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Expression of Branching Enzyme I of Maize Endosperm in *Escherichia coli*[1]

Han Ping Guan, Tadashi Baba, and Jack Preiss*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824 (H.P.G., J.P.); and Institute of Applied Biochemistry, University of Tsukuba, Ibaraki 305, Japan (T.B.)

The gene encoding for mature branching enzyme (BE) I (BEI) of maize (*Zea mays* L.) endosperm has been expressed in *Escherichia coli* using the T7 promoter. The expressed BEI was purified to near homogeneity so that amylolytic activity and bacterial BE could be completely eliminated from the BE preparation. The recombinant enzyme showed properties very similar to those of BEI purified from developing maize endosperm with respect to branching amylose and amylopectin. This result confirmed our earlier report that maize endosperm BEI had a higher rate of branching amylose and a much lower rate (less than 10% of that of branching amylose) of branching amylopectin. This study also showed a great advantage in purifying BEI from the bacterial expression system rather than from developing maize endosperm. Most important, this study has established the system with which to study the structure-function relationships of the maize BEI using site-directed mutagenesis.

Starch consists of two major components: amylose and amylopectin. These two molecules have distinct physical and chemical properties and affect the quality of starch, which, in turn, determines its economic value (Morrison and Karkalas, 1990). Since BE catalyzes the formation of the branch point by cleavage and transfer of α-1,4-linked glucan to α-1,6 branch points (Borovsky et al., 1976), BE plays an important role in starch synthesis (Preiss, 1991).

Multiple forms of BE have been identified in many plants: maize (*Zea mays* L.) endosperm (Boyer and Preiss, 1978), pea seed (Smith, 1988), and rice endosperm (Nakamura et al., 1992; Mizuno et al., 1993). The multiple forms of BE from developing maize endosperm have been purified and characterized (Boyer and Preiss, 1978; Singh and Preiss, 1985; Guan and Preiss, 1993; Takeda et al., 1993). Distinct properties of the BE isoforms from maize endosperm have been noted in the rates of branching amylose and amylopectin and in the structure of the products formed. Maize BEI preferentially transfers longer chains than BEII (Takeda et al., 1993). It has also been found that maize BEI has the highest activity in branching amylose, and its rate of branching amylopectin is less than 10% of that of branching amylose (Guan and Preiss, 1993). Conversely, maize BEII has lower rates in branching amylose (about 9–12% of that of BEI) and has higher rates of branching amylopectin (about 6-fold) than BEI (Guan and Preiss, 1993). These results (Guan and Preiss, 1993; Takeda et al., 1993) suggest that the BE isoforms could play distinct roles in amylopectin synthesis. Recently, cDNA clones of BE have been isolated from maize (Baba et al., 1991; Fisher et al., 1993), pea (Bhattacharyya et al., 1990), potato (Kossmann et al., 1991; Poulsen and Kreiberg, 1993), and rice (Nakamura and Yamanouchi, 1992; Mizuno et al., 1993). To facilitate the study of the structure-function relationships of BE using chemical modification and site-directed mutagenesis, it is necessary to express BE in *Escherichia coli* and to obtain relatively large amounts of the purified enzyme. Therefore, we have expressed a cDNA coding for mature BEI of maize endosperm in *E. coli* and subsequently purified and characterized the recombinant enzyme.

MATERIALS AND METHODS

Assay of BE Activity

BE activity was measured by three different assays as described by Guan and Preiss (1993).

Assay a

The phosphorylase *a* stimulation assay is based on the stimulation by BE of the synthesis of α-D-glucan from α-D-Glc-1-P catalyzed by rabbit phosphorylase *a* (Hawker et al., 1974). This assay was used to monitor branching activity throughout all purification steps. One unit of enzyme activity is defined as 1 μmol of Glc incorporated into α-D-glucan min^{-1} at 30°C.

Assay b

The branching linkage assay determines the number of branching linkages introduced into the reduced amylose (Takeda et al., 1993). One unit of enzyme activity is defined as 1 μmol of the branching linkage formed min^{-1} at 30°C.

Assay c

The iodine stain assay is based on monitoring the decrease in *A* of the glucan-iodine complex resulting from the branching of amylose or amylopectin (Boyer and Preiss, 1978). One unit of enzyme activity is defined as the decrease in *A* of 1.0 min^{-1} at 30°C at the defined wavelength.

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* Corresponding author; fax 1-517-353-9334.

Abbreviation: BE, branching enzyme.

Protein Determination

Protein concentration was determined according to the method of Smith et al. (1985), using the Pierce Chemical Co. prepared bicinchoninic acid reagent and BSA as the standard.

