Investigation of the function of branching enzymes I, IIa, and IIb in the determination of amylopectin structure and regulation of starch biosynthesis

by

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LIST OF ABBREVIATIONS

ae	*amylose-extender*
AEB	Amyloplast extraction buffer
AEC	Anion exchange chromatography
AGPase	ADG-glucose pyrophosphorylase
ADP-Glucose	Adenosine diphosphate-Glucose
AGP-L	AGPase large subunit
AGP-S	AGPase small subunit
AM	Amylose
AP	Amylopectin
APTS	8-amino-1,3,6-pyrenetrisulfonic acid
ATP	Adenosine triphosphate
BE	Branching enzyme
CE	Total soluble crude extract
coIP	Co-immunoprecipitation
DAE	Days after emergence
DAP	Days after pollination
DBE	Debranching enzyme
DEAE	Diethylaminoethyl
DP	Degree of polymerization
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FACE	Fluorophore assisted capillary electrophoresis
GBSS	Granule bound starch synthase
Glc-1-P	Glucose-1-phosphate
GPC	Gel permeation chromatography
GS	Glycogen synthase
HB	Homogenization buffer
H	Hours
IPTG	isopropylthio-β-gaactoside

kDa	Kilodalton
min	Minutes
MOPS	3-[N-morphplino] prpoanesulphonic acid
MOS	Maltooligosaccharide
MOPS	3-[N-Morpholino] propanesulfonic acid
MSC	Multi-subunit complex
nm	Nanometer
PAGE	Polyacrylamide gel electrophorhesis
PL	sample of soluble extract loaded onto column (pre-load)
PMSF	phenylmethylsulfonylfluoride
pIIa	purified native BEIIa
pIIb	purified native BEIIb
Tris	3-N-tris (hydroxymethyl) aminomethane
rIIa	recombinant BEIIa
SEM	Scanning electron microscopy
SS	Starch synthase
UDP-Glucose	Uridine diphosphate-glucose
WSP	Water soluble polysaccharide

ABSTRACT

Starch is one of the most abundant carbohydrates on earth and it serves vital roles as the primary energy reserve of plants, a significant source of calories in the human diet, and a renewable resource for other industrial applications. Glycogen, like starch, is a reserve polysaccharide composed of α-D-glucose units joined by α(1→4) and α(1→6) linkages. These two homopolymers share common chemical features, however, a significant difference between them is the particular molecular architecture of starch that allows it to form semi-crystalline granules. Glycogen biosynthesis utilizes a single enzyme activity for each biosynthetic step in the assembly pathway, whereas starch biosynthesis utilizes multiple isoforms for each activity. The multiplicity of starch biosynthetic enzymes is evolutionarily conserved in plants, particularly in monocots, suggesting their functions are essential in starch biosynthesis. These enzyme functions most likely are determinants of the molecular architecture of starch. The overall goal of this research is to determine the specific molecular functions of the multiple starch branching enzyme (BE) isoforms in maize.

The first question this dissertation addresses is what, if any, are the differences in enzymatic properties of BEIIa and BEIIb. The goal of this research was to purify native BEIIa and BEIIb from maize endosperm cells and compare the activities of the two native branching enzymes to each other and to recombinant branching enzyme BEIIa (rIIa). To assist in the purification of the BEs, two polyclonal antisera were produced, αBEI which identifies BEI specifically, and αBEIIab which identifies both BEII isoforms. Using chromatographic purification techniques, native BEIIa or BEIIb was separated from the other two branching enzymes. The activities of native BEIIa and BEIIb were compared to those of

rIIa in their capacities to modify the structure of amylose by introducing branch points at specific chain lengths. All three BEII enzymes created chains with DP 10 - 25. Recombinant BEIIa enzyme, in addition, produced a high amount of chains DP 6 – 9, suggesting that *in vivo* there are factors affecting the activity of BEs. These results suggest that the evolutionary conservation of BEIIa and BEIIb is not accounted for by their enzymatic differences, at least with respect to product specificity. Another significant finding was that there were differences in the glucan chain length distribution created by native BEIIa and rIIa. It can therefore be hypothesized BE activity is modified *in vivo*.

The next question this dissertation addresses is are branching enzymes involved in multi-subunit complexes (MSCs)? This research provides direct evidence BEIIa and SSI interact with each other in one or more MSC and also that SSIII may be a component of the MSCs. Indirect evidence from GPC analysis suggests that BEIIb may be involved in MSCs. Multiple electrophoretic mobility forms of BEI were identified. Neither the activity nor the mobility was affected by dephosphorylation, giving more support to the multiple bands representing different MSC involving BEI. Pleiotropic effects in branching enzyme mutants on starch synthase (SS) activity revealed at least two SS band activities were affected when BEIIa was mutated. These results are consistent with the hypothesis that BEs are involved in MSC, specifically BEIIa and SSI are involved in at least one MSC. Further research is needed to investigate the functional significance of the complexes involving BEIIa, SSI and possibly SSIII.

The third study of this dissertation addresses what branching enzyme isoforms exist in leaves as compared to endosperm, thus investigating the possibility that tissue-specific gene expression may in part explain the functions of the multiple BE isoforms. This research

established that the same BEI polypeptide present in maize endosperm is present in maize leaves. The leaf BEI was found to be similar to endosperm BEI in predicted molecular weight, approximately 80 kDa, in molecule size as a monomeric protein, and also possessing multiple electrophoretic mobility forms.

In conclusion, this research has demonstrated BEs are involved in MSCs. These findings lead to the next questions, what is the functional significance of the MSCs? Research from Chapter 2 of this dissertation supports a functional significance for the MSCs versus the activities of the individual enzymes. There was no difference between the capacities of native BEIIa and native BEIIb with respect to substrate preferences. However, differences between rIIa and pIIa, in the capacity to branch amylose suggest branching enzyme activities are modified *in vivo*. Further investigation is necessary to determine how BE activity is modified and what factors influence BE activity.

CHAPTER 1: INTRODUCTION

OVERVIEW

Starch is a homopolymer of glucose molecules produced by plants to store energy. The role starch plays in the physiology of the plant is vital toward the plant's survival. During photosynthesis, the energy from the sun is converted into a reduced carbon compound, glucose. Plants store glucose within insoluble starch granules, allowing large amounts of energy to be stored efficiently. As a consequence of its fundamental role in plant physiology starch is one of the most plentiful carbohydrates on earth. This allows starch to serve as an abundant calorie source in the human diet as well as renewable resource that can be utilized in a variety of other industrial applications.

The better we can control the structure and properties of starch, the better we can exploit this resource. The importance of understanding starch metabolism is based upon the benefits starch offers the economy and society as a whole. The economic benefits of a renewable energy resource have become a more desirable alternative due to the society's increasing dependence on fossil fuels as well as the capabilities starch has to be used to improve the quality of health in society.

Because of the potential benefits starch offers society, there is much research focusing on the biosynthetic mechanism of starch. The knowledge obtained could eventually lead to the manipulation of the starch metabolic system to produce designer starches with specific functions. This research addresses a specific aspect of the biosynthetic pathway, the functions of maize branching enzymes I, IIa and IIb in the determination of amylopectin structure and regulation of starch biosynthesis.

In this introduction, I will: (1) review industrial applications of starch; (2) review starch structure and physiology; (3) compare starch to a glucan polymer that occurs in non-plants; and, (4) review starch biosynthetic enzymes including biochemical and genetic analysis and one component, starch branching enzymes, will be discussed in detail.

This introduction will then address information specifically related to the subject of the dissertation. An emphasis will be on the functions of the multiple enzyme activities of branching enzymes, which have generally been conserved evolutionarily in plants, involved in the biosynthesis of starch. Additionally, the coordination of the conserved multiple enzyme activities in the determination of the final starch architecture will also be addressed.

LITERATURE REVIEW

Industrial uses of starch

Starch is the carbohydrate storage product synthesized by plants from the energy of the sun. Most of the starch utilized by the world's population is produced by a small group of crops including maize, potato, rice, tapioca and wheat (Wang 1998). Starch is also a renewable resource with a variety of industrial applications. The amount of starch synthesized in organs harvested from maize, potato, rice and wheat exceeds 10^9 tons per year and about 2×10^7 tons per year are isolated for industrial applications (Koβmann and Lloyd 2000).

The primary role of starch in the human diet is as a calorie source. Starch can also be utilized to improve the functional properties of food like gelling and pasting. Starch gels and pastes are used to control the consistency of sauces and soups (Wang 1998). Starch is also used to make sweeteners in the food and beverage industry. Starch high in amylopectin is

used in salad dressings (Wang 1998). Though the greatest portion of processed starch is used by the food industry, there is a significant market for starch in non-food industries.

There is a diverse list of industrial markets that use starch in a variety of applications. Biodegradable plastics is a developing industry that use starches with high amylose content and small granules (Wang 1998). Small starch particles are also used in the cosmetic products, i.e. face and talcum powders (Ellis et al. 1988). Gelatinization which is the disruption of the molecular order within the starch granule is important to the textile industry in finishing, printing, sizing and fire resistance properties (Ellis et al. 1988). Other industries that use starch products include: pharmaceuticals (e.g. drug delivery and diluent), paper (e.g. binding, coating and sizing), medical (e.g. plasma extender, transplant organ preservation and adhesive) and agrochemical (e.g. surfractants) (Ellis et al. 1988).

One of the goals of starch research is to gain a complete understanding of how the enzymes interact to produce starch and how alterations within starch structure affect its functionality. Transgene technology has enabled researchers to genetically manipulate the starch biosynthetic pathway resulting in the generation of novel starches (Slattery et al. 2000). In the future, transgene technology and other genetic engineering techniques will be used to manipulate biosynthetic enzymes and be able to create novel starches for specific uses.

Starch accumulation and storage

Starch accumulates during plant development in almost every tissue found in higher plants, and it is synthesized as densely packed, semi-crystalline and water insoluble granules. The shape and size of the starch granules varies between 2 –175 microns (μm) depending

upon botanical source and tissue type (Preiss and Sivak 1998). Two types of starch are synthesized by the plant, transitiory and storage (or reserve), and they are classified by how the plant utilizes them. In leaves, transitory starch accumulates in granules in the chloroplasts as a short-term carbohydrate reserve, and it is synthesized by photosynthesis during the day (light period) and degraded by respiration at night (dark period), providing metabolites to the whole plant. In comparison, storage starch accumulates in storage organs (e.g. roots, seeds and tubers) and is used during embryo germination providing food and energy for the growing plant. In other tissues like roots and stems, starch has a role in the plant gravitational response during plant development (Preiss and Sivak 1998). In pollen grains, starch granules provide energy needed during germination and tube growth. The roles of the various starches located throughout the plant confirm that starch participates in roles fundamental to the plants growth, development and survival.

Starch physiology and structure

Starch is composed of 2 α-D-Glucose homopolymers: amylose and amylopectin. The ratios of amylose and amylopectin vary between plant species (Jenkins and Donald 1995). In native wild type plants, the major polysaccharide, amylopectin accounts for 70-80% of the starch granule. Amylopectin is a large and highly branched polymer. The remaining 20-30% of the starch granule is composed of amylose, which is a small and relatively unbranched polymer. Although the starch granule is simplistic in composition, containing one type of sugar residue, α-D-glucose, and two types chemical linkages, it is its hierarchical structure that clarifies its function.

Amylose component

Amylose is essentially a linear chain of α(1→4) linked glucose residues (approximately 1,000 molecules), with a molecular weight of 10^5–10^6 Da (Daltons), and composes 20-30% of the starch granule. In most plant species, amylose is primarily unbranched, but it may contain a low-level (0.3–0.7%) of α(1→6) branch points (Preiss and Sivak 1996; Hamada et al. 2001). Amylose composition has been shown to be dependent upon the plant organ, developmental age of the organ, the growth conditions of the plant, and to some extent, affect the overall rate of starch biosynthesis (Clarke et al. 1999; Denyer et al. 2001). Amylose content is also dependent on the plant species. The carbohydrate composition of storage starch in maize, potato and wheat have been found to vary between 18–36 % (Shannon and Garwood 1984). The chemical nature of amylose facilitates the formation of complexes with hydrophobic molecules. The single helices of amylose are stabilized by phospholipids and fatty acids in some starches (Morrison et al. 1984). For example, in cereals, the amylose fraction is complexed with a high proportion of lipids (Morrison and Karkalas 1990; Morrison 1995).

Amylopectin component

Amylopectin is the major component of starch, making up 70% of the granule. It is a large and highly branched molecule. In amylopectin, α(1→4) linked glucose units form chains about 20 glucose units long and are joined together by α(1→6) glycosidic branch linkages. Approximately five percent of the glucose units in the amylopectin are joined by α(1→6) branch linkages. An average amylopectin molecule is 200-400 nm long and about 15 nm wide (Smith and Martin 1993).

Both amylose and amylopectin are organized in the starch granule into a highly organized semi-crystalline structure. Amylose-free granules from *waxy* mutants in variety of plant species show unchanged degrees of crystallinity, therefore demonstrating the crystallinity of the starch granule is associated exclusively with the amylopectin (Smith et al. 1997). The crystallinity of the starch varies with the plant species, between 15-45% (Zobel 1988). The crystalline nature of the starch granule allows for dense packing of glucose, the fixed carbon product of photosynthesis, without water.

Composition and structure of starch

The most accepted theory for the molecular structure of starch is the "cluster" model proposed by French (1984). The cluster model suggests that the macromolecular structure of amylopectin in composed of different classes of glucan chains. Hizukuri (1986) demonstrated the characteristics of amylopectin chain lengths gave a polymodal distribution, which was consistent with the "cluster model" (Myers et al., 2000). The model proposes that amylopectin is composed of 3 classes of chains: 'A', 'B', and 'C' (Figure 1.1). 'A' chains bind in clusters only to 'B' chains at the C^6 position. 'B' chains bind to other 'B' chains or to a 'C' chain. There is only one 'C' chain per molecule and it has the reducing end of the amylopectin molecule (Wang 1998). Hizukuri (1986) analyzed debranched amylopectin by size exclusion chromatography. Five fractions of glucose chains of varying chains lengths eluted and were labeled A, B1, B2, B3 and B4. Fraction A contained the shortest chain lengths with 11-16 glucose residues. Longer chain lengths of 20 to 24, 42-48, 69-75 and 101-119 glucose residues were found in fractions B1-B4, respectively (Hizukuri 1986). 'A' chains were proposed to span one cluster while B1-B4 were proposed to span 1, 2, 3, and 4

clusters, respectively (Hizukuri 1986). The arrangement of the individual glucan chains and the location of the branch linkages define the first level of organization in amylopectin within the starch granule.

The glucan chains are oriented with their non-reducing ends pointing toward the surface of the granule (French 1984). When amylopectin is enzymatically debranched, the distribution of chain lengths and distance between branch points were found to be non-random, suggesting the branches are clustered (Morrell et al. 1998). This structural characteristic is conserved in all plants suggesting it is required for the formation of the starch granule.

At the next level of structural organization in the starch granule, on a 10-nm scale, are alternating regions of double helices of amyolpectin chains. Regions containing crystalline lamellae are resistant to acid hydrolysis and contain tightly packed double helices of 'A' chains and the unsubstituted lengths of 'B' chains. Alternating with the crystalline lamellae are amorphous lamellae, which are vulnerable to acid hydrolysis. Amorphous lamellae contain the branch linkages, and therefore are less densely packed. The alternating amorphous and crystalline lamellae, a 9 - 10 nm structure, in the starch granule, is conserved among plant species (Jenkins et al. 1993) (Figure 1.1B). As a result of the hierarchical order of organization of amylopectin within the crystalline lamellae, there are two types of crystalline structures based on X-ray diffraction: A-type is found in cereal starches and B-type is found in tuber plant starches (Gerard et al. 2000). The two crystallinites differ with respect to the packing of the amylopectin double helices in the granule and by the quantity of water molecules stabilizing the double helices (Gallant et al. 1997). Hizurki (1986) characterized native cereal starches like maize, rice and wheat, as A-type starches where the

double helices are densely packed with minimal water bound. There is also evidence suggesting starch granules appeared to be composed of small spherical "blockets". Scanning electron microscopy (SEM) data has demonstrated that the granule size varies between 20–500 nm depending upon the botanical source and its location within the granule (Gallant et al. 1997).

At the highest level of structural organization, blockets varying in size (50-500 nm) compose amorphous and semi-crystalline zones or shells (Koβmann and Lloyd 2000) (Figure 1.1C). One repeat of an amorphous zone and a semi-crystalline zone is known as a growth ring. Treatment of the granules with acid or degradative enzymes, like α-amylases, allows growth rings to be observed by atomic force microscopy, light microscopy, and scanning and transmission electron microscopy (SEM and TEM) (Figure 1.1D). Rings of concentric layers reveal layers of high/low refractive index, density, crystallinity and resistance to chemical and enzymatic attack (Buttrose 1960; Gallant and Guilbot 1969; Hall and Sayre 1973; Baker et al. 2001; Pilling and Smith 2003). To date, growth ring development is not completely understood. Studies have suggested that growth ring formations are controlled by the diurnal cycle, day and night variations in the environment (Meyer 1895; Buttrose 1960; Buttrose 1962; Pilling and Smith 2003). Other studies have suggested an additional mechanism controlling the formation growth rings involving circadian rhythm, in the absence of environmental cycles (Roberts and Proctor 1954; Buttrose 1962). Recent studies in potato tubers (Pilling and Smith 2003), suggest both biological and physical factors could contribute to the control of granule ring formation and the interaction between them leads to the complexity of the starch granule.

Amylose is an important component of starch, and how it is packs into the framework of the amylopectin matrix and how it interacts with amylopectin is not well understood. Amylose molecules exist as single helical forms within the starch granule (Gidley and Bociek 1988). It has been thought that the amylose helices are randomly dispersed throughout the granule, however, amylose is predominantly found in the amorphous lamellae of the amylopectin matrix where it interacts with amylopectin (Jenkins and Donald 1995; Gallant et al. 1997). X-ray diffraction experiments performed by Jenkins and Donald (1995) used varieties of barley, maize and pea to analyze the effect of varying amylose content on the internal granular structure. A correlation was observed between increasing amylose content and the increasing size of the crystalline portion of the cluster, suggesting amylose acts to disrupt the packing of the amylopectin chains (Jenkins and Donald 1995).

Comparison of glycogen and amyolpectin

Glycogen and starch are reserve polysaccharides of α-D-glucose, containing α(1→4) and α(1→6) linkages that function as chemical energy forms in animals and plants, respectively. Though they share these common features, glycogen and starch differ in a variety of aspects. Glycogen is an amorphous water-soluble structure and starch is stored as insoluble semi-crystalline structure, the granule, containing the densely packed amylopectin glucan chains (Ball et al. 1998; Ball and Morrell 2003). In glycogen synthesis (in mammalian cells), glycogen synthase is specific for UDP-glucose (uridine 5' diphosphate–glucose). Bacterial glycogen synthase and plant starch synthases use ADP-glucose (adenosine 5' diphosphate–glucose) as the glucose donor to elongate the α(1→4) glycosidic chains (Sivak and Preiss 1998). Both polymers have high molecular weights. The major

component of starch, amylopectin, is 10^7-10^9 Da, approximately 10^7 DP. In comparison, glycogen is 10^7 Da, which is approximately 10^5 DP. Glycogen contains more branch linkages (8-12%) that are regularly spaced every 6-8 glucose residues, and amylopectin contains branch linkages (5%) that are non-randomly distributed, every 12-20 glucose residues (Manners 1991). Multiple enzyme activities also differentiate the two metabolic pathways. Glycogen metabolism has one form of each biosynthetic enzymes: ADP-glucose pyrophosphorylase, starch synthase, branching enzyme and debranching enzyme. The plant starch metabolic pathway contains multiple forms of the enzyme activities. These isoforms are conserved evolutionarily and appear to possess unique functions. Another difference between glycogen and starch is when each is enzymatically debranched, differences in the chain length distributions and ratios of chains observed are discernable. The chain length distributions of glycogen reveals an exponential curve and a greater ratio of 'B' chains to 'A' chains. In contrast, the chain length distributions of amylopectin reveal a polymodal distribution of chains and a greater ratio of 'A' chains to 'B' chains. The most distinguishing characteristic between the two reserve carbohydrates is the final product. Glycogen, though a large molecule, is limited in size because of its steric hinderance. Starch has a negligible osmotic pressure allowing plants to store large amounts of energy, glucose, without disturbing the water relations in the cell (Sivak and Preiss 1998).

Enzymes involved in the biosynthesis of starch

The starch biosynthesis is a complex metabolic network involving the actions multiple enzyme activities. Obvious steps in the starch biosynthetic pathway involve the generation of a sugar donor, polymerization of the sugar units into linear chains, and branching. The

obvious steps in starch biosynthesis are shared with glycogen biosynthesis. In addition, there are some not so obvious steps of starch synthesis that were identified using forward genetic screens indicating that debranching enzymes and the D-enzyme are play important biosynthetic roles. The enzymes involved in the biosynthesis of starch include: ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27), starch synthase (SS, EC 2.4.1.21), starch branching enzyme (BE, EC 2.4.1.18) debranching enzymes (DBE, EC 3.2.1.41 and EC 3.2.1.68) and disproportionating enzyme (D-enzyme, EC 2.4.1.25). The involvement of each of the enzymes and their contributions to starch biosynthesis will be discussed, except the starch branching enzymes. Starch branching enzymes are the focus of this dissertation and an in-depth review in Chapter 1is located in the following sections.

ADP-glucose pyrophosphorylase

ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step in starch biosynthesis. The synthesis of ADP-glucose from glucose-1-phosphate and ATP (adenosine triphosphate) liberates pyrophosphate and serves as a soluble precursor and substrate for starch synthases. In higher plants, AGPase is found as a heterotetramer, consisting of two large subunits (AGP-L) and two small catalytic subunits (AGP-S) that are encoded by two different genes (Preiss and Sivak 1996). Recent biochemical research in cereal endosperm tissues has indicated the presence of two distinct AGPase enzymes, cytosolic and plastidial isoforms in barley (Thorbjornsen et al. 1996), maize (Denyer et al. 1996), rice (Sikka et al. 2001) and wheat (Tetlow et al. 2003). In the developing endosperms of barley, maize, rice and wheat, the cytosolic (extra-plastidial) isoform accounts for 65-95% of the total AGPase activity. This implies the majority of storage starch in storage tissues occurs through the

import of ADP-glucose into the amyloplast. In barley and maize, ADP-glucose is produced through an alternative pathway (James et al. 2003). In wheat amyloplasts, an extra-plastidial AGPase manufactures ADP-glucose directly in the cytoplasm from glucose-1-phosphate and exports it into the amyloplast using it as a substrate for starch synthesis. ADP-glucose in cereal endosperm, is synthesized in the cytosol and is transported into plastids through an inner envelope protein (James et al. 2003). In maize, the *Brittle1* gene codes for an amyloplastidic ADP-glucose transporter; it has the capacity to transport ADP-glucose from the cytosol into the plastid (Shannon et al. 1998). In higher plants, multiple genes encode AGP-L and AGP-S subunits and they are differentially expressed in plant organs. The large subunit of AGPase exhibits strong specificity in expression, e.g. barley and wheat expression is restricted to endosperm, leaf or root (Olive et al. 1989; Villand et al. 1992a; Villand et al. 1992b), and induction from increased glucose or sucrose levels in potato (Muller-Rober et al. 1990). Isoforms of the small subunit of AGPase also demonstrate organ-specific expression patterns, one found specifically in leaves and the other found in both cotyledons and leaves (Weber et al. 1995). In maize, different cDNAs encode the AGP-S subunit and have different expression patterns (Giroux et al. 1994). AGPase is considered an important enzyme in determining the flux of carbon into starch and the expression of it is vital in the determination of starch utilization with the plant.

Starch synthases

Starch synthases (SS) catalyze the transfer of a glucose residue from ADP-glucose to the non-reducing end of an $\alpha(1\rightarrow4)$ linked glucan chain (Figure 1.2). The role of SS is to elongate the linear chains of amylose and amylopectin by the formation of $\alpha(1\rightarrow4)$

glycosidic linkages. Plants contain up to five isoforms (GBSS, SSI, SSII, SSIII and SSIV) that are grouped according to conserved sequence relationships. Cereal endosperms contain at least five SS isoforms: GBSS, SSI, SSIIa, SSIIb and SSIII (Cao et al. 2000). The major classes of SS genes are also highly conserved among monocots and dicots (Ball and Morrell 2003). The first class, which is primarily associated with amylose biosynthesis, includes granule-bound starch synthases, GBSSI and GBSSII. Encoded by the *Waxy* (*wx*) locus in cereals, GBSSI specifically functions in the elongation of amylose (Nelson and Rines 1962; Shure et al. 1983). *Waxy* mutants are amylose-free and synthesize starch comprised soley of amylopectin (for a list of *wx* mutants see: (Koβmann and Lloyd 2000)). *Waxy* dosage and GBSS activity have a linear correlation, but *Waxy* dosage has not been demonstrated to be proportional to amylose content (Tsai 1974; Fujita et al. 2001). Evidence suggests other factors can influence amylose content. GBSSI is stimulated by malto-oligosaccharides (MOS), using them as primers to synthesize amylose (Denyer et al. 1996; Denyer et al. 2001). GBSSI has also been found to be involved in amylopectin biosynthesis. In *in vivo* and *in vitro* experiments, GBSSI was responsible for the extension of long glucans with the amylopectin fraction (Delrue et al. 1992; Maddelein et al. 1994; van de Wal et al. 1998). The two GBSS differ in their expression patterns and are encoded by separate genes (Vrinten and Nakamura 2000). GBSSI is specifically expressed in storage tissues, while GBSSII is expressed in storage tissues and other non-storage tissues that accumulate transient starch (Fujita and Taira 1998; Vrinten and Nakamura 2000).

The second class of starch synthase genes are exclusively involved in amylopectin biosynthesis. These isoforms are soluble and their distribution within the plastid between the stroma and starch granule varies between developmental stages, species and tissues (review

in (Tetlow et al. 2004b)). In theory, each SS isoform has a unique role in amylopectin biosynthesis. Analyses of SS mutants supports the hypothesis that each SS isoform has a specific role *in vivo*. There has been no SSI mutant reported to date, but evidence suggests that SSI is primarily responsible for the synthesis of those glucan chains with degrees of polymerization (DP) of 10 glucosyl units or less (Commuri and Keeling 2001). Further extension of glucan chains to produce longer chains results from the activities of the SSII and SSIII isoforms.

The SSII genes, SSIIa and SSIb, are found in monocots. SSIIa is predominately found in cereal endosperm and SSIIb is found in photosynthetic tissues (James et al. 2003). The role of SSIIb has not been able to be determined and with no mutants identified, the role of SSIIb in starch biosynthesis remains unknown. There are effects observed when SSIIa is absent. The loss of SSIIa (in monocots) and SSII (in dicots) reduces starch content, reduces amylopectin chain-length distribution, alters granule morphology and reduces crystallinity (James et al. 2003). These effects suggest that SSII isoforms have similar functions in starch biosynthesis across species (Tetlow et al. 2004b). It has been suggested that SSIIa, in monocots, is specifically responsible for the elongation of short chains of DP less than 10, synthesizing intermediate-sized glucan chains (DP 12-24) (James et al. 2003).

SSIII also has a role in amylopectin biosynthesis. In potato, antisense suppression of SSIII affected amylopectin biosynthesis by modifying chain length distribution and decreasing starch content (Edwards et al. 1999). In comparison, maize SSIII mutants (*du1*) have slight phenotypes (Gao et al. 1998). The last SS, SSIV, has no known mutant and its role in starch biosynthesis remains unknown at this time.

Starch branching enzymes

Starch branching enzymes catalzye the following reaction in starch biosynthesis. An α(1→4) bond is cleaved within an amylopectin chain, then the glucosyl chain is transferred to another α(1→4) linked-glucan chain and connected by an α(1→6) bond, therefore, creating a branch point (Figure 1.2). In maize, as in other cereals, there are multiple isoforms of branching enzymes present within the plant. Maize starch branching enzymes are described in detail in later sections of the introduction.

Debranching enzymes

There are two types of debranching enzymes (DBE) found in plants: isoamylases and pullulanases (also called limit-dextrinases) that hydrolzye, or debranch, α(1→6) linkages in amylopectin and pullulan, a fungal polymer of maltotriose residues (Figure 1.2). An expected function of DBEs is in the degradation of starch. Mutations affecting isoamylase-type DBE are called *sugary1* (*su1*) and are correlated with the accumulation of a highly branched, water-soluble polysaccharide called phytoglycogen (PG). *Sugary1* mutants have been identified in maize (James et al. 1995) and rice (Nakamura et al. 1996), as well as in the unicellular alga, *Chlamydomonas* (Mouille et al. 1996). Evidence supporting a biosynthetic role for isoamylases has been observed in the expression patterns of *iso1*, the wheat ortholog of *su1*. The *iso1* cDNA has maximum expression in developing endosperm and is undetectable in mature grains (Genschel et al. 2002).

The pullulanase DBE in maize, ZPU1, has been hypothesized to participate in both starch synthesis and degradation (Dinges et al. 2003). Biochemical characterization of ZPU1 has resulted in it being classified it as an endo-acting enzyme that cleaves very short chains

and is affected by changes in the oxidation-reduction state and high concentrations of sugar (Wu et al. 2002).

In summary, genetic analyses have suggested that DBEs function in both starch biosynthesis and degradation. In DBE mutants, the formation of starch is reduced, and there is a build up of glycogen-like material in DBE mutants. DBEs are therefore needed to produce a starch like branching pattern versus a glycogen-like pattern. It is a balancing act between the BEs and DBEs that results in the starch-like pattern versus glycogen-like pattern.

Scientific models have been proposed to explain the function of DBEs in starch synthesis and phytoglycogen accumulation. The glucan-trimming model suggests DBEs are directly involved in amylopectin biosynthesis, removing any inappropriately positioned branches generated at the surface of the growing starch granule. The resulting debranched structure would favor formation of double helices and crystallize on the granule surface (Myers et al. 2000). Recent research supporting this model identified numerous chains, DP 6-11, at the surface of immature granules (Nielsen et al. 2002). An alternative model, the water-soluble polysaccharide (WSP) clearing model, suggests an indirect role for DBEs in starch biosynthesis. DBEs remove ("clear") soluble glucan from the stroma, which in turn removes a pool of substrates from the SSs and BEs. Loss of DBE would cause an accumulation of phytoglycogen at the expense of amylopectin. Evidence to support this model has been observed in an Arabidopsis isoamylase mutant (Zeeman et al. 1998). The exact roles of DBEs in starch biosynthesis have not been completely elucidated and are currently being investigated.

Disproportionating enzyme

Activity of the disproportionating enzyme (D-enzyme) was first described in the mid-1950s in potato extracts (Peat et al. 1956) and later in other plant species (review in (Koβmann and Lloyd 2000)). D-enzyme functions by rearranging α(1→4) bonds in starch-like polymers, in molecules the size of maltotriose to larger starch-like molecules (Figure 1. 2). Evidence has suggested that D-enzyme works in conjunction with starch phosphorylase and contributes to starch synthesis (Takaha et al. 1998). In accordance with the glucan-trimming model, short chain MOS are liberated by the DBEs and converted to longer-chain glucans by the D-enzyme. These longer-chain glucans would then be available to starch phosphorylase for phosphorolysis liberating glucose-1-phosphate that would be used to synthesize ADP-glucose. Evidence of the D-enzyme stimulating the phosphorolytic starch phosphorylase reaction supports a direct role of the D-enzyme in starch biosynthesis (Colleoni et al. 1996).

