Title: Identification of Multiple Phosphorylation Sites on Maize Endosperm Starch Branching Enzyme IIb, a Key Enzyme in Amylopectin Biosynthesis

Running title: Phosphorylation of Starch Branching Enzyme IIb

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TITLE: Identification of Multiple Phosphorylation Sites on Maize Endosperm Starch Branching Enzyme IIb, a Key Enzyme in Amylopectin Biosynthesis


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2Abbreviations used are: ADP-Glc, ADP-glucose; Ala, alanine; Arg, arginine; bp, base pairs; CPK, Corey, Pauling, Koltun; DBE, debranching enzyme; DP, degree of polymerization; EGTA, ethylene glycol tetraacetic acid; FPLC, fast protein liquid chromatography; GBSSI, granule-bound starch synthase; GH, glycoside hydrolase family; Glc1P, α-D-glucose 1-phosphate; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MD, molecular dynamics simulation analysis; MS, mass spectrometry; NDP, nucleotide diphosphate; Pro, proline; RP-HPLC, reverse-phase high-performance liquid chromatography; SBE, starch branching enzyme; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; Ser, serine; SP, starch phosphorylase; SS, starch synthase; Thr, threonine; Tyr, tyrosine
Capsule

**Background:** Starch is the major component of cereal yield, yet the biochemical regulation of its synthesis is poorly understood.

**Results:** Starch branching enzyme IIb is phosphorylated at three sites by two Ca\(^{2+}\)-dependent protein kinases.

**Conclusion:** Two phosphorylation sites represent a general mechanism of control in plants, the third is cereal specific.

**Significance:** Identification of post-translational regulatory mechanism offers possibilities for targeted manipulation of starch.

SUMMARY

Starch branching enzyme IIb (SBEIIb) plays a crucial role in amylopectin biosynthesis in maize endosperm by defining the structural and functional properties of storage starch and is regulated by protein phosphorylation. Native and recombinant maize SBEIIb were used as substrates for amyloplast protein kinases in order to identify phosphorylation sites on the protein. A multi-disciplinary approach involving bioinformatics, site-directed mutagenesis and mass spectrometry identified three phosphorylation sites at Ser residues; Ser\(^{649}\), Ser\(^{286}\) and Ser\(^{297}\). Two Ca\(^{2+}\)-dependent protein kinase activities were partially purified from amyloplasts, termed K1, responsible for Ser\(^{649}\) and Ser\(^{286}\) phosphorylation, and K2, responsible for Ser\(^{649}\) and Ser\(^{297}\) phosphorylation. The Ser\(^{286}\) and Ser\(^{297}\) phosphorylation sites are conserved in all plant branching enzymes and are located at opposite openings of the 8-stranded parallel β-barrel of the active site which is involved with substrate binding and catalysis. Molecular dynamics simulation analysis indicates that phospho-Ser\(^{297}\) forms a stable salt bridge with Arg\(^{665}\), part of a conserved Cys-containing domain in plant branching enzymes. Ser\(^{649}\) conservation appears confined to the enzyme in cereals and is not universal, and is presumably associated with functions specific to seed storage. The implications of SBEIIb phosphorylation are considered in terms of the role of the enzyme and the importance of starch biosynthesis for yield and biotechnological application.
INTRODUCTION

Starch is a water-insoluble polyglucan, providing higher plants and green algae with an osmotically inert carbon store that is adapted for both short-term storage (e.g. over a diurnal cycle in leaves), or longer-term for the next generation (e.g. storage starch of seed endosperms). From a nutritional perspective, starch is the major caloric component of human and many livestock diets, and its varied physical properties are exploited for numerous industrial purposes. In common with other polysaccharides in Nature, starch is composed of linear α-(1→4)-O-linked glucan chains and α-(1→6)-O-linked branches; the organized positioning and frequency of branch points is a distinguishing feature of starches, and contributes to their water-insolubility (1, 2). α-(1→4)-O-linked glucan chains are formed by NDP-glucose-dependent transferases; in plants these are multiple isoforms of glucan chains being found in amylopectin clusters (6). Many starch metabolism have been characterized in this date, none of the regulatory proteins involved in biosynthesis in plants is the identification of amylopectin-synthesizing enzymes in protein complexes (19-21) within starch-synthesizing plastids, and the important regulatory role played by protein phosphorylation. The SBEII class forms a functional trimeric protein complex withSSI and SSIa, and this complex has been implicated in amylopectin cluster biosynthesis (20, 22). The components of the trimeric protein complex eventually become entrapped within the starch granule through the glucan-binding capacity of SSIa (22). In addition, SBEIIb has also been detected in a protein complex with SBEI and starch phosphorylase (SP, E.C. 2.4.1.1) (19). Assembly and disassembly of a number of heteromeric protein complexes involved in amylopectin biosynthesis is regulated by protein phosphorylation (19, 20, 23). In the trimeric protein complex found in cereal endosperm amyloplasts SBEIIb is phosphorylated. In addition un-complexed SBEIIb is also found phosphorylated in the plastid stroma (19, 23, 24). The catalytic activity of SBEII forms in monocot plastids has also been shown to be modulated by the enzyme’s phosphorylation state (19). Regulation of the amylopectin synthesis pathway (and of heteromeric protein complexes) by protein phosphorylation is axiomatic of a carefully controlled metabolic process, ultimately impacting the carbon budget of the plant.

A prerequisite to understanding the complex signal transduction system regulating amylopectin biosynthesis in plants is the identification of phosphorylation sites on identified phosphoproteins in order to determine the effects of phosphorylation on target proteins and protein complexes, and identify protein kinases and protein phosphatases involved. To date, none of the regulatory proteins involved in starch metabolism have been characterized in this
manner. This paper reports on the identification of multiple Ser phosphorylation sites on SBEIIb, a key enzyme in amylopectin biosynthesis, and component of a number of heteromeric protein complexes whose activity influences the functional properties of starch. Two distinct Ca²⁺-dependent protein kinase activities have been partially purified which show differential specificity for Ser residues on SBEIIb.

**EXPERIMENTAL PROCEDURES**

Plant Material and Isolation of Amyloplasts from Developing Endosperm- Maize plants (a common maize inbred background termed CG102) were grown in a field in Guelph, Ontario and tagged at pollination. Whole cobs were harvested 20-25 days after pollination and used to prepare amyloplasts within 4h of harvest. Maize endosperm amyloplasts were isolated using a modification of the methods described by Tetlow et al. (25). Plastids were osmotically lysed in a buffer containing 100mM Tricine/KOH, pH 7.8, 1 mM Na₂-EDTA, 1 mM dithiothreitol (DTT), 5 mM MgCl₂, and a protease inhibitor cocktail (ProteCEASE (G-Biosciences) used at 10 μl per cm³) and stored at -80°C until future use. Stromal proteins were separated from membranes and other particulate material by high speed centrifugation according to previously described methods (25). In general 5ml of amyloplasts (1-2 mg protein per ml) was yielded from 1 maize cob.