N-Terminal Sequencing

The purified recombinant BEI of maize endosperm was spotted on a polyvinylidene fluoride-based membrane (Millipore), desalted in double-distilled H_2O, and sequenced on an Applied Biosystems model 477A protein sequencer.

Construction of Maize BEI Expression Vectors

For expression of maize BEI in *Escherichia coli*, the maize BEI cDNA in pUC19 (Baba et al., 1991) was subcloned into pBluescript SK(−) at the *Eco*RI site. The PCR method was used to modify the N terminus of maize BEI using the following oligonucleotides: primer A, 5′-ATACTAGTCCATGGCTACTGTGCAAGAAGATAAAAC-3′); primer B, 5′-TGTGTACCTTTCAGAAGCAGGAG-3′. Primer A includes the *Nco*I site, the Met codon, and BEI sequence 191 to 214 as well as an *Spe*I site in front of the *Nco*I site; primer B is the complement of sequence 695 to 719 (Baba et al., 1991). The resulting (approximately 500 bp) PCR product, which contained the modified N-terminal sequence, was recombined with the rest of the BEI sequence by replacing the *Spe*I/*Bam*HI fragment of the BEI-pBluescript SK plasmid with the *Spe*I/*Bam*HI fragment of the PCR product. The gene coding for the mature maize BEI was subcloned from pBluescript into the *Nco*I/*Xho*I site of the bacterial expression vector pET-23d (Novagen). The reconstructed gene coding for mature BEI was resequenced to verify that no mutations had occurred during the modification step.

Expression of Maize BEI in *E. coli*

An overnight culture of the transformed cells [BL21(DE3)] with the maize BEI gene was diluted 1:20 (v/v) in fresh Luria broth containing 100 μg mL^{-1} ampicillin. The cells were grown at 37°C for about 2 h to $A600$ = 0.6 before the expression of maize BEI was induced by adding isopropyl β-D-thiogalactoside to 0.5 mM. Following growth at 25°C for 12 h, cells were harvested in a refrigerated centrifuge.

Purification of Maize BEI Expressed in *E. coli*

The recombinant maize BEI was purified by modification of the procedure used to purify the enzyme from developing maize endosperm (Guan and Preiss, 1993). Cell paste (64 g) was resuspended and lysed by sonication in 50 mM Tris-acetate buffer (pH 7.5) containing 10 mM EDTA and 5 mM DTT. The lysed suspension (homogenate) was then centrifuged at 10,000*g* for 15 min. The supernatant fraction was taken to 40% saturation by adding crystalline ammonium sulfate. The precipitate collected by centrifugation was dissolved in a minimum volume of buffer A (50 mM Tris-acetate buffer [pH 7.5] containing 10 mM EDTA, 2.5 mM DTT) and dialyzed overnight against buffer A. The dialyzed BEI fraction was applied to an aminodecyl agarose column (Sigma) as described by Guan and Preiss (1993). The fractions with branching activity were pooled and concentrated by ammonium sulfate precipitation at 50% saturation. The BEI precipitate from the aminodecyl agarose column was dissolved and dialyzed overnight in buffer B (20 mM triethanolamine [pH 8.5] containing 1 mM EDTA, 2.5 mM DTT). The dialyzed fraction was applied to a Mono-Q HR 10/10 column (Pharmacia) equilibrated with buffer B. After the column was washed with 30 mL of buffer B, the BEI was eluted with a linear gradient (160 mL, 0–250 mM KCl in buffer B) at a flow rate of 2 mL min^{-1}. The fractions with branching activity were dialyzed and rechromatographed on a Mono-Q HR 5/5 column (Pharmacia) in buffer B at a flow rate of 0.5 mL min^{-1} as described by Guan and Preiss (1993).

SDS-PAGE and Western Blotting

SDS-PAGE was performed on 9% polyacrylamide gels according to the method of Laemmli (1970). Western blotting was carried out according to the method of Burnette (1981). The primary rabbit antibodies, anti-maize BEI (purified from endosperm; Guan and Preiss, 1993) and anti-bacterial BE (Holmes et al., 1982), were diluted 1:250 and 1:500 (v/v), respectively, in 3% (v/v) gelatin. The antigen-antibody complex was detected using anti-rabbit Ig conjugated with alkaline phosphatase (1:10,000, United States Biochemical) with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates.

Table I. *Purification of maize endosperm BEI expressed in E. coli*

Enzyme activity was measured by the phosphorylase stimulation assay (assay a). One unit of enzyme activity is defined as 1 μmol Glc incorporated into α-D-glucan min^{-1} at 30°C.

