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Consequences of antisense RNA inhibition of starch branching enzyme activity on properties of potato starch

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Antisense constructs containing cDNAs for potato starch branching enzyme (SBE) were introduced into potato (*Solanum tuberosum* L.). A population of transgenic plants were generated in which tuber SBE activity was reduced by between 5 and 98% of control values. No significant differences in amylose content or amylopectin branch length profiles of transgenic tuber starches were observed as a function of tuber SBE activity. Starches obtained from low SBE activity plants showed elevated phosphorous content. ^{31}P n.m.r. analysis showed that this was due to proportionate increases in both 3- and 6-linked starch phosphates. A consistent alteration in starch gelatinisation properties was only observed when the level of SBE activity was reduced to below ~5% of that of control values. Starches from these low SBE activity plants showed increases of up to 5°C in d.s.c. peak temperature and viscosity onset temperature. Studies on melting of crystallites obtained from linear (1 → 4)-α-D-glucan oligomers suggest that an average difference of double helix length of about one glucose residue might be sufficient to account for the observed differences in gelatinisation properties. We speculate that the modification of gelatinisation properties at low SBE activities is due to a subtle alteration in amylopectin branch patterns resulting in small changes in double helix lengths within granules.

Abbreviations: SBE, starch branching enzyme, d.s.c., differential scanning calorimetry.

INTRODUCTION

Starch is the major storage carbohydrate of higher plants, existing in the form of partially crystalline granules within plastids. Starch consists of two major components, namely amylose, a predominantly linear molecule of α-1,4-linked D-glucose units and amylopectin, a branched molecule consisting of α-1,4-linked D-glucan backbone with α-1,6-linked branches. The amylose to amylopectin ratio and the number, position and length of branches in the amylopectin molecules are presumed to be determinants of the physical properties of the starch granules such as gelatinisation, viscosity development and retrogradation.

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Amylopectin is synthesised from ADP–glucose by the concerted action of soluble starch synthase and starch branching enzymes (SBE). SBE hydrolyses α-1,4-linkages within a chain and rejoins the cleaved portion via an α-1,6-linkage to produce a branched structure. The activity of starch branching enzyme is therefore crucial in determining the structure and, hence, physical properties of the synthesised starch.

In most plant storage organs, multiple forms of SBE have been found, e.g. pea embryos (Matters and Boyer, 1981), rice endosperm (Smyth, 1988), maize endosperm (Boyer and Preiss, 1978). In pea, two isoforms (SBE I and SBE II) exist which differ in terms of substrate specificity and in the nature of the products they synthesise in in vitro systems (Smith, 1988). Thus SBE I is less active with amylose than SBE II and makes a less soluble glucan in in vitro

phosphorylase stimulation assays than SBE II. In maize, three SBE isoforms (I, IIa and IIb) have been identified and these have been reported to have distinct properties in respect of rates of branching amylose and amylopectin and in the structure of products formed in vitro (Takeda et al., 1993). Thus, SBE I preferentially transfers longer chains than SBE IIa/IIb. SBE I has the highest activity in branching amylose compared to amylopectin, whilst conversely SBE II has lower rates in branching amylose and higher rates with amylopectin. These results suggest that SBE isoforms could play distinct roles in amylopectin biosynthesis.

Direct evidence that expression levels of SBE affect the nature of synthesised starch comes from analysis of genetic mutants. Wrinkled (*rr*) peas contain a mutation in the r locus which results in complete loss of SBE I activity and the amylopectin content of these peas is decreased from about 70% to about 30% of starch content (Bhattacharyya et al., 1990). The amylose extender (*ae*) mutation of maize results in a reduction of SBE activity, specifically via loss of isoform IIb, and a marked increase in amylose content of endosperm starch (Boyer and Preiss, 1978, Stinard et al., 1993).