Cloning, expression and purification of recombinant SBEIIb and its truncation products- The plasmid vector (pET 29, Novagen Cat. No. 69871-3) containing SBEIIb (amino acids 62-799 referring to its full length cDNA sequence, GenBank accession no. L08065) with fused N-terminal S-tag was kindly provided by Dr. Alan Myers (Iowa State University, USA). Various segments of the coding sequence of SBEIIb and its truncation products- the SBEIIb cDNA were PCR amplified from the pET 29 vector. PCR reactions were set up to produce fragments of different length, and products used to create truncated versions of recombinant SBEIIb. Individual forward primers were designed to initiate amplification at bp 385, 642 and 880 of the SBEIIb cDNA sequence. The sequences of the respective primers were: 5’TTCCATGGCTCAAGGCTATAAGTACC3’, 5’TTCCATGGCTCTGCTAACAATGC3’, 5’GGCCATGGCTCGGAATGAGTAGC3’. A sequence 5’AGGTCGACTCACTCCACTGGAGCATAG’ at 2400 bp was used as a forward primer for all three fragments resulting in N-terminal truncated versions of SBEIIb: 128-799aa (ΔN1), 214-799aa (ΔN2) and 291-799aa (ΔN3) (Fig. 1). Three reverse primers were designed to amplify PCR products at bp 2196, 1935 and 1683. The sequence of the respective primers was: 5’TACGTCGACTCAGCTGTGGTGACAGT3’, 5’GGGTCGACTCATTGCGACCTTTG3’, 5’CCGTCGACTCATTTCTCAAACCACACT3’. The same forward primer 5’GATCTTCCCATGTTCTGAGG3’ at 184 bp was used for all three fragments resulting in C-terminal truncated versions of SBEIIb: 62-732aa (ΔC1), 62-646aa (ΔC2) and 62-562aa (ΔC3) (Fig. 1). All forward primers create a Nco1 restriction site (underlined), while all reverse primers create a Sal1 restriction site (underlined). All reverse primers create stop codon (bold). PCR was performed in a 50μl solution that contained 50 ng of plasmid, 125 ng each of forward and reverse primers, 1 μl Pfu Turbo polymerase (Stratagene Cat. No. 600250), 5μl of 10 x reaction buer, and 1μl of a 25 mM stock solution of all four deoxyribonucleoside 5’triphosphates (Invitrogen Cat. No. 18427013). The PCR conditions were: 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR products were digested with Nco1 and Sal1 restriction enzymes, purified on a 1% agarose gel and extracted using a Gel Extraction Kit (Qiagen Cat. No.28704 ). These fragments were ligated into the pET29 vector that had been digested with Nco1 and Sal1. The resulting pET29 vector constructs with various inserts were transformed and amplified in *Escherichia coli* XL1-Blue competent cells (Stratagen Cat. No.200249) and sequenced to confirm synthesis of the correct constructs. Recombinant plasmids containing full-length and truncated versions of SBEIIb were transformed into ArcticExpress competent cells (Stratagene Cat. No. 230193) and proteins expressed by inducing with 1mM isopropyl-D-thiogalactopyranoside (IPTG) at 10°C, 250rpm for 24h. *Escherichia coli* (ArcticExpress) cells were collected by centrifugation and lysed using ‘BugBuster Protein Extraction Reagent’ (Novagen Cat. No.70584-4). Recombinant proteins were purified from inclusion bodies using a Protein Refolding Kit (Novagen Cat. No.70123) according to the manufacturer’s instructions. Recombinant proteins were further purified by size-exclusion chromatography according to previous protocols (22) in order to separate catalytically active monomers from inactive aggregates. Monomeric (non-aggregated) forms were used in all experiments. Recombinant proteins were stored either at -20°C for...
1 month or at -80°C in 40% (v/v) glycerol for 2-3 months and catalytic activity was monitored by zymogram (in gel assay) before experimental work according to Liu et al. 2009 (23).

Site-directed mutagenesis- The QuikChange site-directed mutagenesis kit (Stratagen, Cat. No. 200518) was used to make point mutations in the recombinant SBEIIb. Serine residues were replaced by alanine in the multiple sites: Ser¹⁴⁷, Ser²⁰⁴, Ser²⁸⁶, Ser²⁹⁷, Ser²⁹⁸, Ser⁵⁶⁸, Ser⁵⁹⁸, Ser⁶⁴⁹, Ser⁶⁵⁹, Ser⁶⁹⁹ and Ser⁷⁰⁵. Double and triple mutants were created by replacing Ser residues by Ala in the Ser⁶⁴⁹ recombinant mutant protein. The QuikChange site-directed mutagenesis was performed using PfuTurbo DNA polymerasell 2.5U per 50µl of reaction mix which also contained 5 µl of 10x buffer from the kit, 50 ng of vector, 125 ng of each forward and reverse primers, 1µl of 50mM MgSO₄ and 1µl of 10mM of dNTP mix (Invitrogen). Cycling parameters were: 18 cycles, 95°C - 30 sec, 55°C - 1 min, 68°C – 12 min followed by the final extension at 72°C for 10 minutes. PfuTurbo DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. A supercoiled double-stranded DNA vector pET 29 with an insert of SBEIIb and a pair of oligonucleotide primers for each mutant containing the desired mutation were utilized. The coding sequence for Ser was substituted for coding sequence for Ala in each set of primers. The primer sequences used for site-directed mutation of SBEIIb were as follows:

5'CTCTATAGAAAGAATCGCTGCAAGACATTGAT GAAC3'-Forward,  5'GTTCATCAATGTCTGACCGGATTCTCATA GAG3'-Reverse for Ser¹⁴⁷,  5'CCAAATGCACTCATGCTGGCCAAAATGAG 3'-Forward and  5'CTCATTTTTGGCCATACATCTGCATTGGG3' -Reverse for Ser²⁰⁴,  5'CTCAAACGGACAAAAAGCATTCGGATATAT G3'-Forward and  5'CATAATCCGAATGTTTGGTCTGTTAGG 3'-Reverse for Ser²⁸⁶,  5'GAACTGCTAGCCGGAACCAGAGATAAAC AC3'-Forward and  5'GTTTATATTTTCTGCATTCGGGTG 3'-Reverse for Ser²⁹⁷,  5'GGAATGAGTCCGGGAACCAGAGATATAA CAC3'-Forward and  5'GTTTTATCTTCCGTCCGGGGCACTCATTC C3'-Reverse for Ser²⁹⁸,  5'GTTAACTTATGCTGAAAGCTCATGATCAAG C3'-Forward and  5'GCTTGATCATGAGCTTCAGCATAAGTTACA C3'-Reverse for Ser⁵⁶⁸,  5'CCCTCGATAGCCTACAACCTCCTACCATGATG 3'-Reverse for Ser⁵⁹⁸,  5'CGAAAGACTCTCCAGCTGTAAGTTATCC C3'-Forward and  5'GGAATACCTTACCAGCTGGTAAGTTATCC G3'-Reverse for Ser⁶⁴⁹,  5'CCAGGGAAATAAACCAGCTTATGACAAATGG 3'-Forward and  5'CGACATTGTCTAAGCTGTTGTTATCCCTG 3'-Reverse for Ser⁶⁵⁹,  5'GAACTCATGACAGCTGACACCAGATATTCC CCC3'-Forward and  5'GGAAATATATCTGGTACGCTGTCGTAGA ATTC3'-Reverse for Ser⁶⁹⁹,  5'CCAGCATTATATTGCCGGAAACATGAGGAG G3'-Forward and  5'CCTCCTCATGTCTTCCGCAATATACTCTGTC 3'-Reverse for Ser⁷⁰⁵ (mutated nucleotides are in bold and codons for Ala are underlined). Incorporation of the oligonucleotide primers generated a mutated plasmid containing staggered nicks. Following temperature cycling, the product was treated with 1µl Dpn I (10U/µl) per 10µl of PCR product, at 37°C for 2 h. The Dpn I endonuclease (target sequence: 5´-Gm6ATC-3´) is specific for methylated and hemi-methylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all Escherichia coli strains is dam methylated and therefore susceptible to Dpn I digestion. 4µl of nicked vector DNA incorporating the desired mutations was then transformed into 50µl of Epicurian Coli® XL1-Blue competent cells. 200µl of each transformation reaction was plated on LB–ampicillin agar with 20 µl of 10% (w/v) X-gal and 20 µl of 100 mM IPTG. Plates were incubated at 37°C for 16-18 h. White colonies were screened for mutated sequences, and isolated DNA samples from white colonies were sequenced to confirm the mutation sites. Samples of the pET 29 vector containing inserts of SBEIIb mutants were transformed into Arctic Express cells and expression and purification of mutant recombinant proteins was carried out as for expression and purification of recombinant SBEIIb described above.