Fraction	Volume	Protein	Activity	Specific Activity	Yield
	mL	*mg*	*units*	*units mg^{-1} protein*	%
Homogenate[a]	455	5733	13,013	2.2	100
Supernatant (10,000 *g*)	405	1498	11,866	7.9	91
0–40% $(NH_4)_2SO_4$	32	480	9,417	19.6	72
Aminodecyl agarose	9.8	123.5	6,402	51.8	49
Mono Q HR 10/10	9	10.8	5,743	532	44
Mono Q HR 5/5	1.6	2.2	2,932	1332	23

[a] From 64 g wet weight of cells (12-L culture).

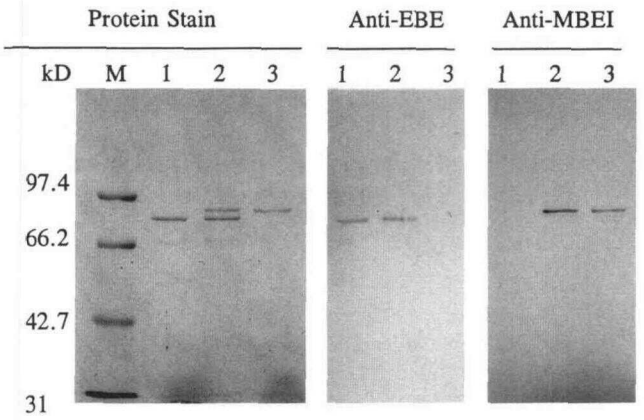

Figure 1. SDS-PAGE and western blot analysis of *E. coli* BE and maize BEI expressed in *E. coli*. Lane 1, *E. coli* BE (0.5 μg) purified from *E. coli* B; lane 2, a mixture of *E. coli* BE (0.5 μg) and recombinant maize BEI (0.5 μg); lane 3, maize BEI (0.5 μg) purified from the *E. coli* expression; lane M, molecular mass markers, phosphorylase *b* (97.4 kD), BSA (66.2 kD), ovalbumin (42.7 kD), and carbonic anhydrase (31 kD). The proteins were stained with Coomassie brilliant blue. The primary rabbit antibodies used were anti-*E. coli* BE (Anti-EBE, 1:500) and anti-maize BEI purified from endosperm (anti-MBEI, 1:250). Goat anti-rabbit Ig conjugated with alkaline phosphatase (1:10,000) was used as a secondary antibody.

Neutralization of BE Activity

Neutralization of BE activity was done according to the method of Fisher and Boyer (1983). The reaction mixture contained 0.05 unit of BE in 0.1 M sodium citrate (pH 7.0), 1 mg mL^{-1} BSA, 2.5 mM DTT, and varying amounts of antiserum diluted with preimmune serum. After incubation at room temperature for 30 min, the mixture was stored on ice for 2 h. The mixture was then centrifuged at 10,000*g* for 15 min. Aliquots (10 μL) of the supernatant fluid were assayed for BE activity using the phosphorylase stimulation assay.

RESULTS AND DISCUSSION

Expression of Maize BEI in *E. coli*

The transit peptide of plastid-targeted proteins is typically processed during import into the plastid (Keegstra et al., 1989). The transit peptide and N terminus of maize BEI have been identified by Baba et al. (1991). The transit peptide of BEI was completely removed from the BEI cDNA clone (Baba et al., 1991), and a Met was added in front of the N terminus of mature BEI. The gene coding for mature maize BEI was expressed in *E. coli* BL21(DE3). The specific activity for BEI in the supernatant fraction was 7.9 units mg^{-1} protein, whereas the cells transformed with the native plasmid showed only 0.15 unit mg^{-1}. Although no BE activity was found in the pellet (after centrifugation of the sonicated mixture), western blotting did show that about 80% of the expressed maize BEI protein was present in an insoluble form (data not shown). Lowering the growth temperature from 37 to 25°C after induction resulted in a 2-fold increase in the specific activity of BEI.

Antibody neutralization studies showed that antibody raised against maize BEI (purified from endosperm; Guan and Preiss, 1993) neutralized the expressed activity efficiently. In a control experiment, the antibody raised against maize BEI did not neutralize *E. coli* BE activity even with higher amounts of antiserum per unit enzyme. The amount of antiserum causing 50% inhibition of the recombinant maize BEI was 4 μL per unit of enzyme, which is very similar to that corresponding to the enzyme purified from maize endosperm (3.4 μL per unit of enzyme).

Table II. *Properties of the products resulting from branching amylose or amylopectin by maize BEI expressed in E. coli*

Products were formed by incubation of 1 mg mL^{-1} amylose (chain length 405) or amylopectin (chain length 21) with 5 units mL^{-1} (assay a) maize BEI expressed in *E. coli* for 2 h, at pH 7.0 and 30°C. Decrease in *A* of the glucan-iodine complex was measured at 660 and 530 nm for amylose and amylopectin, respectively.