SBE isoforms from various sources have recently been classified into two families on the basis of structural relatedness (Burton et al., 1995). Members of each family are more similar to each other than they are to other SBE isoforms of the same species. Thus, members of family A include maize SBE II, pea SBE I and rice SBE III, whilst family B includes maize SBE I, pea SBE II and SBE I from rice.

In potato tuber, only a single form of SBE has so far been identified (Borosvsky et al., 1975, Vos-Scheperkeuter et al., 1989). This enzyme is most closely related to SBE 1 of maize and SBE II of pea and, accordingly, falls in the B family of SBEs (Burton et al., 1995). In potato, no SBE or high amylose mutants are known, although amylose-free mutants have been obtained, by X-ray irradiation (Hovenkamp-Hermelink et al., 1987) and by transformation with antisense-GBSS constructs (Visser et al., 1991a). A potential approach to increasing amylose levels would be to down-regulate branching enzyme activity using antisense constructs. cDNA coding for potato starch branching enzyme has been cloned (Kossmann et al., 1991, Poulsen and Kreiberg, 1993), but previous reports on using antisense constructs have not resulted in significant modification of starch composition or properties (Willmitzer et al., 1993, Müller-Röber and Kossmann, 1994) for transgenic plants containing 10%–20% of wild-type SBE levels (Willmitzer et al., 1993) or having "an almost complete reduction" of SBE (Müller-Röber and Kossmann, 1994).

Here we describe the transformation of potato (*Solanum tuberosum* L.) with antisense potato SBE cDNA constructs and the observation that tuber SBE activity has to be depressed below a threshold value, approximately 5% of wild-type activity, before any detectable alteration in physical properties of synthesised starch is obtained. Starches extracted from such low SBE activity plants show altered gelatinisation and viscosity development, without a significant change in either amylose content or amylopectin branch length profile.

MATERIALS AND METHODS

Plant materials

Stock nodal cutting cultures of potato (*Solanum tuberosum* L., cv Desiree) were maintained on Murashige and Skoog basal media (MS) containing 1% sucrose at 22°C in an illuminated culture room (40 μJ m^{-2} h^{-1}) with a 16 h day. Cuttings were taken every three weeks with five plantlets grown in each Magenta vessel to produce nodes with large leaves. Tuberisation was achieved by transfer of single nodes to MS media containing 8% sucrose and 2.5 mg l^{-1} benzylaminopurine (BAP) and incubating in darkness at 22°C. After tuberisation had proceeded to approximately 1 cm diameter tubers, the explants could be transferred to Magenta vessels containing the same media for storage of up to 6 months.

In vitro rooted transgenic plantlets 1–2 cm high, were transferred to compost (50% Levingtons/50% grit) and grown under high illumination (400 μJ m^{-2} h^{-1}) at 20°C (day) and 18°C (night) with a 16 h day period. After 10–12 days, plantlets were transferred to 3″ pots containing Arthur Bowes Universal Compost. After establishment (40 days), four plants from each clone were repotted together in 10″ pots with the same compost. Day length was reduced to 11 h after approximately 100 days growth. Tubers were harvested after foliage senescence (approximately 120 days).

Construction of antisense starch branching enzyme plant transformation vectors

A 2.3 kb partial length cDNA clone (from an internal *Eco*RI site to the 3′ end, including a poly(A) tail) of potato SBE was subcloned in an antisense orientation between the duplicated cauliflower mosaic (CaMV) virus 35S promoter and the CaMV polyadenylation signal in the vector pJIT 60 (Guerineau et al., 1992). The promoter-antisense potato SBE-poly(A) fragment was then cloned into the plant transformation vector pBIN19 (Bevan, 1984).

The same 2.3 kb fragment of potato SBE was subcloned in an antisense orientation between the patatin promoter and nopaline synthase (NOS) polyadenylation signal in pBI140.5. pBI140.5 is a BIN19 derivative containing a 3.5 kb patatin type I promoter (*Hin*dIII to *Dra*I of clone PAT21, Bevan et al., 1986) and the NOS polyadenylation signal (Bevan et al., 1983).

