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Characterization of the 97 and 103 kDa forms of starch branching enzyme from potato tubers

Jamshid Khoshnoodi*, Bo Ek, Lars Rask, Håkan Larsson

Uppsala Genetic Center, Department of Cell Research, Swedish University of Agricultural Sciences, Box 7055, S-75007 Uppsala, Sweden

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N-Terminal analysis, peptide mapping and partial peptide sequencing of the 97 and 103 kDa forms of starch branching enzyme from potato tubers showed that the two forms are highly related. A comparison with sequence data in the literature showed that these forms belong to the starch branching enzyme isoform I family. An internal cDNA fragment was obtained using PCR technology on potato tuber RNA with two oligonucleotide primers constructed from the peptide sequence data. Southern blot analysis using the PCR fragment as probe showed that there is only one gene locus encoding this isoform of the enzyme in *Solanum tuberosum* as well as in *Solanum commersonii*.

Starch branching enzyme; Q-enzyme; Allozyme; Potato tuber; *Solanum tuberosum*; *Solanum commersonii*

1. INTRODUCTION

Starch is synthesized via three enzymatic reactions in which ADP glucose pyrophosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21) and starch branching enzyme (EC 2.4.1.18) are involved [1]. Starch branching enzyme (SBE, also called Q-enzyme) is believed to have two different enzymatic activities. It catalyzes both the hydrolysis of α-1,4 glucosidic bonds and the formation of α-1,6 glucosidic bonds during synthesis of the branched component in starch, amylopectin.

Isoforms of SBE have been purified from leaves of spinach [2], maize endosperm [3], sorghum seeds [4], developing embryo of pea [5] and developing rice endosperm [6]. Differences in the catalytic properties between the SBE isoforms from pea [5] as well as from maize [7] have been reported. cDNA clones for SBE I from rice seed [8] and for isoform I as well as II from maize endosperm have been isolated [9,10]. Recently the isolation of a gene for SBE I from rice was reported [11].

SBE isolated from potato tubers (*Solanum tuberosum*) is assumed to be encoded by one gene, although the molecular mass of the enzyme varies extensively between different reports, 70 + 20 kDa [12], 85 kDa [13], 79 kDa [14], 86, 97 and 103 kDa [15] and 64 kDa [16]. In this work was have investigated the relationship between the two large potato tuber SBE forms of 97 and 103 kDa.

*Corresponding author. Fax: (46) (18) 673 279.

2. MATERIALS AND METHODS

2.1. *Preparation of SBE from potato tubers and SDS-PAGE*

Potato tubers (*S. tuberosum* cv Bintje) were purchased from the local market. 'Crude preparations' of SBE were prepared according to preparation method 1 in [15], except that nicotine was excluded. SDS polyacrylamide gel electrophoresis was performed as previously described [17]. Molecular weight markers were from Pharmacia Biosystems (Uppsala, Sweden) and gels were stained with Coomassie brilliant blue (CBB).

2.2. *N-terminal sequencing of SBE*

The 97 and 103 kDa enzyme forms (approximately 70 μg of a crude preparation) were separated on an 8% SDS polyacrylamide gel (see Fig. 1) and electrotransferred to a polyvinyldifluoride (PVDF) membrane in 10 mM CAPS buffer (3-[cyclohexylamino]-1-propanesulfonic acid) pH 11, 10% methanol, 0.02% SDS at 300 mA for 3 h at 4°C. The membrane was stained with 0.1% CBB and destained in 50% methanol and 10% acetic acid. The protein bands were cut out and directly sequenced on a protein gas phase sequencer (Applied Biosystems 470 A, Foster City, CA, USA) equipped with on line PTH analyzer.