Enzyme and Protein Assays and Kinetic Studies- SBE activity was assayed indirectly by stimulation of
incorporation of $^{14}$C from [U-$^{14}$C] α-D-glucose 1-phosphate (Glc1P) into glucan by glycogen phosphorylase $\alpha$ according to methods previously described in (20) and also by native-PAGE zymograms according to the methods reported previously (23). SBEIIb kinase activity was determined by direct phosphorylation of recombinant maize SBEIIb (see below) and using a synthetic peptide kinase assay. In vitro peptide kinase assays were performed as previously described (26) with reaction conditions for SBEIIb kinase optimized for all peptides; all reactions were linear with respect to time and protein content (data not shown). Assay mix (25 µl) containing 50mM Tris-HCl (pH 7.0), 1mM DTT, 200µM of $[^{32}$P]$-\text{ATP}$, 5mM MgCl$_2$ 200 µM of synthetic peptide and 10µl of amyloplast lysate or partially purified kinase was incubated at 25°C for 10 min. Following incubation the reaction was terminated by removing 15µl of the reaction mixture and pipetting onto 2cm$^2$ of phospho-cellulose P-81 paper (Whatman) and allowing the sample to soak into the paper. The phospho-cellulose paper was washed 3 times (5min/wash) by swirling in 0.5 L of 1% (v/v) orthophosphoric acid, followed by a final wash in 0.5L of acetone. P-81 paper was dried on paper towels and placed in scintillation vials with 4 cm$^3$ Ecoscint (Dia-Med) and radioactivity quantified using a Beckman Coulter LS6500 Multi-Purpose liquid scintillation counter. Protein concentrations were determined using the Coomassie Blue G-250 dye binding method using bovine γ-globulin (Sigma) as the protein standard (27).

**Synthetic peptides**- Synthetic peptides based on amino acid sequences of SBEIIb were purchased from CanPeptide Inc. (Pointe-Claire, Québec, Canada) and used as substrates for in vitro protein kinase assays (peptide sequences are summarized in Table 1). Each peptide contained an additional three Arg residues at the C-terminus in order to bind to the phospho-cellulose paper.

In vitro phosphorylation of recombinant SBEIIb and amyloplast stromal proteins- In vitro phosphorylation of recombinant proteins was performed by two methods: either phosphorylating recombinant proteins followed by attaching them to S-protein agarose beads (Novagen) or by attaching recombinant proteins to S-agarose beads first prior to phosphorylating them; in both cases using amyloplast lysates as a source of protein kinase. For the first method 20µg of monomeric SBEIIb was incubated for 30 min with 100 µl of amyloplast lysate (1-1.5 mg protein /ml) or partially purified protein kinase in the presence of 0.1 mM $[^{32}$P]$ \text{-ATP}$ (0.5µCi, Perkin-Elmer), 10µl/ml protease inhibitors (Plant ProteaseArrest) and 10µl/ml protein phosphatase inhibitors (PhosphataseArrest, G-Biosciences) in a total volume of 135 µl, then 100 µl of 50% (w/v) S-agarose slurry was added to each sample and the mixture incubated for one hour in order to bind recombinant SBEIIb to the beads. In the second method 20 µg of monomeric recombinant SBEIIb was incubated with 100µl of 50% (w/v) S-protein agarose slurry at room temperature on a rotator for 1 hour in the presence of protease inhibitors (10 µl/ml). Unbound proteins were removed by washing with 10ml of buffer A containing, 20mM Tris-HCl (pH 7.8), 0.15M NaCl, 0.1% (v/v) Triton-X100, 1mM DTT. The washed beads containing recombinant SBEIIb were then incubated with a source of protein kinase and $[^{32}$P]$-\text{ATP}$ as for the phosphorylation reactions described above at room temperature with gentle agitation for 30 min. In both experiments reactions were stopped by adding Na$_2$-EDTA to a final concentration of 20mM and unbound proteins and non-incorporated ATP were washed out from samples with 10 ml of wash buffer B containing 50mM Tris-HCl (pH7.5), 150mM NaCl, 0.1% (v/v) Tween-20, 1mM DTT. 50µl of SDS loading buffer was added to each sample and samples were heated at 95°C for 5 minutes prior to SDS-PAGE. Amyloplast stromal proteins (100 µl, 1-1.5 mg protein/ml) were phosphorylated in vitro by incubation with 0.1mM $[^{32}$P]$-\text{ATP}$ (0.5µCi) in a total volume of 135µl. Reactions were incubated at room temperature with a gentle agitation for 30-45 min and terminated by adding Na$_2$-EDTA to a final concentration of 20mM.

**Phosphoamino Acid Analysis**- Phosphorylated SBEIIb was separated by SDS-PAGE and extracted from gels (5 to 10 gel slices per sample), and partial acid hydrolysis of the sample performed in 5.7 N HCl for 1 h at 110°C. The released phosphoamino acids were resolved by 2D thin-layer electrophoresis and autoradiography using methods described previously (28).

**In-gel Trypsin Digestion**- In-gel trypsin digestion of $^{32}$P-labeled SBEIIb was conducted according to methods described previously (19). Silver-stained gel pieces were first washed with 30 µL of 15mM potassium ferricyanide/50 mM sodium thiosulphate followed by successive rinses with deionized water, 50mM ammonium hydrogen carbonate (NH$_4$HCO$_3$) buffer, and acetonitrile. This destaining step was omitted in the processing of Coomassie Blue-stained bands. Cys residues were reduced with 10mM DTT.
and derivatized by treatment with 100 mM iodoacetamide. After further washing with NH$_4$HCO$_3$ buffer, the gel pieces were dehydrated with acetonitrile and dried at 60°C before addition of modified trypsin (10 μl of a solution containing 6.5 ng protein/μl in 25 mM NH$_4$HCO$_3$; Promega, Madison, WI). Digestion proceeded for 1 h at room temperature (to rehydrate the gel slice in the trypsin solution) followed by incubation at 37°C for 16-18 hours, and products were recovered by sequential extractions with 25 mM NH$_4$HCO$_3$, 5% (v/v) formic acid, then acetonitrile. The pooled extracts were lyophilized and dissolved in 0.1% (v/v) formic acid either for MS analysis or for purification of phosphopeptides by reversed-phase high performance liquid chromatography (RP-HPLC, see below).

**Purification of Phosphopeptides by RP-HPLC**

Tryptic digestion products of phosphorylated SBEIIb were separated by RP-HPLC using a Gemini-NX 3μm C$_{18}$ 110A column on an Agilent 1100 Series HPLC pump according to the manufacturer’s instructions. The mobile phase was a gradient of HPLC grade water with 0.1% (v/v) formic acid and acetonitrile with formic acid, which was converted from 95% to 0% water over 38 min. Absorbance at 217 nm was measured to determine peptide elution. Fractions containing $^{32}$P-labelled phosphorylated peptides were determined by Geiger counter and liquid scintillation counting and subsequently analyzed by mass spectrometry (MS).

**MS analyses-** For MS-based analysis of SBEIIb phosphorylation sites the protein was separated by SDS-PAGE and SBEIIb subjected to in-gel digestion prior to MS analysis. Coomassie Blue stain was removed from the isolated bands with water containing 50% acetonitrile (v/v). The samples were washed twice with 100 mM NH$_4$HCO$_3$ and reduced with 10 mM dithiothreitol in 100 mM NH$_4$HCO$_3$ for 30 minutes at 60°C then cooled to 21°C. Cysteine alkylation was performed with 20 mM iodoacetamide in 100 mM NH$_4$HCO$_3$ in the dark for one hour. Excess iodoacetamide was removed with two washes of 100 mM ammonium bicarbonate. The proteins were digested at 37°C using 0.4 μg of endoproteinase Glu-C (Endo-Glu-C, Sigma-Aldrich) per gel band for 16 h, then with 0.4 μg of endoproteinase Lys-C (Endo-Lys-C, Sigma-Aldrich) per sample for 16 h. Gel bands were washed with 100 μl volumes of water then 50% (v/v) acetonitrile, and washes pooled, dried in a centrifugal concentrator, dissolved in aqueous 0.1% (v/v) formic acid and purified using C$_{18}$ Zip Tips (EMD Millipore, Darmstadt, Germany). Purified Endo-Glu-C/Lys-C digests were analyzed by online nanoflow HPLC-MS/MS using a NanoLC-Ultra 2D HPLC pump coupled to a Nanoflex chHiPLC system and a TripleTOF 5600 mass spectrometer (AB Sciex, Concord ON, Canada). Peptides were loaded and washed on a 0.5 mm long, 200 μm diameter trap (AB Sciex) and resolved on a 150 mm long, 75 μm diameter chromatographic column (AB Sciex). The trap and the analytical column packing material consisted of 3 μm diameter C$_{18}$ particles with 120Å pores. Peptide separation was performed using a binary mobile phase gradient. Mobile phases A and B contained 0.1% (v/v) formic acid in water and acetonitrile respectively. The following mobile phase B compositions were used: 2% at 0 min, 35% at 45 min, 80% from 46 to 50 min, 2% from 51 to 60 min. The mass spectrometer was operated in positive ion mode with an electrospray voltage of 2300 V. The MS data acquisition cycle consisted of a single MS scan with a range of 400 to 1250 Th followed by MS/MS scans from 100 to 1800 Th of the most abundant ions detected in the preceding MS scan. The number of MS/MS scans per MS scan was limited to twenty. Peptides and phosphopeptides were identified from the tandem mass spectra using ProteinPilot™ software with phosphorylation emphasis and amino acid substitutions specified in the search parameters. Separate searches of the dataset were conducted using the RefSeq Zea mays library which contained 22444 sequences representing 7089707 residues for peptide identifications (downloaded November 14, 2012).