Starch Branching Enzymes

Chemical Reaction of Branching Enzymes

Branching enzymes are classified as (1,4-α-D-glucan:1-4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-tranferases; EC 2.4.1.18). A starch branching enzyme cleaves an α(1→4) linkage within an amylopectin chain and transfers the glucosyl chain to another α(1→4) linked-glucan chain by an α(1→6) bond, creating a branch point (Figure 1.2). A proposed chemical mechanism for the starch branching enzyme transfer reaction involves the formation of a covalent enzyme-intermediate (Robyt 1984; Robyt 1998) (Figure 1.3). The intermediate complex is formed when the α(1→4) glycosidic bond in the first glucan chain is cleaved by

the branching enzyme. The C^6-hydroxyl group of the second glucan chain attacks the covalent enzyme-complex forming an α(1→6) branch linkage (Figure 1.3) (Robyt 1998). Branches occur about every 21 glucose units (Kainuma 1988). The end result is the creation of an α(1→6) branch glycosidic linkage which increases the number of non-reducing ends, and, in turn, facilitates starch synthesis. Therefore, starch branching enzymes play an important role in determining the quality and quantity of starch produced by the plant.

Similarities of branching enzymes and α-Amylases

BEs catalyze two enzymatic reactions involving glycosidic bonds, cleavage of an α(1→4) bond and formation of an α(1→6) bond. Both reactions are also characteristic of α-amylases. A correlation was found between branching enzyme function catalyzing α-amylase reactions and the similarity between branching enzyme structure and α-amylase. Structure homology between the glycogen branching enzymes and amylolytic enzymes was first reported in the late 1980's (Romeo et al. 1988). Conserved amino acid sequences of branching enzymes have been identified among four catalytic regions that are characteristic of and highly conserved in amylolytic enzymes (Baba et al. 1991). Analysis of the primary amino acid sequences of plant branching enzymes revealed an evolutionary relationship between branching enzymes and amylolytic enzymes (e.g. α-amylase, cyclodextrin glucanotransferase, glucosyl transferase and pullulanase) (Jespersen et al. 1993). The crystal structures of α-amylase and glucanotransferase have been determined and contain a catalytic $(\beta/\alpha)^8$-barrel domain consisting of eight parallel β-strands surrounded by eight parallel α-helices, with βα/βα connectivity (Jespersen et al. 1991).

Evidence suggests that the conserved functional amino acids of α-amylases may also be functional in the actions of branching enzymes. Amino acid replacement experiments with site-directed mutagensis determined that conserved aspartate (Asp) residues in regions 2 and 4 and a conserved glutamate (Glu) residue in region 3 were required for branching enzyme catalytic activity (Kuriki et al. 1996), however, it remains unclear how these residues function in catalysis. Alignment of branching enzyme homologues revealed two conserved arginine residues. Chemical modification of the arginine residues suggested they were important for branching enzyme activity (Cao and Preiss 1996).

To understand the structure-function relationship in branching enzymes between the amino (N-) and carboxyl (C-) terminus and branching enzyme specificities, chimeric enzymes of maize branching enzyme I and II were constructed. The C-terminus was determined to control substrate specificity and catalytic capacity, and the N-terminus was found to control the size of the glucan chain transferred (Kuriki et al. 1997). Recent experiments involving the C- and N- termini of maize branching enzyme II have begun to elucidate the role of important amino acids in branching enzyme activity and chain transfer pattern. Truncating the amino terminus of BEII altered the branching pattern (Binderup et al. 2002), whereas the deletion of 58 amino acids from the C-terminus had no effect on branching enzyme activity (Hong and Preiss 2000). The deletion of 39 amino acid residues from the N-terminus did have an effect on catalytic activity of maize branching enzyme II, whereas, a single amino acid substitution ($Glutamine^{270} \rightarrow Arginine^{270}$) was responsible for the loss of branching enzyme activity (Hong and Preiss 2000). The $Glutamine^{270}$ residue is located at the beginning of the $(\beta/\alpha)^8$-barrel domain and may be required for maximum enzyme activity.

Branching enzymes in higher plants

In higher plants, branching enzymes are divided into two classes based upon their primary amino acid sequence (Burton et al. 1995). There are distinguishing characteristics for each branching enzyme class. The members in family A possess an extra N-terminal domain of 60-100 amino acids. Also, members of family A, except pea SBEI, lack 50 amino acids at the C-terminus. The family A enzymes are characterized by the presence or absence of a specific 12-residue sequence at the C-terminus, whereas, family B enzymes are characterized by the presence or absence of a specific 13-residue sequence at the C-terminus (Burton et al. 1995). A variety of botanical sources have been identified to contain starch branching enzymes.

Branching enzyme isoforms have been identified in various higher plants including: maize[1] (mBEI, mBEIIa and mBEIIb) (Boyer and Preiss 1978a; Boyer and Preiss 1978b; Boyer and Fisher 1984; Dang and Boyer 1988; Guan and Preiss 1993; Fisher et al. 1996), pea (SBE A and SBE B) (Bhattacharyya et al. 1990), rice[1] (rBEI, rBEIIa and rBEIIb) (Mizuno et al. 1992; Yamanouchi and Nakamura 1992; Kawasaki et al. 1993; Mizuno et al. 2001), wheat (wBEI, wBEIIa and wBEIIb) (Denyer et al. 1995; Nair et al. 1997; Repellin et al. 1997; Rahman et al. 2001), potato (BEI and BEII) (Borovsky et al. 1976; Vos-Scheperkeuter et al. 1989; Blennow and Johansson 1991), Arabidopsis[1] (AtBEIIa and AtBEIIb) (Fisher et al. 1996), kidney bean (PvSBE1 and PvSBE2) (Nozaki et al. 2001), cassava (SBE1) (Salehuzzaman et al. 1992) and barley (SBEIIa and SBEIIb) (Jansson et al. 1997; Sun et al.

[1] For maize, rice and Arabidopsis, see appendix, Table 1.1: Branching enzyme isoform information and nomenclature.

1998; Sun et al. 1999). Branching enzyme (BE) isoform information is summarized using the classification standards set by Burton et al. (1995) in Table 1.2.

This dissertation will use terminologies when referring to enzymes as well as the various forms of the enzymes, and these terms will be defined as follows and used throughout the dissertation. Isoforms are different forms of an enzyme that possess the same enzymatic activity, but do not contain the same catalytic peptide, e.g. BEI, BEIIa, and BEIIb in maize. Isozymes are different forms of an enzyme that contain the same catalytic peptide, e.g. the multiple electrophoretic mobility forms of BEI.

Branching enzymes in maize have been identified in reproductive and vegetative tissues. All three isoforms in maize are expressed in endosperm tissue, mBEIIb has seed-specific expression and found primarily in the embryo (Gao et al. 1997), and mBEI and mBEIIa are found in leaf tissue (Dang and Boyer 1988; Gao et al. 1997); and mBEIIa is also expressed in root, stem and tassel tissue (Gao et al. 1997). In pea, BEI and BEII are both expressed in embryo and leaf tissues (Burton et al. 1995; Denyer et al. 1995). Similar to maize, all three branching enzyme isoforms[1] (rBEI, rBEIIa and rBEIIb) in rice are expressed in endosperm (seed) tissue (Mizuno et al. 2001), rBEIIb has seed-specific expression, rBEI and rBEIIa are both expressed in leaf tissue (blade and sheath), and rBEIIa is also expressed in the culm and root tissues (Mizuno et al. 1992; Yamanouchi and Nakamura 1992; Nakamura et al. 1992b). The three branching enzymes in wheat, wBEI, wBEIIa and wBEIIb, are expressed in endosperm tissue (Rahman et al. 1995; Morrell et al. 1997; Rahman et al. 2001); wBEI and wBEIIa are present in leaf (Morrell et al. 1997). Potato branching enzymes have been studied since the mid-1970's (Borovsky et al. 1975; Borovsky et al. 1976; Borovsky et al. 1979). Potato branching enzyme II (BE-II) is expressed in leaf tissue

exclusively and branching enzyme I (BE-I) is expressed exclusively in tubers (Larsson et al. 1998). Arabidopsis has two isoforms of SBE II: SBE2.1 and SBE2.2. Both are expressed in seedlings, inflorescences, rachis, mature leaves and flowers (Fisher et al. 1996; Khoshnoodi et al. 1998). In kidney beans, SvBE1 and SvBE2, are expressed in the seed (Hamada et al. 2002). The cassava branching enzyme (SBE1) is expressed in the petiole, stem, and tuber (Salehuzzaman 1994). In barley, all three branching enzyme isoforms (SBEI, SBEIIa and SBEIIb) are expressed in endosperm tissue (Sun et al. 1997; Sun et al. 1998), SBEIIb has seed-specific expression, and SBEIIa is also expressed in the embryo, leaf and root. Table 1.3 contains a summary of branching enzyme isozyme expression patterns.

The focus of this dissertation research was the branching enzyme isoforms in maize endosperm. With the large body of starch branching enzyme homologue information, narrowing the focus of comparisons of maize with other representative plant species was necessary. The cereal family includes barley, maize, rice and wheat. Maize and rice BEs share high degree of homology and sequence identity among the cereals. To compare monocot branching enzymes with those of a dicot, Arabidopsis was chosen to analyze and compare with maize. Table 1.4 contains BESTFIT comparison data of maize, rice and Arabidopsis branching enzyme isoforms. A summary of the data is presented in Table 1.1. The two isoforms of branching enzyme II (BEII) in all three of the plant species (maize, rice and Arabidposis) have between 80-86% amino acid identity within each species. The BEII isoforms when compared to the BEI from the same species contain 41%, 58% and 60% identical amino acids in Arabidopsis, rice and maize, respectively. Between plant species, identical amino acids in BEII isoforms are more conserved than in BEI isoforms. The proposed BEI in Arabidopsis has 38% and 39% amino acid identity with mBEI and rBEI,

respectively. In comparison, mBEI (maize) and rBEI (rice) have 85% amino acid identity. Further investigation of the putative Arabidopsis BEI is needed to determine it is function in starch biosynthesis.

Branching enzyme isoforms in maize

Three genes have been identified in maize that code for the branching enzyme isoforms termed BEI, BEIIa, and BEIIb (Fisher et al. 1993; Fisher et al. 1995; Gao et al. 1997). The gene names are *sbe1*, *sbe2a*, and *amylose-extender* (*ae*), respectively. The cDNA sequences of *sbe2a* and *sbe2b* have divergent 5' and 3' ends, however the predicted amino acids sequences share 78% identity over a 2.1 kb region (Blauth et al. 2001) of the cDNA. From the cDNA sequences, maize BEI is predicted to be slightly different from BEIIa and BEIIb in molecular size. The predicted molecular weights are are 80 kDa for BEI, 89 kDa for BEIIa, and 85 kDa for BEIIb (Fisher et al. 1993; Fisher et al. 1995). The expression patterns of the maize BEs are distinct in that all three of them are expressed in endosperm tissue (Gao et al. 1996), whereas BEI and BEIIa are also expressed in leaves but BEIIb is not (Fisher et al. 1996; Gao et al. 1996). Also, the cDNA for maize endosperm BEI and BEIIb have been cloned and expressed in *E. coli* (Guan et al. 1994a; Guan et al. 1994b). The recombinant enzymes demonstrated properties (i.e. substrate specificity) similar to branching enzymes isolated from maize endosperm.

There are also distinct properties that have been identified of the maize endosperm BEs with respect to chain-length transfer patterns and substrate specificities. Maize BEI transfers long chains (degree of polymerization, DP 40-100), whereas the BEII-containing peak transferred shorter chains (DP 6-14) (Takeda et al. 1993). As a substrate *in vitro*, mBEI

prefers branching amylose to amylopectin and mBEIIa/mBEIIb preferentially branches amylopectin (Guan and Preiss 1993). These data led researchers to hypothesize that mBEI first branches amylose, transferring longer chains that mBEIIa and mBEIIb use as substrates for further branching to amylopectin. In comparison, a recent study (Seo et al. 2002), obtained different results pertaining to the roles of BEI, BEIIa and BEIIb. Seo et al. (2002) heterologously expressed all three of the maize branching enzymes in yeast and observed SBEIIa and SBEIIb acting on a precursor polymer before SBEI. The discrepancies between the various studies could be the result of different host organisms or the interaction of SBEI is different in *E. coli* glycogen synthase than the yeast enzyme. These studies support the theory the three maize endosperm BEs play distinct roles in amylopectin biosynthesis, however, further investigations are needed to clarify the roles of starch branching enzymes in starch biosynthesis.

Branching enzyme mutants

Branching enzyme deficient mutants have been found in a variety of plant species. The *amylose-extender* (*ae*) mutants have been studied in the endosperm tissue of maize (Dang and Boyer 1989; Stinard et al. 1993) and rice (Mizuno et al. 1993), and in the *rugosus* mutant of pea embryo (Bhattacharyya et al. 1990). The *ae*$^{-}$ maize mutant was first studied in the 1950's (Vineyard and Bear 1952) and was observed to have increased amylose content in the endosperm, specifically 60% (Detherage et al. 1954), as compared to the wild type value of approximately 25% (Manners 1991). In gene dosage experiments with the *ae* gene, a correlation was seen with increasing amylose content (Ferguson et al. 1966) and a linear relationship observed between increasing the gene dosage of the dominant *Ae* allele and

branching enzyme IIb activity (Hedman and Boyer 1982). Based on these results, the dominant *amylose-extender* (*Ae*) allele was hypothesized to be the structural gene for branching enzyme IIb (Hedman and Boyer 1982). Subsequent molecular cloning analysis demonstrated directly that the BEIIb isoform is coded for by the *ae* gene (Fisher et al. 1993; Stinard et al. 1993).

Mutants deficient in BEI and BEIIa activity have not been detected using standard genetic screens. Mutagenesis, a reverse genetics approach, was used to isolate a BEIIa-lacking mutant from maize (Blauth et al. 2001). Endosperm starch from the *sbe2a::Mu* mutants had branching patterns indistinguishable from wild type patterns. This suggests that mBEIIb possibly compensates for the lack of mBEIIa activity and mBEIIa may not be required in determining endosperm starch structure.

Mutator insertional mutagensis was also used to isolate a BEI-lacking mutant in maize (Blauth et al. 2002). Endosperm starch of mBEI mutants was unaltered when compared to wild type endosperm starch. In comparison, a BEI-deficient mutant in rice was generated using N-methyl-N-nitrosourea (Satoh et al. 2003). The lack of the rBEI protein in rice was determined to be controlled by a single gene, *sbe1*. Phenotypically, there was no difference between *sbe1* and wild type rice plants and the starch content was unaffected, however, the absence of rBEI did modify the fine structure of amylopectin and the physiochemical properties, and also affected the gelatinization temperature of starch in rice endosperm (Satoh et al. 2003). In summary, the existing research on starch branching enzyme mutants from various plant species has provided evidence that the branching enzyme isoforms have specific roles in starch biosynthesis in some plant species, whereas in others the function of BEIIa appears to be redundant.

OUTSTANDING PROBLEMS

Protein – Protein interactions

There is an evolutionary conservation of the multiple enzymatic activities of the starch biosynthetic enzymes seen in higher plants. The general evolutionary trend for branching enzymes is that each starch producing plant possesses at least one form of BEI and BEII. However, Arabidpoisis is an anomaly, apparently possessing only BEII isoforms and a BE-like protein that does not bare high similarity to any known branching enzymes. The conservation of the multiple enzyme activities could be related to the determination of starch's architecture

The most prevalent hypothesis suggests that there is coordination among the multiple enzyme activities, e.g. starch synthases and branching enzymes. One theory suggests that BEs and SSs interact directly creating linear chains with branch points simultaneously. Alternatively, BEs and SSs could be in the same complex but not directly in contact, therefore the spatial orientation of the SSs and BEs would allow them to function and work in coordination creating linear chains and inserting branch points.

To address this hypothesis, both the abundant circumstantial and small pool of direct evidence need to be considered. In plants, there is a broad conservation both starch branching enzyme (BE: I and II) classes (Smith et al. 1997; Cao et al. 1999; Li et al. 1999), and all four-sequence classes of starch synthases (SS: I, II, III and GPSS) that supports isoform-specifc functions. Mutations in genes coding for specific BEs or SSs indicate non-overlapping functions of the multiple isoforms (Shannon and Garwood 1984). Recent biochemical and genetic evidence supports the hypothesis that the synthesis of starch involves the coordination of enzyme activities through direct protein-protein interactions.

Pleiotropic effects offer indirect evidence supporting the idea that branching enzymes participate in multi-subunit complexes. One example involves mutation of the maize gene *dull1*, which is known to code for the starch synthase isoform SSIII (Gao et al. 1998). When proteins isolated from plants carrying the *dull1* mutation were fractionated, the SSIII activity peak was missing, as expected (Boyer and Preiss 1978b; Boyer et al. 1981). Also seen in *dull1* mutants, however, is the decrease in activity of the BEIIb-containing peak. The reason the SSIII mutation affects BEIIb activity is not known, but one explanation might be SSIII and BEIIb area binding partners, and SSII may be required for BEIIb activity.

Another example of pleiotropic effects involving mutations in starch biosynthetic enzyme is the *amylose-extender* (*ae*) mutation. The *ae* gene codes for BEIIb (Fisher et al. 1993; Stinard et al. 1993). Zymogram analysis of the proteins of maize *ae* mutants reveals the absence of mBEIIb activity, as expected, and also has indirect effects on mBEI activity. Two electrophoretic forms ("red" bands) of BEI are absent (Colleoni et al. 2003). More specifically two of the four prominent electrophoretic mobility forms presumed to be BEI are absent in the *ae*⁻ mutant strains. Pleiotropic effects are also seen in the rice *ae* mutant. There is a 50% reduction observed in soluble SSI activity, as well as the lack rBEIIb of activity. This suggests that rBEIIb and SSI proteins possibly interact (Nishi et al. 2001).

DBE mutants not only affect the activities of debranching enzymes, but also have pleiotropic effects on other starch biosynthetic enzymes, i.e. the loss of mBEIIa activity. Both in maize and rice, molecular defects in isoamylase-type DBE have been known to secondarily reduce the activity of the pullulanase-type DBE (Pan and Nelson 1984; Nakamura et al. 1996). In maize, mutations affecting both a pullulanase-type DBE (*zpu1*-

204) and an isoamylase-type DBE (*su1-st*) both cause a loss of mBEIIa activity, although normal levels of the mBEIIa polypeptide accumulate (James et al. 1995; Dinges et al. 2001).

It has been hypothesized that coordination of branching, debranching and starch synthase activities is a required for starch synthesis, and the coordination of multiple enzyme activities is accomplished through physical interactions of these enzymes in a complex or complexes in the amyloplast (Ball et al. 1996; Ball and Morrell 2003). The coordination of SSs and BEs activities would determine the chain length distributions in amylopectin, and the coordination of BEs and DBEs multiple enzyme activities would determine branch frequency and branch point placement. There are two independent lines of indirect evidence that support the existence of functional interactions between starch biosynthetic enzymes. First, the addition of purified BEI or II to maize kernel extracts stimulated SSI activity (Boyer and Preiss 1979). Second, another approach suggesting individual branching enzyme isoforms interacting directly with other starch biosynthetic enzymes comes from a study of heterologous expression of maize enzymes in yeast (Seo et al. 2002). BEI had no effect on glucan structure without BEIIa and BEIIb present. One explanation is that BEI requires both BEII isoforms and interacts with them to function in starch biosynthesis. Also, using the heterologous yeast system a functional interaction was observed between SSIII, specifically the amino terminal region of this protein that is not involved in the enzymatic activity, and BEIIa (Kim et al. 2005).

Recent biochemical analyses have provided evidence for the association of branching enzymes with other starch biosynthetic enzymes in stable complexes (Tetlow et al. 2004a). The activity of the starch biosynthetic enzymes was regulated by protein phosphorylation, presumably by a protein kinase in the plastid. Also noted was that protein phosphorylation in

the plastid stimulated starch biosynthesis. Co-immunoprecipitation experiments demonstrated directly BEI, BEIIb, and phosphorylase interact forming a complex, while BEIIa is in its own complex. Furthermore, this phosphorylation was essential for the formation of the complexes, because treatment with a general protein phosphatase destabilized the complex (Tetlow et al. 2004a). In summary, it appears that protein phosphorylation regulates complex assembly and starch biosynthesis.

RATIONALE

The overall question posed by this research is what is the molecular explanation for the existence of multiple isoforms of branching enzymes in maize endosperm. This research set out to answer three questions pertaining to the three branching enzyme isoforms in present maize endosperm. The first question was what, if any, are the differences in enzymatic properties of branching enzymes IIa and IIb. The second question was if there are specific binding peptide partners for the branching enzymes in maize. The third question was what branching enzyme isoforms and polypeptides exist in leaves.

With regards to question one, what, if any, are the differences in enzymatic properties of branching enzymes IIa and IIb, the following is known. BEI and II in maize possess different biochemical properties (Guan and Preiss 1993; Takeda et al. 1993); enzyme kinetics and immunological properties (Boyer and Preiss 1978b; Fisher and Boyer 1983; Singh and Preiss 1985; Preiss 1991; Fisher et al. 1996). In cereals, BEIIa and BEIIb are conserved, implying that each of the BEII isoforms possess specific functions in starch biosynthesis. Supporting specific isoform functions is tissue-specific expression observed in the branching enzyme isoforms (see Table 1.2) and the timing of expression within maize tissues (Gao et

al. 1996). What is not known are the enzymatic differences, if any, between BEIIa and BEIIb in maize endosperm. The two BEII isoforms, IIa and IIb, have not previously been purified from each other to accurately determine how each contributes to starch biosynthesis.

With regards to question two, if there are specific peptide partners for the branching enzymes in maize, the following is known. There are six or more electrophoretic mobility forms identified as branching enzymes in maize kernels (20 DAP) that have been partially purified (Colleoni et al. 2003). Three genes encode for the three maize branching enzyme isoforms: I, IIa and IIb. The two BEII isoforms, IIa and IIb, have also been shown to be products of two separate genes (Fisher et al. 1996). The structural gene for BEIIb, *amylose-extender* (*ae*) has been identified (Fisher et al. 1993; Stinard et al. 1993) in maize. It has been hypothesized that the branching enzymes along with other starch biosynthetic enzymes are involved in multi-protein complexes that are regulated through post-translational modifications. What is not known is the reason for the multiple electrophoretic forms of branching enzymes and if the branching enzyme isoforms interact with each other or with other starch biosynthetic enzymes in complexes to synthesize starch.

With regards to question three, what branching enzyme isoforms and polypeptides exist in leaves, the following is known. Analyzing maize leaf and kernel branching enzymes, Dang and Boyer (1988) compared chromatographic, kinetic and immunological properties. Leaf branching enzyme I was determined to be similar to kernel branching enzyme I, and leaf branching enzyme II similar to kernel branching enzyme IIa (Dang and Boyer 1988). BEIIa is expressed in leaves while BEIIb is not (Dang and Boyer 1989). RNA for BEI has been identified in maize leaf tissue (Gao et al. 1996). Blauth et al (2001) observed in the maize BEIIa mutant, *sbe2a::Mu*, no apparent branching enzyme IIa polypeptide or protein was

detected. In *sbe2a::Mu* leaf, it appears that the BEI polypeptide or protein is not present, but the starch is still altered. It is possible that BEI is not required to synthesize transient starch in leaf, an unknown branching enzyme could be present, or there is a technical explanation that branching enzyme BEI is present but the BEI protein or polypeptide has not been detected.

DISSERTATION ORGANIZATION

All of the experiments of this dissertation were performed by me, Rebekah Suzanne Marsh. The three recombinant expression plasmids used in this study were constructed by previous members of our laboratory group, Drs. Heping Cao and Seungtaek Kim.

This dissertation was written by me, with editorial assistance from my co-major professor, Alan Myers. Figure 1.3 taken from Robyt (1998), and is used with permission of the author.

This dissertation is organized into four chapters. Chapter one contains a literature review and an introduction to the specific research questions of the dissertation. Chapters 2, 3, and 4 are research chapters, each addressing one of the major questions around which this study is organized. Chapter 4 presents preliminary studies on leaf BEI and further studies will need to investigate the role of BEI in transitoty starch biosynthesis in comparison to the role of BEI in storage starch biosynthesis.

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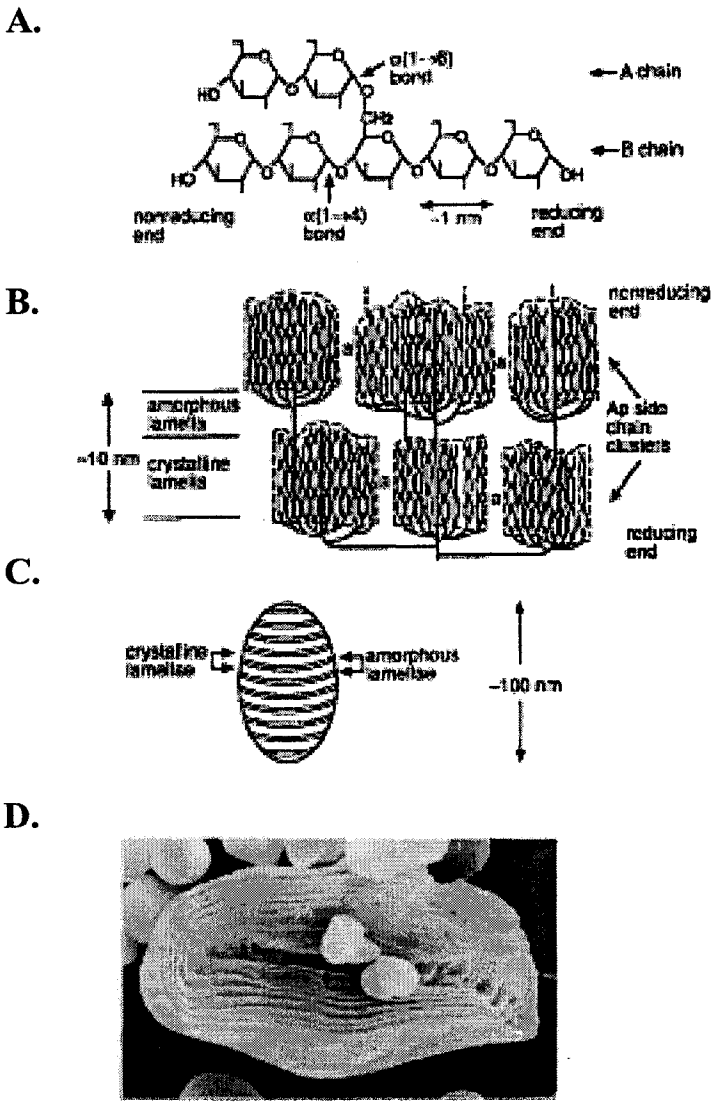

Figure 1.1. The hierarchial organization of amylopectin structure (adapted from Myers et al., (2000)). (A) Individual glucose units are connected through α(1→4) and α(1→6) glycosidic bonds. (B) On a 10-nm scale, the repeating unit of amorphous and crystalline lamallae is depicted. Solid lines indicate glucan chains, and intersections between them indicate branch linkages. Dotted lines indicate the boundaries of amylopecin side chain clusters where unbranched chains are primarily associated in tightly packed double helicies. a, Amorphous regions that separate amylopectin side chain clusters. (C) On a 100-nm scale, a "blocklet" is a discrete unit composed of amylopectin side chain clusters. (D) Growth rings of the starch granule are seen in a scanning electron micrograph of a wheat starch granule exposed to mild α-amylase treatment (adapted from Ball and Morrell (2003)).

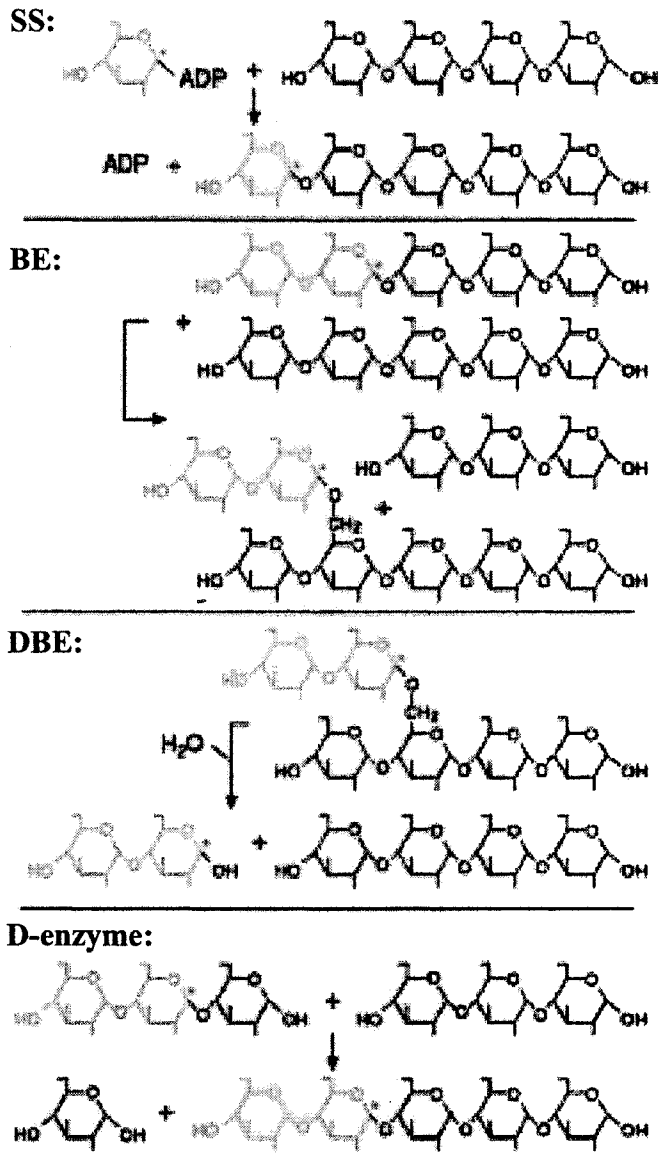

Figure 1.2. The reactions catalyzed by the starch biosynthetic (adapted from Myers et al., (2000)). The enzymes are as follows: starch synthase, SS; branching enzyme, BE; debranching enzyme, DBE; and disproportionating enzyme, D-enzyme.