2.3. *Cleavage of SBE by endoproteinase Lys-C, isolation of peptides and amino acid sequence determination*

Approximately 3 mg of a crude preparation was applied to an 8% SDS-PAGE and the proteins were electroblotted onto a nitrocellulose filter with 0.005% SDS in the electrotransfer buffer (48 mM Tris, pH 9.1, 39 mM glycine, 20% methanol). The filter was stained with Ponceau S and the 97 and 103 kDa protein bands were cut out from the filter and treated with 0.5% polyvinylpyrrolidone (PVP-40) dissolved in 100 mM acetic acid [18]. The nitrocellulose strips were washed 7 times with water to remove excess PVP-40 and then cut into pieces of approximately 1 × 1 mm. The proteins on the nitrocellulose pieces were digested in 100 μl of 100 mM Tris-HCl pH 8.5/acetonitrile (95:5 v/v), containing 0.04 μg/ml endoproteinase Lys-C from *Achromobacter lyticus* (Wako Pure Chemical Industries, Osaka, Japan) and incubated at 37°C overnight. The digested proteins were reduced by addition of 0.1% β-mercaptoethanol (v/v) and incubated at room temperature for 1 h. Alkylation was carried out by the addition of 0.3% 4-vinyl-pyridine (v/v) followed by incubation at room temperature for 2 h. Additional peptides were obtained by directly digesting a crude preparation of the enzyme with endoproteinase Lys-C.

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All endoproteinase Lys-C digests were acidified with 1% trifluoroacetic acid (TFA) and separated on an HPLC (Beckman Instruments, San Ramon, CA, USA) equipped with a C_4 column (Aquapore BU-300, 2.1 × 30 mm, Brownlee Labs., Applied Biosystems, Santa Clara, CA, USA) and a diode array UV monitor (Waters 990, Millipore Corporation, MA, USA), using a linear gradient of 2–60% acetonitrile followed by a wash 60–90% acetonitrile in 0.1% TFA for 60 and 5 min, respectively, at a flow of 100 μl/min. The peptides were monitored at 214 nm and peaks were collected manually and directly sequenced in the protein sequencer or frozen.

2.4. *Cleavage of SBE with cyanogen bromide*

A crude preparation of the enzyme was treated with CNBr as described elsewhere [19]. The cleaved material was separated on a 15% SDS-PAGE and electroblotted onto a PVDF membrane in a semi-dry electrophoresis apparatus in 125 mM Tris, 125 mM boric acid and 10% methanol for 35 min at 20 V and 140 mA. The membrane was stained with 0.1% CBB and destained as described above. One major peptide band was cut out from the membrane and sequenced in the protein sequencer.

2.5. *Synthesis of oligonucleotide primers*

The degenerated oligonucleotide primers 5′-TTYGGITAYCAYG-TIACIAAYTTYTT-3′ and 5′-YTCGCRTAIGCIATRCAY-TT-3′ as upstream and downstream primer, respectively, were constructed on basis of two peptide sequences, P5 (FGYHVTNFF) and P1 (CIAYAE). Since endoproteinase Lys-C cleaves at the carboxyl-end of Lys residues, the second primer (P1) could be prolonged with three bases by including a Lys residue in front of the Cys residue. The oligonucleotides were synthesized using a DNA synthesizer (Applied Biosystems 381A).

2.6. *RNA extraction and PCR experiments*

Total RNA was extracted from potato tubers as described by others [20]. PCR amplification was achieved using Thermostable rTth Reverse Transcriptase RNA PCR Kit (Perkin Elmer Cetus Instruments, Norwalk, CT, USA) and 1 μg total RNA as template. The downstream primer (20 nmol) was annealed to the RNA at 50°C to avoid unspecific annealing. After synthesis of the first cDNA strand the PCR amplification was initiated by adding upstream primer (20 nmol) and running the thermal cycler (Techne PHC-3, Princeton, NJ, USA) for 35 cycles with the thermal cycle condition as follows: 94°C for 1 min, 45°C for 30 s and 60°C for 1 min. The PCR reaction mixture was run on a 4% preparative agarose gel and the PCR fragment was cut out from the gel and used as template in new PCR reactions to increase the amount of the fragment, which finally was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA, USA).