**Partial Purification of SBEIIb Kinase-** SBEIIb kinase was partially purified from amyloplasts via various column chromatography steps at 4°C using an AKTA Explorer FPLC (Amersham Biosciences). Between 5-10 mg of amyloplast stromal proteins (10-12 ml plastid lysate) were loaded onto a 1ml HiTrapDEAE FF column (GE Healthcare) at a flow rate of 0.5 ml/min. The column was washed with running buffer containing 100 mM Tricine-NaOH (pH 7.5), 7.5mM MgCl$_2$, 1mM DTT until the A$_{280}$ had returned to baseline and SBEIIb kinase activity was eluted using a 25 ml linear gradient from 0 to 1M KCl in running buffer. Catalytically active fractions were determined using both the peptide kinase assay and phosphorylation of recombinant SBEIIb. Active fractions were pooled, desalted on a PD-10 column (Amersham Biosciences) and loaded onto a 1ml HiTrap Blue HP column (GE Healthcare). The column was washed with running buffer until the A$_{280}$ had returned to baseline then SBEIIb kinase activity
Electrophoresis and Immunoblotting- Protein samples were separated on 10% SDS PAGE and Western blot analyses performed using methods described previously (19). Proteins were identified by cross-reaction with either anti-SBEIIb or anti-S-tag antibodies (AbCam Cat. No. ab24838) in dilution of 1:5000 or 1:3000 respectively. Peptide-specific polyclonal rabbit antisera targeted to maize SBEIIb was prepared and purified according to methods described by Liu et al. (23)

Homology modeling of SBEIIb- The structural and sequence homologs of maize SBEIIb were identified using TM-align program (29) and Blastp (30) respectively. In this analysis, only the mature protein sequence (coding for 738 amino acids) was used and the 61 amino acid N-terminal transit peptide region was not considered. Sequence alignment was performed using MAFFT, Tcoffee-EBI, and ClustalW (http://www.ebi.ac.uk/Tools/msa/). Maize SBEIIb structure prediction was carried out using six tools: CPPhmodels3,4 (31), ESyPred3D5 (32), SWISS-MODEL Workspace6 (33), Robetta Beta7 (34), I-TASSER8,9 (35) and Phyre 2.010 (36). These tools used one or a combination of homology, ab initio, or threading/ fold recognition modeling methods. Out of these six, the homology structure produced by I-TASSER was chosen for subsequent MD analysis.

Molecular dynamics simulations of SBEIIb- Molecular dynamics simulation of the maize SBEIIb homology structure was performed using the GROMACS software package (37) version 4.5.5 and the Gromos96 ffG53a6 force-field (38). This force-field was parameterized to recognize phosphorylated Ser residues, as was previously done for the Gromos96 forcefield (http://www.gromacs.org/Downloads/User_contributions/Force_fields), with partial charges and Van der Waals values for the atoms in the phosphate group obtained from Hansson et al. (39). All simulations were run on the Compute Canada/SHARCNET high performance computing cluster (www.sharcnet.ca). Eight different SBEIIb structural models were used in these experiments: (i) unmodified SBEIIb, (ii) SBEIIb phosphorylated at Ser286 (SBEIIb_phos286), (iii) SBEIIb phosphorylated at Ser297 (SBEIIb_phos297), (iv) SBEIIb phosphorylated at Ser649 (SBEIIb_phos649), (v) SBEIIb doubly phosphorylated at Ser286 and Ser297 (SBEIIb_phos286_297), (vi) SBEIIb doubly phosphorylated at Ser286 and Ser649 (SBEIIb_phos286_649), (vii) SBEIIb doubly phosphorylated at Ser297 and Ser649 (SBEIIb_phos297_649) and (viii) SBEIIb triply phosphorylated at Ser286, Ser297 and Ser649 (SBEIIb_phos286_297_649). In all cases, the appropriate Ser residues were phosphorylated using SYBYL-X 1.3 molecular modeling suite (SYBYL, Tripos Associates, St. Louis, MO) and the charge on each phosphate group was -2. Each model was prepared in an identical way for molecular dynamics simulation. The protein was centered in a virtual cubic box, allowing at least a 1 nm gap between the protein and the edge of the box. The box was solvated with water molecules using the spc216 water model (40), and ions Na+ and Cl- ions were added such that the final salt concentration was 0.15 M and the overall net system charge was neutral. This solvated and neutralized system was energy minimized using the steepest decent minimization algorithm to a maximum overall force of <1,000 kJ mol⁻¹ nm⁻¹, and this was followed by two 100 ps steps in which the system was equilibrated at 298 K and 1 bar whilst the protein position was restrained. Molecular dynamics simulation were then carried out for a total of 100 ns utilizing velocity rescaling with a stochastic term for temperature coupling (41) and Berendsen isotropic pressure coupling (42). The trajectories were visualized using VMD (43) version 1.9.1 and analysis was done using a number of GROMACS utilities.

Statistics- Data were analysed using the Student’s t test, and deemed significant at p < 0.05.

RESULTS

32P-labelling of N- and C-terminal truncations of recombinant maize SBEIIb

Previous in vitro 32P-labelling studies in maize endosperm amyloplasts have shown that SBEIIb is rapidly phosphorylated by a plastidial protein kinase activity (24). Immobilized recombinant SBEIIb was also readily phosphorylated in the presence of amyloplast lysates, a source of protein kinase, and [γ32P]-ATP (Fig. 1B) and formed the basis for phosphorylation experiments with truncated forms of SBEIIb (see below). Phosphoamino acid analysis of both native and recombinant forms of phosphorylated SBEIIb indicated that phosphorylation occurred on one or more serine (Ser) residues (data not shown).

In order to locate the approximate positions of phosphorylation sites on the mature protein (i.e. the...
form lacking the putative transit peptide predicted to be in amyloplasts) a series of N- and C-terminal truncations of recombinant maize SBEIIb was produced (Fig. 1A) and heterologously expressed in E.coli and used as substrates for SBEIIb kinase(s) present in amyloplasts. The recombinant maize SBEIIb used in these experiments was the mature form, lacking the 61-amino acid transit peptide. The truncations were designed to give maximum coverage of the mature sequence of SBEIIb, although none of the truncated products were catalytically active (data not shown). Fig.1B shows no loss of phosphorylation of SBEIIb with the ΔC1 truncation when compared to the full-length, mature protein, but almost complete loss of 32P-labelling in the ΔC2 and ΔC3 truncations.