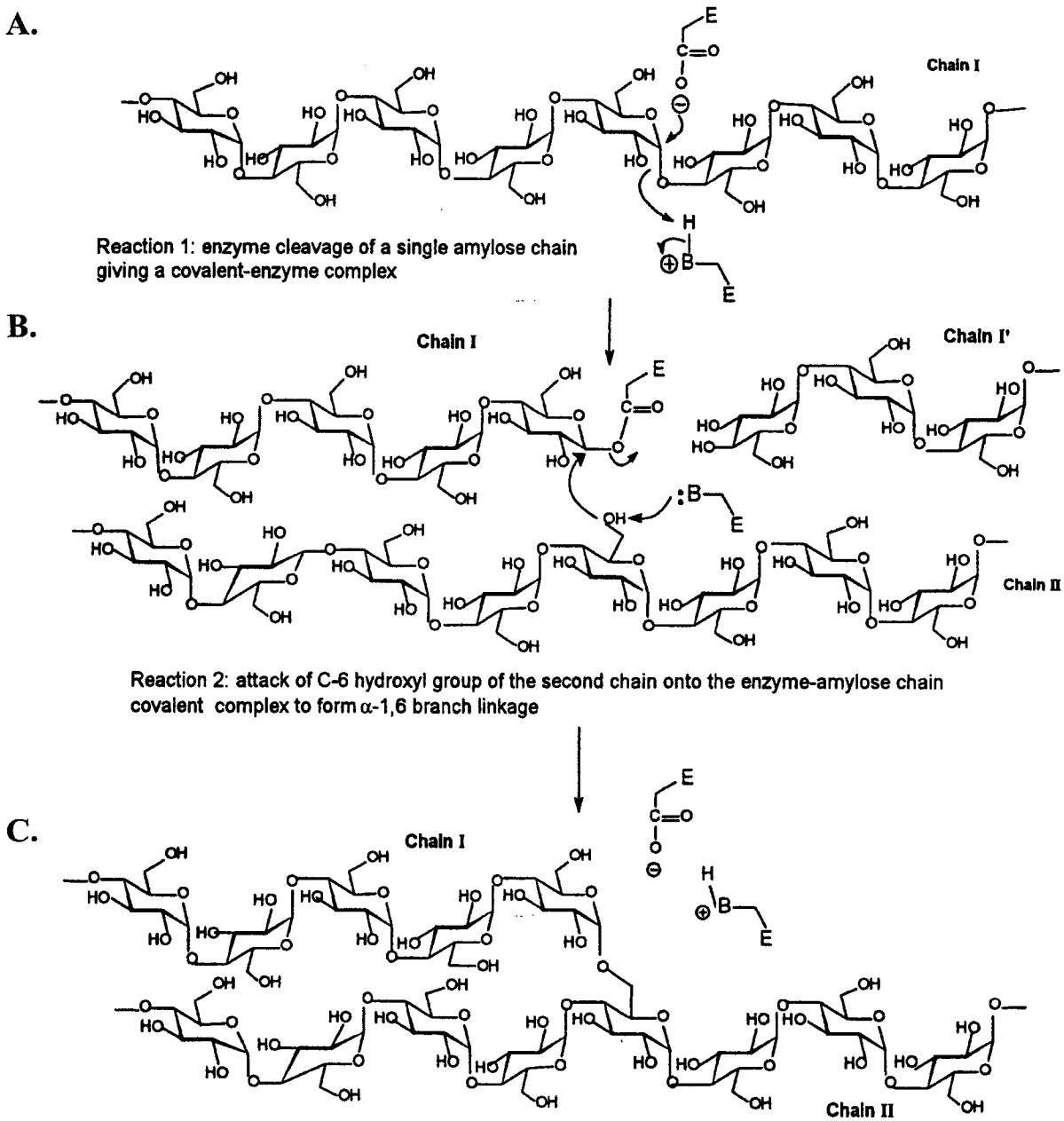

Figure 1.3. The molecular mechanism of the branching enzyme reaction, from Robyt, J. (1998). (A) Branching enzyme cleaves α(1 4) bond in one amylose chain, forming a covalent-enzyme complex. (B) The C^6-hydroxyl group of an adjacent amylose chain attacks the convalent-enzyme complex, releasing the enzyme and forming an α(1 6), a branch point. (C) The end result is the formation of an α(1 6) between two adjacent amylose chains.

Table 1.1. Dissertation nomenclature and starch branching enzyme isoform information

Species	Dissertation nomenclature	Literature nomenclature	Genbank accession number	Locus ID	Literature gene name	Reference
Zea mays L. (maize)	mBEI	BEI	D11081	MZBE1		
	mBEIIa	BEIIa	U65948	ZMU65948		
	mBEIIb	BEIIb	L08065	MZEGLUCTRN	*ae*	Stinard et al. (1993); Fischer et al.
Oryza sativa (rice)	rBEI	RBE1	D11082	RICBE1	*sbe1*	Satoh et al. (2003)
	rBEIIb	RBE3	D16201	RICBCE3	*ae*	Mizuno et al. (1993)
	rBEIIa	RBE4	AB023498	AB023498		
Arabidopsis thaliana L.	AtBEI		AK118785	At3g20440		
	AtBEIIb	SBE2.1	U18817	ATU18817		
	AtBEIIa	SBE2.2	U22428	ATU22428		

Table 1.2. Branching enzyme isoforms in higher plant species

Species	Branching enzyme family		Reference
	A	B	
Zea mays L. (maize)[1]	mBEIIa, mBEIIb	mBEI	Boyer and Preiss (1978a), (1978b); Boyer and Fisher (1984); Singh and Preiss (1985); Dang and Boyer (1988); Guan and Preiss (1993); Takeda et al. (1993); Fisher et al. (1997); Gao et al. (1997)
Pisum sativum (pea)	pea BEI (SBE A)	peaBEII (SBE B)	Bhattacharyya et al. (1990); Tomlinson et al. (1997)
Oryza sativa (rice)[1]	RBE3, RBE4	RBE1	Mizuno et al. (1992); Yamanonchi and Nakamura (1993): Kawasaki et al. (1993); Mizuno et al. (2001)
Triticum aestivum (wheat)	wBEIIa, wBEIIb	wBEI	Denyer et al. (1995); Repellin et al. (1997); Rahman et al. (2001)
Solanum tuberosum (potato)	BE-II	BE-I	Borovsky et al. (1975); Vos-Scheperkeuter et al. (1989); Blennow and Johansson (1991)
Arabidopsis thaliana L. [1]	SBE2.1, SBE2.2		Fisher et al. (1996)
Phaseolus vulgaris L. (kidney bean)	PvSBE2	PvSBE1	Nozaki et al. (2001); Hamada et al., (2001)
Manihot esculenta Crantz (cassava)		SBE1	Salehuzzaman et al., (1992)
Hordeum vulagare L. (barley)	SBEIIa, SBEIIb	SBEI	Jansson et al. (1997); Sun et al. (1996)

[1]Branching enzyme isoform nomenclature is defined in Table 1.

Table 1.3. Expression patterns of branching enzyme isoforms in higher plants

Species	Branching enzyme isoform	Tissue expression	Reference
Zea mays L. (maize)[1]	mBEI mBEIIa mBEIIb	embryo, endosperm, kernel, leaf, root and tassel embryo, endosperm (low level), kernel, leaf, root and tassel embryo (low level), endosperm (seed-specific) and tassel	Gao et al. (1996); Dang and Boyer (1988); Dang and Boyer (1989)
Pisum sativum (pea)	BEI BEII	embryo and leaves embryo and leaves	Burton et al. (1995); Denyer et al. (1996)
Oryza sativa (rice)[1]	rBEI rBEIIa rBEIIb	endoperm, leaf and seed endoperm, leaf, culm and root endosperm (seed-specific)	Mizuno et al. (1992); Nakamura et al. (1992b); Yamanonchi and Nakamura (1993); Mizuno et al. (2001)
Triticum aestivum (wheat)	wBEI wBEIIa wBEIIb	endosperm and leaf endosperm and leaf endosperm (seed-specific)	Morrell et al. (1997); Rahman et al. (1999); Rahman et al. (2001)
Solanum tuberosum (potato)	BE-I BE-II	tuber leaf	Larsson et al. (1998); Jobling et al. (1999)
Arabidopsis thaliana L. [1]	AtBEIIa AtBEIIb	flowers, inflorescence, mature leaves and seedlings flowers, inflorescence, mature leaves and seedlings	Fischer et al. (1996); Khoshnoodi et al. (2001)
Phaseolus vulgaris L. (kidney bean)	PvSBE1 PvSBE2	seed seed	Nozaki et al. (2001); Hamada et al. (2001)
Manihot esculenta Crantz (cassava)	SBE1	petiole, tubers and stem	Salehuzzaman et al. (1994)
Hordeum vulagare L. (barley)	SBEI SBEIIa SBEIIb	endosperm embryo, endosperm, leaf and root endosperm (seed-specific)	Sun et al. (1996); Sun et al. (1997)

[1]Branching enzyme isoform nomenclature is defined in Table 1

Table 1.4. Amino acid identity among branching enzyme proteins in Maize, Arabidopsis and Rice

		Percent Amino Acid Identity[a]								
		Zm [b]			*At* [b]			*Os* [b]		
		mBEI	mBEIIa	mBEIIb	AtBEI	AtBEIIb	AtBEIIa	rBEI	rBEIIb	rBEIIa
Zm [a]	mBEI	100								
	mBEIIa	59	100							
	mBEIIb	59	86	100						
At [a]	AtBEI	39	42	40	100					
	AtBEIIa	59	85	81	41	100				
	AtBEIIb	57	73	79	42	85	100			
Os [a]	rBEI	85	60	60	40	60	58	100		
	rBEIIb	60	80	87	41	81	72	61	100	
	rBEIIa	59	91	85	42	83	75	61	81	100

[a]Full length amino acid sequences of each protein were compared pairwise using standard parameters of the GCG program BESTFIT. Values indicated are the percentage of identical amino acids as determined by the algorithm. Genbank acession numbers of each amino acid sequence are listed in Table 1.

[b]Abbreviations are as follows: *Zm* , *Zea mays* (maize); *At* , *Arabidopsis thaliana* ; *Os* , *Oryza sativa* (rice)

CHAPTER 2: ENZYMATIC PROPERTIES OF MAIZE STARCH BRANCHING ENZYMES BEIIa AND BEIIb

ABSTRACT

Multiple enzyme activities of starch branching enzyme are involved in the starch biosynthesis in plants. Dicots in general possess two BE isoforms, BEI and BEII, whereas monocots possess three BE isoforms, BEI, BEIIa and BEIIb. Evolutionary conservation of BEIIa and BEIIb in monocots suggests that each isoform is functionally significant. To date, however, there is no direct evidence allowing comparison of the enzymatic functions BEIIa and BEIIb. This study set out to purify BEIIa and BEIIb from maize endosperm cells, and also used recombinant techniques to generate recombinant BEIIa. Two polyclonal antisera were produced to facilitate purification, one specific for BEI, and the other recognizing both BEIIa and BEIIb. Protein purification techniques were used to separate BEIIa or BEIIb away from the other two isoforms. Purified native BEIIa, purified native BEIIa, and purified recombinant BEIIa were each tested in their capability to modify the structure of amylose by generation of branch chains of specific length distributions. All three forms of BEII showed a preference for creating chains in the range of DP 10-25. The recombinant enzyme also produced a much larger percentage of chains of DP 6 and DP 7, which was not observed for either native enzyme. No differences were observed in the glucan chain length distributions created by either native BEIIa or native BEIIb. The data suggest that distinct enzymatic properties, at least with regard to product specificity, do not account for the evolutionary conservation of BEIIa and BEIIb. The data also indicate that some modification of BEIIa occurs in its native environment in endosperm cells that modifies its enzymatic properties.

INTRODUCTION

Starch, one of the most abundant storage carbohydrates produced on earth, is composed of two homopolymers of α-D glucose: amylose and amylopectin. Amylose is an essentially linear polymer comprising of α1→4) linked glucose residues, whereas amylopectin is a branched glucan polymer containing approximately α(1→6) branch linkages in addition to the linear α1→4) linkages between glucose residues (Manners 1991). The biosynthesis of starch involves the actions of multiple enzyme activities, including isozymes of ADP-glucose pyrophosphorylase (AGPase), starch synthases (SS), starch branching enzymes (BE) and starch debranching enzymes (DBE). Branching enzymes hydrolyze an α(1→4) linkage in a chain of amylopectin and transfer the glucosyl chain to another α(1→4) linked glucan chain in amylopectin through an α(1→6) linkage. This reaction creates a branch point within a linear chain of glucose residues and increases the number of non-reducing ends in starch, thereby, facilitating starch biosynthesis. Additionally, branching enzymes determine the placement of branch linkages, and also affect chain length distribution. An important fundamental question about starch biosynthesis is the role played in determination of polymer architecture by the individual isoforms of a given enzymatic activity. This study addressed the issue of potential isoform-specific functions of two closely related forms of branching enzyme II in maize, BEIIa and BEIIb.

Three genes have been identified in maize that code for the branching enzyme isoforms termed BEI, BEIIa, and BEIIb, respectively (Fisher et al. 1993; Fisher et al. 1995; Gao et al. 1997). The gene names are *sbe1*, *sbe2a*, and *amylose-extender* (*ae*), respectively. The cDNA sequences of *sbe2a* and *ae* have divergent 5' and 3' ends, however the predicted

amino acids sequences share 78% identity over a 2.1 kb region (Blauth et al. 2001) of the cDNA. From the cDNA sequences, the three maize BEs are slightly different from each other. The predicted molecular weights are are 80 kDa for BEI, 89 kDa for BEIIa, and 85 kDa for BEIIb (Fisher et al. 1993; Fisher et al. 1995). The expression patterns of the maize BEs are distinct in that all three of them are expressed in endosperm tissue (Gao et al. 1996), whereas BEI and BEIIa are also expressed in leaves but BEIIb is not (Dang and Boyer 1988; Gao et al. 1996).

One means of assaying whether or not an isoform has a specific function is to eliminate that enzyme by mutation and examine for effects on starch structure. From such analyses it is clear that BEIIb has a function that cannot be replaced by BEI or BEIIa. The *ae*⁻ mutations of maize, and analogous mutations affecting rice endosperm or pea embryo have been utilized to determine the effects of eliminating BEIIb (Dang and Boyer 1989; Bhattacharyya et al. 1990; Mizuno et al. 1993; Stinard et al. 1993). The *ae*⁻ maize mutant was first studied in the 1950's (Vineyard and Bear 1952) and was observed to have increased amylose content in the endosperm (Detherage et al. 1954), specifically 60% as compared to the wild type value of approximately 25% (Ball et al. 1998). In gene dosage experiments with the *ae* gene, a correlation was seen with increasing amylose content (Ferguson et al. 1966), and a linear relationship was observed between increasing the gene dosage of the dominant *Ae* allele and BEIIb activity (Hedman and Boyer 1982). Based on these results, the dominant *amylose-extender* (*Ae*) allele was hypothesized to be the structural gene for branching enzyme IIb (Hedman and Boyer 1982). Subsequent molecular cloning analysis directly demonstrated that the *ae* gene codes for the BEIIb isoform (Fisher et al. 1993; Stinard et al. 1993).

Mutants deficient in BEI and BEIIa activity have not been detected using standard forward genetic screens. In a reverse genetics approach, however, transposon mutagenesis was used to isolate a BEIIa-lacking mutant from maize (Blauth et al. 2001). Endosperm starch from the *sbe2a::Mu* mutants had branching patterns indistinguishable from those of wild type plants. This suggests that BEIIb possibly compensates for the lack of BEIIa activity, and that BEIIa may not be essential in determining endosperm starch structure. *Mutator* insertional mutagenesis was also used to isolate a BEI-lacking mutant in maize (Blauth et al. 2002). Endosperm starch of these mutants was unaltered as compared to wild type plants. A BEI-deficient mutant in rice was generated using N-methyl-N-nitrosourea (Satoh et al. 2003). The lack of the BEI protein in rice was determined to be controlled by a single gene termed *sbe1*. In contrast to maize, there was an obervable phenotypic difference between the starches of *sbe1*$^-$ and wild type rice plants. The absence of BEI resulted in modified fine structure of amylopectin, and also affected the physiochemical properties of the mutant starch including the gelatinization temperature (Satoh et al. 2003). In summary, the existing research on starch branching enzyme mutants from various plant species has provided, in some instances, evidence for specific roles in starch biosynthesis, whereas, in other instances, the function of BEIIa appears to be redundant.

Biochemical analysis of BE activities is a necessary complementary approach to the genetic studies. In maize endosperm, branching enzymes have been resolved using diethylaminoethyl (DEAE)-cellulose (Boyer and Preiss 1978a; Boyer and Preiss 1978b; Boyer and Preiss 1981) revealing two peaks, the first containing BEI and the second peak designated as containing BEII. The uncharacterized second peak clearly contains BEIIb, and most likely also BEIIa. Using these column peaks as the source of enzyme, distinct

properties were noted for maize endosperm BEs with respect to chain-length transfer patterns and substrate specificities. Maize BEI transfers long chains (degree of polymerization, DP 40-100), whereas the activity of the BEII peak transfers shorter chains (DP 6-14) (Takeda et al. 1993). As a substrate *in vitro*, BEI prefers amylose to amylopectin and the BEII peak preferentially branches amylopectin (Guan and Preiss 1993). These data led researchers to hypothesize that BEI first branches amylose, transferring relatively long chains, which BEIIa and BEIIb subsequently use as substrates for further branching in order to form amylopectin.

In addition to purified native enzyme, recombinant BEI and BEIIb have also been characterized regarding their enzymatic properties. The cDNA for maize endosperm BEI and BEIIb have been expressed in *E. coli* (Guan et al. 1994a; Guan et al. 1994b). Each recombinant enzyme, BEI and BEII, demonstrated properties similar to the respective branching enzyme column fraction isolated from maize endosperm. Both BEI and BEII were purified and free from contaminating amylolytic activity. BEI has the highest activity for branching amylose, however, its rate of branching amylopectin was less than 10% of the rate of branching amylose (Guan et al. 1994a). Similar techniques revealed BEII has highest activity branching amylopectin, which was twice that of branching amylose (Guan et al. 1994b).

This research set out to investigate what, if any, were the differences in enzymatic properties of BEIIa and BEIIb in maize. To date there has been no direct comparison of the *in vitro* properties of BEIIa to those of either BEIIb or BEI. The reasons for this gap in our understanding are that IIa and IIb have not previously been purified from each other, nor has there been any report of the expression of recombinant BEIIa. In this study, BEIIa and BEIIb from developing maize kernels were effectively separated by chromatographic procedures.

In addition, maize BEIIa was expressed in abundant quantity in *E. coli*. Analyses of properties of the separate isoforms showed that chain lengths transferred using amylose as the substrate did not differ between the partially purified native forms of BEIIa and BEIIb. However, significant differences were observed in the chain length transfer preferences between native BEIIa and recombinant BEIIa. The results indicate that differences in the products of BEIIa and BEIIb are not responsible for the evident evolutionary conservation of these two isoforms among monocots. Further, these data suggest that the biosynthetic properties of BEIIa are not entirely intrinsic to the polypeptide, but instead that external factors present in the native environment also influence the products of BEIIa activity.

MATERIALS AND METHODS

Expression of recombinant proteins and purification of recombinant BEIIa

In preliminary experiments the induction conditions were optimized, specifically IPTG concentration, culture time after addition of the inducer, and growth temperature. *E. coli* host strain BL21(DE3) transformed with the expression plasmid pHC16 (described in Results) were grown in LB-KAN medium (LB medium containing 30 μg/mL kanamycin [Sigma-Aldrich, St. Louis, MO; catalog no. K-4000]). Overnight cultures were inoculated into fresh LB medium at a 1:200 dilution and grown in 400 mL LB-KAN in a 2 L flask at 37°C until the A_{600} was between 0.6 - 0.9. Expression was induced by addition of isopropylthio-β-galactoside (IPTG) to a final concentration of 1 mM, and the cultures were then grown at room temperature overnight (16 - 20 h). Cells in each flask were collected by centrifugation, and suspended in 10 mL sonication buffer (20 mM Tris-hydrochloride, pH 7.5, 150 mM Sodium chloride (NaCl), 5 mM dithiothreitol (DTT), 0.1% Triton X-100, and 1 mM

phenylmethylsulfonyl fluoride (PMSF)). The cells were lysed by treatment with lysozyme (100 μg/mL) followed by sonication. Lysates were centrifuged at 20,000 rpm for 20 min and the supernatant was collected. These soluble *E. coli* extracts were stored at -80°C.

Recombinant BEIIa, containing the S-tag affinity peptide, was affinity purified according to the manufacturer's protocol (Novagen). Briefly, the total soluble extracts were incubated with S-protein agarose beads at room temperature with gentle rocking for 1 h. (2 mL bead slurry mixed with 5 mL soluble *E. coli* extract). The beads were washed three times with 1X Bind/Wash buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Trition X-100), then suspended in 1 mL 1X Thrombin cleavage buffer (Novagen). Biotinylated thrombin (5 μL, 1U/μL) was added to cleave the recombinant protein between the S-tag sequence and the BEIIa sequence. The mixture was incubated at room temperature for 2 h with gentle rocking. Biotinylated thrombin was removed from the cleaved purified protein by treatment with streptavidin-agarose (100 μL) for 30 min at room temperature with gentle rocking. The supernatant, after removal of the S-agarose beads and the streptavidin-agarose by centrifugation, contained recombinant BEIIa (referred to as "rIIa") that was used in further studies as an antigen and a source of BEIIa enzyme.

A similar expression approach was used in efforts to produce BEI and BEIIb. The expression plasmids were pKST1, for BEI, (see Results) and pHC17, for BEIIb, (data not shown). In preliminary expression studies following the general conditions outlined for BEIIa, total *E. coli* cell lysates, and soluble and insoluble fractions generated by centrifugation at 20,000 rpm for 20 min, were tested for the presence of the recombinant BE protein using S-protein-alkaline phosphatase conjugate (Novagen) in a protein blotting assay.

For both BEI and BEIIb, all of the recombinant protein was in the insoluble phase (data not shown).

Polyclonal antibody production

Standard methods were used to raise antisera in rabbits, using either recombinant BEIIa or synthetic peptides from BEI as the antigen (Cooper and Paterson 1997). Antibodies reactive with BEIIa were produced in two New Zealand white rabbits using protocols approved by the Laboratory Animal Resource Facility of Iowa State University. Purified rIIa protein (see previous section) (0.5 mg in 1 mL of 1X Thrombin Cleavage Buffer [20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$]) was emulsified in an equal volume of Freund's complete adjuvant (Sigma, F-5506) and administered by injection. Subsequent booster injections of rIIa (0.25-0.5 mg) were emulsified in Freund's incomplete adjuvant (Sigma, F-5881) three times at two-week intervals and serum was harvested two weeks after the final injection. Pre-immune serum was collected prior to the first injections to serve as a negative control. Subsequent sera were tested against maize and *E. coli* proteins to monitor titer strength and determine protein band identities. Two antisera were produced, in two different rabbits designated as #3434 and #3435, both of which recognize BEIIa and BEIIb in maize. There is equal recognition of BEIIa and BEIIb by the serum from rabbit #3434, and a stronger recognition of BEIIa then BEIIb by the serum from rabbit #3435. The serum from rabbit #3434 was used throughout this dissertation research, and will be referred to hereafter as αBEIIab.

Efforts to produce BEI to use as antigen were not successful due to insolubility of the recombinant protein. Alternatively, two peptides from BEI were chemically synthesized. To

determine regions specific in the sequence of maize BEI in comparison to the two BEII isoforms, computational analyses were performed using GCG Sequence Analysis Software Package (Genetics Computer Group, Madison, WI). Selection of peptide sequences followed guidelines from Cambridge Research Biochemicals (http://crb.gb/com/antibodies-antigenic.htm) including choosing regions with few hydrophobic residues, avoiding multiple glutamines or two cysteines in a row, and containing prolines and tyrosines within the sequence. Two peptides of 20 amino acids each, specific to the C-terminal region of BEI, were chosen. These were amino acids 776-795 (YRVDEAGAGRRLHAKAETGK) and residues 811-830 (KEDKEATAGGKKGWFARQP). The synthetic peptides were synthesized as MAP (Multiple antigenic peptide) conjugates. Briefly, MAP peptides are synthesized on a resin that has an alanine with a four to eight lysine core that branches from the resin. The peptides are synthesized on each of the branches, creating a large molecule without a carrier protein.

Antibodies against BEI were produced in rabbits using university-approved protocols. A mixture of the two peptides was used antigen. The same injection protocol employed for BEII was used for BEI. The initial and booster injections included 0.5 mg of a mixture of the two peptides in 0.5 mL of PBS. Complete Freund's adjuvant as was used for the initial injections, and incomplete adjuvant for the booster injections. Pre-immune serum was again collected prior to the first injection to serve as a negative control. The serum from one of the two rabbits, #3672, exhibited a stronger reaction against BEI in maize extracts (see Results) than the other, #3673. The serum from rabbit #3672, designated as αBEI, was used throughout this study.

Extraction of proteins from plant tissue

All maize plants (*Zea mays* L.) used for analyses were in the inbred W64A genetic background. Plants were grown under normal field conditions in the summer at Iowa State University in Ames, Iowa, USA, or grown in the greenhouse under standard conditions with supplemental lighting on a 14/10h day/night cycle. Seeds for maize *sbe1::Mu* and *sbe2a::Mu* homozygous mutants were provided by Dr. Mark Guiltinan (Pennsylvania State University, University Park, PA). The *ae*$^{-}$ mutant line was provided by Dr. Martha James and was congenic in the W64A background.

Kernels were harvested at 20 days after pollination (DAP), quick-frozen in liquid nitrogen and stored at -80°C. Frozen kernels were ground into a fine powder in a chilled mortar and pestel under liquid nitrogen. Ground tissues were mixed with homogenization buffer (HB) (50 mM Tris-acetate, pH 7.5, 10 mM EDTA, 2.5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1X Protease Inhibitor cocktail [Sigma-Aldrich, catalog no. P-2714]). HB was added to the ground kernels at the ratio of 1-mL/g kernel weight. The mixture was allowed to stand for approximately 5 min on ice, and then centrifuged at 20,000 rpm for 20 min at 4°C. The supernatant, referred to "crude soluble extract", was kept on ice until further analysis. All further steps were performed either on ice or at 4°C, unless otherwise noted.

Ammonium sulfate precipitation and desalting

Ammonium sulfate was added to the crude soluble extract to the concentration of 40%, and proteins were allowed to precipitate during an 1 h incubation at 4°C, with gentle rocking. The mixture was centrifuged at 20,000 rpm for 20 minutes to pellet precipitated proteins, and

the supernatant was discarded. The pellet was suspended in approximately 10 mL of Buffer A (50 mM Tris-acetate, pH 7.5, 1 mM DTT) and then passed through a 0.22 μm pore diameter nitrocellulose syringe filter. The protein solution was then loaded onto a Sepharose G25 desalting column using the ÄKTA FPLC system (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The column was eluted in Buffer A while simultaneously monitoring protein in the eluate by measuring absorbance at 260 nm and salt in the eluted fractions by measuring conductivity. The protein-containing fraction was concentrated by filter centrifugation to approximately 5 mL using Amicon® Ultra-4 filter concentrators (Millipore, Billercia, MA) (molecular weight cutoff of 50 kDa).

Anion exchange chromatography

Buffer A and buffer B (50 mM Tris-acetate, pH 7.5, 1 M sodium chloride (NaCl), 1 mM DTT) were prepared with double deionized water and vacuum filtered through a 0.22 μm nitrocellulose filter prior to being run over a MonoQ column and using the ÄKTA FPLC system. The desalted extract sample, approximately 5 mL, was loaded onto the Mono Q column, and proteins were eluted using a flow rate of 1 mL/min. The elution program first applied 100% Buffer A (20 mL), then a gradient of 0 to 40% Buffer B (20 mL; 0 - 400 mM NaCl), then a high salt wash of 100% Buffer B (10 mL). Fractions (1.3 mL) were collected and tested for BE activity as described in a following section. Fractions containing BE activity were pooled and concentrated to approximately 2 mL by filter centrifugation as described previously.

Gel permeation chromatography

Gel permeation chromatography (GPC) was performed at 4°C using a Sephacryl S300 column (16 mm x 60 cm; 120 mL bed volume; Amersham Pharmacia). One column bed volume of S300 running buffer (50 mM Tris-acetate, pH 7.5, 8 mM $MgCl_2$ and 1 mM DTT) was applied at a flow rate of 0.3 mL/min. Fractions (1.4 mL) were collected, and characterized for branching enzyme activity as described in following sections, or for the presence of BEIIa, BEIIb and/or BEI protein by immunoblot analysis. Fractions containing either BEIIa or BEIIb were pooled and then concentrated by filter centrifugation to less than 500 µL.

Measurement of BE activity in solution by the phosphorylase *a* stimulation assay

The basis of the phosphorylase *a* stimulation assay is the stimulation of the synthesis of α-D-glucan from α-D-Glucose-1-phosphate (Glc-1-P), catalyzed by phosphorylase *a* (Guan and Preiss 1993). Addition of glucose units occurs at the non-reducing end of the substrate polymer. Branching enzymes increase the number of non-reducing ends in a glucan molecule by introducing branch points. The more branching enzyme activity present, the more non-reducing ends that are available to be acted upon by phosphorylase *a*. This assay was used to monitor branching enzyme activity throughout all purification steps.

The assay followed a previously published protocol (Guan and Preiss 1993). The assay mixture (total reaction volume 105 µL) contained 100 mM sodium citrate buffer, pH 7.0, 1 mM AMP, 2 µg/µL phosphorylase a (Sigma-Aldrich, catalog no. P-1261), 50 mM ^{14}C-Glc-1-P (approximately 50 cpm/nmol) and proteins from extracts or purification fractions (5 µL). The reaction was initiated upon the addition of ^{14}C-Glc-1-P, then vortexed, and incubated at

30°C for 1 h. To terminate the reaction and precipitate the glucan polymers present, 1 mL of a 75% methanol/1% KCl was added, and then 100 µL of a 10 µg/µL glycogen solution was added as a precipitation carrier. The precipitate was collected by centrifugation in a microfuge at full speed for 5 min, and the pellet was suspended in 200 µL of water and washed once in 1 mL of 75% methanol/1% KCl. The pellet dissolved in 500 µL of water, and added to 10 mL of scintillation fluid. The amount of radioactivity incorporated into precipitated glucan was then quantified by liquid-scintillation counting (1600 TR, Tri-Carb; Packard/PerkinElmer, Downers Grove, IL). This value indicates the total BE activity present, calculated in units of cpm incorporated/µg of protein/min.

In-gel enzyme BE activity analysis (zymograms)

Protocols followed were described previously (Dinges, et al 2001). Proteins present in soluble crude extracts (25-50 µg) or in specific chromatography fractions (approximately 30 µL of each fraction) were first separated on a native polyacrylamide gel (7 cm length by 1.5 mm thickness). The resolving gel contained 7% acrylamide (29:1 acrylamide-bisacrylamide) and 375 mM Tris-HCl, pH 8.8. The stacking gel contained 4% acrylamide and 63 mM Tris-HCl, pH 6.8. Electrophoresis was conducted at 4°C for 1 h at 150 mA in electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.8, 2 mM DTT). At the end of electrophoresis, the gel was transferred (20V, overnight at 4°C) by electroblotting to a non-denaturing polyacrylamide gel of the same size and specifications with the addition of 0.3% potato starch (Sigma-Aldrich, catalog no. S-2630). During the transfer the proteins in the separation gel have the opportunity to act on the starch in the recipient gel, thus modifying its structure and propensity for staining with iodine. After transfer, the recipient gel was stained

with I_2/KI solution (0.1% I_2, 1% KI in water) to reveal the presence of starch metabolic activities, and photographed immediately.

Protein blot analysis

Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of 0.1% SDS using the method of Laemmli (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes by electroblotting in Western transfer buffer (25 mM Tris, 192 mM glycine, pH 7.5, and 20% methanol) for 1 h at 170 V. For immunoblot analysis the nitrocellulose membranes were probed with isoform-specific primary antibodies, either polyclonal antiserum αBEIIab, or αBEI. The antibody dilution ranged from 1:250 to 1:5000, depending on the application. Secondary antibody was goat anti-rabbit alkaline phosphatase conjugate (1:3,000) (Bio-Rad Laboratories, Hercules, CA., catalog no. 170-6518). An alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, catalog no. 170-6432) was used for color detection, following manufacturers instructions.

In some instances the nitrocellulose membranes were probed with S-protein-alkaline phosphatase conjugate (Novagen, Madison, WI, catalog no. 69598-3) at a 1:5,000 dilution, in order to reveal the presence of S-tagged fusion protein. Alkaline phosphatase activity detection was the same as for the immunoblots.

Structural characterization of the products of BE activity

Branching of amylose

A 1% amylose solution in 25 mM 3-[N-Morpholino] propanesulfonic acid (MOPS), pH 7.5, was prepared as follows. Amylose (0.5 g) (Sigma-Aldrich, catalog no. A-7043) was dissolved by adding a small amount of 0.1 N NaOH, dropwise, until a gel-like substance was

formed. Approximately 2.5 mL of 0.1 N NaOH was required. A 25 mL volume of 50 mM MOPS was added, and then the solution was brought to a volume of approximately 40 mL by addition of water. The solution was brought to pH 7.5 with 1.0 N HCl, and then to the final volume of 50 mL by addition of water. This amylose solution was used as the substrate for modification by various BE enzyme preparations. The amylose was prepared fresh for each assay.