A full-length 3.0 kb cDNA of potato SBE was isolated from a λZap cDNA library and subcloned in an antisense orientation between the granule-bound starch synthase promoter (0.8 kb *Hin*dIII–*Nsi*I fragment, Visser et al., 1991b) and NOS polyadenylation signal in the plant transformation vector pGPTV-HYG (Becker et al., 1992).

The antisense SBE plasmids were transferred into *Agrobacterium tumefaciens* using a direct DNA uptake method (An et al., 1988).

Agrobacterium transformation

Halved in vitro tubers were incubated with log phase *A. tumefaciens* cells for 10 min, after which the explant tissue was removed, blotted on filter paper and transferred onto nurse plates. Nurse plates were prepared by plating 2 ml *Nicotiana plumbaginofolia* suspension cells onto regeneration media (0.8% Bactoagar, MS salts, 1% sucrose, 0.2 mg l^{-1} indole acetic acid (IAA), 5 mg l^{-1} zeatin). Explants were incubated under illumination for 2 days before transfer to fresh regeneration media containing 500 mg l^{-1} cefotaxime. Five days later explants were transferred to the same media containing 100 mg l^{-1} kanamycin. After 4 weeks (two transfers) explants were transferred onto expansion media (MS salts, 1% sucrose, 1.0 mg l^{-1} gibberellic acid (GA3) containing cefotaxime and kanamycin. After a total of 8 weeks, regenerating shoots were removed and transferred to basal media (MS salts, 1% sucrose) containing cefotaxime and kanamycin.

Starch branching enzyme (SBE) assay of transgenic tubers

Sample tubers from each plant were taken after harvest, washed and stored at 20°C until assay. Frozen tubers were crushed in a mortar and pestle in 2 vol. extraction buffer cooled to 4°C. The buffer contained 100 mM 2-amino-2-(hydroxymethyl)-1,3 propanediol (Tris), pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM dithiothreitol (DTT), 0.1% (w/v) sodium metabisulphite and 10% (w/v) polyvinyl-polypyrrolidone (PVPP). When completely homogenised, the crude homogenate was clarified by centrifuging at 10 000 × *g* for 10 min. The supernatant was retained for the assay of starch branching enzyme activity. The standard SBE assay reaction mixture, in a volume of 0.2 ml, was 200 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, pH 6.5, 50 mM[^{14}C]glucose 1-phosphate (100 nCi), 0.05 mg rabbit phosphorylase A and potato tuber extract. Incubations were performed at 30°C for 60 min. Negative controls contained either (a) no phosphorylase, or (b) the potato tuber extract boiled for 30 min to destroy enzyme activity. The reaction was terminated and glucan polymer precipitated by the addition of 1 ml of 75% (v/v) methanol, 1% (w/v) potassium hydroxide (KOH) and then 0.1 ml of glycogen (10 mg/ml). Insoluble glucan polymer was pelleted by centrifugation and washed with a further 1 ml of methanol/KOH before being redissolved in water and the incorporated radioactivity measured in a Beckman LS 3800 liquid scintillation counter. Activity was expressed as units, with one unit defined as 1 μmol of glucose incorporated per minute. All measurements were taken during the phase of the assay when the rate of glucose incorporation was linear.

Starch extraction

Potato tubers were homogenised in water for 2 min in a Waring blender operating at high speed. The homogenate was washed and filtered (initially 2 mm, then 1 mm filters) using approximately 4 l of water per 100 g of tubers (six extractions). Washed starch granules were finally extracted with acetone and air dried.

Amylose content determination

Starches were analysed for amylose content by either potentiometric iodine titration or colorimetrically using the method of Morrison and Laignelet (1983).