2.7. *DNA sequencing*

Nucleotide sequencing of both strands of the PCR-fragment was carried out by the cycle sequencing method using a Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) in the PCR thermal cycler. The sequencing reactions were run on a DNA sequencer (Applied Biosystems 373 A).

2.8. *Preparation of genomic DNA and Southern blotting analysis*

Genomic DNA was isolated from fresh leaves of *S. tuberosum* and *S. commersonii* as described [21]. The DNA samples (20 μg) were digested with *Eco*RI, *Taq*I and *Xho*I, separated by electrophoresis in a 0.7% agarose gel, and then transferred onto Hybond N+ nylon filter (Amersham, UK). The filter was incubated in prehybridization solution (0.26 M Na_2HPO_4 pH 8.0, 1% bovine serum albumin, 1 mM EDTA, 7% SDS, 5% dextran sulphate and 0.2 mg/ml salmon sperm DNA) at 65°C for 3 h. The PCR fragment was labelled by random priming using [α-^{32}P]dCTP (Amersham) and added to the hybridization solution to a specific activity of 1×10^6 cpm/ml. The filter was incubated a 50°C for 20 h and then washed twice in 2 × standard sodium citrate (1 × SSC: 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS at room temperature for 15 min and twice in 1 × SSC, 0.1% SDS at 50°C for 20 min. The filter was analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

3. RESULTS

3.1. *Isolation of SBE from potato tubers*

Crude preparations of starch branching enzyme were obtained from stored potato tubers using the polyethylene glycol precipitation procedure introduced by Blennow and Johansson [15]. Analysis by SDS-polyacrylamide gel electrophoresis showed the 97 and 103 kDa forms of the enzyme and a few additional faint bands migrating between 85–90 kDa (Fig. 1). The 103 kDa form was the most abundant component in our preparations.

3.2. *N-terminal sequencing of SBE*

To establish that the isolated proteins indeed were SBE their N-terminal sequences were determined. To accomplish this, the two components were electrotransferred from an SDS-polyacrylamide gel to a PVDF membrane and then sequenced. The N-terminal sequence obtained from the 103 kDa component was Val-Leu-Thr-Asp-Asp-Asn-Ser-Thr-Met-Ala-Pro-Leu-Glu-

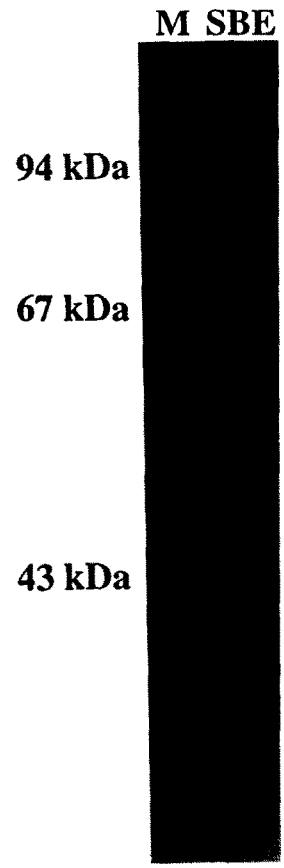

Fig. 1. SDS-PAGE gel showing a crude preparation of SBE I from potato tubers. Molecular weight markers (M) are, from top, phosphorylase *b*, serum albumin and ovalbumin.

Glu-Asp-Val-Lys-Thr-Glu-Asn. A shorter sequence was obtained for the 97 kDa component (data not shown) showing that the N-termini of the two components were identical. The N-terminal sequence was in turn identical to a region starting 77 amino acid residues from the N-terminal Met residue in an unprocessed form of potato tuber SBE I of 103,243 Da encoded by a full-length cDNA clone (BE7) [22,23].