Site-Directed Mutagenesis of Putative Ser Residues in the C-terminus

Results from 32P-labelling of the C-terminal truncations of recombinant maize SBEIIb (Fig.1B) suggest that the phosphorylation site(s) responsible for most of the 32P-labelling are present in the C-terminus, specifically from amino acid residues 646-732. A series of site-directed mutants of SBEIIb were produced in which all Ser residues within this C-terminal region were individually mutated to Ala, and the resulting recombinant proteins treated with [γ-32P]-ATP and amyloplast lysates as a source of protein kinase (Figure 2B). The results show that the Ser649→Ala mutation caused substantial loss of 32P-labelling compared to the wild-type protein, whereas other Ser→Ala mutations in the C-terminus caused no loss in labelling (Figure 2B). Loss of the Ser649 residue and its replacement by Ala caused no measurable change in the catalytic activity of the mutant SBEIIb compared to the wild-type protein as determined by phosphorylase a-stimulation assay and zymogram analysis (data not shown).

It is notable, however, that the Ser649→Ala mutation showed some residual phosphorylation at one or more other sites. Because phosphorylation of Ser649 appears to account for much of the radiolabelling of SBEIIb in vitro, we therefore mutated additional candidate Ser residues in the Ser649→Ala recombinant protein. Fig. 2D shows that mutation of Ser286 or Ser297, in this background, caused further reduction in 32P-labelling of the recombinant proteins. Notably, mutation of the adjacent Ser residue, Ser298, did not reduce the labelling of SBEIIb further. Phosphorylation of SBEIIb was not detectable in the Ser649-Ser286-Ser297 triple mutant (Fig. 2D).

32P-labelling of native and recombinant SBEIIb is inhibited in the presence of a synthetic peptide containing Ser649

The data in Fig. 2 show that phosphorylation of Ser649 is responsible for most of the 32P-labelling of SBEIIb when amyloplast lysates are incubated with the recombinant protein in the presence of [γ-32P]-ATP. A 19-amino acid synthetic peptide was generated containing the amino acid sequence surrounding Ser649 which also included three C-terminal Arg residues to allow the peptide to be employed as a protein kinase substrate in in vitro assays (26) (see below). The peptide was also used as an antagonist to the protein kinase activity responsible for SBEIIb phosphorylation (Fig. 3). Phosphorylation of both recombinant maize SBEIIb and the native protein in amyloplast lysates was strongly inhibited by micromolar concentrations of the Ser649-synthetic peptide (Fig. 3). Fig.3B shows that many amyloplast stromal proteins are phosphorylated following 30-min incubation with 0.1 mM [γ-32P]-ATP, but notably only phosphorylation of SBEIIb is inhibited by the Ser649-synthetic peptide.

Partial purification of SBEIIb protein kinase activity shows SBEIIb is phosphorylated at multiple sites by more than one plastidial protein kinase

The protein kinase(s) responsible for phosphorylation of SBEIIb were partially purified from amyloplast stromal preparations using anion exchange (DEAE-sepharose) and Blue-sepharose chromatography columns. SBEIIb protein kinase activity was assayed using the Ser649-synthetic peptide (PRGPQLPSGKFIPGR) and recombinant SBEIIb proteins as substrates, and eluted as a single activity peak from the DEAE column in 0.28-0.45M KCl (data not shown). Following binding and elution from a Blue-sepharose column, the SBEIIb kinase-active fraction resolved into two peaks of protein kinase activity designated K1 and K2 (Fig. 4). SBEIIb kinase activity in the eluted fractions was measured using 32P-labelling of recombinant maize SBEIIb and the Ser649-synthetic peptide assay (Fig. 4); both assays were in agreement in terms of their detection of SBEIIb kinase activity eluting from the different chromatography columns. When using recombinant SBEIIb as a substrate for protein kinase activities eluting from the column, it can be seen that 32P-labelled bands of lower molecular mass are also observed in fractions active for SBEIIb kinase (Fig. 4). These minor bands represent truncated, expressed
forms of the recombinant maize SBEIIb whose identity was confirmed by western blotting, but are not evident from Ponceau-S staining, suggesting they are minor contaminants (Fig. 2C, Fig. 3A). In order to examine the possibility of other phosphorylation sites on SBEIIb, in addition to Ser$^{649}$, we also assayed the Blue-Sepharose column eluant fractions using the recombinant Ser$^{649}$-Ala SBEIIb mutant as substrate for the SBEIIb kinase(s). The elution profile of protein kinase activity eluting from the Blue-Sepharose column using the site-directed mutant lacking the Ser$^{649}$ phosphorylation site was similar to that of the wild-type protein. By contrast, phosphorylation of the Ser$^{649}$-Ala mutant was observed in the fractions corresponding to K1, with very low phosphorylation observed in the fractions corresponding to K2 (Fig. 4B).

Identification of putative phosphorylation sites on SBEIIb

Phosphorylation of the Ser$^{649}$-Ala mutant by the K1 protein kinase fraction (Fig. 4B) indicates that SBEIIb has more than one phosphorylation site. We employed a combination of a bioinformatics-driven approach and MS/MS to identify other phosphorylation sites on SBEIIb. A number of putative SBEIIb phosphorylation sites were predicted by NetPhos2.0, and sites with high probability (P > 0.9) were tested as possible substrates for the SBEIIb kinase(s) using synthetic peptides with triple C-terminal Arg tails as for the Ser$^{649}$ peptide (Table 1). The maximum catalytic activity of the SBEIIb kinase was determined using the various synthetic peptides under optimal conditions previously determined for the Ser$^{649}$ synthetic peptide. Table 1 shows that measurable kinase activity was found with three peptides, Ser$^{649}$, Ser$^{286}$ and Ser$^{297}$ using amyloplast lysates as a source of protein kinase, but others as well. Importantly, the phosphorylation sites on these three peptides, phosphorylated by amyloplast lysates, were also identified in the mature protein using MS/MS (see below). Mutation of either of Ser$^{286}$ and Ser$^{297}$ residues to Ala in recombinant SBEIIb resulted in enzymes with significantly reduced (approximately 4-fold) catalytic activities based on the phosphorlyase $a$-stimulation assay (data not shown).

The three synthetic peptides representing putative phosphorylation sites at Ser$^{649}$, Ser$^{286}$ and Ser$^{297}$ were employed in peptide kinase assays of amyloplast extracts separated by Blue-Sepharose chromatography. Protein kinase activities responsible for phosphorylation of peptides Ser$^{286}$ and Ser$^{297}$ were found, respectively, in K1 and K2 fractions (Fig. 4C). Kinase activity phosphorylating both Ser$^{286}$ and Ser$^{297}$ peptides was noticeably lower than the activity with the Ser$^{649}$ peptide for both K1 and K2 fractions (Fig. 4C). For comparison, and as a control, there is no significant kinase activity towards a synthetic peptide containing Ser$^{147}$ by any fraction. Kinetic analyses of the properties of the two protein kinase fractions K1 and K2 with respect to phosphorylation of the Ser$^{649}$, Ser$^{286}$ and Ser$^{297}$ peptides is summarized in Table 2. Protein kinase activities K1 and K2 showed high specificities for their substrates ($K_m$ values for ATP and synthetic peptides were in the micromolar ranges).

Both kinase activities showed marked inhibition by the protein kinase inhibitor K252a (staurosporin), although K2 was less sensitive to the inhibitor than K1 (Table 2). Catalytic activities of K1 and K2 for the Ser$^{649}$, Ser$^{286}$ and Ser$^{297}$ peptides were dependent on Ca$^{2+}$ ions. Addition of 1 mM Na$_2$-EGTA reduced kinase activities of both K1 and K2 with the Ser$^{649}$ synthetic peptide by up to 400-fold (activities of K1 and K2 in the presence of 1mM Na$_2$-EGTA were 0.4 and 2.6 nmol/mg protein/h respectively, compared with 411 and 358 nmol/mg protein/h in the presence of 50μM Ca$^{2+}$ respectively), and full activity was restored on addition of 5 μM or greater Ca$^{2+}$ ions.

MS/MS analysis of phosphorylation sites on SBEIIb

Phosphorylation at Ser$^{286}$ was detected in trace amounts following K2-treatment and in greater abundance in K1-treated samples by LC-MS/MS. Serine and tyrosine in the Glu-C/Lys-C peptide SLRIYE are both potential phosphosites, however product ion spectra showed serine to be the modified residue (Fig. 5A). A complete y-ion series matched that expected of the unmodified peptide, while all $a$-ions and b-ions present were phosphorylated. A peak at m/z 136.1 was attributed to an immnomion ion of unmodified tyrosine.