The amylose branching protocol of Guan et al. (1997) was followed. In this assay, the largely linear polymer amylose is treated with a BE, and the presence of newly formed branches is detected by the FACE method described in the following section. In a 1.0 mL reaction volume, 1% amylose in 25 mM MOPS, pH 7.5, was incubated with a BE preparation at 30°C for 2 h. After incubation, the reaction was terminated by boiling for 2 min. The pH was adjusted by addition of 0.25 mL of 1 M sodium acetate, pH 3.5, and 2 μL of *Pseudomonas* isoamylase (285 U/mg; Megazyme, County Wicklow, Ireland, catalog no. E-ISAMY) was added. The debranching reaction was run overnight (16 - 20 h) at 45°C, then stopped by boiling for 2 min and kept on ice until further analysis. As a control, the same reaction was carried out without the addition of any enzyme.

Determination of glucan chain length distributions by FACE

Glucan chain length distributions of the treated amylose were determined by fluorophore-assisted capillary electrophoresis (FACE) using previously published procedures (O'Shea et al. 1998; Dinges et al. 2003). Briefly, 10 μL of the debranching reaction described in the previous section was evaporated to dryness in a Speed-Vac. The reducing ends of the liberated oligosaccharide chains were derivatized with the fluorescent compound

8-amino-1,3,6-pyrenetrisulfonic acid (APTS) (Sigma-Aldrich, catalog no. 0934) as follows. The dried sample was suspended in 2 μL of 1 M sodium cyanoborohydride in tetrahydrofuran (Sigma-Aldrich, catalog no. 29681) and 2 μL of APTS (0.1 mg/μL in 15% acetic acid). The reaction was incubated overnight at 42°C, diluted with 46 μL of purified water, vortexed, and centrifuged briefly in a microfuge. A 5 μL aliquot was added to 195 μL of purified water, and this sample was applied to a Beckman P/ACE capillary electrophoresis instrument. The sample injection parameters were 5 s at 0.5 psi. Separation was accomplished at 23.5 kV in an uncoated capillary using Carbohydrate Separation Gel Buffer N (Beckman Coulter, catalog no. 477623). Assays were repeated in triplicate from three separate branching reactions.

Elution times were correlated with specific degrees of polymerization (DP) using a known standard. To accomplish this, test samples were spiked with maltotriose (Sigma-Aldrich, catalog no. M8378-5G), thus identifying the DP3 peak and allowing standardization for other DPs based on this elution time. The fluoresence units in each peak were determined by integration using software provided with the P/ACE instrument. For each DP, the value in the peak obtained from the standard with no enzyme was subtracted from the value obtained with enzyme, thus revealing the quantity of new chains of that DP formed through the action of the enzyme. Chain length distribution data was then normalized based on the total fluorescence units present in chains of DP 6-55. The normalized data allows comparison between strains of the percentage of total chains transferred for each DP. To better visualize the differences in normalized data between strains, difference plots were generated in which for each DP the value for one strain was subtracted from the corresponding value in the other.

RESULTS

Expression of maize BEs and purification of recombinant BEIIa

As a first step towards investigating the differences in enzymatic properties of the maize branching enzymes, a recombinant form of BEIIa (referred to as rIIa) was generated and purified. The expression vector, pHC16 (constructed by H. Cao), based in the plasmid pET-29a(+) (Novagen), contains the complete coding sequence of maize BEIIa, amino acids 21 to 847, lacking the organelle targeting peptide, as specified by the available cDNA sequence (GenBank accession #U65948) (Figure 2.1A). This cDNA is incomplete at the 5' end (Gao et al. 1997), so the actual codon numbers according to the full-length sequence are not known. However, amino acid 21, as labeled here, is known to be the mature amino terminus of BEIIa after cleavage of the targeting peptide during the import into the plastid (Fisher et al. 1993). The BEIIa coding region is fused at the N-terminus to a 15-amino acid S-tag sequence, followed by a thrombin-cleavage site sequence, both provided by pET-29a(+), and the gene fusion is expressed from the bacteriophage T7 promoter.

Typical expression results are seen in Figure 2.2. Increased expression of a protein matching the expected molecular weight of the recombinant BEIIa was observed under induced conditions (Figure 2.2, lanes 1, 5) versus uninduced conditions (Figure 2.2, lanes 6, 7). This protein was not observed in control cells containing the expression host vector lacking any BEIIa sequence (Figure 2.2, lane 8). Even though some of the recombinant BEIIa was present in the insoluble phase (Figure 2.2, lane 5), a substantial quantity of the protein was present in the soluble phase (Figure 2.2, lane 1). The size of the induced protein was as expected, slightly larger than 88kDa, the predicted molecular weight of native BEIIa. To purify the expressed recombinant protein, the S-tag was bound to bound to S-protein

agarose beads. Unbound proteins were removed (Figure 2.2, lane 2). The S-protein agarose beads were washed (Figure 2.2, lane 3) and the recombinant protein was cleaved from the S-tag sequence by biotintylated thrombin. The resulting recombinant BEIIa was the size of native BEIIa (Figure 2.2, lane 4). The purification of recombinant BEIIa was verified by binding to S-protein AP-conjugate. The major S-protein binding band expressed in induced conditions (Figure 2.2, lane 1) disappeared in the purified protein fraction, thus verifying removal of the amino terminal S-tag after thrombin cleavage (Figure 2.2, lane 4). The purified recombinant protein (Figure 2.2, lane 4) was used as an antigen, as described in Materials and Methods, to produce the polyclonal antiserum named αBEIIab.

Similar methods using an analogous expression plasmid were used in an effort to express BEIIb. The expressed protein, however, was located entirely in the insoluble phase (data not shown). Also, the total level of BEIIb accumulation was far less than that of BEIIa, even though the same host vector and expression methods were used. Thus efforts to produce recombinant BEIIb were discontinued at this point.

The same approach was again used in an effort to express recombinant BEI, making use of recombinant plasmid, pKST1 (Figure 2.1B) (constructed by S. Kim). In all expression tests, however, the great majority of BEI was found in the insoluble fractions of the *E. coli* lysates (data not shown). This protein fraction, dissolved in 8M urea, was useful as a test of antiserum specificity, as described in the following section.

Generation and verification of isoform-specific BE antibodies

Polyclonal antisera that recognize either BEIIa and BEIIb (referred to as αBEIIab), or BEI (referred to as αBEI), were produced in rabbits. For the BEII antiserum, the antigen was

the full-length recombinant form of BEIIa, affinity purified from *E. coli* as described in the previous section. For the BEI antiserum it was necessary to use synthetic peptides as the antigen. The sequences of BEI, BEIIa, and BEIIb were aligned in order to allow identification of amino acid sequences unique to BEI (Figure 2.3). Two such peptides were selected, synthesized, and administered in a mixture as antigen.

The specificity of each antiserum was demonstrated by immunoblot analysis against maize kernel extracts, and against *E. coli* extracts from cells expressing specific recombinant maize BEs. Extracts from maize mutants known to lack a specific BE isoform were used as controls. For the *E. coli* extracts, negative controls were provided by cells not exposed to induction conditions, and by cells containing the expression vector lacking any maize cDNA sequence.

The αBEI antiserum bound to a protein of approximately 80 kDa present in wild type kernel extracts (Figure 2.4B, lane 1), matching the molecular weight predicted from the known gene sequence (Fisher et al. 1995). The presence of the reacting protein was unaffected by mutation of the gene coding for BEIIa or BEIIb (Figure 2.4B, lanes 2, 3); however, this signal was absent from extracts of an *sbe1*$^{-}$ mutant (Figure 2.4B, lane 4). The αBEI antiserum did not react with *E. coli* extracts from cells harboring the BEIIa expression plasmid (Figure 2.4B, lanes 5, 6), but did react with an 80 kDa protein from cells expressing BEI (Figure 2.4B, lanes 7, 8). Taken together, these data demonstrate definitively that αBEI reacts with BEI and not with BEIIa or BEIIb. The antibody is highly specific, because the BEI band is the only signal observed in immunoblot analysis of total maize kernel extracts.

The αBEIIab antiserum bound two proteins of approximately 88 kDa and 85 kDa, respectively, present in wild type kernel extracts (Figure 2.4C, lane 1), matching the

molecular weights predicted from the known gene sequences of BEIIa and BEIIb (Fisher et al. 1993; Fisher et al. 1996). The presence of reacting proteins was not affected by mutation of the gene coding for BEI (Figure 2.4C, lane 4). However, the upper signal was absent and the lower signal remained unchanged in extracts of an *sbe2a*$^-$ mutant in which the gene coding for BEIIa is inactive (Figure 2.4C, lane 3). In *sbe2b*$^-$ mutant extracts, the upper band remained unchanged by the mutation, whereas the signal of the lower band was less intense (Figure 2.4C, lane 2). The αBEIIab antiserum did not react with *E. coli* extracts harboring the BEI expression plasmid (Figure 2.4C, lanes 7, 8), but did react with an 88 kDa protein (migrating at a slightly higher molecular weight than native BEIIa) from cells expressing BEIIa (Figure 2.4C, lanes 5, 6).

Collectively taken, these data demonstrate that the αBEIIab antibody reacts with both BEIIa and BEIIb and not BEI. The upper band recognized in wild type kernel extracts, also present in *sbe1*$^-$ and *sbe2b*$^-$ mutant extracts, and absent in the *sbe2a*$^-$ mutant, is defined as BEIIa. The lower band, present in wild type, *sbe2a*$^-$ and *sbe1*$^-$ extracts and faintly recognized in *ae* (*sbe2b*$^-$) extracts, is defined as BEIIb.

A possible reason that the *ae* mutation reduces but does not eliminate the band identified here as BEIIb may be that the *ae*$^-$ allele is a point mutation as opposed to a transposon insertion (Fisher et al. 1996). Therefore, a reduced amount of BEIIb may be produced, even though it is lacking in enzymatic activity. An alternative explanation that must be considered, however, is that a fraction of the BEIIa protein is proteolyzed such that the fragment runs in SDS-PADE at the same position as BEIIb.

Partial purification and separation of native BEIIa and native BEIIb

To analyze differences in enzymatic properties between BEIIa and BEIIb, a series of purification steps was performed to separate the two different isoforms. Total soluble proteins extracted from 20 DAP kernels were fractionated by ammonium sulfate precipitation, and the 40% pellet was retained because immunoblot analysis with αBEIIab serum revealed the presence of both BEIIa and BEIIb in this fraction. Proteins in the fraction were suspended in MonoQ A buffer, desalted, and separated by anion exchange chromatography on a MonoQ column. Immunoblot analysis identified the fractions that contained BEIIa and BEIIb protein (Figure 2.5A). In addition, each fraction from the MonoQ column was tested for BE activity using the phosphorylase *a* stimulation assay (Figure 2.6A).

Immunoblot analysis with αBEI demonstrated that the fractions containing BEIIa and BEIIb were free of BEI protein. The BEI signal was clearly detected early in the gradient, in fractions D4-D7, but was completely absent in the BEII-containing fractions, D12-E3 (data not shown). The anion exchange step partially purified BEII from other kernel proteins, but was not effective in separating BEIIa and BEIIb from each other (Figure 2.5A). Those MonoQ fractions exhibiting both BEIIa/BEIIb immunoblot signal and BE enzyme activity were pooled, concentrated by filter centrifugation, and then further fractionated by gel permeation chromatography using an S300 column.

In gel permeation chromatography, proteins are expected to elute based on their molecular size, with larger molecules eluting early in the column and smaller molecules later. Again, the fractions were probed with αBEIIab (Figure 2.5B) and assayed for total BE activity (Figure 2.6B), thus revealing the presence of BEIIa and BEIIb. There was a general

correlation of BE activity with BEIIa and BEIIb protein detected by immunoblot analysis (compare Figure 2.5B with Figure 2.6B). Compared to standards, the BEIIa protein eluted near its expected molecular weight as a predicted monomeric protein in fractions E1-E9. In contrast, the BEIIb protein eluted in the later fractions H1 to H5, as if it were a very small molecule. The size of BEIIb appears to be far less than its predicted molecular weight, around the size of a 10 kDa protein as indicated by comparison to standards run in the same chromatographic separation.

A possible explanation for the anomalous behavior is that the BEIIb protein has affinity for the matrix of the S300 column, thereby retarding its elution. Regardless of the explanation, the S300 chromatography has the unexpected, yet useful, result of separating BEIIa and BEIIb (Figure 2.5B). It should be noted that there was a very small amount of BEIIa activity, verified by zymogram analysis, contaminating the purified BEIIb fraction. Those S300 fractions containing either BEIIa and BEIIb protein and enzyme activity were pooled and concentrated by filter centrifugation and used in further analyses of the actions of BEIIa and BEIIb in branching amylose.

The same purification scheme was applied to *ae-Ref* (mutated in BEIIb) and in *sbe2a::Mu* (lacking BEIIa) mutant lines. To further characterize band identity for BEIIa and BEIIb, *sbe2a*$^-$ and *ae*$^-$ mutant soluble extracts were separated by gel permeation chromatography and compared to wild type. In the *ae*$^-$ mutant, the later eluting, faster migrating band is absent, and the earlier eluting, slower migrating band is the only BE band detected (data not shown). Conversely in *sbe2a*$^-$, the earlier eluting upper band is absent, and the only band detected is the later eluting lower band. These data confirm the identities of

the partially purified BEs. Furthermore, the fact that the purified lower band is completely missing in the *ae*⁻ mutant rules out the possibility that it is explained by breakdown of BEIIa.

Branching capacity differences between native BEIIa and BEIIb

Purified BEs were used to branch amylose in order to determine if there are differences in the branching capacities between native BEIIa (pIIa) and native BEIIb (pIIb), or between native BEIIa and recombinant BEIIa (rIIa). The amylose substrate is only lightly branched, thus allowing observation of new chains formed as the result of BE activity. Figure 2.7 shows that from a fixed amount of amylose, debranching of untreated sample released a certain number of linear chains in the range of DP 6-55, and treatment with any of the BE preparations significantly increased the number of chains present. Subtraction of the values for the untreated sample from that of the treated samples indicated total BE activity (Figure 2.7, right column).

Preliminary experiments indicated that the enzyme was in excess, so that final products of branching treatment could be compared between enzyme sources. The incubation time was varied in order to confirm that the reactions proceeded to completion. No differences were observed in the number of chains released among a 2 h, 4 h, or 16 h treatment (data not shown). Also, when the substrate concentration was reduced, proportionally reduced numbers of chains were observed (data not shown), indicating enzyme excess. Finally, one reaction was incubated for 2 h, then a sample was removed for analysis, then more enzyme was added and the incubation continued for another 2 h. The 2 h and 4 h samples yielded the same number of chains released (data not shown), again indicating that in the first 2 h incubation the enzyme was in excess and the reaction had proceeded to completion.

The purified fractions pIIa and pIIb yielded nearly identical profiles of chain length transfer distribution. In other words, chains of DP 6 represented approximately 1% of the total chains formed by pIIa treatment and about the same percentage of chains formed by pIIb treatment, and this equivalence held for all DPs analyzed (Figure 2.8, right panel, pIIa-pIIb). Thus, it appears that there are no inherent differences in the capability of native BEIIa and native BEIIb to affect the structure of amylopectin.

In contrast, significant differences were observed in the branching products of native BEIIa compared to the recombinant form of the enzyme. Specifically, rIIa produced a much larger percentage of chains of DP 6 and DP 7 (Figure 2.8). Excess frequency of DP 8 and DP 9 was also produced by rIIa as compared to pIIa, and the recombinant enzyme produced a lower frequency of chains in the range of DP 18-27 (Figure 2.8). As expected, the same general trends were observed in the comparison between rIIa and pIIb. From these data it is evident that BEIIa as it exists in its native state in maize endosperm is functionally different than the enzyme as it is produced in recombinant form in *E. coli*.

DISCUSSION

Expression of BE isoforms

Of the three BE isoforms, BEIIa was by far the most amenable to recombinant expression. BEIIa expresses at high levels in *E. coli*, is found in high quantity in the soluble phase of *E. coli* extracts, and retains enzyme activity over time when stored frozen. Thus, BEIIa was an excellent candidate for further detailed biochemical studies based on recombinant expression, and was also useful as a plentiful antigen for generating the αBEIIab antiserum.

In contrast to BEIIa, BEI and BEIIb proved to be refractory to recombinant expression in *E. coli*, even though a variety of induction and expression conditions were tested. BEI was completely insoluble in the cell extracts, in comparison to BEIIa and BEIIb, even after treatment with 8M Urea. Improper folding of the recombinant BEI protein could have contributed to its insolubility. About half of the expressed BEIIa protein was in the soluble phase, even though it accumulated at high levels. The expression of recombinant BEIIb was very different from that of BEIIa, which was unexpected considering that BEIIa and BEIIb share 78% identity at the amino acid level. The level to which recombinant BEIIb accumulated was far lower than that of BEIIa and the small amount of BEIIb protein that did accumulate was entirely insoluble.

The explanation for the different expression behaviors of BEIIa and BEIIb is not obvious considering the two isoforms have high identity between them. One possible explanation for the differences in the expression levels of recombinant BEIIa and BEIIb could be rare codon usage in the BEIIb mRNA that is not compatible with the *E. coli* strain used to express the protein. Analysis of the codon usage frequencies in BEIIa and BEIIb, however, revealed very few differences between the two isoforms. Another explanation could be that factors required for BEIIb to fold properly are present in the native environment in plant cells but not within the bacterial host cells. In contrast, BEIIa might be able to fold into a stable state without any other required factors, or make use of host factors for folding. The fact that BEIIa and BEIIb interact with other proteins is suggested by many observations in the literature, and is addressed directly in Chapter 3 of this dissertation. Such interactions could influence protein folding and thus their absence in *E. coli* might affect solubility and

ultimately the expression level. In this view, BEIIb would be dependent on extraneous host factors, whereas BEIIa would fold to some extent independently of exogenous interactions.

Antibody generation

The technical objective of producing isoform-specific antibodies was met for the most part, by obtaining sera that distinguish between BEI and either of the BEII isoforms. In other words, the αBEI antiserum recognized BEI but neither BEIIa nor BEIIb, and the αBEIIab antiserum recognized both BEII isoforms, but not BEI. The specificity of all the sera was definitively confirmed both in negative controls in which one of the isoforms was eliminated by mutation, and in positive controls in which a recombinant form of each isozyme was added to *E. coli* extracts by exogenous gene expression. Sera specific for either BEIIa or BEIIb was not obtained, most likely owing to the fact that BEIIa used as antigen is so highly conserved with the BEIIb amino acid sequence. The αBEIIab serum is still useful to recognize BEIIa or BEIIb specifically, at least in SDS-PAGE gels, because the two isoforms migrate at different rates.

Enzymatic comparison

As described in Results, the unexpected late elution of BEIIb from the Sephacryl S300 column afforded the ability to separate BEIIa from BEIIb. The separation of native BEIIa and native BEIIb involved various purification steps, each of which was analyzed for the presence of BE proteins using the isoform-specific antibodies and tested for total BE activity using the phosphorylase *a* stimulation assay. During anion exchange chromatography, BEI eluted early in the salt gradient separate from BEIIa and BEIIb, both of which eluted later in the gradient. As expected due to the high similarity between the two BEII isoforms, native

BEIIa and BEIIb, co-purified together in anion exchange chromatography. However the unexpected, yet useful, separation of native BEIIa from native BEIIb during gel permeation chromatography using a Sephacryl S300 column allowed for the analysis of native BEIIa activities independent of the activities of native BEIIb. With the separation of native BEIIa and native BEIIb and recombinant BEIIa, analysis of their enzymatic activities could be performed.

Three enzyme preparations could thus be tested for differences in chain length transfer during reaction with amylopectin, those being pIIa (free of pIIb), pIIb (mixture with small amount of pIIa contamination) and rIIa. In these chain transfer assays, the final observation shown in Figure 2.7 indicates the total number of chains transferred when a given amount of amylose is modified to completion by extended treatment with the BE. It should be noted that this is not a specific activity measurement, because the number of chain transfer events per unit time per unit mass of enzyme is not what is being measured while enzyme is rate limiting. Specific activity measurements are difficult to consider for BEs, because each time the enzyme acts the structure of the substrate is modified, although extrapolation of initial rate measurements could be used to approximate this parameter. The current study, however, is restricted to the final products after branching to completion. Complete branching was indicated by varying the enzyme to substrate ratio and observing that increase in substrate resulted in a proportional increase in the number of branches formed. Thus, the enzyme was in excess. Also, adding more enzyme to a branching treatment and continuing the incubation failed to change the amount of branches present in the final product. Using branching to completion as a measure of "activity", it appears from the raw data as if pIIb was more active

than pIIa while branching amylose (Figure 2.7, left panel). The recombinant enzyme rIIa was the most active for branching of amylose among the three enzyme preparations tested.

Normalizing the data of the products of pIIa, pIIb and rIIa allowed direct comparison between the distributions of specific chain lengths transferred, and thus reveals chain length preferences for the three enzyme preparations. Both pIIa and pIIb yielded nearly identical chain length distribution profiles as indicated in the negligible variation from the zero value in the difference plot of Figure 2.8. These data suggest that there are no inherent differences in the capacities of native BEIIa and native BEIIb to affect the structure of amylopectin. Thus, the hypothesis that conservation of BEIIa and BEIIb in monocots results from unique aspects of their enzymatic capabilities is not supported by these results. Other explanations should be sought. One possibility is that gene duplication and subsequent variation in tissue- or temporal-specific gene expression patterns of BEIIa and BEIIb provides some selective advantage. Another possibility that needs to be considered is how critical the total level of BEII activity is, and duplication of the two genes simply allows for greater total activity of BEII.

A potentially significant conclusion from the chain transfer preference data is that the branching properties of BEIIa may not be entirely inherent to the BEIIa polypeptide. The conclusion comes from the fact that the chain length transfer preference of pIIa, representing the native enzyme, is clearly different than that of rIIa, which represents the BEIIa polypeptide in the absence of any other maize protein. The key observations are that rIIa has a much higher preference for transferring shorter chain lengths of DP 6-9, in comparison to pIIa or pIIb (Figure 2.8). In contrast, the native enzymes both transfer chains of DP 19-25 at a higher frequency than does rIIa. A possible explanation for this result is that there are other

factors within the plant cell that affect the activity of the branching enzymes e.g., other proteins with which BEIIa and/or BEIIb may interact. Another possible explanation could be that branching enzymes are post-translationally modified *in vivo* which affects their activities. The existence of such protein-protein interactions and potential post-translational modifications of BEs is investigated in Chapter 3 of this dissertation.

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A.

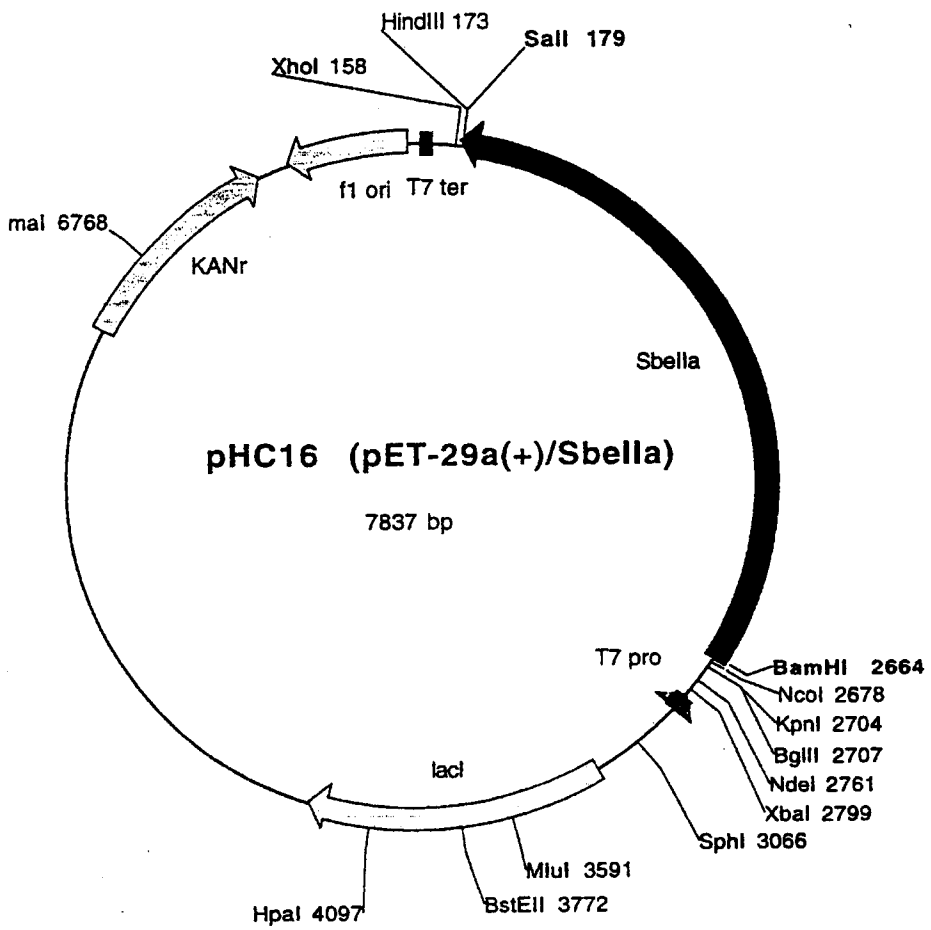
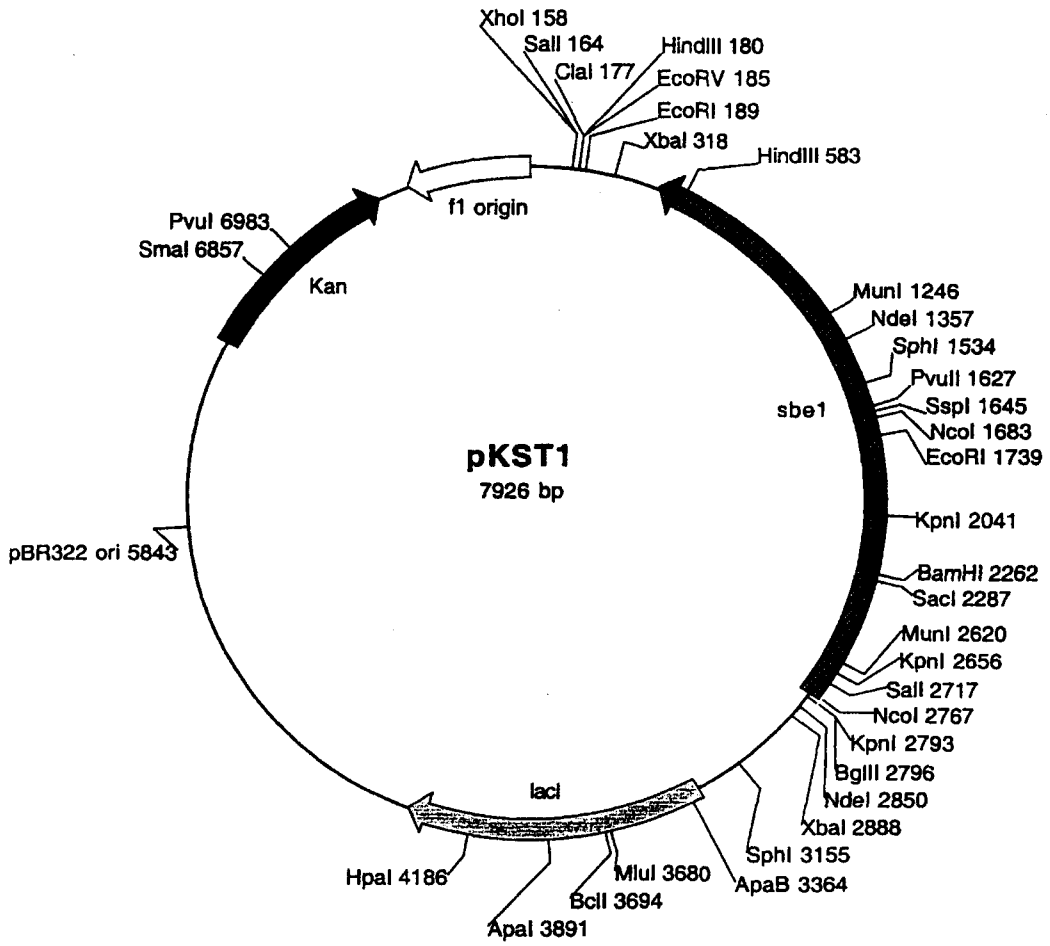


Figure 2.1. Plasmids for recombinant expression of maize BEs in *E. coli*. (A) Plasmid pHC16. The coding region of mature BEIIa (black bar) was cloned in expression vector pET-29a. The sequence of the BEIIa cDNA inserted into the vector is available as GenBank Accession No. U65948), and modifications made during cloning are described in Results. Expression of the recombinant gene is directed by the bacteriophage T7 promoter (labeled "T7 pro"), and transcription termination was controlled by the T7 terminator sequence ("T7 ter") present in the vector. This plasmid was constructed by Dr. H. Cao. (B) Plasmid pKST1. The full length coding sequence of the maize BEI cDNA (GenBank Accession No. U17897) was cloned in expression vector pET-29b. The T7 promoter and T7 terminator are present in the analogous locations to those shown in panel A for pHC16. This plasmid was constructed by Dr. S. Kim.

B.