3.3. *Peptide mapping of SBE and amino acid sequence determination of internal peptides*

In order to further determine the relationship between the 97 kDa and 103 kDa forms of SBE, the two components were electrotransferred from an SDS-polyacrylamide gel to a nitrocellulose filter and separately digested with endoproteinase Lys-C as described in section 2. The peptides obtained from each form were separated by HPLC (Fig. 2). The two HPLC peptide maps were very similar except for a significant extra double peak eluting late in the chromatogram of the 103 kDa component. Four large peaks, two pairs of corresponding peptides, were selected for sequence analysis (P1 and P2 from the 103 kDa form and P3 and P4 from the 97 kDa form). This showed that the corresponding peptides, P1/P3 and P2/P4, were identical (Table I). The P1/P3 and P2/P4 sequences were identical to two regions in the amino acid sequence deduced from the BE 7 cDNA clone mentioned above (Table II).

Because of the great similarity between the 97 and 103 kDa enzyme forms, we have not succeeded in separating the two components in quantitative amounts by conventional chromatographic techniques. To generate further peptides for collecting additional sequence data, two preparations of the enzyme (containing mainly a mixture of the 97 and 103 kDa components as shown in Fig. 1) were subjected to treatment with either CNBr or endoproteinase Lys-C. Partial amino acid sequences of one peptide (P5) from the CNBr-cleavage and of four peptides (P6–P9) from the digestion with endoproteinase Lys-C could be determined (Table I). Three of these peptide sequences (P5–P7) were identical to regions encoded by the BE 7 cDNA clone, whereas peptides P8 and P9 differed from the deduced amino acid sequence of the cDNA clone (Table II). Interestingly, P8 was identical to a sequence corresponding to another

Table I

Amino acid sequences of peptides P1–P9 from potato SBE I

Peptide	Sequence	Origin*	Cleavage
P1	CIAYAESHDQSIVG	103 kDa	Lys-C
P3	CIAYAESHDQ	97 kDa	Lys-C
P2	WIDYLK	103 kDa	Lys-C
P4	WIDYLK	97 kDa	Lys-C
P5	EHSYYGSFGYHVTNFFAVS	CP	CNBr
P6	LWDSRLFNYA	CP	Lys-C
P7	NDEDWSM	CP	Lys-C
P8	FAAPYDGVYXDPPPSERYHF	CP	Lys-C
P9	FRRQXNLADSEHLRYK	CP	Lys-C

*The peptides were isolated and sequenced after cleavage of either electrophoretically separated 97 and 103 kDa components or crude preparations (CP) containing mainly a mixture of the two components (see Fig. 1).