Amylopectin branch length profiles

To obtain an amylopectin-enriched fraction, potato starches (0.3 g) were suspended in water (30 ml), heated to boiling (microwave oven) and sheared extensively (Ultra-Turrax T25, Janke and Kunkel) to eliminate granular structure. *n*-Butanol (3 ml) was added to the hot solution and the mixture shaken and then cooled quiescently to room temperature. The resultant amylose/butanol precipitate was removed by centrifugation (2000 × *g* for 1 h) and the supernatant poured into an equal amount of acetone containing NaCl (2 mg/ml). The mixture was stored quiescently for 2–3 h and the supernatant decanted. The precipitated amylopectin-rich fraction was washed with ethanol (× 2) and diethyl ether (× 2) and air-dried. This fraction was dissolved (5 mg/ml) in sodium acetate (100 mM, pH 4.0) and incubated with isoamylase (0.13 u ml^{-1}, 40°C, 20 h). The resultant solution was desalted with Dowex-MR 3 mixed bed resin, NaOH added to 0.1 M and filtered (0.45 μm, Millex HV, Millipore). A 50 μl sample was injected onto a Dionex DX-300 LC series HPLC system (Sunnyvale, CA, USA) equipped with a Dionex CarboPac PA-100 column (250 × 4 mm) in combination with a CarboPac PA-100 guard column, run at approximately 20°C. Elution conditions were as in Gidley et al. (1995). Oligoglucan chain lengths were calibrated by spiking duplicate samples with maltoheptaose.

Phosphorous contents and form

Elemental phosphorous was determined following ashing (550°C, 16 h), re-dissolution of the ash in hydrochloric acid (3 M) and analysis at 213.6 nm by inductively coupled plasma optical emission.

^{31}P n.m.r. was used to identify the chemical nature of phosphorous-containing components following the method of Mette Bay-Smidt et al. (1994). A Bruker AMX 400 machine operating at 161.98 MHz was used with starch samples gelatinised in D_2O (25%, w/v). An integration standard of methylene diphosphonic acid in D_2O was present in

a co-axial capillary within the sample tubes. A pulse width of 90°, an acquisition time of 1 s and a relaxation delay of 5 s were used to ensure full signal intensity for integration purposes.

Differential scanning calorimetry of transgenic starches

Starch powders isolated from a range of transgenic potato plants were analysed using the Perkin-Elmer DSC 7 instrument. One to 4 mg of starch were accurately weighed into an aluminium sample pan, and water added so that the starch concentration was less than 25% (w/v) to give a total sample weight of 10–15 mg. An empty reference sample pan was used. A heating rate of 10°C/min was used to heat the test and reference samples from 25 to 95°C. Data analysis was performed using the instrument software. A number of temperature parameters can be obtained from such plots, the most accurate being the peak temperature.

Viscosity development of transgenic starches

Starches were analysed for viscosity development using the Rapid Visco Analyser 3C (Newport Scientific, Sydney, Australia). Starch (2.5 g) was weighed into an instrument sample holder, and water (22.5 g) added so that the final concentration was 10% (w/w) starch. Suspensions were equilibrated for 2 min at 50°C and heated under standard stirring conditions at 1.5°C min^{-1} from 50 to 95°C, then held at 95°C for 15 min. The viscosity developed was measured in instrument stirring number units (SNU). The broad maximum observed as a function of temperature makes the accurate determination of a peak temperature difficult, but the fact that viscosity starts from a very low level and rapidly rises allows an accurate determination of a viscosity onset temperature, defined as the temperature at which viscosity is at least 50% higher than at all lower temperatures above 50°C.