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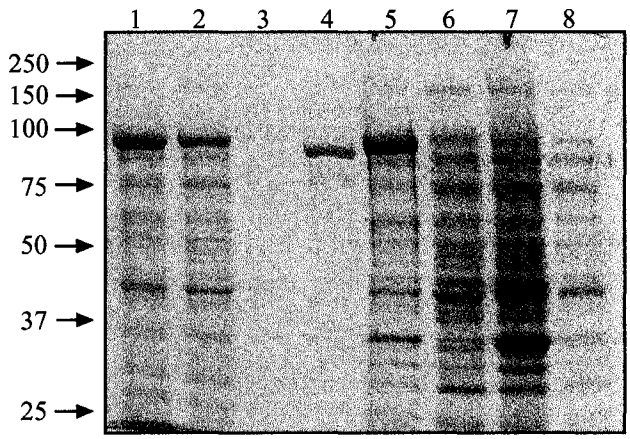
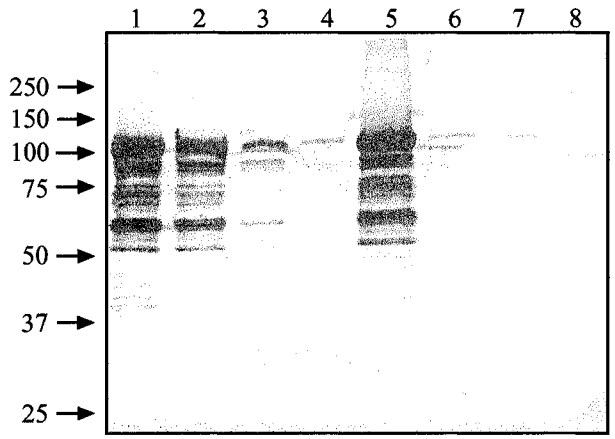


Coomassie blue stain



Protein blot probed with S-protein-AP conjugate

Figure 2.2. Purification of recombinant BEIIa. Proteins in the indicated fractions were separated by SDS-PAGE and then either stained with Coomassie blue or probed in a Western blot procedure with S-protein-alkaline phosphatase conjugate (1:5,000 dilution). Lane 1, total soluble *E. coli* extract from cells harboring plasmid pHC16 induced with IPTG. Lane 2, unbound protein, i.e., the supernatant after incubation with S-protein agarose and removal of the beads. Lane 3, wash of S-protein-agarose beads. Lane 4, final protein preparation obtained after cleavage of the S-tag sequence. Lane 5, insoluble pellet from *E. coli* cells harboring plasmid pHC16 induced with IPTG. Lane 6, total soluble *E. coli* extract from uninduced cells harboring plasmid pHC16. Lane 7, insoluble pellet from uninduced *E. coli* cells harboring plasmid pHC16. Lane 8, total soluble *E. coli* extract from untransformed cells subjected to a mock induction with IPTG.

```
                1                                                   50
maize_Sbe2a     dlpsvlfrrk dafsrtvlsc agapgkvlvp gggsddllss aepvvdtqpe
maize_Sbe2b     ---------- mafrvsgavl ggavraprlt gggegslvfr htglfltrga
 maize_Sbe1     ---------- ---------- ---------- ---------- ----------

               51                                                  100
maize_Sbe2a     elqipeaelt vektsssptq ttsavaeass .gveaeerpe lsevigvggt
maize_Sbe2b     rvgcsgthga mraaaaarka vmvpegendg lasradsaqf qsdelevpdi
 maize_Sbe1     -------mlc lvspsssptp lppprrsrsh adraappgia gggnvrlsvl

               101                                                 150
maize_Sbe2a     ggt.kidgag ik......ak aplveekprv ipppgdgqr. .iyeidpmle
maize_Sbe2b     see.ttcgag v.......ad aqal.nrvrv vpppsdgqk. .ifqidpmlq
 maize_Sbe1     svqckarrsg vrkvkskfat aatvqedktm atakgdvdhl piydldpkle

               151                                                 200
maize_Sbe2a     gfrghldyry seykrlraai dqheggldaf srgyeklgft rsaegityre
maize_Sbe2b     gykyhleyry slyrrirsdi deheggleaf srsyekfgfn rsaegityre
 maize_Sbe1     ifkdhfryrm krfleqkgsi eenegslesf skgylkfgin tnedgtvyre

               201                                                 250
maize_Sbe2a     wapgaysaal vgdfnnwnpn adamarneyg vweiflpnna dgspaiphgs
maize_Sbe2b     wapgafsaal vgdfnnwdpn adrmsknefg vweiflpnna dgtspiphgs
 maize_Sbe1     wapaaqeael igdfndwnga nhkmekdkfg vwsiki.dhv kgkpaiphns

               251                                                 300
maize_Sbe2a     rvkirmdtps gv.kdsipaw ikfsvqapge i..pyngiyy dppeeekyvf
maize_Sbe2b     rvkvrmdtps gi.kdsipaw ikysvqapge i..pydgiyy dppeevkyvf
 maize_Sbe1     kvkfrf.lhg gvwvdripal iryatvdask fgapydgvhw dppaserytf

               301                                                 350
maize_Sbe2a     khpqpkrpks lriyeshvgm sspepkinty anfrdevlpr ikklgynavq
maize_Sbe2b     rhaqpkrpks lriyethvgm sspepkinty vnfrdevlpr ikklgynavq
 maize_Sbe1     khprpskpaa priyeahvgm sgekpavsty refadnvlpr irannyntvq

               351                                                 400
maize_Sbe2a     imaiqehsyy asfgyhvtnf fapssrfgtp edlkslidka helgllvlmd
maize_Sbe2b     imaiqehsyy gsfgyhvtnf fapssrfgtp eelkslidra helgllvlmd
 maize_Sbe1     lmavmehsyy asfgyhvtnf favssrsgtp edlkylvdka hslglrvlmd

               401                                                 450
maize_Sbe2a     ivhshssnnt ldglngfd.. .gtdthyfhg gprghhwmwd srlfnygswe
maize_Sbe2b     vvhshassnt ldglngfd.. .gtdthyfhs gprghhwmwd srlfnygnwe
 maize_Sbe1     vvhshasnnv tdglngydvg qstqesyfha gdrgyhklwd srlfnyanwe
```