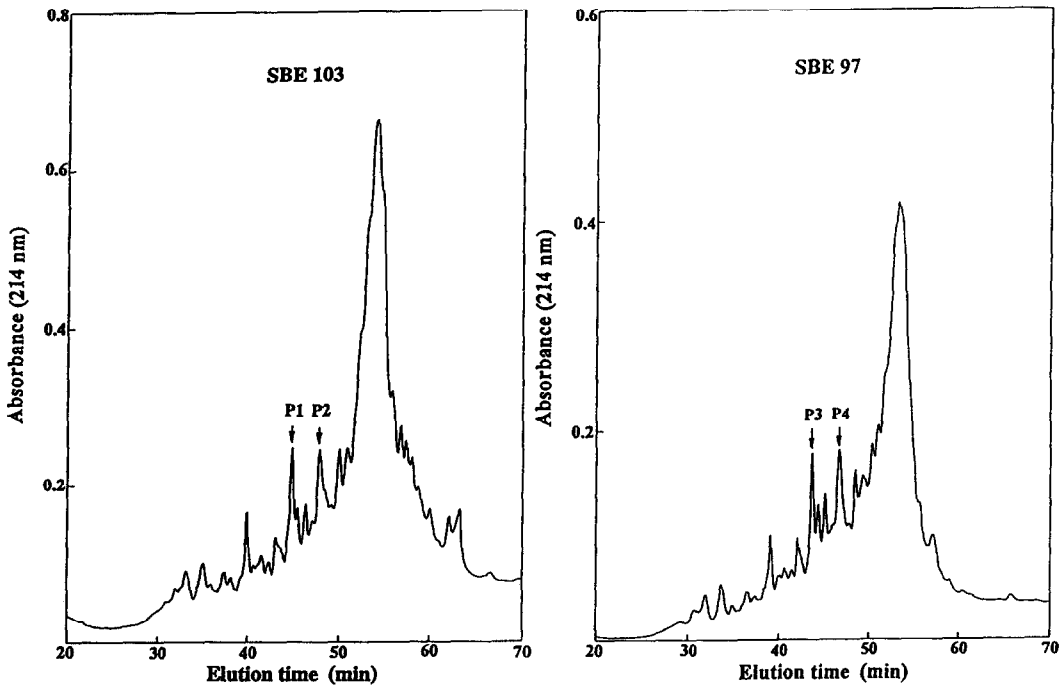

Fig. 2. HPLC separation of peptides from 103 kDa (SBE 103) and 97 kDa (SBE 97) SBE I forms digested with endoproteinase Lys-C. Peptides P1 and P2 correspond to peptides P3 and P4, respectively. The major peak in both chromatograms is due to residual PVP-40.

cDNA clone (BE 71) encoding a truncated allelic form of the enzyme assumed to have a molecular weight of about 64 kDa after removal of the transit peptide [23] (Table II). Peptide 9 differed from the deduced amino acid sequences of the BE 7 as well as of the BE 71 clone by having a Phe residue instead of a Cys residue in the first position.

Our sequence data show that the 97 kDa and 103 kDa forms of potato branching enzyme are highly similar to isoform I of the enzyme from maize endosperm [9] (Table II). Three out of four of the internal peptides (P1, P2 and P6) from the middle third of the enzyme are identical to corresponding sequences in the maize enzyme. The remaining peptides (P5, P7, P8 and P9) were 95%, 57%, 75% and 67% identical to the maize enzyme, respectively. The N-terminal sequence of the isolated potato tuber SBE I, however, showed no similarity with maize SBE I.

3.4. *Isolation of an internal cDNA fragment coding for potato SBE using PCR*

Two peptides (P1 and P5) from the conserved middle region of SBE were selected for synthesis of degenerated oligonucleotides. The two oligonucleotides encoded 7 amino acid residues from P1 and 9 amino acid residues from P5 (see section 2). The downstream primer was constructed complementary to the mRNA strand to enable direct PCR amplification on total RNA isolated from potato tubers. A PCR fragment with the expected length (approximately 700 bp) could be isolated after analyzing the PCR reaction by agarose gel electrophoresis. The fragment was subcloned into pBluescript SK+ and sequenced. The amino acid sequence deduced from the PCR-fragment (699 bp) contained the sequence of three peptides (P2, P6 and P7) in addition to those of the two peptides (P1 and P5) from which the primers were constructed. The nucleotide sequence of the fragment differed from the BE 7 and BE 71 cDNA clones [23] at 4 and 3 bases, respectively, leading to two conserved amino acid replacements in both cases (Fig. 3). These discrepancies might be due to misincorporation by the Taq polymerase but they could also reflect polymorphism (see section 4). The deduced amino acid sequence of the PCR fragment was 82% and 80% iden-

Table II

Comparison of peptides P1–P9 of potato tuber SBE I with the corresponding amino acid sequences deduced from the BE 7 and BE 71 potato cDNA clones [23] and a maize SBE I cDNA clone (M1) [9]