The Ser$^{297}$ phosphopeptide was observed exclusively in K2-treated SBEIIb, from which it was identified with 99% confidence by a ProteinPilot search against the whole Zea mays library. The peptide THVGMSSPEPK, which contains the Ser$^{297}$ site, was observed from Glu-C/Lys-C digests in both methionine-oxidized and unmodified forms with retention times between 12 and 13 min into the 60-min chromatographic run. The Glu-C/Lys-C peptides containing Ser$^{297}$ had consistently low LC-MS signals. For instance, an untargeted MS/MS analysis of K2-treated wild-type SBEIIb, resulted in relative signal
intensities of 1 and 8 and 78 for the monoisotopic precursor ions of oxidized THVGMSSPEPK, phosphorylated WIDFPRGPQRLPSGK (Ser\textsuperscript{649}) and non-phosphorylated WIDFPRGPQRLPSGK respectively. The concentrations of these species should be comparable since all originated from a single protein, however signals of the Ser\textsuperscript{649} containing peptide and phosphopeptide were orders of magnitude higher than those of the Ser\textsuperscript{297} containing peptide, suggesting the analytical platform had differential sensitivity for these analytes. Chromatograms of the oxidized and unmodified peptide THVGMSSPEPK were compared to those of other peptides observed in the same runs in order to determine if sensitivity was limited by low HPLC performance. Oxidized THVGMSSPEPK and unmodified THVGMSSPEPK had chromatographic qualities comparable to other analytes. Despite considerable difference in their areas, the peaks of the oxidized Ser\textsuperscript{297}-containing peptide and the unmodified Ser\textsuperscript{649}-containing peptide were 0.2 min and 0.22 min full width at half maximum (FWHM) respectively, suggesting that HPLC performance did not selectively reduce either signal. As evidenced by its early elution time, THVGMSSPEPK is a hydrophilic peptide and may not be effectively retained by the C\textsubscript{18} trap. The grand averages of hydropathy for the Glu-C/Lys-C peptide THVGMSSPEPK and the corresponding tryptic peptide IYETHVGMSSPEPK were -0.945 and -0.764 respectively, indicating both were hydrophilic (44). The low signals of THVGMSSPEPK suggest observation of the corresponding phosphopeptide was unfavored, since MS sensitivity for phosphopeptides is lower than for peptides.

Phosphorylated Ser\textsuperscript{297} was not observed in Glu-C/Lys-C digests of SBEIIb, but was identified by LC-MS/MS of tryptic digests previously fractionated offline by RP-HPLC. Consistent with its higher calculated hydrophobicity, IYETHVGMSSPEPK eluted later than the Glu-C/Lys-C equivalent digest, with a retention time of 21 min. Phospho-IYETHVGMSSPEPK had a retention time of 23 min. The monoisotopic ion of the triply charged tryptic phosphopeptide had a maximum signal of 65000 counts in the precursor ion scan. While the signal may have increased due to C\textsubscript{18} HPLC pre-fractionation, the sensitivity of the LC-MS system for this species was apparently much higher than for the Glu-C/Lys-C equivalent digest.

Potential kinase targets within the peptide IYETHVGMSSPEPK include two Ser, one Tyr and one Thr residue. Product ion spectra showed the phosphate to be absent from all but the Ser\textsuperscript{297} site (Fig. 5B). Abundant a\textsubscript{3}, b\textsubscript{2}, and b\textsubscript{3} ions indicated that tyrosine was not phosphorylated while b\textsubscript{5}, a\textsubscript{6}, b\textsubscript{6} and b\textsubscript{7} ions suggested both Tyr and Thr were unmodified. The Ser closest to the C-terminal of the peptide was observed in an unmodified state within a y\textsubscript{5} ion having a mass accuracy of 0.0037 Da and s/n between 5.5:1 and 11:1. All product ions containing the Ser\textsuperscript{297} site also contained a phosphate. Two series of phosphorylated ions, from y\textsubscript{5} to y\textsubscript{12} and b\textsubscript{11} to b\textsubscript{13}, supported the assignment of the ninth residue within the peptide as the phosphorylation site.

A Ser\textsuperscript{649} phosphopeptide was present in both K1- and K2-treated recombinant SBEIIb samples. A missed Glu-C cleavage was consistently observed around the Ser\textsuperscript{649}site, resulting in a peptide with the sequence WIDFPRGPQRLPSGK, which was identified with 99% confidence in searches against the Zea mays protein library. Low digestion efficiency at the third Asp of the Ser\textsuperscript{649} peptide may be due to the Pro located two residues C-terminal to it, since Pro at this position has been shown to inhibit protease activity (45). A Ser residue is the only potential phosphorylation site within this sequence and MS/MS data acquired from phospho-WIDFPRGPQRLPSGK featured product ions consistent with serine phosphorylation (Fig. 5C). The fragments y\textsubscript{12}, y\textsubscript{13} and y\textsubscript{14}, containing both the C-terminus and the phosphate, were detected with s/n values of 56:1, 150:1 and 29:1 respectively. Six prominent peaks corresponded to fragments having undergone phosphoric acid loss, an energetically favored fragmentation pathway of phosphopeptides. A series of N-terminal fragments which did not contain the Ser were without modifications. Using the combined MS/MS evidence, the presence and location of the phosphate at Ser\textsuperscript{649} in both K1-treated and K2-treated SBEIIb were deduced.

Untreated SBEIIb, as well as protein kinase-treated SBEIIb Ser\textsuperscript{649}→Ala and a SBEIIb Ser\textsuperscript{286}→Ala/Ser\textsuperscript{297}→Ala/Ser\textsuperscript{649}→Ala triple mutant were digested then analyzed by LC-MS/MS under conditions identical to those used for kinase-treated wild-type SBEIIb to further assess the kinase-dependence of the phosphorylation. While unmodified peptides containing Ser\textsuperscript{649}, Ser\textsuperscript{286} and Ser\textsuperscript{297} were observed in the untreated controls, no phosphorylation of these sites was detected in these samples (data not shown).
Modeling and molecular dynamics simulation analysis of SBEIIb

Structural homology modeling and molecular dynamics simulations (MD) were carried out on SBEIIb in order to determine the 3-D location of the phosphorylation sites at Ser^{286}, Ser^{297} and Ser^{649} and to gain insights into the possible effects of phosphorylation on the conformation of the enzyme. SBEIIb is produced as a nuclear-encoded protein with 799 amino acids including a 61-residue N-terminal transit-peptide, which targets it to the plastid before being subsequently cleaved. The mature protein thus has 738 amino acids and it is this sequence that was used in homology modeling. Of all proteins with published structures, starch branching enzyme I (SBEI) from rice (Oryza sativa L.) was identified as the top sequence homolog with 86% identity, as well as the top structural homolog (Protein Data Bank ID: 3AML) (46) with a TM-score of 0.904 from the TM-align program (30). This SBEI structure was also chosen as the top homolog for structure prediction by all six structural prediction tools that we used (see Experimental Procedures). The core and C-terminal regions of all six predicted structures agree quite well, however, there is variability at the N-terminus of the protein likely due to poor sequence alignment between the first 55 N-terminal residues of maize SBEIIb and rice SBEI. Due to this variability observed at the N-terminus, we decided to exclude these 55 residues before structural refinement by MD. In these MD experiments, the I-TASSER (47, 35) structure was used as it was the only one of the six tools which permitted us to assign both deposited structures of SBEI (PDB code: 3AML and 3AMK) (46) as templates for structure prediction.

In the 100 ns MD experiments, most of the global structural refinement occurs within the first 10 ns of the simulation with root mean squared deviation of ~5Å at 10 ns compared to the starting structure. Subsequent to the first 10 ns of the simulation, there is very little global change and the conformation appears to be in a steady state (data not shown). The structure of SBEIIb at the end of the 100 ns simulation consists of three distinct domains: a central domain containing parallel β-strands arranged in a barrel surrounded by α-helices in a (β/α)₈ arrangement, along with N- and C-terminal domains which each fold into an anti-parallel β-sandwich structure (Figure 6A). All three phosphorylation sites are in disordered loop regions and are solvent exposed. Both Ser^{286} and Ser^{297} are close to the opening of the β-barrel on opposite sides.