Substrate + BE	λ_{max}	Decrease in *A*	β-Amylolysis Limit	
			Before isoamylolysis	After isoamylolysis
	nM	%	%	%
Amylose only	638		84	100
+ BEI	520	94	53	100
+ BEI[a]	530	89	44	100
Amylopectin only	530		60	100
+ BEI	486	61	53	100
+ BEI[a]	495	54	53	100

[a] BEI purified from developing maize endosperm (Guan and Preiss, 1993).

Purification and Characterization of Maize BEI Expressed in *E. coli*

To fully characterize the expressed maize BEI, we purified the enzyme. Table I summarizes the purification of maize BEI from 64 g (12-L culture) of transformed *E. coli* cells and highlights the advantage over purifying BEI from maize endosperm (Guan and Preiss, 1993). The purified recombinant BEI had a similar or slightly higher specific activity (1300 units mg^{-1} protein) than that purified from maize endosperm (1200 units mg^{-1} protein; Guan and Preiss, 1993).

Table III. *Specific activities (units mg^{-1} protein) of maize BEI expressed in E. coli*

BE Assay	BEI	BEI[a]
Phosphorylase stimulation (a)	1332	1196
Branching linkage assay (b)	2.4	2.6
Iodine stain assay (c)		
Amylose (c_1)	574	800
Amylopectin (c_2)	47	24
Ratio of activity		
a/b	555	460
a/c_1	2.3	1.5
a/c_2	28	49.8
c_2/c_1	0.08	0.03

[a] BEI purified from developing maize endosperm (Guan and Preiss, 1993).

Purification of BEI from the expression system resulted in higher activity yield (23%) than purification from maize endosperm (3–4%; Guan and Preiss, 1993) with fewer purification steps, and the BE isoforms from maize endosperm are not separated until DEAE-Sepharose chromatography (Guan and Preiss, 1993). Sequencing the N terminus of the purified enzyme indicates that the N-terminal sequence of the recombinant BEI starts as ATVQEDKTMA. . ., which is exactly what is expected (Baba et al., 1991), and it is different from that of *E. coli* BE (Baecker et al., 1986). The Met introduced in front of the mature BEI was apparently cleaved by *E. coli*. The purified maize BEI (about 86 kD) migrated slower than purified *E. coli* BE (about 85 kD; Boyer and Preiss, 1977) on a 9% SDS-PAGE gel (Fig. 1).

Western blotting showed that antibody raised against maize BEI purified from endosperm (Guan and Preiss, 1993) recognized the 86-kD but not the 85-kD polypeptide. On the other hand, antibody prepared against *E. coli* BE (Holmes et al., 1982) recognized the 85-kD but not the 86-kD polypeptide (Fig. 1). Thus, both N-terminal sequencing and immunoblotting indicate that the recombinant maize BEI preparation is free of *E. coli* BE. Furthermore, no amylolytic activity could be detected in a 3-h incubation at 30°C with mixtures containing 5 units (assay a) BE mL^{-1} and 5 mg mL^{-1} of amylose or amylopectin as substrate, using a modified Park-Johnson method to determine reducing power (Takeda et al., 1993).

Separation of BEI from other amylolytic activities enabled us to characterize the catalytic properties of the maize BEI expressed in *E. coli* and to compare them with BEI purified from developing maize endosperm (Guan and Preiss, 1993). The recombinant maize BEI showed very similar properties to those of BEI purified from developing maize endosperm (Tables II and III). The recombinant BEI further branched amylose and amylopectin, causing a greater decrease in *A* and in the wavelength maxima of the glucan-iodine complexes (Table II).

To further characterize the recombinant maize BEI, three different methods were used to assay the activity of the purified enzyme. The results (Table III) confirmed the earlier report that BEI had a higher rate in branching amylose and the rate of branching amylopectin by BEI was less than 10% of that of branching amylose (Guan and Preiss, 1993).

This study demonstrates that the maize BEI expressed in *E. coli* not only has the same molecular mass as that purified from developing maize endosperm but also possesses immunological and catalytic properties similar to those of BEI purified from developing maize endosperm (Fig. 1, Tables II and III). The earlier results (Guan and Preiss, 1993) suggested that BEI and BEII may play different roles in the synthesis of amylopectin in vivo, i.e. BEI could be involved mainly in the synthesis of the B chains of amylopectin, whereas BEII could play a major role in the synthesis of A chains. Understanding of the structure-function relationships in the cause of this apparent specificity may lead to modification of the gene. Subsequent transformation of the modified gene into plants could produce altered starch granules desirable for specific industrial uses. The expression system described here makes it possible to perform site-directed mutagenesis studies on maize BEI and will facilitate the understanding of the structure-function relationships of maize BEI.

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