Peptide	Sequence
P1/3	C I A Y A E S H D Q S I V G
BE7	C I A Y A E S H D Q S I V G
BE71	C I A Y A E S H D Q S I V G
M1	C I A Y A E S H D Q S I V G
P2/4	W I D Y L K
BE7	W I D Y L K
BE71	W I D Y L K
M1	W I D Y L K
P5	E H S Y Y G S F G Y H V T N F F A V S
BE7	E H S Y Y G S F G Y H V T N F F A V S
BE71	E H S Y Y G S F G Y H V T N F F A V S
M1	E H S Y Y A S F G Y H V T N F F A V S
P6	L W D S R L F N Y A
BE7	L W D S R L F N Y A
BE71	L W D S R L F N Y A
M1	L W D S R L F N Y A
P7	N D E D W S M
BE7	N D E D W S M
BE71	N D E D W S M
M1	D D S E W S M
P8	F A A P Y D G V Y X D P P P S E R Y H F
BE7	S L Q T Y D G V Y W D P P - S E R Y H F
BE71	F A A P Y D G V Y W D P P P S E R Y H F
M1	F G A P Y D G V H W D P P A S E R Y T F
P9	F R R Q X N L A D S E H L R Y K
BE7	C R R Q W N L A D S E H L R Y K
M1	C R R Q W S L V D T D H L R Y K