Single phosphorylation at Ser^{286}, Ser^{297}, and Ser^{649}, as well as double and triple phosphorylation are well accommodated with minimal change in global structure (data not shown). There are extensive salt-bridge interactions between the phosphate group (-2 charge) and several Arg and Lys residues within the protein (Fig. 6B,C,D). These salt-bridges tend to form very early in the simulations (within the first 10 ns) and persist throughout the simulation. The phosphorylated Ser^{297} site was shown to be stabilized by interaction with Arg^{665} (Fig. 6C), part of a conserved Cys-containing region (Fig. 7). Generally, the pattern of salt-bridges observed at each phosphorylation site was very consistent and was unaffected by phosphorylation at any of the other two sites.

DISCUSSION

Branching enzymes were the first class of proteins in the starch biosynthetic pathway shown to be directly regulated by protein phosphorylation (19). However, the role played by protein phosphorylation in regulating and coordinating the many enzymes of starch metabolism is not well understood. The aim of the current work was to identify the phosphorylation sites on maize SBEIIb, the major branching enzyme involved in amyllopectin synthesis, in order to understand how this enzyme’s activities are coordinated with other components of the starch biosynthetic pathway through protein phosphorylation, and to begin to characterize the protein kinases and phosphatases that regulate SBEs.

Phospho-amino acid analysis of HCl-digested maize SBEIIb indicates that the protein is phosphorylated at Ser residues, in common with previous studies with wheat endosperm SBEIIa and SBEIIb (19). Truncated forms of recombinant SBEIIb were helpful in determining the location of the most phosphorylated region of the protein (containing the Ser^{649} site), and bioinformatics was then employed to guide site-directed mutagenesis and biochemical analysis of likely phosphorylation sites. Of the number of probable phosphorylation sites predicted by bioinformatics, likely in vivo phosphorylation sites (i.e. those with scores of >0.9) were selected based on in vitro protein kinase assays using isolated amyloplasts as a source of protein kinase, and site-directed mutagenesis of specific Ser sites to Ala. Mutation of three Ser sites, Ser^{649}, Ser^{286} and Ser^{297} to Ala each resulted in reduced phosphorylation of the mutant recombinant SBEIIb (Fig. 2), making them likely candidate in vivo phosphorylation sites. Since
the phospho-Ser$^{649}$ site accounted for much of the $^{32}$P-label on the native and recombinant proteins following incubation with [γ-$^{32}$P]-ATP and protein kinase, detection of the loss of $^{32}$P-label in Ser$^{286}>$Ala and Ser$^{297}>$Ala mutants was only possible in double and triple mutants containing the Ser$^{649}>$Ala mutation (Fig. 2D). Synthetic peptides containing each of the three putative phospho-Ser phosphorylation sites proved to be effective substrates for plastidial protein kinases at micromolar concentrations (Table 2). Confirmation that the three Ser sites are phosphorylated came from MS analysis of native SBEIib and recombinant SBEIib following treatment with protein kinase and ATP.

Synthetic peptides based on the three phosphorylation sites of SBEIib and the recombinant protein all proved to be effective substrates for measuring and characterizing the plastidial protein kinase activities responsible for phosphorylation of SBEIib. Amyloplast lysates used for phosphorylation experiments and for subsequent purification were highly enriched from other sub-cellular contaminants (typically <0.3% cytosolic contamination and <0.2% contamination from other compartments determined using marker enzyme assays); it is therefore unlikely that protein kinases from compartments other than the amyloplast were responsible for phosphorylation of SBEIib or the synthetic peptides. Two chromatographically distinct protein kinase activities were isolated from amyloplasts which showed distinct substrate preferences for the different synthetic peptides, and presumably the different phosphorylation sites on SBEIib in vivo, and both protein kinase activities showed a dependence on Ca$^{2+}$ ions. Whilst the elution profiles are consistent with the separation of two SBEIib protein kinase activities, this knowledge of yield in cereals, and the resolution of two, site-specific phosphorylation sites on one of the enzymes of starch synthesis (19-24). The formation of multi-enzyme complexes involving the interaction of SBEIib with other enzymes of starch synthesis (19-24). The formation of stabilizing salt-bridges between the phosphate groups and basic residues within disordered loop regions could play an important role in constraining these loops, possibly orientating them for binding interactions. Additionally, Ser$^{286}$, Ser$^{297}$ and Ser$^{649}$ are all on the surface of the protein in highly disordered regions (Fig. 6) making them highly accessible to protein kinases. Although phosphorylation at these sites did not result in major conformational change in SBEIib, phosphorylation will alter the surface charge of the enzyme which could be critical in modulating protein-protein interactions. This is particularly pertinent given previous observations that protein phosphorylation stimulates the formation of stabilizing salt-bridges between the phosphate groups and basic residues within disordered loop regions which could play an important role in constraining these loops, possibly orientating them for binding interactions. Additionally, Ser$^{286}$, Ser$^{297}$ and Ser$^{649}$ which are located on opposite ends of the central catalytic β-barrel could be ideally positioned to interact with a glucan substrate, a process that would undoubtedly be affected by phosphorylation. Studies of the wheat enzyme suggest that enzyme activity is modulated by protein phosphorylation (19). Mutation of Ser$^{286}>$Ala or Ser$^{297}>$Ala caused significant (more than 4-fold) loss in catalytic activity, reinforcing the critical role of these Ser residues in enzyme function. Overall, our modeling and MD studies show that SBEIib has the typical fold of SBEs within the GH13 family and demonstrates that phosphorylation at the three identified sites does not cause major structural change but could play an important role in modulating protein interactions and catalysis.

The results presented constitute direct evidence of specific phosphorylation sites on one of the enzymes associated with the pathway of starch biosynthesis. Given the centrality of the latter to the determination of yield in cereals, and the resolution of two, site-specific, protein kinase activities, this knowledge provides new possibilities for the targeted
manipulation of a major metabolic pathway with potential application for the food and non-food industries in which starch is widely exploited.
REFERENCES


FIGURE LEGENDS

FIGURE 1. In vitro phosphorylation of recombinant SBEIIb and its truncation products. A. Diagrammatic representation of the full-length (F.L.) sequence of maize SBEIIb including the 61-amino acid transit peptide (green) and the range of N- and C-terminal truncation products (ΔN and ΔC, respectively) used as affinity ligands for phosphorylation by amyloplast protein kinase(s). Also shown (orange) are the 4 conserved catalytic domains (I-IV) common to all branching enzymes. B. The mature sequence of maize SBEIIb was expressed in E.coli along with various truncation products, as recombinant proteins with S-tag fusions, which facilitated binding to resin. Equal amounts of immobilized recombinant proteins (5 μg, visualized by Ponceau-S staining, left panel) were incubated with amyloplast lysates (1 mg/ml protein) as a source of protein kinase and 0.1 mM [γ-32P]-ATP for 30 min at 25°C, purified, and separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The right panel is an autoradiograph showing phosphorylation of SBEIIb and its various truncation products following labeling with 32P.

FIGURE 2. 32P-labelling of site specific SBEIIb mutants. Recombinant maize SBEIIb and a series of site-directed Ser>Ala SBEIIb mutants which covered all C-terminal Ser residues from amino acids 646-732 were immobilised on S-agarose beads and incubated with 0.1 mM [γ-32P]-ATP and amyloplast stroma (0.1 mg protein) as a source of protein kinase for 30 min at 25°C. A and C. Immobilized proteins on beads were washed and 5µg separated by SDS-PAGE, electroblotted to a nitrocellulose membrane and visualized using anti-S-tag antibodies. B and D. 32P-labelling of the electroblotted recombinant proteins visualized by autoradiography.