Fractionation and crystallisation of debranched glycogen

Debranched glycogen was prepared as described by Gidley and Bulpin (1987) and dissolved in 20 mM ammonium hydrocarbonate (15 mg in 5 ml). The solution was applied to a column (2.6 × 100 cm) of Bio-Gel P6 (Extra Fine <45 μm mesh) at room temperature and eluted at 20 ml h^{-1} with the same buffer. Eluants were monitored by continuous differential refractometry (Bio-rad 1755) and fractions (5 ml) collected. Selected fractions were analysed by high-performance anion-exchange chromatography as described for debranched amylopectin. Products from several fractionations were pooled for study of crystallisation behaviour. Typically, selected fractions (10–20 mg) were dissolved in deionised water (to 5%, w/v) with heating and stored either at 1°C or subjected to freezing (18°C) and thawing (25°C) cycles. Samples which showed precipitate formation were analysed by d.s.c. as described above. For some materials, glucans in precipitate and supernatant phases were analysed by high-performance anion-exchange chromatography. In all cases, chain length profiles were very similar for material from each of the two phases.

RESULTS

Transformation of potato with antisense partial potato SBE construct

A BIN 19 construct containing a partial (2.3 kb) potato SBE cDNA in an antisense orientation between the duplicated CaMV 35S promoter and CaMV polyadenylation signal was used to transform potato in vitro tubers via *Agrobacterium*-mediated infection. Transgenic lines were regenerated and positive transformants were identified by PCR amplification (data not shown).

Tubers were harvested from independent transformed plants and assayed for SBE activity. Fig. 1 shows the tuber SBE activities obtained from one such experiment (31 plants) and it can be seen that, relative to control values, SBE activities had been reduced by between 5 and 98%. Several of the transformed plants were found to have SBE activities less than 0.75 u g^{-1} tuber (below 5% of average control values).

Starch molecular analysis

Estimation of amylose content of transgenic starches by potentiometric titration or spectrometric iodine-binding assay (Morrison and Laignelet, 1983) resulted in values ranging from 22.6 to 27.8% and no apparent correlation with SBE activity levels in tubers.

Amylose-rich and amylopectin-rich fractions were separated by selective complexation with *n*-butanol for eight starches covering the range of tuber SBE activities. Each amylopectin-rich fraction was treated with isoamylase and debranched products analysed by high-performance anion-exchange chromatography. Example chromatograms are shown in Fig. 2. For each starch, the highest intensity peaks were for DP 13 and 14 with a subsidiary maximum at DP 45–50. Within the accuracy of peak height measurements, no significant differences were found in branch

Table 1. Phosphorous content (ppm) as a function of tuber SBE activity (u g^{-1}) in transgenic potato lines

Starch no.	SBE activity	P (ppm)
7	0.40	1080
11	0.44	790
33	0.53	1140
12	0.53	1210
15	0.53	1150
1	0.75	1020
26	5.50	640
16	12.2	750
32	12.50	690

length profiles for the starches analysed. The very small apparent differences between individual starches did not correlate with tuber SBE activities.

Phosphorous contents did, however, show differences correlating with tuber SBE activities (Table 1) with a 50%–100% increase in phosphorous content between low and 'normal' enzyme activities, with only one exception. This increase has been noted previously (Kossmann, 1994). In order to establish the molecular nature of phosphorous, ^{31}P n.m.r. spectra were obtained. Examples for increased and 'normal' phosphorous content starches are shown in Fig. 3. The only starch-based signals observed are at 0.85 and 1.48 ppm, assigned (Mette Bay-Smidt et al., 1994) to starch-bound 6-phosphate and 3-phosphate, respectively. Comparison with an integration standard showed that the ratio of 6-phosphate to 3-phosphate was conserved in all samples, but that the total levels increased proportionately with the elemental phosphorous content.

Starch gelatinisation properties

During gelatinisation of starch, molecular/crystalline order is lost with subsequent granule swelling and polymer leaching which result in rheological changes. The loss of molecular/crystalline order is conveniently monitored by differential scanning calorimetry (d.s.c.) which records the enthalpy and temperature range associated with the disordering of double helices within granules (Atwell et al., 1988, Cooke and Gidley, 1992). Examples of d.s.c. traces for starches from transgenic potatoes with low and high residual tuber SBE activity are shown in Fig. 4: all features of the d.s.c. trace are displaced to higher temperature for the low SBE activity sample. As a convenient single point measure, peak temperatures for each transgenic line are shown in Fig. 5, plotted against SBE activity. Below a threshold value of ca 0.75 u g^{-1} tuber, peak temperatures are elevated above the range of 69–71°C characteristic of higher SBE activity tubers. Replicate analyses of the same line show a spread of peak temperature values of typically less than 1°C. No significant differences in endotherm enthalpy were found as a function of tuber SBE activity levels.