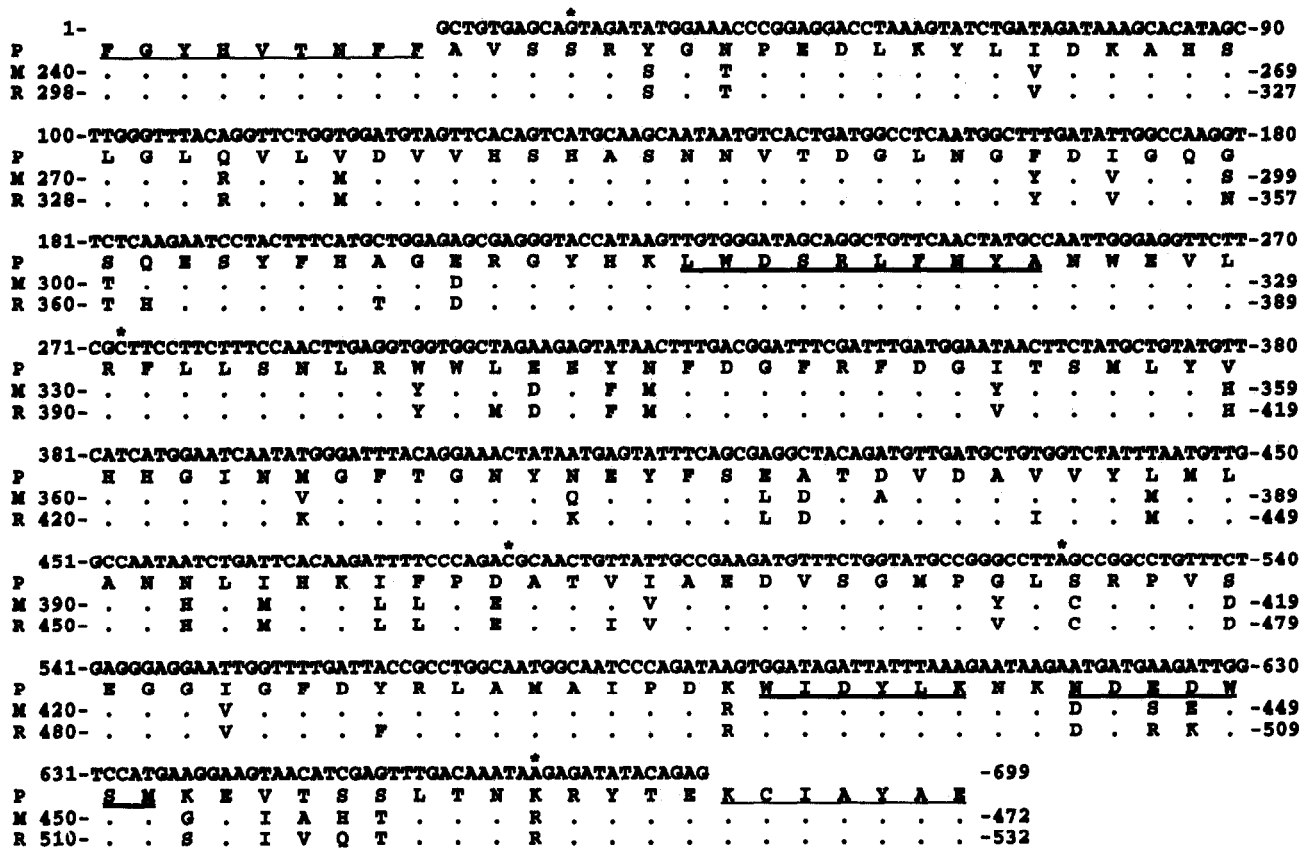

Fig. 3. Nucleotide sequence and the deduced amino acid sequence (P) of a 699-bp PCR-fragment encoding a part of potato SBE I aligned with the corresponding amino acid sequences of maize (M) [9] and rice (R) [8] SBE I. The two peptides (P1 and P5) from which the PCR oligonucleotide primers were synthesized are underlined once. The additional isolated and sequenced peptides (P2, P6 and P7), included in the PCR-fragment, are underlined twice. The differences between the PCR-fragment nucleotide sequence and the BE 7 and BE 71 cDNA clones [23] are marked (*). Of these differences three resulted in amino acid substitutions: Ser (nt 38) for Asp in BE 71, Ser (nt 526) for Gly in BE 7 and Lys (nt 665) for Arg in both BE 7 and BE 71.

tical to the primary sequence of SBE I from maize [9] and rice [8], respectively (Fig. 3).

3.5. *Southern blotting analysis*

To investigate the number of SBE genes in the potato genome hybridizing to the PCR fragment, Southern blotting analysis was performed using the PCR-fragment as the probe. Hybridization to *S. tuberosum* DNA digested with three different restriction enzymes resulted in a single band even under non-stringent hybridization and washing conditions (Fig. 4). Furthermore, the probe was used for hybridization to *S. commersonii* DNA. Also in this case a single band was obtained.

4. DISCUSSION

Isolation of SBE from potato tubers has resulted in reports giving the enzyme different molecular weights (see section 1). This variation might be due to the following. First, it could be the result of degradation of the enzyme, depending on the choice of purification protocol, as suggested by others [15]. Second, it could reflect the expression of allozymes of different size, as recently was reported from the characterization of cDNA clones of potato SBE I [23]. Third, the presence of distinct isoforms, encoded at different gene loci, can not be excluded after the reported failure to reduce the amount of amylopectin in transgenic potato plants containing an antisense construct for isoform I of the enzyme [24].

We have investigated the 97 and 103 kDa forms of SBE from potato tubers. The two very similar peptide maps and the sequence data show that the two forms are highly related. Furthermore, the single band obtained in the Southern blot analysis suggest that they are encoded from the same gene locus. We have so far been unable to identify the peptides giving rise to the additional double peak present solely in the HPLC peptide map of the 103 kDa form. These extra peptides presumably account for the difference in size between the two enzyme forms observed on SDS gels. Since the N-termini of the enzymes are identical, the difference between the two forms might be due to proteolysis at the C-terminus resulting in the 97 kDa form. Alternatively, the 97 and 103 kDa forms could be allozymes of different length. As mentioned above, the BE 7 cDNA clone encodes an enzyme including the N-terminal transit