Figure 2.3. Amino acid sequence alignment of maize BEI, BEIIa, and BEIIb. The predicted amino acid sequence of each protein was obtained by translating the cDNA sequence. The relevant GenBank accession numbers are U17897 for BEI, U65948 for BEIIa, and AF072725 for BEIIb. The sequences were aligned using the program PILEUP from the GCG software package. The sequences in BEI that were synthesized as peptides for use as antigen are boxed.

```
            451                                                        500
maize_Sbe2a  vlrfllsnar wwleeykfdg frfdgvtsmm ythhglqvtf tgnygeyfgf
maize_Sbe2b  vlrfllsnar wwleeykfdg frfdgvtsmm ythhglqvtf tgnfneyfgf
 maize_Sbe1  vlrfllsnlr ywldefmfdg frfdgvtsml yhhhginvgf tgnyqeyfsl

            501                                                        550
maize_Sbe2a  atdvdavvyl mlvndlirgl ypeavsiged vsgmptfcip vqdggvgfdy
maize_Sbe2b  atdvdavvyl mlvndlihgl ypeavtiged vsgmptfalp vhdggvgfdy
 maize_Sbe1  dtavdavvym mlanhlmhkl lpeatvvaed vsgmpvlcrp vdeggvgfdy

            551                                                        600
maize_Sbe2a  rlhmavpdkw iellkqsdey .wemgdivht ltnrrwlekc vtyceshdqa
maize_Sbe2b  rmhmavadkw idllkqsdet .wkmgdivht ltnrrwlekc vtyaeshdqa
 maize_Sbe1  rlamaipdrw idylknkdds ewsmgeiaht ltnrrytekc iayaeshdqs

            601                                                        650
maize_Sbe2a  lvgdktiafw lmdkdmydfm aldrpstpri drgialhkmi rlvtmglgge
maize_Sbe2b  lvgdktiafw lmdkdmydfm aldrpstpti drgialhkmi rlitmglgge
 maize_Sbe1  ivgdktiafl lmdkemytgm sdlqpaspti drgialqkmi hfitmalggd

            651                                                        700
maize_Sbe2a  gylnfmgnef ghpewidfpr gpqslpngsv ipgnnnsfdk crrrfdlgda
maize_Sbe2b  gylnfmgnef ghpewidfpr gpqrlpsgkf ipgnnnsydk crrrfdlgda
 maize_Sbe1  gylnfmgnef ghpewidfpr .......... .egnnwsydk crrqwslvdt

            701                                                        750
maize_Sbe2a  dylryrgmqe fdqamqhleg kyefmtsdhs yvsrkheedk viifergdlv
maize_Sbe2b  dylryhgmqe fdqamqhleq kyefmtsdhq yisrkheedk vivfekgdlv
 maize_Sbe1  dhlrykymna fdqamnalde rfsflssskq ivsdmndeek vivfergdlv

            751                                                        800
maize_Sbe2a  fvfnfhwsns yfdyrvgcfk pgkykivlds ddglfggfsr ldhdaeyfta
maize_Sbe2b  fvfnfhcnns yfdyrigcrk pgvykvvlds daglfggfsr ihhaaehfta
 maize_Sbe1  fvfnfhpkkt yegykvgcdl pgkyrvalds dalvfgghgr vghdvdhfts

            801                                                        850
maize_Sbe2a  ........dw phdnrpcsfs vyapsrtavv yapagaede- ----------
maize_Sbe2b  ........dc shdnrpysfs vytpsrtcvv yapve----- ----------
 maize_Sbe1  pegvpgvpet nfnnrpnsfk vlspprtcva yyrvdeagag rrlhakaetg

            851                                                   893
maize_Sbe2a  ---------- ---------- ---------- ---------- ---
maize_Sbe2b  ---------- ---------- ---------- ---------- ---
 maize_Sbe1  ktspaesidv kasrassked keataggkkg wkfargpsdq dtk
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Figure 2.3. (continued).

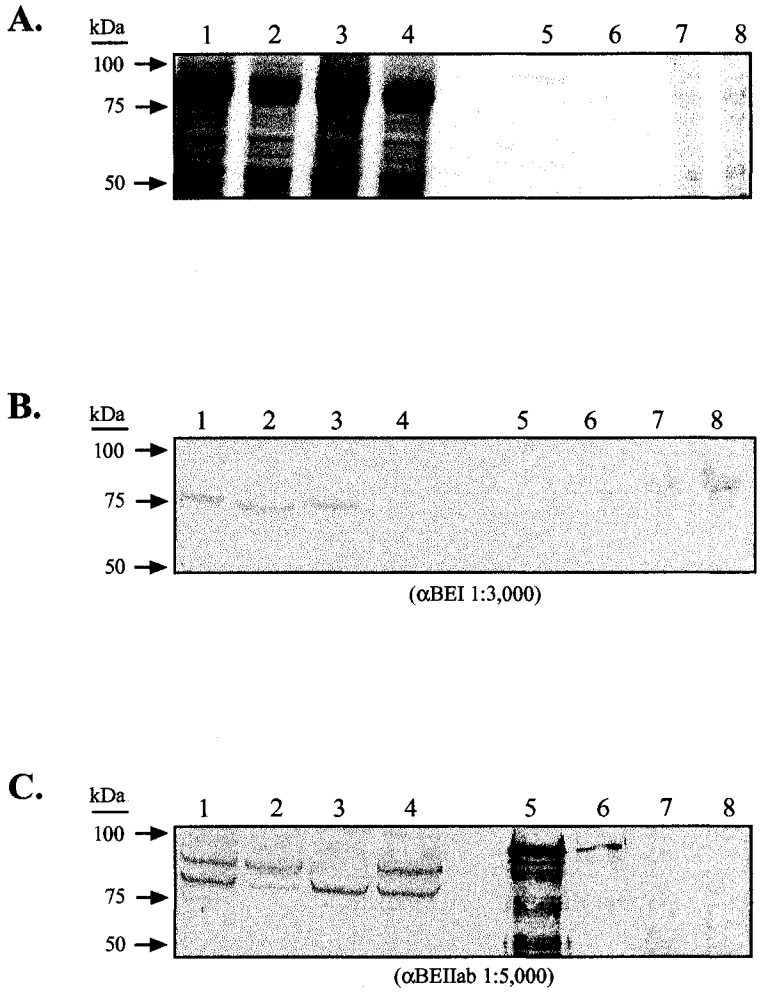


Figure 2.4. Antibody specificity tests. Total soluble protein extracts from wild type maize, and mutant maize lines deficient in either BEI, BEIIa, or BEIIb, as well as *E. coli* extracts from cells expression either BEIIa or BEI, were probed by immunoblot using either αBEI or αBEIIab antiserum. (A) Coomassie blue stained gel. (B) Immunoblot probed with αBEI. (C) Immunoblot probed with αBEIIab. Lanes designations are the same in all three panels. Lane 1, wild type maize; Lane 2, *ae*$^-$ mutant maize, deficient in BEIIb; Lane 3, *sbe2a::Mu* mutant maize, deficient in BEIIa; Lane 4, *sbe1::Mu* mutant maize, deficient in BEI; Lane 5, Soluble *E. coli* extract containing recombinant BEIIa; Lane 6, Soluble *E. coli* extract from the same strain as lane 5, but not subjected to induction; Lane 7, Insoluble *E. coli* fraction containing recombinant BEI dissolved in 8M Urea; Lane 8, Insoluble *E. coli* extract from the same strain as lane 7, dissolved in 8M Urea but not subjected to induction.

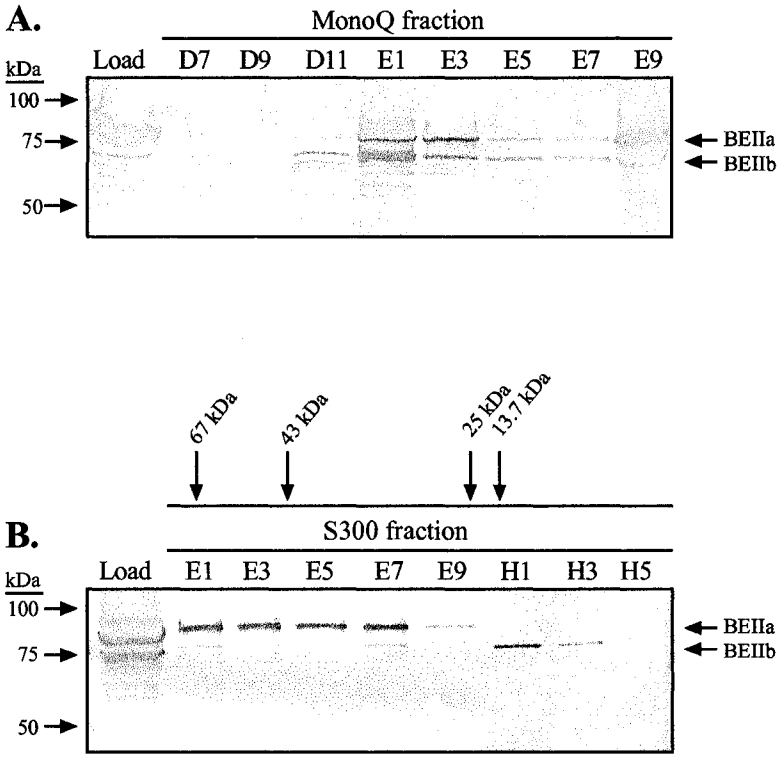
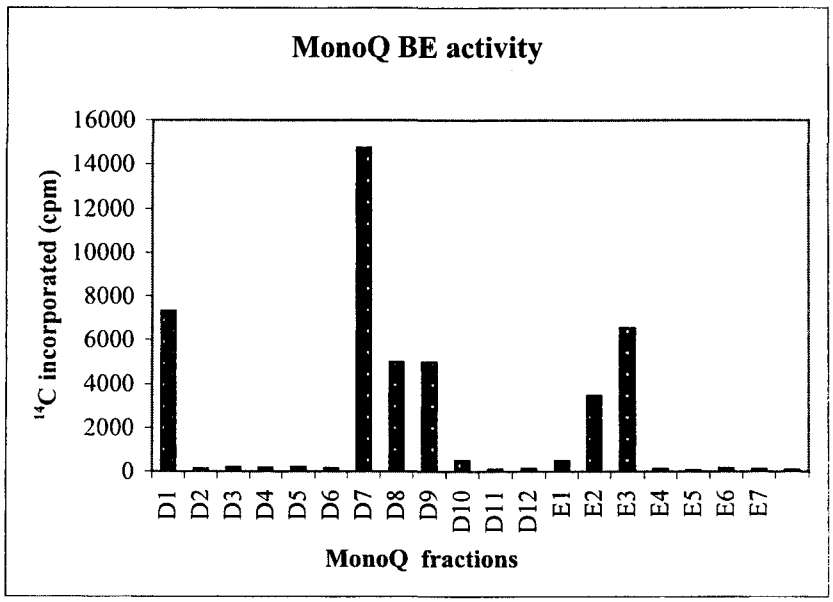


Figure 2.5. Purification of BEIIa and BEIIb from maize endosperm. (A) Anion exchange separation. Proteins present in the 40% ammonium sulfate pellet from a total soluble kernel extract were separated by chromatography on a MonoQ column. Gradient fractions of increasing salt concentration were probed in immunoblot analysis with αBEIIab antibody at a dilution of 1:5,000. Load indicates the ammonium sulfate pellet fraction applied to the column. The positions of molecular weight standards migrating in the same gel are indicated. (B) Size exclusion separation. Pooled MonoQ fractions containing the greatest amount of BEIIa and BEIIb, and the least amount of contaminating proteins, were applied to a Sephacryl S300 column. Eluted fractions increasing in elution volumne were probed with αBEIIab antibody as in panel A. Load indicates the pooled MonoQ fractions applied to the column. The elution points of molecular weight standards chromatographed under identical conditions are indicated. Standards were as follows: thyrogobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsin, 25 kDa; ribonuclease A, 13.7 kDa.

A.



B.

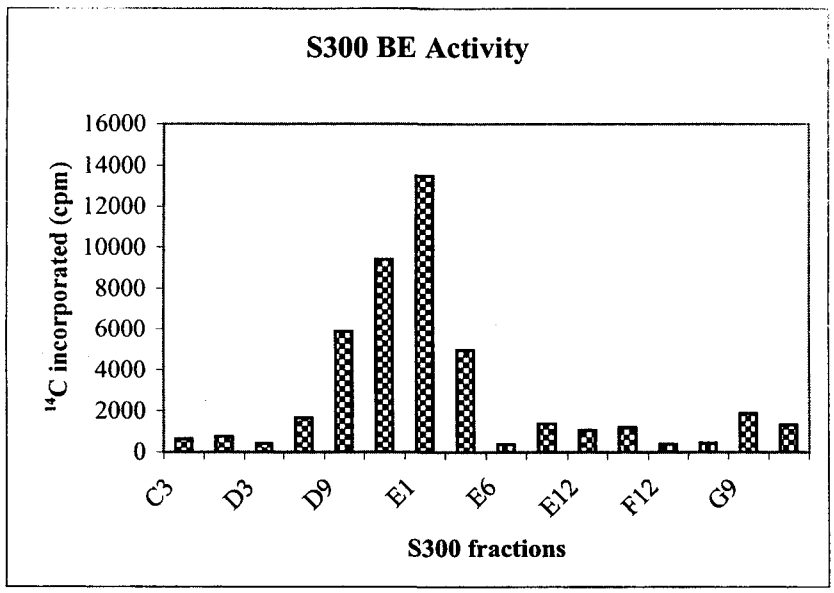


Figure 2.6. Total BE activity assays. (A) Fractions from MonoQ chromatography were analyzed for total BE activity using the phosphorylase *a* stimulation assay. (B) Total BE activity present in Sephacryl S300 fractions.

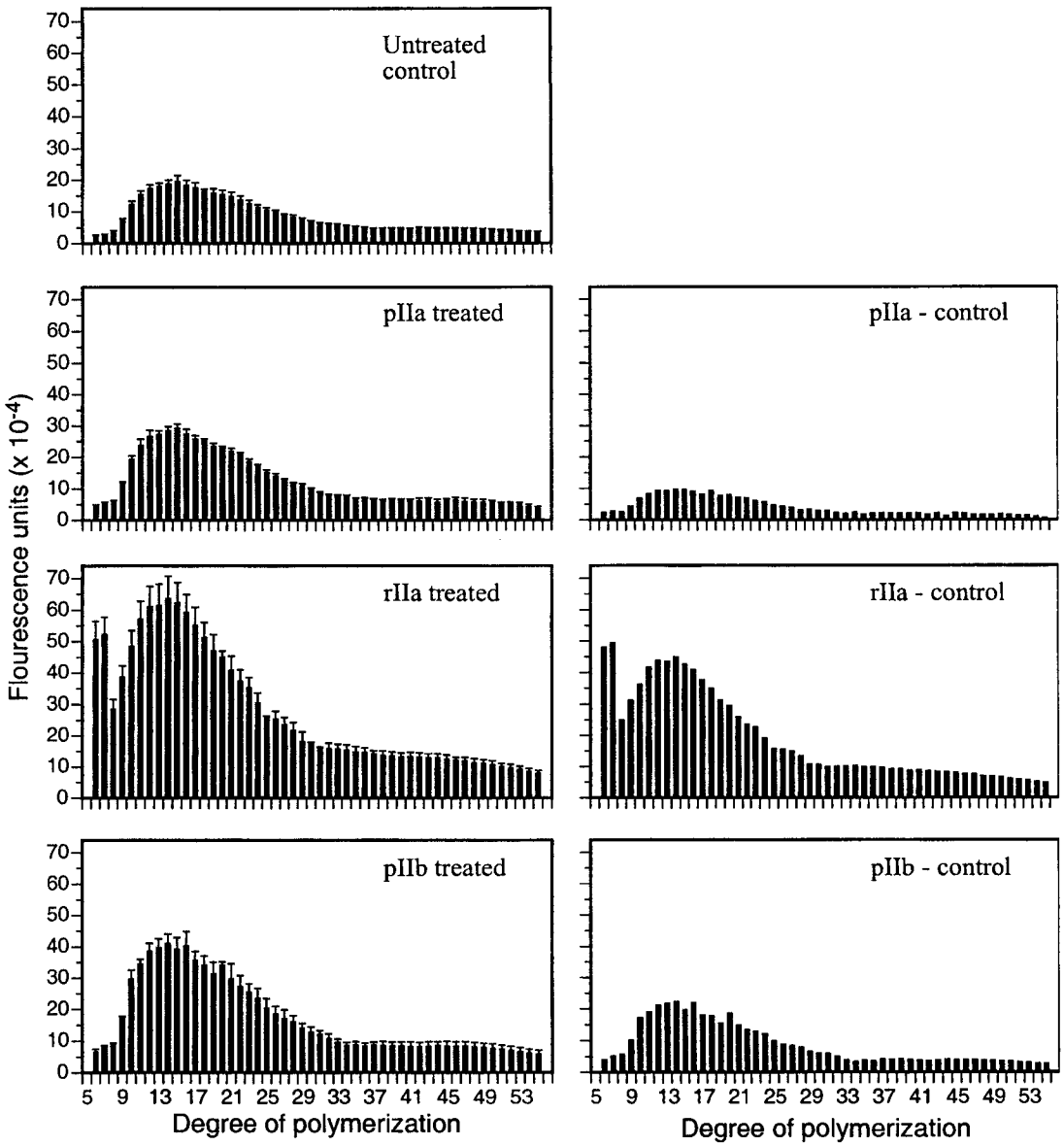


Figure 2.7. Chain length distribution of the products of BE activity. Each BE enzyme source was incubated in the presence of commercial amylose. After treatment the product polymer was debranched and the number of linear chains of each indicated DP in a specific reaction volume was determined by FACE analysis. The number of chains present in untreated amylose was determined (uppermost panel), and for each DP this values was subtracted from the number of chains observed in treated samples. Each sample was analyzed in triplicate from separate reactions, and standard errors are indicated. All plots are drawn to the same scale.

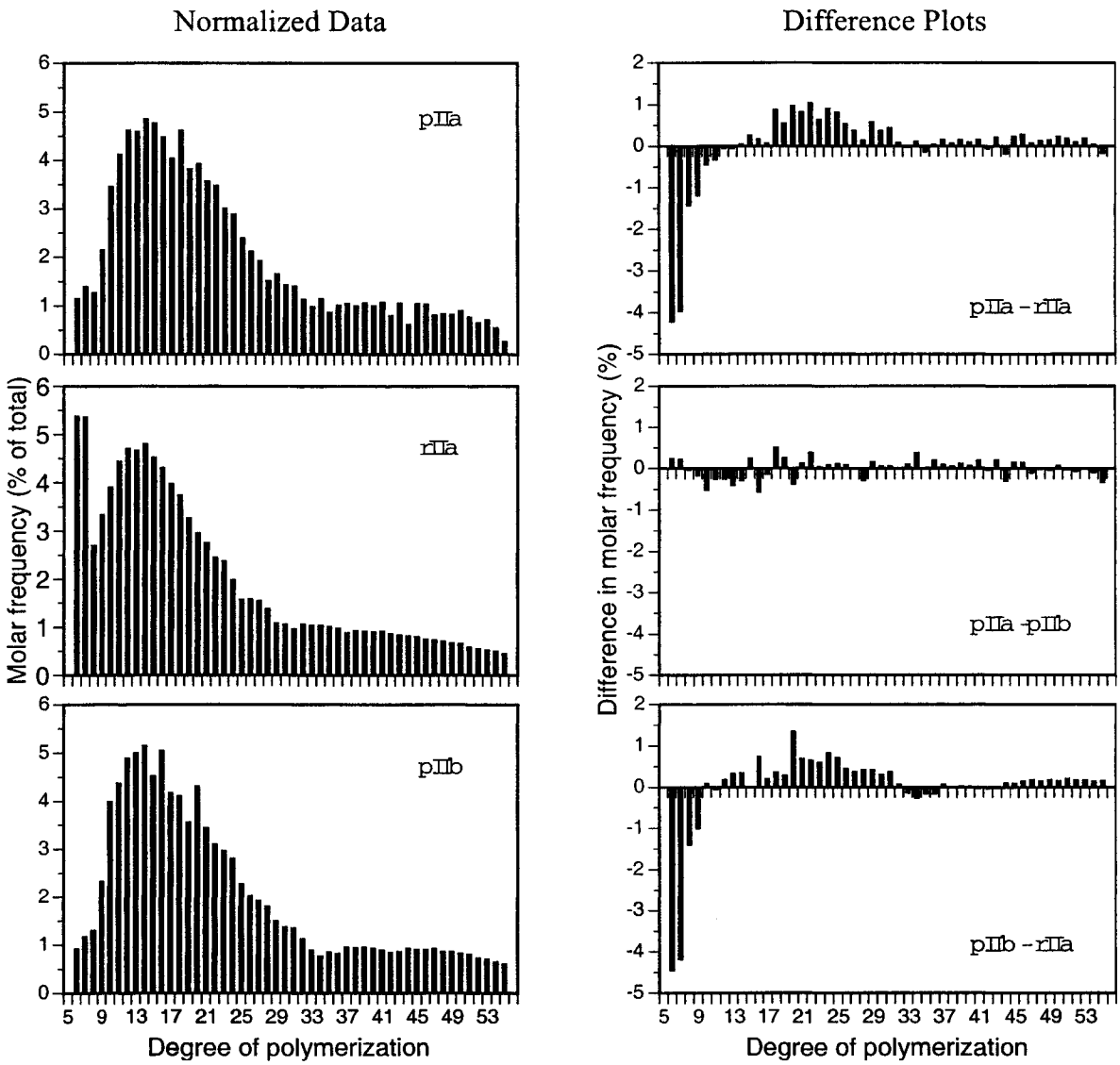


Figure 2.8. Normalized chain length distribution of the products of BE activity, and difference plots. The data of Figure 2.7, after subtraction of the values for untreated amylose, were normalized to the total number of chains present. The normalized data was compared between samples by subtracting the value for each DP in one of the samples from the corresponding value in the other.

CHAPTER 3: GENETIC AND BIOCHEMICAL EVIDENCE FOR INVOLVEMENT OF MAIZE BRANCHING ENZYMES IN MULTI-SUBUNIT COMPLEXES

ABSTRACT

There is substantial indirect genetic evidence suggesting that coordination of multiple biosynthetic enzyme activities in multi-subunit complexes (MSCs) produces the final starch structure. There is little direct evidence, however, indicating protein-protein interactions among these enzymes, particularly BEs and SSs. This study took several approaches to obtain new evidence, both direct and indirect in nature, for involvement of all of the maize BEs in MSCs. CoIP results provide strong direct evidence that SSI and BEIIa are involved with each other in one or more MSCs. The coIP analysis suggested further that SSIII may also be a component of one of these complexes. GPC analysis revealed a major change in the BEIIb elution profile when the enzyme was obtained by a gentle procedure including plastid isolation, as compared to harsh grinding of whole tissue, which could be explained by its involvement in MSCs *in vivo*. BEI was proven to exist in multiple electrophoretic mobility forms, again consistent with MSCs. The multiple forms of BEI were not affected by dephosphorylation. Finally, pleiotropic effects on SS activities were observed in branching enzyme mutants, revealing that three bands of SS activity in zymograms were affected when BEIIa was mutated. These data demonstrate that BEIIa participates in at least one MSC also containing SSI, and implicates BEI and BEIIb as participants in such complexes.

INTRODUCTION

Chapter 2 of this dissertation introduced the concept that multiple isoforms of branching enzymes might each impart specific functions in starch biosynthesis, in particular with regard to determination of starch architecture. In that study, the focus was on the individual enzymatic properties of specific branching enzyme isoforms. In this chapter, the dissertation investigates the hypothesis that distinct branching enzyme isoforms participate in various multi-subunit complexes. This hypothesis suggests that spatial coordination between the site of action of a starch synthase and a branching enzyme, accomplished by the quaternary structure of a multi-component complex, may be a determinant of chain length distribution and/or branch linkage placement.

Pleiotropic effects offer indirect evidence supporting the idea that branching enzymes participate in multi-subunit complexes. One example involves mutation of the maize gene *dull1*, which is known to code for the starch synthase isoform SSIII (Gao et al. 1998). When proteins isolated in plants carrying the *dull1* mutation were fractionated, the SSIII activity peak was missing, as expected (Boyer and Preiss 1978b; Boyer et al. 1981). Also seen in *dull1* mutants, however, is the decrease in activity of the BEII-containing peak. The reason the SSIII mutation affects BEII activity, both BEIIa and BEIIb, is not known, but one explanation might be SSIII and BEIIa and BEIIb area binding partners, and SSIII may be required for BEIIa and BEIIb activity.

Another example of pleiotropic effects involving mutations in starch biosynthetic enzymes is the *amylose-extender* (*ae*) mutation. The *ae* gene codes for BEIIb (Fisher et al. 1993; Stinard et al. 1993). Zymogram analysis of the proteins from the maize *ae* mutant reveals the absence of BEIIb activity, as expected, and also indirect effects on BEI activity.

More specifically, two of the four prominent electrophoretic mobility forms presumed to be BEI are absent in the *ae*⁻ mutant strains lacking BEIIb (Colleoni et al. 2003). Furthermore, an isoamylase-type debranching enzymes also has altered properties as an indirect effect of the mutation of BEIIb (Colleoni et al. 2003). Pleiotropic effects are also seen in the rice *ae* mutant. There is a 50% reduction observed in soluble SSI activity, in addition to the loss of BEIIb activity, suggesting BEIIb and SSI proteins possibly interact (Nishi et al. 2001).

Another example in maize involves mutations affecting both a pullulanase-type DBE (*zpu1-204*) and an isoamylase-type DBE (*su1-st*), both of which cause a loss of BEIIa activity. In these instances, normal levels of the BEIIa polypeptide accumulated, indicating a post-translational effect (James et al. 1995; Dinges et al. 2001; Dinges et al. 2003).

Two independent lines of indirect evidence support the existence of functional interactions between starch biosynthetic enzymes. First, the addition of purified BEI or II to maize kernel extracts stimulated SSI activity (Boyer and Preiss 1979). Second, another approach suggestive of individual branching enzyme isoforms interacting directly with other starch biosynthetic enzymes comes from a study of heterologous expression of maize enzymes in yeast (Seo et al. 2002). BEI had no effect on glucan structure without BEIIa and BEIIb present. One explanation is that BEI requires both BEII isoforms and interacts with them to function in starch biosynthesis. Also using the heterologous yeast system a functional interaction was observed between SSIII, specifically the amino terminal region of this protein that is not involved in the enzymatic activity, and BEIIa (Kim et al. 2005).

Recent biochemical analyses have provided evidence for the association of branching enzymes with other starch biosynthetic enzymes in stable complexes in wheat (Tetlow et al. 2004a). The activity of the starch biosynthetic enzymes was regulated by protein

phosphorylation, presumably by a protein kinase in the plastid. Also noted was that protein phosphorylation in the plastid stimulated starch biosynthesis. Co-immunoprecipitation experiments demonstrated directly that endosperm BEI, BEIIb, and phosphorylase interact forming a complex, while BEIIa is in its own complex. Furthermore, this phosphorylation was essential for the formation of the complexes, because treatment with a general protein phosphatase destabilized the complex (Tetlow et al. 2004a). In summary, it appears that protein phosphorylation regulates complex assembly and starch biosynthesis.

The research presented in this chapter investigated specific binding peptide partners for the branching enzymes in maize, testing the overall hypothesis that physical interactions between components of the starch assembly pathway influence their enzymatic activities and properties. Using the BEII-isoform specific antibody, co-immunoprecipitation experiments determined if BEIIa and/or BEIIb interact directly with other starch biosynthetic enzymes. The multi-subunit complex theory was tested using size-exclusion chromatography, and potential pleiotropic affects on starch synthases in branching enzyme mutants were analyzed using in-gel activity assays. Positive results were obtained in the co-immunoprecipitation experiments, which demonstrated one or more stable complex(es) exist, containing some combination(s) of BEIIa, SSI and SSIII.

MATERIALS AND METHODS

In-gel enzyme activity assays

The methods used for zymogram analyses to identify starch-metabolizing activities in native polyacrylamide gels impregnated with commercial potato starch were described in Chapter 2. Briefly, total soluble extracts from kernels harvested 20 DAP were separated by

native PAGE, electroblotted to a starch-containing gel, incubated so as to allow modification of the starch by any enzymes that might be present, then stained with iodine solution.

SS activities were also analyzed by an in-gel activity assay. The protocols followed were previously described by Zhang et al. (2004), with minor modifications. Crude soluble extract from 20 DAP kernels of wild type maize plants and various mutant plants lacking specific BEs was prepared as described in Chapter 2. The supernatant was passed through a 0.22 μm nitrocellulose syringe filter, and proteins were separated by anion exchange chromatography on a MonoQ HR 5/50 column, as described in Chapter 2. Proteins eluted in the gradient fractions were separated by non-denaturing PAGE (6% polyacrylamide, 375 mM Tris-HCl, pH 8.8) containing 0.3% rabbit glycogen (Sigma-Aldrich, catalog no. G-8876). Following electrophoresis gels were incubated at 30°C overnight in incubation buffer designed to allow SS activity (0.1 M bicine, pH 8.5, 5 mM ADP-glucose, 0.5 M sodium citrate, 0.5 mg/mL BSA, 25 mM potassium acetate 133 mM $(NH_4)_2SO_4$, 7 mM $MgCl_2$, 10 mg/mL rabbit liver glycogen, 25 mM β-mercaptoethanol). After incubation, gels were equilibrated in water and starch synthase activities were observed by staining the gels with fresh I_2/KI solution.

Immunoblot analysis

Proteins separated by non-denaturing PAGE were analyzed by immunoblot analysis using αBEI and αBEIIab antisera. Electrophoresis conditions were those used for starch zymogram analysis (Chapter 2). After the electrophoretic separation, the gels were incubated in 1X Western transfer buffer (25 mM Tris, 192 mM glycine, pH 7.5, 20% methanol) for 30 min, and then transferred to a nitrocellulose membrane under electroblot conditions as

described in Chapter 2. The nitrocellulose membrane was probed according to immunoblot protocols using the antisera αBEI (rabbit # 3672) or αBEIIab (rabbit #3434), as described in Chapter 2. The same αBEI and αBEIIab antisera were used to analyze immunoprecipiated proteins separated by SDS-PAGE, along with antisera against SSI (Drs. H. Guan and P. Keeling, BASF Plant Sciences, personal communication) and SSIII (Cao et al. 1999)

Alkaline phosphatase treatment

The protocol of Tetlow et al., (2004a) was followed. Commercial calf intestinal alkaline phosphatase (1 U/μL, Promega, Madison, WI, catalog no. M1821] was used to treat partially purified maize extracts. The 40% ammonium sulfate pellet suspended and desalted in MonoQ buffer A (Chapter 2) was used as the protein sample. In a 60 μL total reaction volume, 46 μL of protein solution (approximately 5 μg/μL) was incubated with 14 μL of the alkaline phosphatase. In the control reaction without alkaline phosphatase 14 μL of the enzyme storage buffer specified by the supplier was added. The reactions were incubated at room temperature for 1 h. From the 60 μL sample, 30 μL was used for zymogram analysis, 20 μL for immunoblot analysis, and 5 μL for measurement of total BE activity by the phosphorylase *a* stimulation assay.

Plastid isolation

Amyloplasts were partially purified from developing maize kernels harvested 17-18 DAP, following a previously published protocol (Tetlow et al. 2004a). Endosperm tissue picked from developing kernels and immersed in ice cold amyloplast extraction buffer (AEB) (50 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 1 mM KCl, 2 mM $MgCl_2$ and 0.5 M

sorbitol, 10 μL/mL of protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, catalog no. P-9599]) in a glass petri dish (approximately 20 mL AEB for 500 kernels). Keeping the solutions on ice, endosperm tissue was finely chopped with a razor blade, and the mixture was then filtered through eight layers of Micracloth. The filtrate was collected in a centrifuge tube, and an equal volume of AEB containing 3% Histodenz (Sigma-Aldrich, catalog no. D2158-1006) was layered underneath the endosperm extract. The solution was centrifuged at 100*g* at 4°C for 5 min. The pellets containing the amyloplasts were suspended in a small volume of HB homogenization buffer (Chapter 2), approximately 1-1.5 mL. Intact amyloplasts were lysed by three freeze-thaw cycles in liquid nitrogen. Starch granules were removed by centrifugation at 14,000*g* for 5 min at 4°C, and the supernatants were collected. These samples, defined as amyloplast extracts, were stored in 500 μL aliquots at -80°C.

Gel permeation chromatography

For analysis of total endosperm extracts, kernels were collected 20 DAP from wild type maize inbred W64A and stored at -80°C. Total soluble kernel extracts in HB buffer were prepared after grinding the tissue in liquid nitrogen, as described in Chapter 2. The lysates were filtered through a 0.22 μm syringe filter, then concentrated five-fold by centrifugal filtration. This fraction (2 mL, approximately 5 mg/mL) was applied to a Sephacryl S300 column (Amersham Biosciences, Inc.) following conditions described in Chapter 2. Fractions (1.4 mL) were collected and characterized for the presence of BEI and/or BEIIa/BEIIb by immunoblot analysis (Chapter 2). For analysis of plastid extracts, the amyloplast lysate fractions described in the preceding section were loaded directly onto the Sephacryl S300 column (1 mL, approximately 1 mg/mL). Molecular weight standards

chromatographed on the same column were obtained from Amersham Biosciences, Inc. (catalog nos. 17-042-01 and 17-0441-01).

Co-immunoprecipitation

Protocols followed were adapted from Current Protocols in Protein Science (Bonifacio et al. 1997) with modifications. Protein-A Sepharose CL-4B beads (Sigma-Aldrich, St. Louis, MO, catalog no. P-3391) were swelled and then equilibrated in HB homogenization buffer (Chapter 2) according to manufacturer's instructions. The bead slurry was distributed into microfuge tubes in 100 µL aliquots. Antiserum (5 µL) was added to one aliquot of the beads and incubated on ice for one hour. The bead-antibody complexes were washed three times with HB buffer to remove unbound serum proteins. Crude soluble kernel extract (100 - 200 µL containing approximately 500 µg protein), prepared as described in Chapter 2, was added to the antibody-bead slurry, mixed and incubated on ice for one hour. The protein-antibody-bead mixture was centrifuged for 5 min at 10,000 *x* g in a microfuge to remove unbound proteins, and the supernatant (fraction S) was saved for further analyses. The protein-antibody-bead mix was washed six times with HB buffer (1 min at 10,000 *x* g) to remove residual unbound proteins, and the final wash (fraction W) was saved for analysis. Bound proteins and antibody were released from the Protein A-Sepharose by incubation in low pH elution buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 3.0) followed by centrifugation in a microfuge (1 min at 10,000 *x* g). The supernatant (fraction P) was saved for further analysis. Collected fractions (S, W, and P) and the starting crude soluble extract were then analyzed by immunoblot analysis.

RESULTS

Identification of multiple electrophoretic forms of BE that contain the BEI protein

To test the hypothesis that physical interactions exist between branching enzymes and other components of the starch biosynthetic pathway, specific binding partners of maize branching enzymes were investigated. As a first step in characterizing the native state of BEI, zymogram analysis was coupled with immunoblot detection to learn whether a series of four migratory forms of BE activity all posses the same polypeptide as the catalytic factor.

Zymogram analysis of proteins isolated from developing endosperm of wild type maize plants revealed a series of four BE bands predicted to be forms of BEI (Colleoni et al. 2003) (Figure 3.1A). Identification of these four bands as putative BEI bands was based on observations that they co-fractionate in anion exchange separation, and that all four are missing in the *sbe1*$^{-}$ mutant (Colleoni et al. 2003). However, to date there is no direct evidence for co-migration of the BEI polypeptide with these activity bands, and so the possibility has not been ruled out that some other catalytic subunit is providing enzyme activity. The availability of αBEI antiserum (Chapter 2) allowed direct analysis of this question.

Whole kernel proteins were separated by anion exchange chromatography as described in Chapter 2. MonoQ fractions of wild type whole kernel extracts known to contain large quantities of the BEI protein were loaded onto a native PAGE gel in duplicate. One set of the analyzed fractions was transferred to another native polyacrylamide gel containing 0.3% starch, for the zymogram analysis. The other set of lanes was equilibrated in Western transfer buffer and transferred to a nitrocellulose membrane by standard immunoblot procedure. After incubation, the starch gel was stained with iodine, thus visualizing the

starch metabolic enzyme activities (Figure 3.1A). In such analyses, BE activity shows up as a pink or red band, owing to the nature of the glucan-iodine complex that forms after modification of the starting starch substrate by the BE. The nitrocellulose membrane was probed with either αBEI or αBEIIab antibodies. From these parallel analyses, the presence of any of the known BE polypeptides in each specific BE activity band could be determined.

The zymogram analysis of total crude extract showed, as expected, the two bands previously identified as BEIIa and BEIIb (Colleoni et al. 2003), and, in addition, a series of red staining bands of lower mobility (Figure 3.1A, lane CE). The series of red bands co-fractionated on the MonoQ column, and separated to a large extent from the BEIIa and BEIIb activities. The five red bands are most clearly evident in gradient fraction D7 (Figure 3.1A), which also appears to contain a trace amount of BEIIa activity beginning to elute in this portion of the gradient. The immunoblot analysis also showed multiple mobility forms of the BEI protein, which corresponded well with the position of the BE activity bands that stained red with iodine. Probing the same protein fractions with αBEIIab in a parallel immunoblot indicated clearly that there was no BEIIa or BEIIb protein co-migrating with any of the red bands (data not shown). The conclusion from this experiment is that multiple BE activities exist, as defined by the fact that each one migrates reproducibly at a different rate in the non-denaturing gel, and that all of these different forms contain BEI as the catalytic subunit.

Effect of protein phosphorylation on BE activities

Tetlow et al., (2004a) demonstrated that the activities of the BE isoforms were regulated by protein phosphorylation, and also that the phosphorylation state of the proteins regulated the interactions of branching enzymes with other proteins in multi-subunit

complexes. Thus, the possibility that differential protein phosphorylation was the cause of the various mobilities of the BEI enzyme forms was tested (Figure 3.2A). The 40% ammonium sulfate fraction from wild type kernel extracts (methods as in Chapter 2) was treated with alkaline phosphatase, and then the presence or absence of the BEI bands was determined by the zymogram method. In this experiment, all five BEI bands were not as evident as was seen in the anion exchange fractions, however, multiple red bands could still be distinguished even after phosphatase treatment. There was no evident shift in the mobility of any band. Although this experiment may not be definitive, because the efficiency of the alkaline phosphatase enzyme cannot be known, the results suggest that differential protein phosphorylation is not the sole explanation for multiple mobility forms of BEI.

The possible effects of phosphorylation and dephosphorylation on the total BE activity present in soluble kernel extracts were also examined. Alkaline phosphatase treatment was again applied to test the effect of dephosphorylation on branching enzyme activity. From whole kernel extracts, branching enzymes were ammonium sulfate precipitated (40%). The ammonium sulfate pellet was suspended in MonoQ A buffer. The protein extracts were then incubated at room temperature in the presence or absence of a general alkaline phosphatase. Branching enzyme activities pre- and post- alkaline phosphatase treatment were analyzed using the phosphorylase a stimulation assay (Chapter 2). The results indicated that total BE activity measured by this assay was decreased by approximately 50% upon dephosphorylation of proteins in the crude extract (Table 3.1).

Table 3.1. Effects of alkaline phosphatase treatment on total BE activity

Assay no.	Counts per minute[a]		% Difference
	+AP Treatment	-AP Treatment	
1	16293	45110	64
2	28736	50573	43
3	34545	49019	30
4	33343	50410	34
5	10198	32683	69

[a] Equivalent amounts of protein were assayed in both the presence and absence of alkaline phosphatase (AP) treatment

The phosphatase treatment experiments also allowed determination of whether or not there was a change in the electrophoretic mobility of either of the BEII isoforms. The zymogram analysis in Figure 3.2 shows the BEIIa and BEIIb band from extracts treated with protein phosphatase, or in untreated controls. No differences were observed in the intensity or mobility of either the BEIIa or BEIIb band.

Size determination of branching enzyme proteins

Gel permeation chromatography (GPC) was used as another method to test whether maize branching enzymes exist as part of multiple subunit complexes. This technique was used to determine if branching enzymes elute from the column at a volume corresponding to a native molecular weight above, at, or below the predicted molecular weights for the monomeric protein. Proteins were separated based on size with the largest protein(s) eluting early, and smaller proteins eluting later. The methods used for this gel permeation chromatography approach are described in Chapter 2. The S300 column fractions were probed with the isoform-specific branching enzyme antibodies, αBEI and αBEIIab.

An important aspect of this analysis was to separate proteins from both whole kernel and plastidial fractions. The idea behind this approach was to try to be as gentle as possible prior to applying the proteins to the column, so that any complexes that exist *in vivo* could hopefully be maintained during the separation procedure. This was attempted by keeping the plastids intact as long as possible, and then rupturing them by a gentle lysis procedure. Also, in contrast to the analyses shown in Chapter 2, here the total soluble extracts were applied directly to the Sephacryl S300 column, without subjecting them to either ammonium sulfate precipitation or anion exchange chromatography.

In the separation of whole kernel proteins, BEIIa was completely separated from BEIIb (Figure 3.3A). BEIIa elutes in a peak that is centered between the 67 kDa and 43 kDa molecular weight markers, thus exhibiting an elution time slightly longer than expected based on the predicted molecular weight of approximately 89 kDa. BEIIb, in contrast, eluted at the size of a very small molecule, after the 13.7 kDa molecular marker, which is less than its predicted molecular weight of approximately 85 kDa (Figure 3.3A). This unexpected separation of BEIIa and BEIIb was utilized in the purification of BEIIa from BEIIb and was discussed previously in Chapter 2 of this dissertation.