A similar thermal shift is seen in viscosity development profiles. Examples are shown in Fig. 6 for a range of SBE activity samples. As with d.s.c., each of the characteristic features of the viscosity development are shifted to higher temperatures for starch from low SBE activity tubers. The broad maximum observed as a function of temperature makes the accurate determination of a peak temperature difficult, but the fact that the viscosity starts from a very low level and rises rapidly allows an accurate determination of a viscosity onset temperature, defined as the temperature at which viscosity is at least 50% higher than at all lower temperatures. The variation of viscosity onset temperature with tuber SBE activity is shown in Fig. 7. Below a threshold SBE activity value of *ca.* 0.75 u g^{-1} tuber, viscosity onset temperatures (and temperatures of other profile features) show a consistent increase. There is a close correlation between increases in d.s.c. peak and viscosity onset temperatures (data not shown).

Effect of chain length on double helix melting temperatures

In order to assess whether small changes in double helix lengths within the amylopectin fractions of transgenic starches could underlie the observed differences in d.s.c. peak and viscosity onset temperatures, the melting behaviour

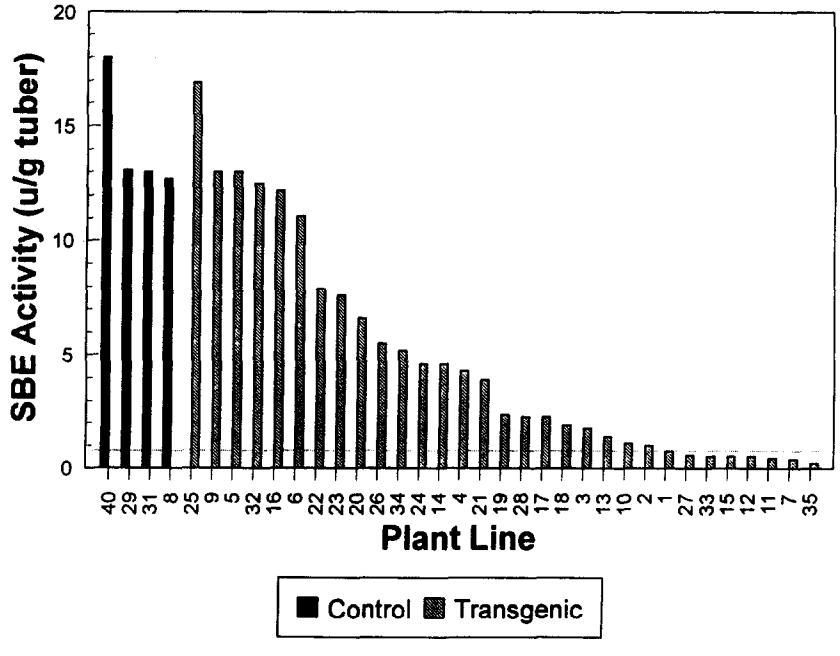

Fig. 1. Tuber SBE activities (u g^{-1} fwt) of independent transgenic plants obtained by transformation with the 2 × CaMV 35S antisense-partial potato SBE construct. The dashed horizontal line delineates an SBE activity of 0.75 u g^{-1} tuber which corresponds to 5% of average control activity.

