.3	97	2.3
Ratio of activity (c_1/c_2)	40	0.066	30

[a] Activity is expressed as U/mg of protein. For details on how the chimeric enzyme was created and how the different types of assays were performed, please see text.

branching-enzymes plays an important role in the size of the oligosaccharide chain transferred.

In all branching enzymes, but not in other amylolytic enzymes, an acidic amino acid such as Glu or Asp follows region three. In *E. coli* this acidic amino acid is Glu-459, which follows the amino acids that form part of the catalytic site and is located in a region predicted as a β-strand–α-helix loop known to host the reaction center of α/β-barrels. However, although the conservation of an amino acid throughout evolution suggests that the amino acid may be significant for the activity of the enzyme, only site-directed mutagenesis can corroborate such a hypothesis. Binderup and Preiss (in press) constructed the mutants E459A, E459D, E459K, and E459Q of the *E. coli* branching enzyme, expressed the mutated genes, and purified the mutant enzymes. The purified enzymes were then characterized using the different activity assays available for branching enzyme. Mutation of Glu-459 did not result in loss of activity. Some activity remained even when Glu-459 was substituted with an amino acid of opposite charge (E459K). The similar responses of activity to changes in pH by the wild type and the E459A mutant also ruled out the possibility that Glu-459 may be involved in acid–base catalysis. Binderup and Preiss (in press) also concluded that Glu-459 is unlikely to be involved in chain transfer, as the HPAEC-PAD chromatograms were practically identical for all constructed mutants.

A three-dimensional structure is required before a specific role can be assigned to Glu-459 and to understand why this residue is a conserved

acidic amino acid in all known branching-enzymes. Although Binderup (1997) has obtained crystals of *E. coli* branching enzymes, an essential step in constructing a reliable three-dimensional model of the enzyme, the crystals are of the needle cluster kind, unsuitable for X-ray diffraction studies. Because the only possible approach in obtaining suitable enzyme crystal is trial and error, it is impossible to predict when (and if) a three-dimensional model will be obtained.

It is worth noting that a conservative change from Glu to Asp resulted in higher specific activities in all three types of assays. It is interesting that all higher plant branching-enzymes have an Asp at the position occupied by Glu-459 in *E. coli.*

The branching enzyme from *Bacillus stearomophilus* decreased the molecular size of synthetic amylose. On studying the product of this reaction, it was found that BE had catalyzed the intramolecular transglycosylation to form a cyclic structure with a side chain. After removing the cyclic part of the molecule (using isoamylase) from the rest of the molecule, its cyclic nature was confirmed by the use of mass spectrometry. The authors proposed a new mechanism for the action of BE and suggested that plant BE may catalyze the cyclization of amylose and amylopectin.

The understanding of how branching enzymes work is progressing quickly, and this progress is likely to lead to applications to biotechnology in the near future, with the transformation of altered branching enzyme genes into crop plants resulting in starch with an altered structure and physical properties that are advantageous to the starch industry.