From the analysis of whole kernel extracts there was no evidence of participation of BEIIa or BEIIb in multi-subunit complexes, however, analysis of purified plastid extracts showed different results. There was a shift in the elution time of both BEIIa and BEIIb in the separation of plastid proteins in comparison to whole kernel proteins. BEIIa again eluted in a broad peak, however, a significant portion of the protein was found earlier in the gradient as compared to the whole kernels extracts, extending to a position near that of 158 kDa marker.

BEIIb from plastids also eluted earlier than it did from whole kernel extracts, near BEIIa and closer to its predicted molecular weight (Figure 3.3B).

BEI was analyzed similarly. In the separation of whole kernel proteins, BEI elutes in a peak centered between the 158 kDa and 67 kDa molecular markers, thus indicating a molecular weight roughly matching the predicted monomer value of approximately 80 kDa (Figure 3.4A). There was no observed difference between the elution peak of BEI from plastid extracts and that from whole kernel extracts (Figure 3.4B). Thus, in contrast to BEIIa and BEIIb, no differences in the native state of the enzyme could be detected for BEI by this method of analysis. The fact that both BEIIa and BEIIb exhibited different elution patterns in the GPC analysis when they were fractionated from plastids as compared to whole kernels, with increases in the apparent native molecular weight in the plastids, might be explained by binding to other proteins in the native configuration.

Identification of BE binding partners by co-immunoprecipitation

Co-immunoprecipitation (coIP) was used as an approach to analyze potential binding partners of maize BEs. The isolation of proteins and the coIP protocols used are described in Materials and Methods. Briefly, IgG antibodies in a particular serum were bound to Protein A-Sepharose beads, residual unbound antibody was rinsed from the beads, and total soluble kernel extract from wild type plants was incubated with the bead-antibody slurry. The beads with bound material were washed extensively. The pH was then lowered in order to release the antibody and bound proteins from the protein-A attached to the Sepharose beads. The presence of specific proteins in the immunoaffinity-purified fraction was then tested by immunoblot analysis using antibodies against BEI, BEIIa and BEIIb, or SSI. The objective

was to identify purified proteins other than those that react directly with the antibody coupled to the Protein A-Sepharose. Such identification would raise the possibility that the protein directly bound to the antibody is a member of a multi-subunit complex, which also contains the second protein. The fractions from these analyses are denoted as follows: S, unbound proteins present in the supernatant after incubation with beads and their subsequent removal by centrifugation; W, sixth wash of the beads; P, purified fraction obtained after eluting antibody and bound proteins from the beads.

As negative controls, pre-immune sera were used to verify that co-precipitation of any particular protein required the presence of a specific binding partner on the beads. Another verification of specificity of protein-protein interactions came from reciprocal co-precipitation experiments. Thus, if antibody to a protein termed X co-precipitates protein Y, and antibody to protein Y co-precipitates protein X, and pre-immune serum fails to precipitate either protein X or protein Y, then the interaction between these two proteins can be taken as specific and significant.

The results showed that, as expected, Protein-A Sepharose beads pre-incubated with αBEIIab serum were able to bind BEIIa from whole kernel extracts (independent analyses are shown in Figures 3.5A, D, E). Little if any BEIIb was found in these same eluted fractions (Figures 3.5A, D, E). Thus, the αBEIIab serum appears to be far more effective at immunoprecipitating BEIIa than BEIIb, even though in immunoblot analysis the two BEII isoforms are both recognized well by the IgG population in this serum. Probing the eluted fraction P with αSSI antiserum revealed an immunoblot signal of the size expected for the SSI protein (independent analyses are shown in Figures 3.5B, C). Neither BEIIa, BEIIb, nor

SSI eluted from the Protein A-Sepharose beads pre-incubated with the pre-immune serum corresponding to the αBEIIab serum (referred to as preBEIIab) (Figure 3.5A, B).

Reciprocal immunoaffinity purifications were then performed. When antibody against SSI was bound to the Protein A-Sepharose beads, SSI was found in fraction P as expected (Figures 3.5C, F). Probing the fraction P eluate from the αSSI Protein A-Sepharose beads with αBEIIab serum revealed a signal of the expected molecular weight for BEIIa and also a weaker signal that corresponded to the expected mobility of BEIIb (Figure 3.5D). Pre-immune serum corresponding to the αSSI serum was not available, so that particular control experiment could not be performed. Still, it is clear that neither SSI nor BEIIa nor BEIIb are affinity purified using Protein A-Sepharose beads pre-incubated with a non-specific serum.

In summary, reciprocal co-purification was observed between BEIIa and SSI, i.e., the αBEIIab beads bound SSI and the αSSI beads bound BEIIa. Pre-immune serum controls bound neither SSI nor BEIIa. Thus, the data strongly indicate that BEIIa and SSI participate in the same multi-subunit complex. The data also revealed that αSSI precipitated BEIIb, however, this interaction was detected only in one orientation. Thus, the data suggest SSI and BEIIb may participate in the same multi-subunit complex, but further investigations are needed.

The same approach was used to identify binding partners for SSIII. An antiserum termed αDU1N, which was raised against the amino terminal region of SSIII (Cao et al. 1999), was pre-incubated with Protein A-Sepharose. The fraction P eluate from these beads was then probed for the presence of either SSIII, BEIIa, BEIIb or SSI. As expected, the

αDU1N serum was able to precipitate SSIII (data not shown). In addition, SSI and BEIIa were both detected in the eluates of the αDU1N precipitate (Figures 3.5E, F). BEIIb could also be detected in fraction P from the αDU1N beads (Figure 3.5E). Again it should be noted that Protein A-Sepharose beads pre-incubated with a non-specific serum purified neither SSI nor BEIIa.

In these instances a reciprocal experiment could not be performed, i.e., it was not possible to test whether αBEIIab or αSSI beads are able to precipitate SSIII. The reason for this problem is that SSIII is difficult to detect by immunoblot analysis, likely due to protein degradation. Because reciprocal co-immunoprecipitation was not demonstrated, the data suggesting that SSIII and SSI, or SSIII and BEIIa, participate in the same multi-subunit complexes are not as comprehensive as is the case for the SSI-BEIIa interaction. Nevertheless, the data are consistent with the hypothesis that SSIII, SSI, and BEIIa are all involved with each other in one or more multi-subunit complexes.

Pleiotropic effects of branching enzyme mutations on starch synthases

In the previous section, co-immunoprecipitation experiments revealed BEIIa interacts with two starch synthases, SSI and SSIII. If direct interactions occur *in vivo* between BEs and SSs, and if these interactions have functional significance, then it could be predicted that mutation of one component of the complex could affect the activity of the other. In-gel activity assays (i.e., zymograms) were used in order to test this hypothesis. The first step in the two-dimensional procedure was to separate proteins using anion exchange chromatography. In the second step, the proteins in each column fraction were separated by non-denaturing PAGE. Finally, SS activity was measured in the polyacrylamide gels, so that

the various SS isoforms, as defined by these separation procedures, could be identified. The rationale of the approach was to seek for altered patterns of SS activity in a BE mutant, as compared to wild type.

Crude soluble extracts from kernels harvested 20 DAP were first fractionated using a MonoQ column, as described in Chapter 2 of this dissertation. A difference from the previously described analysis, however, is that the ammonium sulfate precipitation step was omitted. Subsequently, the proteins that eluted in the column fractions were separated by non-denaturing PAGE in gels containing 0.3% glycogen. The purpose of including glycogen in the gel was to provide a primer for SS activity. Following electrophoresis, the gels were incubated in the presence of the SS substrate ADP-glucose. Long glucan chains synthesized in the gel where an SS isoform is present were visualized by a dark brown staining with iodine, above the background tan stain of the glycogen in the gel. The SS activities from wild type maize kernels are displayed in Figure 3.6A. Three branching enzyme mutants were analyzed, carrying mutations of the gene *ae*, coding for BEIIb (Figure 3.6B), *sbe2a::Mu*, coding for BEIIa (Figure 3.6C), or *sbe1::Mu*, coding for BEI (Figure 3.6D). The sources for these genetic resources are referenced in Chapter 2.

The 2D zymogram patterns of SS activity are complex and can be difficult to compare between genotypes, however, there were consistencies noted between all four strains and some distinctions that can be attributed to loss of a particular BE. The most slowly migrating band, termed Band I, appears in fractions D10 to E7. This band was also apparent in all three BE mutants, except that in all three instances the end of the elution peak shifted slightly and terminated in fraction E5. Thus, it appears that the SS activity of Band I is not evidently affected by loss of any BE. SS activity Band II is present in fractions E4-E7 of wild type,

and essentially is unchanged in any of the mutants. This band has been identified as SSIIa through analysis if mutations in the *sugary2* locus, which codes for this isoform (Zhang et al. 2004). Band V is evident in fractions D10 and D11 of the wild type line, and appears to be consistent throughout for all three mutants. Finally, two weak SS bands present in wild type in fraction D11, denoted as Band VII/VIII, are also evident in the same fraction in all three mutants. In two instances, however, there is only a single resolved band.

Band III may be affected in the BEIIa mutant. This band extends from fraction D10 to E2 in wild type (Figure 3.6A), and D10 to E3 or E4 in the BEIIb mutant (Figure 3.6B) or BEI mutant (Figure 3.6D). In the BEIIa mutant, however, there was a shift in the elution of Band III. The starting elution point for this SS activity is fraction at D12, and the majority of activity elutes significantly later in the gradient than it does for any of the other strains (Figure 3.6C). Another SS activity band that might be affected by the BEIIa mutation is Band IV, present in wild type in fractions D7 and D9 (curiously with no evident activity in the intervening fraction D8). This activity is clearly present in fraction D9 of both the BEIIb and BEI mutant, however, it is missing from that in the BEIIa mutant. A third SS activity band that might be affected by the BEIIa mutation is Band VI, present in wild type in fractions E3-E9. This activity is present in both the BEI and BEIIb mutants with no apparent affects on migration (Figure 3.6). However, differences were observed in the BEIIa mutant. Band VI is apparently normal in fraction E3 in this mutant, but it is strongly reduced in fractions E4 to E9 (Figure 3.6C). A novel band appears in fractions E4-E7 in the BEIIa mutant (marked with as asterisk in Figure 3.6C) that is not seen in wild type or the BEI or BEIIb mutants. These distinctions in 2D zymogram behavior might indicate a difference in the SS isoform represented by Band III, Band IV, and/or Band VI in the absence of BEIIa.

DISCUSSION

This dissertation addressed the central hypothesis that the multiple enzyme activities involved in starch biosynthesis coordinate their actions to produce the final starch structure. A means of enzyme coordination is proposed to be direct interactions in MSCs. To test whether BEs interact in MSCs with other components of the starch biosynthetic pathway, direct and indirect experimental approaches were taken. The major contribution of this section of the dissertation is the direct evidence that certain BEs are involved with SSs in one or more MSCs. New indirect evidence also provided by this research gives additional support for the participation for BEs in MSCs.

Direct evidence

With regard to direct evidence, the strongest data comes from coIP results indicating BEIIa and SSI are members of the same MSC. Essentially the experiments showed that when BEIIa is precipitated from endosperm cell extracts owing to its affinity for an antibody, SSI is also present in the precipitate (Figures 3.5A, B). Similarly, when SSI was precipitated by antibody affinity, BEIIa was also present in the precipitate (Figures 3.5C, D). The reciprocal coIP results are taken as strong evidence that a BEIIa-SSI complex exists *in vivo*. The importance of the reciprocal findings is that the alternative explanation that the same antibodies recognize both SSI and BEIIa, for some unknown reason, becomes very unlikely. In this alternative view αBEIIab would need to non-specifically precipitate SSI, and αSSI would also need to non-specifically precipitate BEIIa. The likelihood of the two coIP findings both being artifactual is very small, and thus the explanation that SSI and BEIIa are in the same complex is well supported. The data could be explained by direct interaction

between the BEIIa and SSI proteins. Alternatively, BEIIa and SSI might not interact directly, but both could be present in a stable complex of three or more proteins.

The coIP data also suggest that BEIIa and SSIII exist together in a MSC, and that the same situation applies for SSI and SIII. The essential observation here is that when SSIII is precipitated by antibody affinity, BEIIa and SSI are both also present in the eluate. It was not possible to discern whether SSIII was present in the precipitate generated by affinity to the αBEIIab or αSSI antibodies, so further analysis is needed in order to absolutely verify this conclusion. Presuming it to be correct, however, the data are consistent with different MSC arrangements involving various combinations of multimers, e.g., several different heterodimers (BEIIa-SSI, BEIIa-SSIII, and SSI-SSIII) or a single heterotrimer (BEIIa-SSI-SSIII). Although further research is needed to define the specific nature of the complexes, this dissertation has directly proven that BEIIa is a component of at least one, and possibly more MSCs.

Throughout the coIP experiments, αBEIIab antiserum was used. In Chapter 2, the degree of isoform-specificity for αBEIIab was characterized and both BEIIa and BEIIb were recognized equally by the αBEIIab antisera in immunoblot analysis. In comparison, when αBEIIab was used as the precipitating antiserum for coIP, the results revealed BEIIa was preferentially pulled down versus BEIIb. The same protein source was used for both the coIP and immunoblot analyses, specifically soluble whole cell extract from developing kernels. Therefore, differences in protein expression cannot explain the differential detection of BEIIb in the two different methods. A likely explanation for these results is that the major epitope on BEIIb is revealed only when the protein is in a denatured state and thus the antibody would not bind to the native protein during the coIP. In contrast, for BEIIa the

major epitopes are presumably exposed both in the native state and in the presence of SDS, and thus the antibody would bind both in the immunoblots and the coIP.

Considering that αBEIIab does not precipitate BEIIb, this approach does not provide information about BEIIb binding partners. To address this question, a specific antibody that has high affinity for BEIIb is needed. The best approach to take in the production of a BEIIb isoform-specific antibody would be to utilize synthetic peptides specific to BEIIb. This is needed owing to the high sequence similarity between the two BEII isoforms in maize. It might be possible to identify BEIIb-specific peptides in the amino terminus of the protein that are not represented in BEIIa (see Figure 2.3).

Indirect evidence

Characterization of BEs by gel permeation chromatography

The lines of indirect evidence identified by this research add to the existing substantial pool of circumstantial genetic data supporting the existence of MSCs containing BEs. Evidence for MSCs involving either BEI or BEIIa was not observed in the GPC data. Both BEI and BEIIa eluted as monomers from whole cell and plastids extracts, whereas eluting at a higher predicted molecular weight could have suggested the protein(s) was involved in a MSC. The GPC data, however, neither confirm nor rule out the possibility that BEI and/or BEIIa are present in MSCs. A possible explanation for the BEI and BEIIa GPC data could be related to the stability of the interactions within the MSC *in vivo* in comparison to *in vitro*. Within the plastid during periods of starch biosynthesis there is presumably a high concentration of glucan polymer. The presence of the higher glucan amounts *in vivo* could effect the chemical environment around the MSCs, therefore affecting the stability of their

interactions. The glucan concentration *in vitro*, i.e., in cell extracts and partially purified fractions, almost certainly is not equivalent to the concentration *in vivo*. This could result in breakdown of interactions within the MSC and elution of the BEI or BEIIa as a monomer, even if *in vivo* the protein is present in a complex. To try to overcome such potential difficulty, in one approach plastids were kept intact as long as possible prior to lysis and application to the GPC column. No differences in the elution profiles were observed, however, in comparison to those obtained from whole kernel lysates.

In comparison to BEI and BEIIa, there was a significant difference in the GPC elution profile of BEIIb observed between plastid and whole cell extracts, which might be explained by the persistence of BEIIb in a MSC in the plastids. In whole kernels soluble extracts BEIIb eluted as a 13 kDa protein, and this could result from BEIIb interacting with the Sephacryl S300 column. In contrast, BEIIb isolated from plastid extracts eluted closer to its predicted molecular weight, approximately 80 kDa. A possible explanation for the difference is that BEIIb in the plastid extracts remains in association with one or more other partners in a MSC, and that this in turn reduces the affinity of BEIIb for the column matrix. Thus, the observation can be taken as evidence in support of the hypothesis that BEIIb in the native state binds to other proteins.

Multiple electrophoretic forms of BEI, and affects of protein phosphorylation state

Another line of indirect evidence came from the analysis of the multiple electrophoretic "red" bands proposed to be BEI (Colleoni et al. 2003). The results of this study demonstrated that all five of these activity bands contain the BEI polypeptide. Thus, the potential explanation for the multiple BEs that they result from a different catalytic subunit,

as yet unidentified, can be definitively ruled out. Knowing that all the red bands are forms of BEI, two general theories need to be considered in order to determine the reason for the existence of the multiple electrophoretic forms. One theory suggests the multiple bands represent various post-translational modification states of BEI. The other theory proposes that each band represents a distinct MSC involving BEI in association with other proteins. Furthermore, these two theories are not mutually exclusive.

Protein phosphatase treatment was used as a means of testing whether post-translational modification by phosphorylation was the source of the various mobilities of the BEI protein. In this view, negative charges carried by the phosphate groups would change the electrophoretic mobility of the BEI isoforms in native PAGE, and removal of the phosphates would collapse the multiple forms into a single band, or at least reduce the number of red bands. This experiment was prompted by recent research reporting that the activities of the BE isoforms in wheat are regulated by protein phosphorylation, and furthermore, that the phosphorylation state of BEI regulates its interaction with other proteins (Tetlow et al. 2004a). Multiple red bands were still distinguishable in the zymograms post alkaline phosphatase treatment and there was no evident shift in the mobility of any of the five bands. These results argue against protein phosphorylation of BEI being the sole explanation for the multiple electrophoretic mobility forms. Thus, the possibility of various MSCs existing that contain BEI remains open, as does the possibility of post-translational modification other than protein phosphorylation.

BEIIa and BEIIb were also analyzed in zymograms for effects of protein phosphorylation. There were no apparent differences in band intensities, band mobilities, or protein concentrations of BEIIa or BEIIb before and after alkaline phosphatase treatment

(Figure 3.2). To further test the effect of protein phosphorylation on BE activity, total crude soluble extract was analyzed using the phosphorylase a stimulation assay. Total BE activity decreased approximately 50% after alkaline phosphatase treatment (Table 3.1). These results demonstrate that total BE activity is not solely regulated by phosphorylation. There is an apparent anomaly, however, because if protein phosphorylation regulated BE activity then it is expected that the some effects of dephosphorylation would be observed in band mobility and intensity in native PAGE. To the contrary, these results revealed no effect on zymogram band activity or mobility. A possible explanation is that the regulation of activity by phosphorylation is indirect, i.e., that a protein affected by the alkaline phosphatase treatment in the total soluble extracts acts as a regulator of BE activity.

Pleiotropic effects of BE mutation on SS activity

Yet more indirect evidence from this research was obtained from the analysis of pleiotropic effects of BE mutations on SS activities using anion exchange chromatography. The key observation is that the elution profile of three SSs, represented by Band III, Band IV, and Band VI in the two-dimensional zymograms of Figure 3.6, are altered when BEIIa is missing owing to the *sbe2a::Mu* mutation. These data are consistent with the hypothesis that BEIIa participates in a MSC with one or more SSs. If proteins are interacting in a complex together, then taking one component away through mutation should have an effect on the remaining components of the complex, in this instance in their anion exchange elution behavior. These data are similar in nature to previous findings that BEIIa activity is affected by mutations in other starch biosynthetic enzymes, particularly the DBEs coded for by *su1* and *zpu1* (Dinges et al. 2001). Other explanations such as indirect regulatory effects on the

SS activities are not ruled out, so these pleiotropic effects do not offer definitive proof for MSCs containing BEIIa, however, the growing body of circumstantial evidence is strong support for this hypothesis.

In summary, this research provided strong, direct evidence that BEIIa and SSI are components of the same MSC, and indirect support for the general hypothesis that BEs and SSs coordinate their actions. This contribution is a starting point for further research and directs future investigation into the nature of the complex or complexes that contain these two enzymes. Of particular interest is MSC purification, determining the stoichiometry of the components of the MSCs, and learning the identity(s) of any other possible constituents of that MSC. In the long term, it will be necessary to learn the functional consequences of the participation of each enzyme in the complex, and how those potential regulatory effects may determine specific architectural features of the amylopectin polymers produced by the coordinated action of the enzymes.

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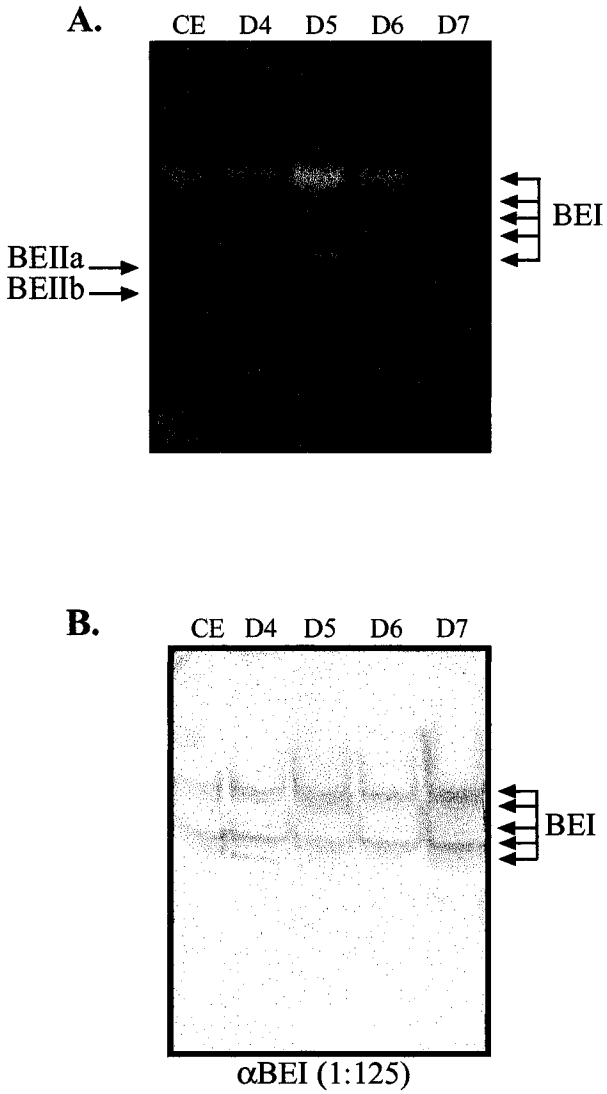


Figure 3.1. Identity determination of multiple branching enzyme bands. (A) Mono Q fractions from wild type kernels were first run on a native PAGE gel. A set of gel lanes was then transferred overnight into another polyacrylamide gel impregnated with 0.3% starch, which was then developed with an iodine solution. (B) A duplicate set of gel lanes produced as in panel A was incubated in Western transfer buffer, electroblotted to a nitrocellulose membrane, and probed in immunoblot analysis with αBEI. Lane designations are as follows: CE, total crude soluble extract; D4 - D7, fractions from the elution of the MonoQ column that contained the greatest amounts of BEI protein and red band activity.

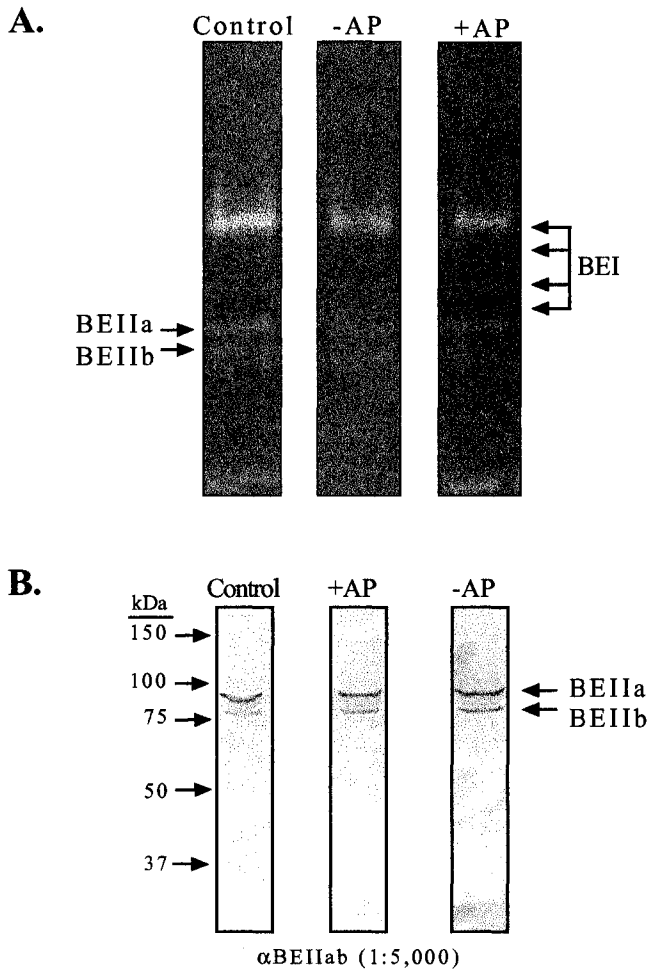


Figure 3.2. Effect of protein dephosphorylation on BE activities and electrophoretic mobilities. (A) Samples were separated by native-PAGE and then subjected to zymogram analysis. Lane designations are as follows: Control, 40% ammonimum sulfate pellet equilibrated in MonoQ A buffer; -AP, a sample of the control fraction diluted and added to a sample of alkaline phosphatase buffer, but lacking any alkaline phosphatase treatment; +AP, a sample of the control fraction diluted and added to a sample of alkaline phosphatase buffer containing 14 U of alkaline phosphatase. (B) Samples equivalent to those analyzed in panel A were separated by SDS-PAGE and subjected to immunoblot analysis using αBEIIab, to reveal possible changes in protein content or mobility resulting from the alkaline phosphatase treatment.

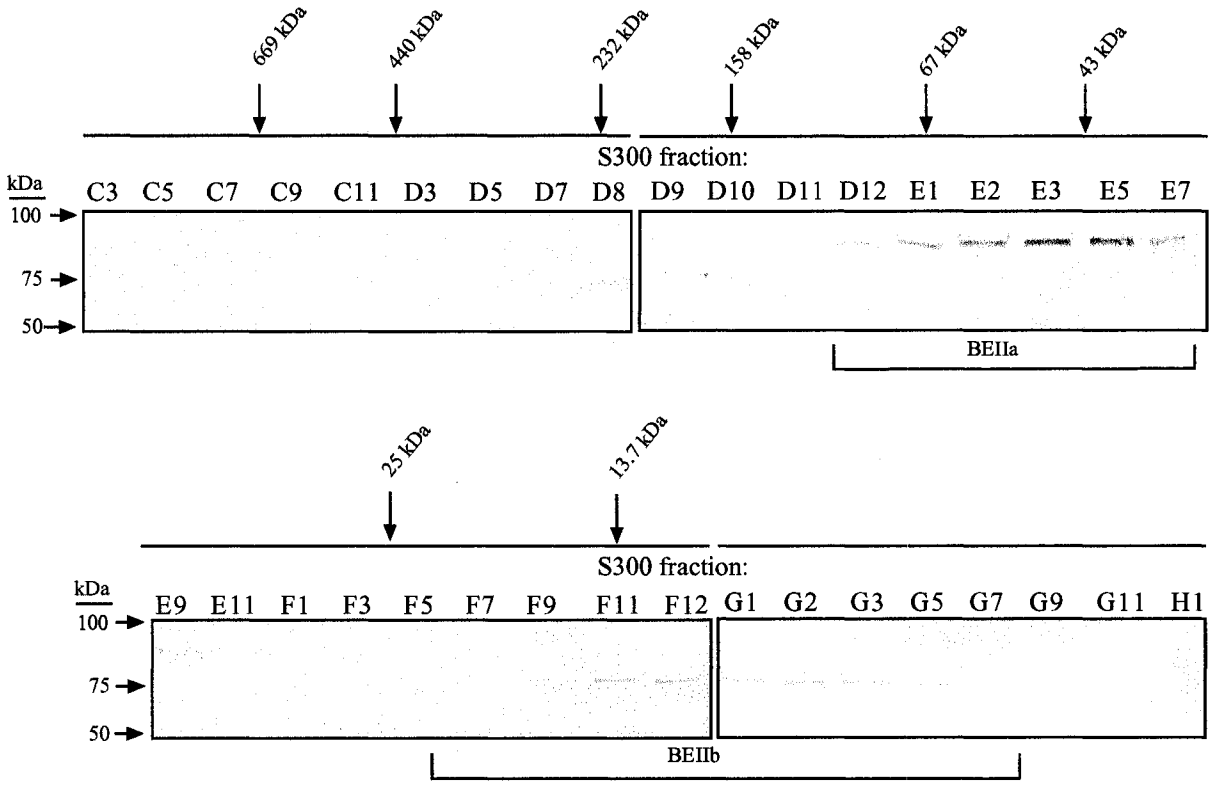


Figure 3.3A. Fractionation of BEIIa and BEIIb by size exclusion chromatography. Total whole kernel soluble extract from wild type inbred W64A was applied to a Sephacryl S300 column, and fractions were probed with αBEIIab at a dilution of 1:5,000. BEIIa and BEIIb eluting from the column were identified based on mobility in the gel as compared to whole kernel extract (see Figure 2.5). Molecular weight standards are as described in Figure 2.5.

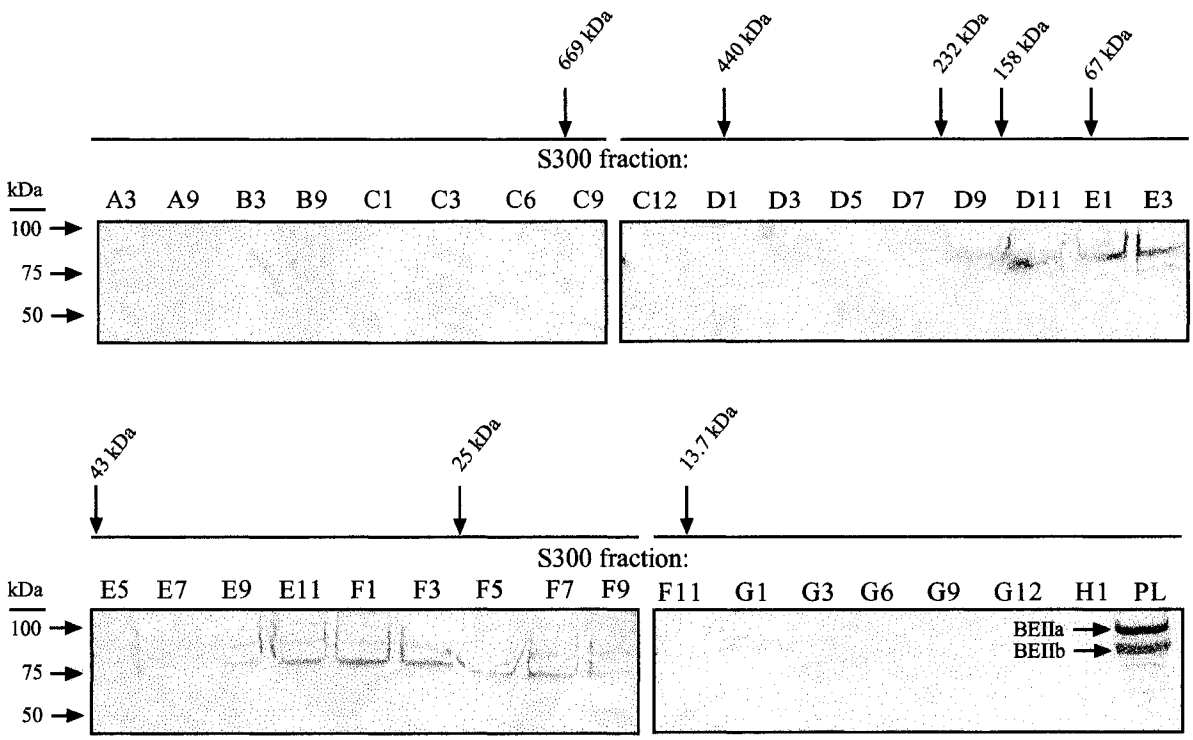


Figure 3.3B. Analysis as in Figure 3.3A, except that the extract applied to the column was from wild type maize plastids from developing endosperm. The fraction designated as PL is a sample of the extract loaded onto the column. Molecular weight standards are as described in Figure 2.5.

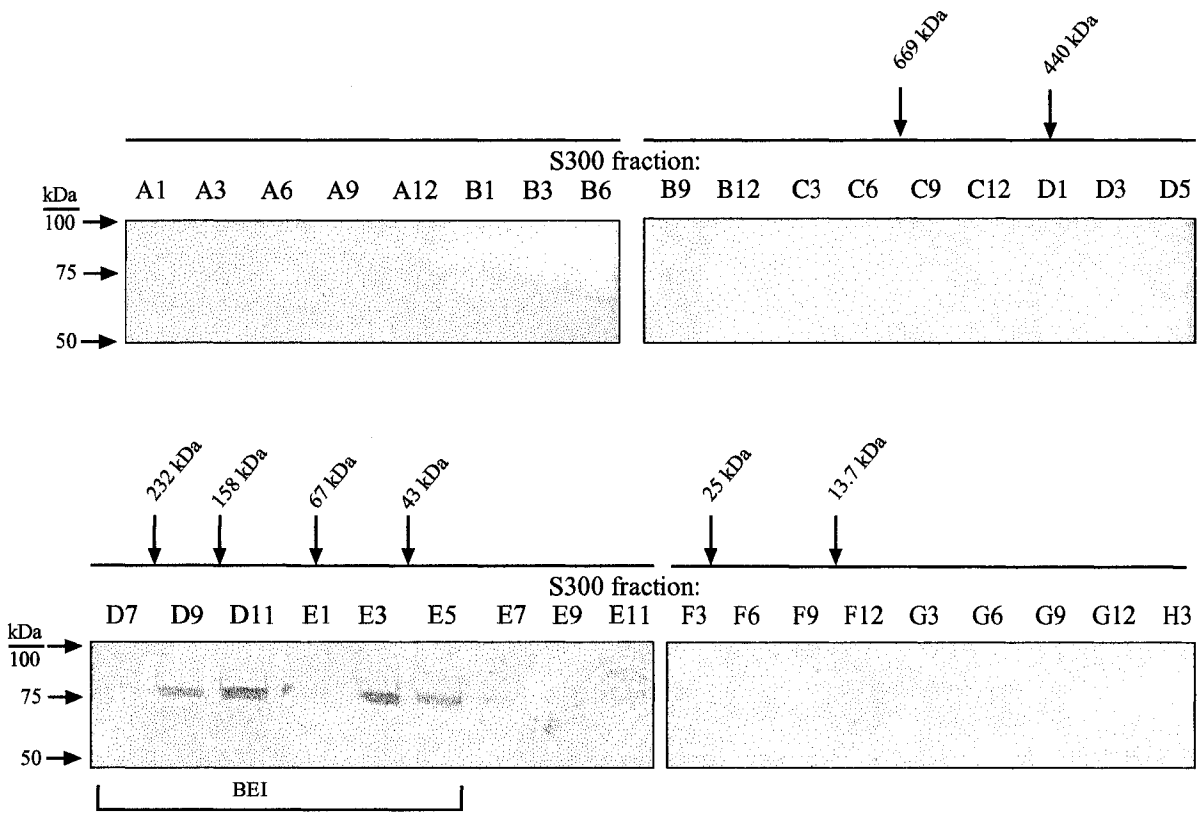


Figure 3.4A. Fractionation of BEI by size exclusion chromatography. Total whole kernel soluble extract from wild type inbred W64A was applied to a Sephacryl S300 column, and fractions were probed with αBEI at a dilution of 1:1,000. Molecular weight standards were as described in Figure 2.5.

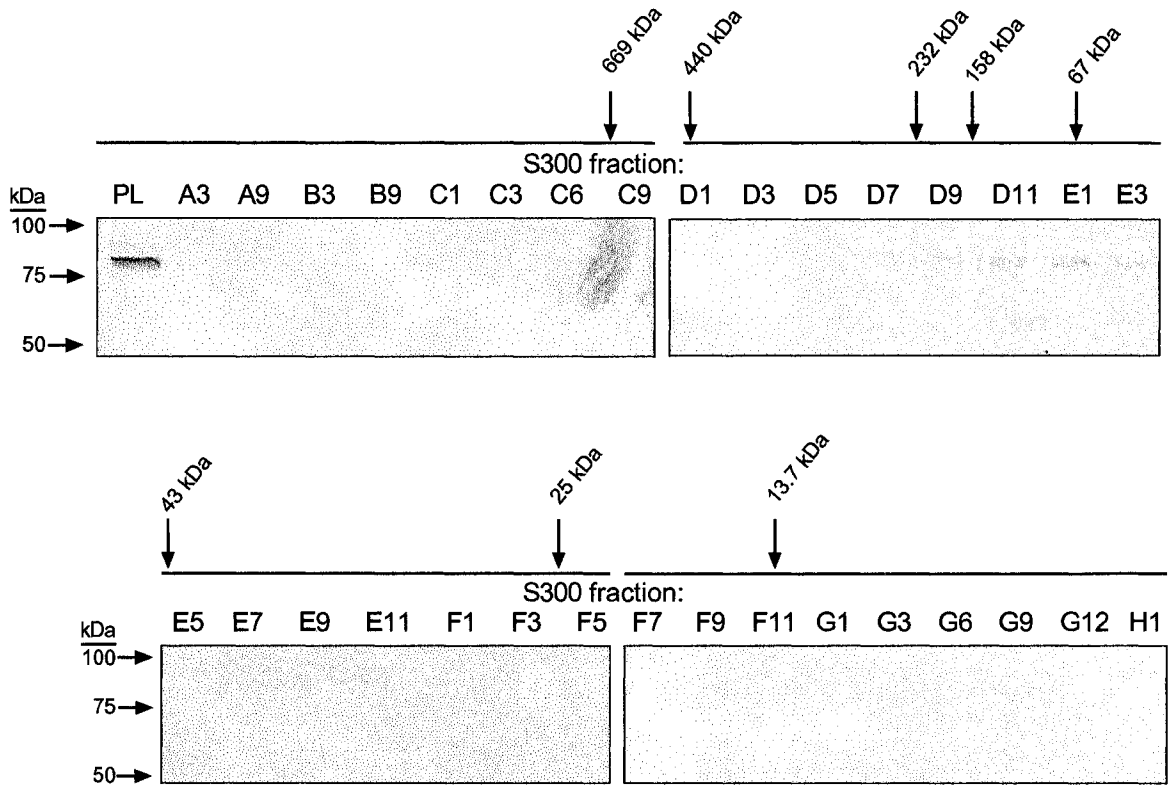


Figure 3.4B. Analysis as in Figure 3.4A, except that the extract applied to the column was from wild type maize plastids from developing endosperm and the fractions were probed with αBEI at a dilution of 1:2,000. The lane designated as PL contains a sample of the extract loaded onto the column. Molecular weight standards are as described in Figure 2.5.

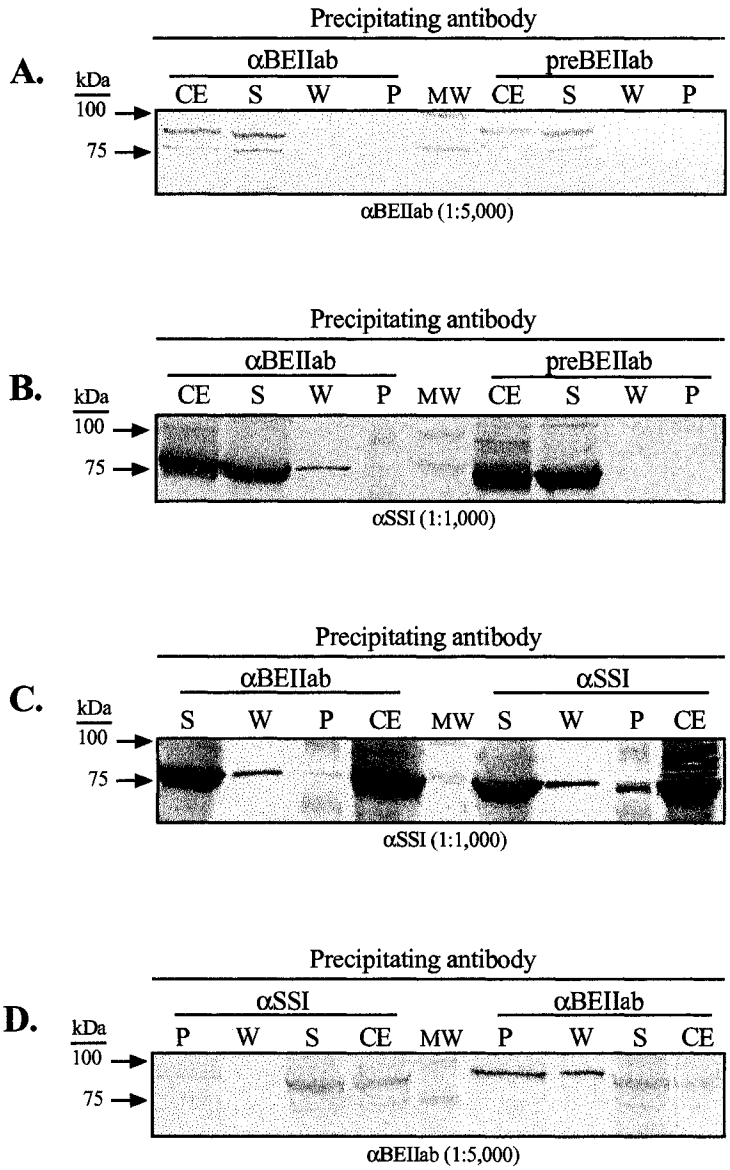


Figure 3.5. Co-immunoprecipitation reactions using the αBEIIab antibody. Total soluble extract of maize endosperm cells harvested 20 DAP was incubated with Protein A-sepharose beads to which polyclonal antibodies had been bound that recognize both BEIIa and BEIIb (αBEIIab), preimmune serume (preBEIIab), SSI (αSSI) or the N-terminus of SSIII (αdul). The beads were pelleted by centrifugation and washed extensively. Proteins bound to the antibody were eluted by incubation of the beads in a buffer at pH 3. The eluted proteins were then separated by SDS-PAGE and probed with polyclonal antibodies specific for SSI, dul, BEIIa, BEIIb and BEI. The fractions collected were: S, unbound proteins; W, 6th bead wash; P, proteins bound to the antibody; CE, crude extract; and, MW, molecular weight standards (Bio-Rad, catalog no. 161-0374).

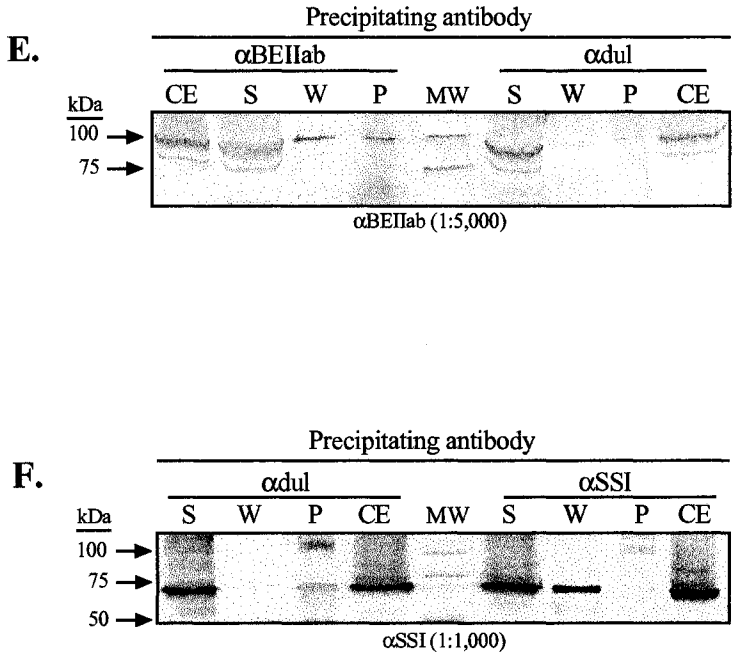


Figure 3.5. (continued)

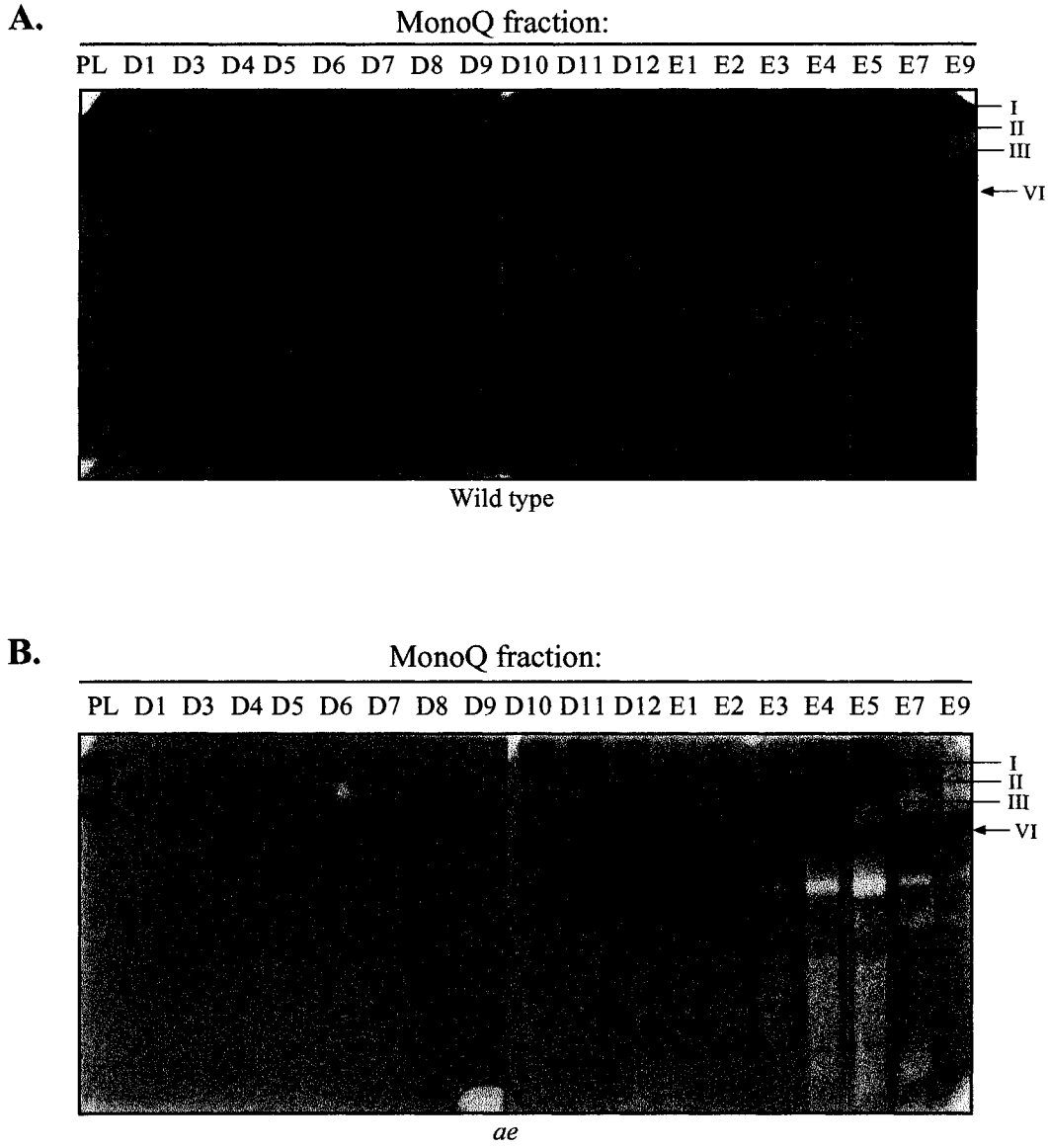
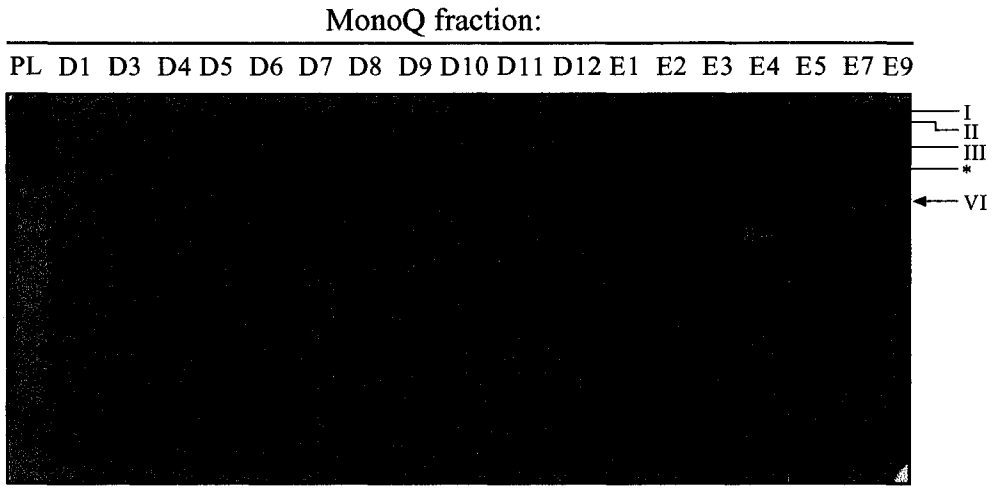


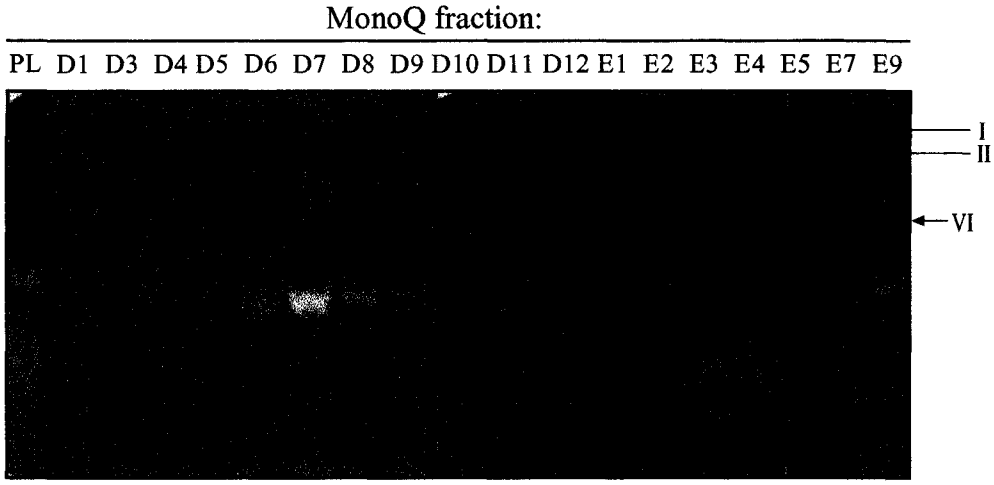
Figure 3.6. Starch synthase in-gel activity assays. Total whole kernel soluble extracts of wild type and branching enzyme mutants (*ae*, *sbe2a::Mu* and *sbe1::Mu*) were separated by anion exchange chromatography using a MonoQ column. Gradient fractions (described in Materials and Methods) were collected and proteins were separated by native PAGE, incubated overnight incubation in a glucose-1-phosphate solution and stained with iodine solution. Analyzed lines were as follows: (A) wild type; (B) *ae*; (C) *sbe2a::Mu*; and, (D) *sbe1::Mu*.

C.



sbe2a::Mu

D.



sbe1::Mu