FIGURE 3. Phosphorylation of recombinant maize SBEIIb and native SBEIIb is reduced following incubation with a synthetic peptide containing the Ser649 phosphorylation site. (A) S-agarose-immobilized recombinant maize SBEIIb (5μg) was incubated with 0.1 mM [γ-32P]-ATP, amyloplast stroma (0.1 mg protein) and increasing concentrations of the peptide containing the Ser649 SBEIIb phosphorylation site (PRGPQRLPSGKFIPGNRRR). Immobilized recombinant protein was washed, separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane and visualised using anti-S-tag antibodies (left panel) or 32P-labelling visualised by autoradiography (right panel). Fig. 3B shows 32P-labelling of amyloplast stromal proteins (same conditions as in Fig. 3A) with increasing concentrations of the Ser649 peptide; left panel, Ponceau-S-stained proteins following electro blotting and right panel, autoradiograph of the corresponding blot. Solid arrow on autoradiograph shows native SBEIIb (previously identified by immunoblotting with anti-SBEIIb antibodies) and open arrow indicates migration of the phosphorylated Ser649 peptide.

FIGURE 4. Partial purification of SBEIIb protein kinase and separation of activities responsible for phosphorylation of peptides representing Ser649, Ser286 and Ser297 phosphorylation sites on SBEIIb. Following separation of SBEIIb kinase activity in amyloplast lysates by DEAE-sepharose chromatography, active fractions were desalted and loaded onto a Blue-sepharose column and eluted with a linear gradient of KCl (0-1.5 M). Fig. 4A shows two peaks of SBEIIb kinase activity, termed K1 and K2, visualized as autoradiograms of 32P-labelled 87.4 kDa recombinant maize SBEIIb phosphorylated by the SBEIIb kinase fractions eluting from the column. Fig. 4B shows phosphorylation of the Ser649-Ala mutant SBEIIb by the K1 and K2 protein kinase fractions. M, molecular mass markers; size shown in kDa on the left. C peptide kinase activities responsible for phosphorylating the Ser649, Ser286 and Ser297 synthetic peptides eluting from a Blue-sepharose column with a linear gradient of KCl (0-1.5 M) following prior separation of amyloplast lysates by DEAE-sepharose chromatography. The recombinant SBEIIb kinase activities, K1 and K2, are coincident with the peaks of phosphorylation of the Ser649 peptide.
FIGURE 5. *Tandem mass spectra of SBEIIb phosphopeptides with phosphates located at amino acid residues corresponding to Ser\textsuperscript{649}, Ser\textsuperscript{286} and Ser\textsuperscript{297}.* A: MS/MS of the doubly protonated Glu-C/Lys-C-generated phosphopeptide SLRIYE with the phosphate located at the Ser\textsuperscript{286} site from the K1-treated sample. B: MS/MS of the of the doubly protonated tryptic phosphopeptide IYETHVGMSSPEPK with the phosphate located at the Ser\textsuperscript{297} site, obtained from the fourth C\textsubscript{18} RP-HPLC fraction of trypsin-digested K2-treated triple mutant of SBEIIb Ser\textsuperscript{286}>Ala/ Ser\textsuperscript{297}>Ala/ Ser\textsuperscript{649}>Ala. This spectrum was acquired from endogenous SBEIIb in the triple mutant sample, co-purifying with the protein kinase preparation. C: MS/MS of the triply protonated Glu-C/Lys-C-generated phosphopeptide WIDFPRGPQRLPSGK from the K1-treated sample with the phosphate located at the Ser\textsuperscript{649} site.

FIGURE 6. *Structure of SBEIIb obtained from homology modelling and molecular dynamics simulations.* The images were rendered in VMD version 1.9.1 (43) and were captured at the end of the 100 ns MD simulation. The protein is represented as a ribbon while all labeled residues are represented as CPK. Panel A shows unmodified SBEIIb and highlights the location of the three identified phosphorylation sites: Ser\textsuperscript{286}, Ser\textsuperscript{297} and Ser\textsuperscript{649} along with the three conserved structural and functional domains. These domains, namely, the central ($\beta/\alpha$)$_8$ structure along with N- and C-terminal anti-parallel $\beta$-sandwich structures are colored yellow, blue and red respectively. Within the central ($\beta/\alpha$)$_8$ domain are four highly conserved regions which are important for catalysis, namely: (I) residues 376-381, (II) residues 443-452, (III) residues 498-505 and (IV) residues 562-570 are colored cyan, orange, magenta and green respectively. Panels B, C and D illustrates that phosphorylation at positions Ser\textsuperscript{286} (PhSer\textsuperscript{286}), Ser\textsuperscript{297} (PhSer\textsuperscript{297}) and Ser\textsuperscript{649} (PhSer\textsuperscript{649}) are stabilized by salt-bridge interactions involving Arg and Lys residues.

FIGURE 7. *Amino acid sequence alignments of SBEII and SBEI and phosphorylation sites on maize SBEIIb.* A, Ser\textsuperscript{286} and Ser\textsuperscript{297} show high sequence conservation within the branching enzymes of the plant kingdom, whereas Ser\textsuperscript{649} is found only in the endosperm-specific SBEIIb isoform of some cereals (B). Molecular simulation indicates that phosphorylation of Ser\textsuperscript{297} forms a salt bridge with Arg\textsuperscript{665}.
TABLE 1

Synthetic peptides used as substrates for SBEIIb kinase

Position of the Ser (highlighted) in the mature SBEIIb sequence. All synthetic peptides contained a triple Arg tail for use in in vitro assays with $^{32}$P-labelled ATP. Prediction scores are for the Ser-containing sequence from SBEIIb based on NetPhos prediction. Activities given are for standard assay using plastid lysates as a source of protein kinase, values represent the mean of at least three separate determinations and are reproducible within ± 20% (S.E.) of the mean.

<table>
<thead>
<tr>
<th>Position of Serine in SBE IIb</th>
<th>Corresponding sequence of peptides with putative phosphorylation sites (Serine)</th>
<th>Predicted scores for putative phosphorylation sites</th>
<th>Vmax (nmol/mg/h)Amyloplast peptide kinase</th>
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<tr>
<td>Ser$^{147}$</td>
<td>LYRRIRS*DIDEHEGGRRR</td>
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<td>ETHVGMAS*PEPKINRRR</td>
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<tr>
<td>Ser$^{297}$, Ser$^{298}$</td>
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<td>0.017</td>
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</tr>
</tbody>
</table>
TABLE 2

Saturation kinetics of SBEIIb kinases K1 and K2

Partially purified K1 and K2 protein kinase fractions from amyloplasts were used in standard synthetic peptide assays in which ATP or the synthetic peptide was varied. K252a was used in the optimized assay. Data represent the mean of at least four separate determinations and are reproducible within ± 15% (S.E.) of the mean.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Kinase</th>
<th>Km for peptide (µM)</th>
<th>Km for ATP (µM)</th>
<th>Vmax (nmol/mg/h)</th>
<th>IC-50 for K252a (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser\textsuperscript{549}</td>
<td>K1</td>
<td>10.7</td>
<td>57.7</td>
<td>402</td>
<td>4.7</td>
</tr>
<tr>
<td>Ser\textsuperscript{549}</td>
<td>K2</td>
<td>11.0</td>
<td>58.9</td>
<td>352</td>
<td>23.8</td>
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<tr>
<td>Ser\textsuperscript{286}</td>
<td>K1</td>
<td>27.6</td>
<td>48.0</td>
<td>59</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Fig. 3A

Fig. 3B
Fig. 4A

<table>
<thead>
<tr>
<th>kDa</th>
<th>M</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>15</th>
<th>16</th>
<th>Fraction number</th>
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<tr>
<td>75</td>
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</tbody>
</table>

Fig. 4B

Fig. 4C

![Graph showing activity vs. fraction number with peaks at different points for different Ser numbers](image-url)
Identification of multiple phosphorylation sites on maize endosperm starch branching enzyme IIb, a key enzyme in amyllopectin biosynthesis

Amina Mahmoudova, Declan Williams, Dyanne Brewer, Sarah Massey, Jenelle Patterson, Anjali Silva, Kenrick A. Vassall, Fushan Liu, Sanjeena Subedi, George Harauz, K. W. Michael Siu, Ian J. Tetlow and Michael J. Emes

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