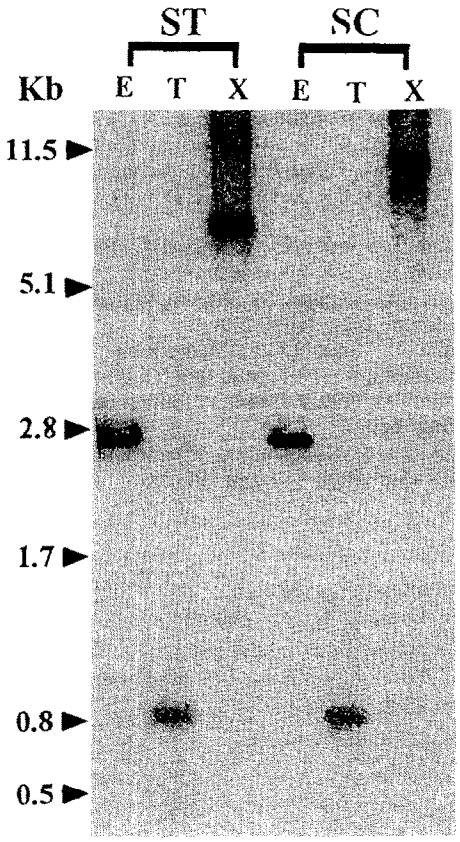

Fig. 4. Southern blotting analysis of genomic DNA from *Solanum tuberosum* (ST) and *Solanum commersonii* (SC). The restriction enzymes used were *Eco*RI (E), *Taq*I (T) and *Xho*I (X).

peptide with a molecular weight of 103,243 [23]. After removal of a 76 amino acid transit peptide an enzyme of about 95 kDa is obtained. This product could very well be identical to the isolated component with a relative molecular mass of 97 kDa on SDS gels. Assuming that the isolated 103 kDa enzyme is synthesized with a transit peptide of the same length, the unprocessed enzyme in this case would be approximately 110 kDa and, thus, not encoded from an allele corresponding to the BE 7 clone. This implies that it should be possible to isolate a cDNA clone, larger than the BE 7 clone, encoding the precursor of the isolated 103 kDa enzyme form.

Two of the sequenced peptides (P8 and P9), obtained after cleavage of a crude preparation (which contained mainly the 97 and 103 kDa components), differed from the amino acid sequence corresponding to the BE 7 clone. This suggests that at least one of the two enzyme forms is encoded by an allele distinct from the BE 7 clone. As mentioned above, P8 was found with perfect match in the amino acid sequence predicted from the BE 71 cDNA clone that was reported to encode a truncated enzyme of about 64 kDa after removal of the transit peptide [23]. Since no proteins of that size could be observed in our crude preparations and, furthermore, P8 was present in high amounts after cleavage of the crude preparation, it is reasonable to assume that P8 emanated from one of the two larger enzyme forms.

The BE 7 and BE 71 cDNA clones [23] were obtained after an initial immuno-screening of a λgt11 potato tuber cDNA library with an antiserum against a denatured 79 kDa form of the enzyme [22,23]. An 85 kDa form of the enzyme has also been reported [13]. A protein of approximately 85 kDa, assumed to be a degradation product [15], is usually present in low amounts in crude preparations of the 97 and 103 kDa forms. The 79 kDa form of the enzyme, on the other hand, has not been observed in our preparations. Whether these forms of SBE of lower molecular weight are active proteolytic products of the larger forms or represent additional allelic forms has to be clarified.

The sequence discrepancies between the PCR-fragment and the two cDNA clones BE 7 and BE 71 presumably reflect the fact that there are at least three different allelic forms of SBE I expressed in potato tubers. As discussed above, the isolated 97 and 103 kDa forms encountered in the variety Bintje might very well be allelic gene products. Nevertheless, Southern blotting analysis using the PCR-fragment as the probe indicated that there is only one gene locus for SBE I in potato. A similar conclusion was reached earlier [22]. This means that although different alleles exist with slightly different nucleotide sequences and also stop codons at different positions, the alleles are sufficiently conserved to make it difficult to resolve them by Southern blotting. The high degree of conservation of the SBE genes is further emphasized by the fact that restriction sites in the SBE gene are conserved between *S. tuberosum* and *S. commersonii*, which is somewhat surprising. *S. commersonii* is a diploid relative of *S. tuberosum*, but is nevertheless a distinct species. Even if only one SBE gene has been identified in potato so far, additional genes encoding SBE with low sequence identity to the identified gene might very well exist in potato. Whether this putative second isoform of the enzyme is expressed in potato tubers remains to be investigated.

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