Figure 3.6. (continued).

CHAPTER 4: ANALYSIS OF BE ACTIVITIES IN MAIZE LEAVES AND COMPARISON TO ENDOSPERM BEs

ABSTRACT

In most plants starch functions both as a long-term source of carbohydrate reserve, e.g., in seeds, and also as a transitory storage molecule that is completely catabolized each day, e.g., in leaves. These various physiological functions are provided by differences in the biosynthetic mechanisms, however, the distinctions in starch biosynthesis between leaves and seeds are not fully understood. In maize, previous research raised the possibility that different forms of starch branching enzyme I (BEI) function in leaves and seeds. The current study tested this hypothesis and established, in contrast to the previous work, that the same BEI polypeptide functions in both tissues. Using immunological techniques the same BEI protein known to be present in maize seeds was clearly identified in leaf extracts. The leaf BEI protein migrates under denaturing conditions at nearly the same molecular weight as the endosperm BEI. Partial purification of leaf BEI characterized it as a monomeric protein of approximately 80 kDa. Initial analyses suggest that there are multiple electrophoretic forms of leaf BEI, similar to endosperm BEI. This research supports the existence of BEI in maize leaf and provides evidence that two BEs, namely BEI and BEIIa, are responsible for all BE activity in the synthesis of transitory starch in maize leaves. Taken together with previous analyses of a mutant lacking BEIIa, the data allow the conclusion that BEI alone can support synthesis of granular starch. Finally, no distinctions in the native state of BEI as it exists in leaf or endosperm could be observed based on chromatographic properties.

INTRODUCTION

The previous sections of this dissertation describe the existence of multiple genes that code for active starch branching enzymes, and characterized the BE enzyme activities as they exist in developing maize endosperm. In this chapter, the focus turns to the question of the complement of branching enzymes in maize leaves responsible for the synthesis of transitory starch. The long-term goal is to determine the identity of the BEs present in leaves, and to compare these isoforms to those present in endosperm tissue. Transient starch produced in leaves serves a different physiological function within the plant as compared to storage starch. Thus, distinctions between the enzymes involved in production of these two starch forms may provide different structures and properties of the products. There is evidence that some of the BE isoforms may function in both endosperm and leaf. Contradictory results have also been published, however, that raise the possibility of novel forms present as part of the transitory starch system (Blauth et al. 2001). Tools developed in the previous chapters of this dissertation were applied here to further clarify the components of the starch biosynthetic system present in maize leaves.

BEs from storage organs have been more thoroughly characterized than those from leaves, however, there is biochemical evidence from pea, rice and maize showing that leaves also contain branching enzyme I and II isoforms. Antibodies raised against the BEI and BEII isoforms of pea embryos were able to neutralize the branching enzyme activity present in pea leaves (Tomlinson et al. 1997). These results indicate that the storage and transitory starch branching enzymes are at least immunologically related, if not identical. Genetic evidence also supports the proposal that the same BEs are shared between leaf and embryo

(Bhattacharyya et al. 1990; Burton et al. 1995). The *rugosus* (r) mutation of pea, which is known as the famous round/wrinkled mutation used by Mendel to identify the concept of genes, codes for the BEII isoform. This mutation affects branching enzyme activity both in leaves and embryos of pea, providing clear evidence for isoform sharing in these two tissues.

Gene expression analysis in maize indicated that there are distinctions in the BEII isoform distribution between leaves and endosperm. Transcripts from the *ae* gene, which codes for BEIIb, accumulate in seeds but are not present in leaves (Gao et al. 1996). In comparison, the transcript for BEIIa was found throughout the plant in all the tissues that were analyzed, including strong expression in leaves (Gao et al. 1996). Therefore, BEIIa appears to be the only BEII isoform in leaves, where as BEIIa and BEIIb are both functional in endosperm.

Genetic analyses have been applied to address the function of each BE isoform in maize. Mutation of the *ae* gene, which codes for BEIIb, has a major effect on branching enzyme activity in endosperm, as well as the structure of storage starch (Boyer and Preiss 1978a; Boyer and Preiss 1978b; Dang and Boyer 1988; Dang and Boyer 1989; Yamanouchi and Nakamura 1992; Mizuno et al. 1993; Fisher et al. 1996; Gao et al. 1996). The effects of *ae*$^-$ on leaf starch have not been examined, however, the prediction is that there would be no effect because the BEIIb transcripts are not present in that tissue. From these data it is clear that there is a function of BEIIb in endosperm starch biosynthesis that cannot be compensated for by the presence of either of the remaining isoforms, BEI or BEIIa. Loss of function mutations in the *sbe2a* gene, which codes for SBEIIa, have also been examined for effects on leaf and endosperm starch (Blauth et al. 2001). No effects were observed on the structure of storage starch, suggesting that all BEIIa functions can be compensated for by

either BEI or BEIIb. The major alteration observed in leaf starch when BEIIa was eliminated by mutation was a lower proportion of short chains of amylopectin (Blauth et al. 2001). This indicates an essential BEIIa function in leaves, in contrast to its role in endosperm.

The relation of BEI isoforms in maize leaf and endosperm is unclear based on the published literature, in particular from the analysis of the *sbe2a*⁻ mutant. During analysis of the effects of the null allele *sbe2a::Mu*, no BEIIa protein or activity was detected, as expected. Because BEIIb is not expressed in maize leaves (Gao et al. 1996), the *sbe2a::Mu* plants were predicted to produce starch in the leaves using the sole remaining isoform, BEI. A surprising result, however, was that no BEI protein was identified in the *sbe2a::Mu* leaves, even though starch was accumulated (Blauth et al. 2001). These results could be explained by the presence of an as yet unidentified BE isoform in the leaves, or by the ability to produce starch without a classical branching enzyme. Alternatively, there may have been a technical difficultly in the branching enzyme identification analyses in this previous study.

In this chapter, new isoform-specific branching enzyme antibodies, characterized in Chapter 2, were used to better define the complement of branching enzymes in maize leaves. The results demonstrated definitively that both BEI, and BEIIa are present in leaf tissue and confirmed that BEIIb is absent. This allows the conclusion that leaf starch in the *sbe2a::Mu* mutant is produced using exclusively BEI. Accordingly, the BEII isoform, even though it is highly conserved in plants, is not essential for the formation of crystalline starch.

MATERIALS AND METHODS

Extraction of proteins from plant tissues

Total soluble kernel extracts from maize were prepared as described in Chapter 2. Leaf protein extracts followed a similar protocol. Leaf blade tissue was harvested at 20 days after emergence (DAE), quick-frozen in liquid nitrogen and stored at -80°C. The frozen leaves were ground in a chilled mortar and pestel under liquid nitrogen into a fine powder. Ground tissues were mixed with HB buffer supplemented with PMSF and protease inhibitor cocktail as specified in Chapter 2. The ratio HB buffer to leaf tissue was 1 mL per gram. The suspension was left on ice for approximately 10 min, and then centrifuged at 20,000 rpm for 20 min. The supernatant, referred to "crude soluble leaf extract", was kept on ice until further analysis. All further steps were performed either on ice or at 4°C, unless otherwise noted.

Anion-exchange chromatography

BEI proteins from leaves were partially purified by anion exchange chromatography using the same MonoQ column protocol described in Chapter 2. Crude soluble leaf extract was filtered and loaded directly onto the column (6 mL at approximately 0.5 mg/mL). Column buffers and the elution gradient conditions were as in Chapter 2. MonoQ fractions were collected, concentrated, and analyzed for the presence of BEI. Due to the low abundance of BEI protein in leaf tissue, samples were concentrated by filter centrifugation (Amicon® Ultra-4 concentrators, molecular weight cutoff 50 kDa [Millipore]). MonoQ fractions of approximately 1.3 mL were concentrated to less than 100 µL.

Gel-permeation chromatography

Gel permeation chromatography (GPC) using a Sephacryl S300 column was performed as described in Chapter 2. The S300 fractions (1.4 mL) were collected, and concentrated to less than 100 μL for further analysis.

Immunoblot analysis and in-gel activity assays (zymograms)

Immunoblot procedures were described in Chapter 2. Blots were probed using isoform-specific polyclonal antisera, either αBEI (rabbit #3672) or αBEIIab (rabbit #3434). The antibody dilution ranged from 1:250 to 1:5,000 depending on the application. The methods used for zymogram analysis of starch metabolizing enzymes, including native PAGE and transfer to starch-containing gels, were described in Chapter 2.

RESULTS AND DISCUSSION

Identification of BEI protein in maize leaves

The previous report of Blauth et al. (2001) indicated that BEI might not be present in maize leaves. To investigate this issue further, immunoblot analyses using an isoform-specific BEI antibody were conducted using wild type and mutant plants. The αBEI antibody used for these analyses was raised against a mixture of two 20-amino acid polypeptide sequences specific to the carboxy-terminus of maize BEI. As shown in Figure 2.4 (Chapter 2), this antiserum identifies a single band in total soluble kernel extracts from wild type, and this band is absent from kernels bearing the null mutation *sbe1::Mu* (Blauth et al. 2002). Thus, the antibody is presumed to be specific for BEI among the three possible BE isoforms.

This immunoblot analysis was extended to leaf tissue. Leaves from wild type and homozygous *sbe1::Mu* or *sbe2a::Mu* mutant plants were used. For all analyses, leaf tissues were harvested at 20 DAE and kernels were harvested at 20 DAP. The results revealed that the BEI protein is detectable in leaf extracts of wild type inbred W64A, although apparently in far lower abundance than in kernels (Figure 4.1B). This band was absent from the *sbe1*⁻ leaf extract, however, it was detected in the *sbe2a*⁻ extract at approximately the same low level as in wild type (Figure 4.1B). The size of this band as judged by comparison to SDS-PAGE standards was near 80 kDa, approximately matching the prediction from the sequence of the BEI cDNA. The size of the reacting leaf protein was slightly different, however, than the band that is detected in kernel extracts. As a control, the same leaf extracts were probed with αBEIIab antibody described in Chapter 2. These data revealed, as expected, that BEIIa is detectable in wild type and *sbe1::Mu* extracts, and not in the *sbe2a::Mu* sample (Figure 4.1C).

Some differences were observed in the detection of the putative BEI protein in leaves between separate replications of the assay. In the data set marked as "Assay 2" in Figure 4.1B the apparent abundance of BEI is greater than in the data set marked "Assay 1". The relative mobility of the BEI band also varied. The apparent molecular weight of BEI in Assay 1was greater than that in endosperm cells, whereas in Assay 2 BEI appeared to have a lower molecular weight in leaves as compared to endosperm.

In addition to SDS-PAGE as a means of separating the leaf proteins prior to immunoblot analysis, native PAGE was also employed (Figure 4.1D). As in the analyses of Chapter 3, duplicate native gels were subjected either to immunoblot analysis or starch zymogram analysis. Two distinct mobility forms of BEI were readily detected in the

immunoblot analysis. The mobility of these two bands coincided with the presence of faint red color staining in the zymogram, indicative of BE activity.

The analysis of BEII isoforms present in leaves revealed a possible discrepancy with previously published data. It had been previously reported by Dang and Boyer (1989) that the one BEII isoform present in maize leaves was identical to BEIIa present in maize endosperm. In addition, the mRNA transcript that codes for BEIIa is known to be expressed throughout the maize plant (Gao et al. 1996), whereas the BEIIb mRNA was found to be endosperm-specific and thus not present in leaf (Gao et al. 1996). It was surprising, therefore, to observe two bands in leaves that were identified by αBEIIab (Figure 4.1C). The upper band migrated the same distance as BEIIa from endosperm extract and the lower band ran slightly faster in comparison to endosperm BEIIb. Both bands were present in wild type and *sbe1::Mu* leaf extracts, however the two bands were both absent in the *sbe2a::Mu* extracts (Figure 4.1C). The fact that mutation of *sbe2a* causes loss of both bands suggests that they are in fact related, and thus the faster migrating band may result from degradation of BEIIa protein in leaves. The key observation is that when *sbe2a* is mutated there is no signal detected by the BEII-specific antiserum. These data support the previous conclusions that the BEII isoform present in maize leaf tissue is BEIIa, and that BEIIb protein is not expressed in leaves (Blauth et al. 2001).

Summary of the BE complement in maize leaves

From the data shown here, it is clear that wild type maize leaves contain BEI and BEIIa, and do not contain BEIIb. The relation of BEI isoforms in maize leaf and endosperm had been unclear based on the published literature. In particular, the BEI protein was not

identified in *sbe2a::Mu* leaf extracts and Blauth *et al.* (2001) suggested, therefore, that the BE activity in maize leaf was attributed to BEIIa along with an unidentified BEI-like protein. Previously published data, however, supported the existence of the same BEI in both leaves and endosperm. Dang and Boyer (1989) reported that BEI in maize leaves was related to but not identical to BEI in maize endosperm. Transcript for BEI was found throughout the plant in all tissues analyzed, including expression in the leaves (Gao et al. 1996). In the current study, the new antiserum conclusively revealed that an isoform either identical or closely related to endosperm BEI is present in leaves. The fact that only a single BEI mRNA and gene has been characterized in maize suggests that the leaf and endosperm forms are in fact identical. A duplicate gene coding for a specific BEI isoform expressed in leaf, however, cannot be definitively ruled out. During the analysis of leaf extracts it was noted that the abundance of BEI protein overall in total leaf extracts was much less than the amount of BEI protein in total endosperm extracts. This fact, together with the use of a less effective antiserum, could have contributed to the difficulties in BEI identification in the earlier study.

BEI in maize leaves resembles the BEI in endosperm with respect to the presence of multiple electrophoretic mobility forms. Like endosperm, the same catalytic subunit is present in multiple BEI forms in leaves. Two forms were observed in the leaf tissue, in comparison to the five distinct forms identified in endosperm (Chapter 3). Another distinction, possibly related to this feature of leaf BE, is that the activity appeared to be very unstable during extract storage as compared to the endosperm samples. Further analyses will be required to learn the explanation for the different electrophoretic mobility forms, especially with regard to the issue of whether the variability in the two tissues results from the same set of conformational or structural changes.

From these results it is possible to deduce that a starch biosynthetic system containing BEI as the sole branching enzyme activity is capable of producing crystalline starch granules. In the absence of BEIIa, in *sbe2a::Mu* mutant leaves, BEI is apparently the only branching enzyme activity present. The starch in *sbe2a::Mu* leaves is abnormal in terms of the amylopectin fine structure, however, granules still form (Blauth et al. 2001). Similarly, BEI can be eliminated by mutation, leaving only BEIIa, and again crystalline starch still forms (Blauth et al. 2002). Presumably, there is a selective advantage of having both BEI and BEIIa present in the biosynthesis of transitory starch, because evolutionary conservation of both genes is evident in all plants studied. A possible explanation for the fact that neither the BEI nor the BEII isoform appears to ever be lost in evolution is that the combination of activities produces an amylopectin structure that is most amenable to the transitory storage function of leaf starch, i.e., the ability to both store large amounts of glucose in a small physical space, and also to relatively quickly be completely degraded to simple sugars during the night.

Partial purification of BEI from maize leaves

BEI from maize leaf was partially purified by anion-exchange chromatography and size exclusion chromatography, towards the aim of comparing the properties of this BE isoform between leaf and endosperm tissue. The overall hypothesis to be tested was that different assembly states of BEI in leaf and endosperm could contribute to distinct functions in production of transitory versus storage starch. The differences in electrophoretic mobility forms, and enzyme stability mentioned in the previous section are suggestive that such distinctions might exist between the leaf and endosperm BEI forms. In addition, partial

purification was used to concentrate specific leaf proteins, thereby facilitating easier recognition of the BEI protein than could be accomplished using total soluble leaf extracts as in Figure 4.1.

Soluble proteins were extracted from wild type leaf tissue and these extracts were then fractionated by anion exchange chromatography using a MonoQ column. Owing to the significantly lower amount of BEI protein in leaf as compared to kernels (Figure 4.1), the MonoQ fractions were concentrated prior to immunoblot analysis using SDS-PAGE (Figure 4.2A). Fractions across the entire elution gradient were analyzed (Figure 4.2A and data not shown). One prominent band was detected by αBEI antiserum, migrating according to a molecular weight of approximately 80 kDa, again corresponding to the predicted mass of BEI. This band was detected in fractions D5-D9, corresponding to salt concentrations of 11-17 mM. The data confirm the presence of BEI protein in leaves, as was originally indicated in analysis of total soluble leaf extracts in Figure 4.1. Also the data show that the anion exchange properties of BEI from leaves are similar to those of the same isoform from endosperm, because both enzymes were found to elute in the salt gradient between 11 and 19 mM NaCl.

Size exclusion chromatography was used to estimate the native molecular mass of the BEI, i.e., to test whether it might be present as a multimer or part of a multi-subunit complex. The MonoQ fractions identified to contain the leaf BEI protein were pooled, concentrated by filter centrifugation and applied to a Sephacryl S300 column. Analyzing each column fraction by SDS-PAGE and immunoblot analysis allows estimation of the native molecular weight of BEI from leaves (Figure 4.2B). The enzyme elutes approximately between the 158 kDa and 67 kDa molecular weight markers. These values correspond roughly to the

expected molecular weight of 80 kDa for BEI, thus indicating that the enzyme elutes from the column as a monomer.

In summary, this data supports the presence of BEI in maize leaves. Initial analyses demonstrated leaf BEI is similar to its endosperm counterpart in predicted molecular size and weight. No clear distinctions between the enzyme in leaf and endosperm that might account for functional differences in the starch product could be discerned by these methods of analysis.

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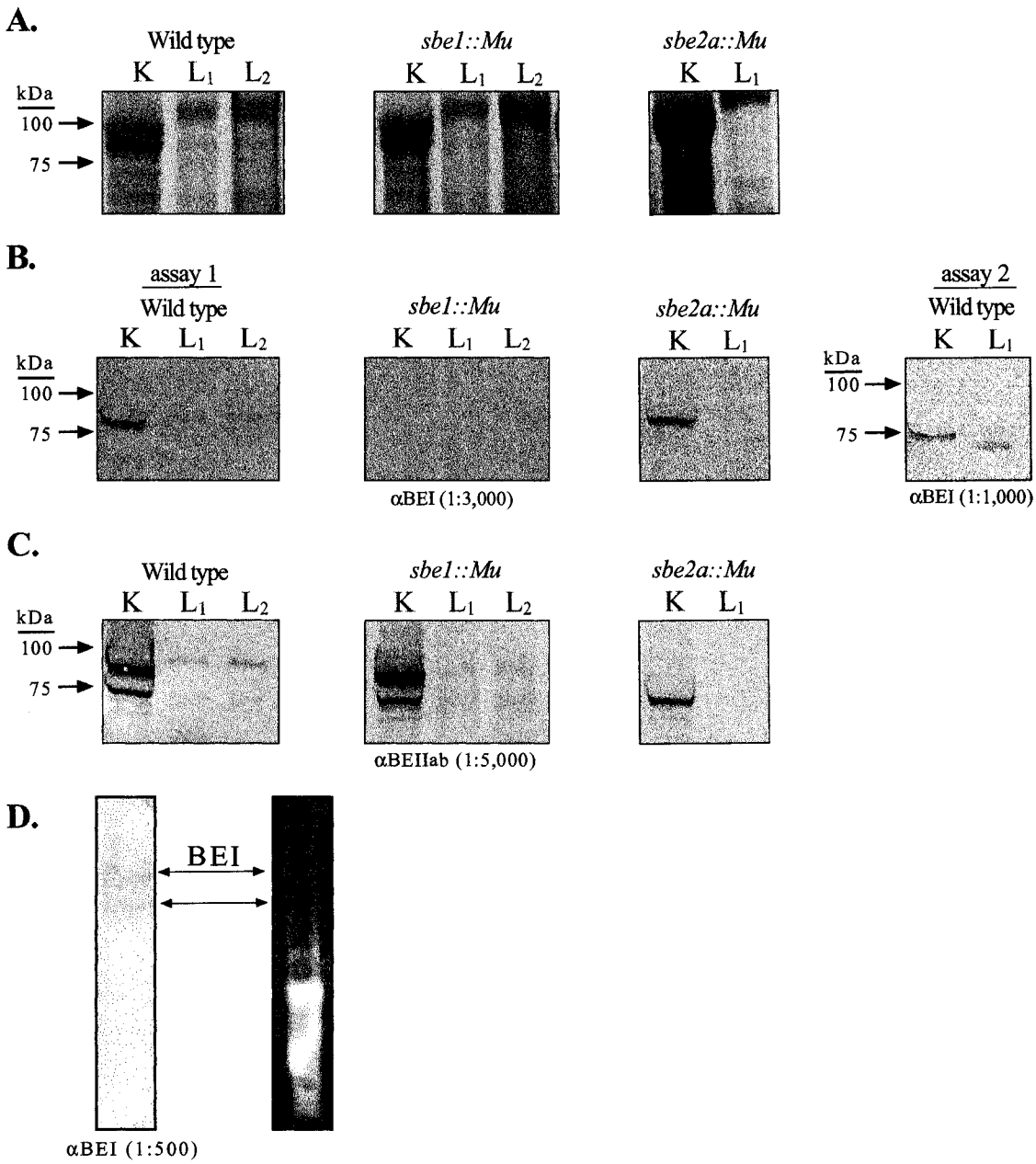

Figure 4.1. Total soluble extracts from wild type maize and mutant lines deficient in either BEI or BEIIa were probed using either αBEI or αBEIIab antiserum. Kernel (K) and leaf (L) proteins were analyzed, and the amount of protein loaded was: K, 10 mg; L_1, 10 mg; and, L_2, 20 mg, respectively. Lane designations are the same in A, B and C. (A) Coomassie blue stained gel. (B) Immunoblot probed with αBEI. A second independent assay blot comparing kernel and leaf BEI probed with αBEI and is shown in the last panel of part B. (C) Immunoblot probed with αBEIIab. (D) Wild type leaf crude extract was loaded onto a native PAGE gel and either transferred overnight to another native PAGE gel containing starch, and developed with iodine (right panel) or, incubated in Western transfer buffer and then transferred overnight for immunoblot protocols in Materials and Methods and probed with αBEI.

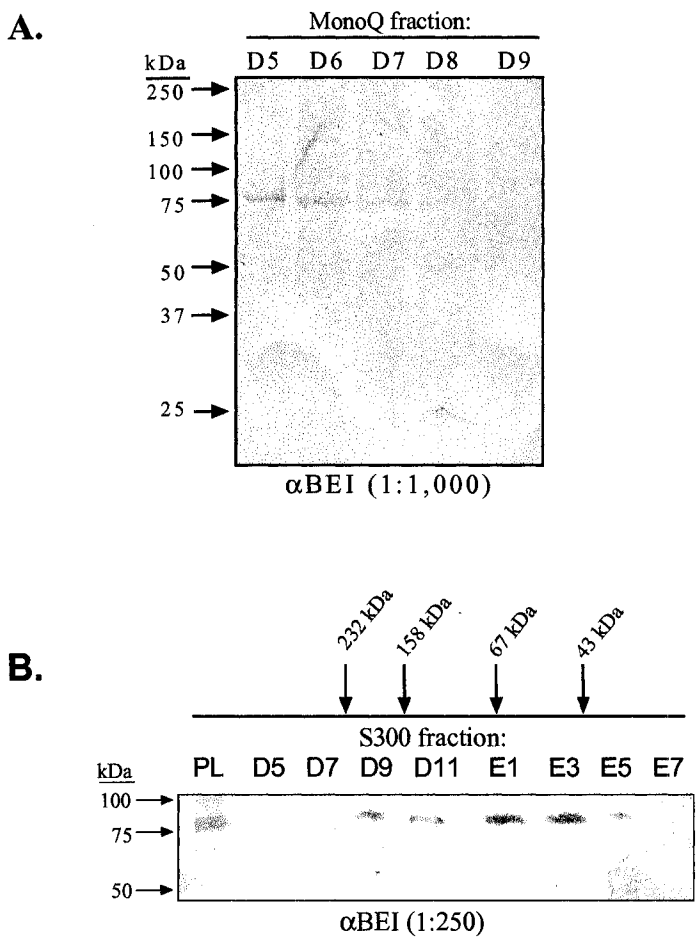

Figure 4.2. Partial purification of leaf BEI. (A) Anion exchange chromatography. Proteins in total soluble leaf extract were separated using a MonoQ column. Fractions from the elution gradient were concentrated and analyzed by immunoblot analysis using αBEI. (B) Size exclusion chromatography. The MonoQ fractions containing BEI protein were pooled and loaded onto a Sephacryl S300 column. Fractions were concentrated and then probed with αBEI. The elution points of molecular weight standards chromatographed under identical conditions are indicated. Standards were as follows: 232 kDa; aldolase, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsin.

CHAPTER 5. GENERAL CONCLUSIONS AND SUMMARY

The overall goal of this research was to investigate the functions of branching enzymes I, IIa and IIb in maize in the determination of amylopectin structure. The biosynthesis of starch, a homopolymer of α-D-glucose, involves the actions of multiple enzymes activities, and the multiple isofoms of branching enzymes have been evolutionary conserved in plants. This conservation suggests that each isoform has a specific function required for starch biosynthesis. To address the significance of multiple isoforms of branching enzymes, the first question this research asked was what, if any, are the differences in enzymatic properties of branching enzymes IIa and IIb? Branching enzymes IIa and IIb had not been previously separated from each other and/or BEI and this was a major goal of this research. Using chromatographic techniques, BEIIa and BEIIb were separated from the other two branching enzyme isoforms in maize endosperm and their activities on amylose were analyzed. This research observed no differences between the two BEII isoforms in their capacities to modify amylose. A difference in branching amylose was observed between native BEIIa and purified BEIIa suggesting the activities of the branching enzymes are modified *in vivo*. Taken together, this research suggests that the evolutionary conservation of the branching enzyme isoforms in plants is not attributed to distinct enzymatic properties, specifically in regard to product specificity.

The second question this dissertation investigated was the involvement of maize branching enzymes in multi-subunit complexes (MSCs). There is an abundant amount of circumstantial evidence suggesting that starch biosynthetic enzymes are involved in protein-protein interactions, however, there was very little direct evidence. The most significant

contribution of this research was the direct evidence that BEIIa and SSI are involved in at least one multi-subunit complex (MSC). Evidence also supports the involvement of SSIII as a possible component in MSCs with BEIIa and SSI. This research also contributed indirect evidence supporting the involvement of BEs in MSCs. Gel permeation chromatography revealed that BEIIb might be a component of an MSC. Multiple electrophoretic mobility red bands were identified to all possess the same catalytic subunit, BEI. This research found the activity and mobility of the various electrophoretic bands of BEI were unaffected by dephosphorylation. Recent research, however, found that not only was the activity of BEI regulated by phosphorylation but also that the ability of BEI to remain in complex with other starch biosynthetic proteins was dependent upon its phosphorylation state (Tetlow et al. 2004a). The findings of this dissertation research do not rule out the possibility that other post-translational modifications could regulate the multiple electrophoretic forms of BEI. Another possible explanation for the multiple electrohporetic forms of BEI is that they could represent BEI in different conformation in various MSCs. Lastly, pleiotropic effects in branching enzyme mutants revealed that two SS activities were affected when BEIIa was mutated, suggesting that BEIIa interacts with SSs.

The third question this dissertation investigated was what branching enzyme isoforms are present in maize leaves. This dissertation research has shown that two branching enzyme isoforms, BEIIa and BEI, are present in maize leaves. These findings are in agreement with previous research that reported that BEI mRNA was expressed in maize leaves (Gao et al. 1996). The BEI protein found in maize leaf was found to be similar to endosperm BEI in molecular weight, approximately 80 kDa. Partial purification revealed leaf BEI to be a

monomeric protein in denaturing conditions and possessed multiple electrophoretic mobility forms, similar to endosperm BEI.

The results of this research lead to specific suggestions for follow-up studies. This research found no enzymatic differences, with respect to product specificity, between BEIIa and BEIIb that could explain the significance behind the evolutionary conservation of the two BEII isoforms in monocots. Other areas need to be explored in order to understand the significance of having two highly homologous proteins, which appear to contribute similar chain lengths to the final starch structure. Using a recombinant BEIIa to compare and contrast activities with native BEIIa and native BEIIb, data revealed that *in vivo* factors could be affecting BE activity. Attempts to express recombinant BEIIb for this research were not successful, however exploring other options to produce soluble recombinant BEIIb that can used and eventually its activites compared to purified native BEIIb. Investigation of potential *in vivo* factors, e.g. ligands, hormones, and other proteins and how they interact BE activity would be beneficial in understanding how the branching enzyme isoforms function in starch biosynthesis.

This research contributed direct and indirect evidence supporting the involvement of branching enzymes in multi-subunit complexes. BEIIa and SSI are directly involved in at least one MSC, and SSIII is most likely another component in the MSC with BEIIa and SSI. Results from the coIP experiments using showed weak bands of BEIIb possibly interacting with BEIIa, SSI and SSIII. An isoform-specific polyclonal antisera specific for maize endosperm BEIIb needs to be produced and used in coIP experiments to determine which MSCs, if any, BEIIb is a component. This research did not observe affects of dephosphorylation on BE activity, however BE activity might be regulated by other post-

translational modifications, and could investigated further using mass spectrometry, e.g. MS/MS. Of particular interest would be MSC purification, determining the stoichiometry of the components of the MSCs, and learning the identity(s) of any other possible constituents of that MSC. In the long term, it will be necessary to learn the functional consequences of the participation of each enzyme in the complex, and how those potential regulatory effects may determine specific architectural features of the amylopectin polymers produced by the coordinated action of the enzymes.

This dissertation research, along with previous research, confirmed that two branching enzyme isoforms, BEI and BEIIa, are responsible for branching amylopectin in transitory starch (Gao et al. 1996). The data from this research along with previous analyses of *sbe1*$^-$ and *sbe2a*$^-$ plants show definitively that BEI alone and BEIIa alone is able to provide the enzyme activity needed for formation of semi-crystalline starch granules (Blauth et al. 2001; Blauth et al. 2002). Further investigation of leaf BEI needs to include a comparison of the sequences of endosperm BEI and leaf BEI to determine their similarity. Distinctions between the BEI enzyme in leaf and endosperm also needs to be investigated as possible explanations for the functional differences between storage starch and transitory starch.

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