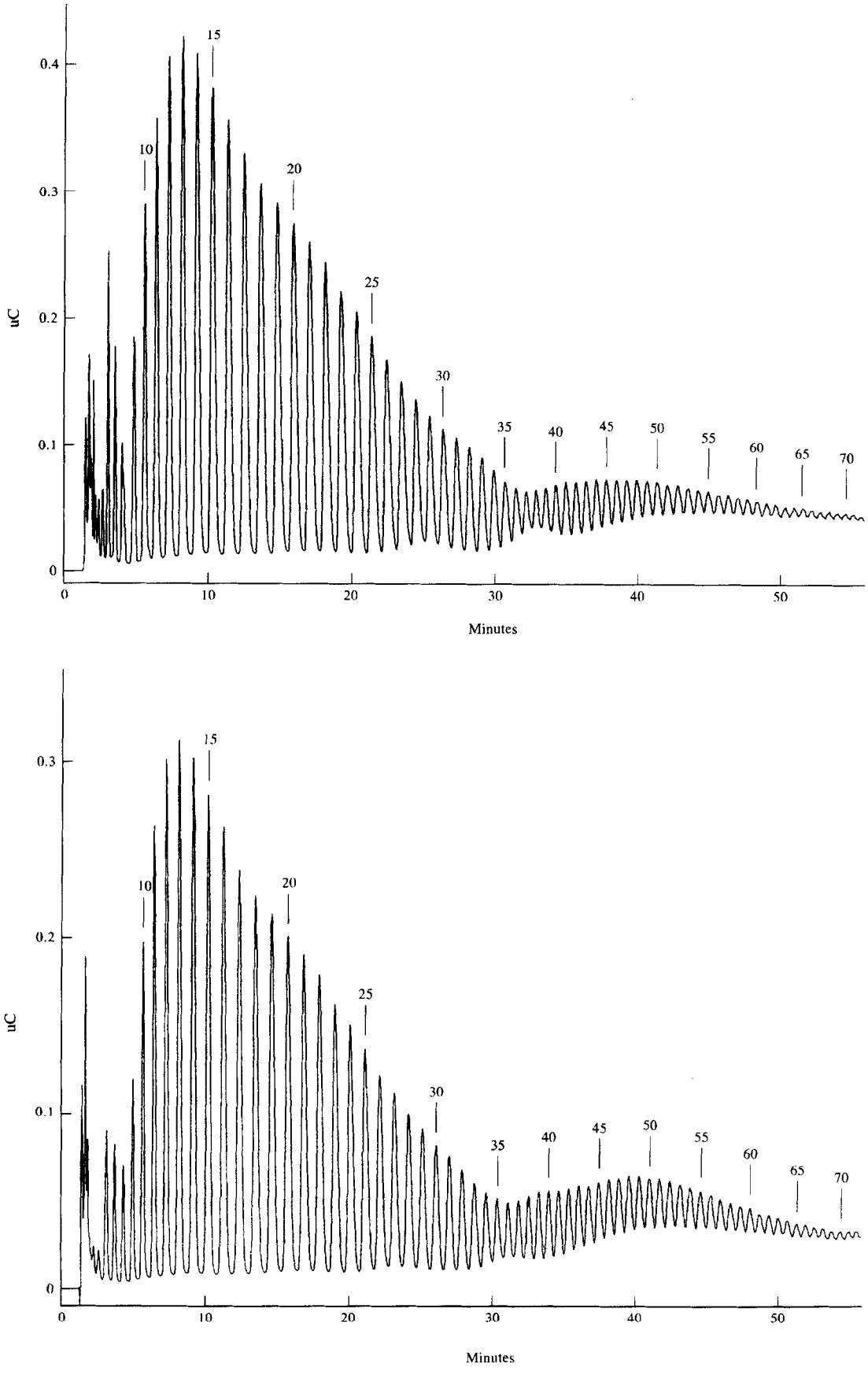

Fig. 2. Chain length profiles of debranched amylopectin fractions from transgenic lines 15 (upper trace, SBE activity 0.53 u g^{-1} tuber) and 20 (lower trace, SBE activity 6.6 u g^{-1} tuber). Numbers above peaks refer to degrees of polymerisation.

of crystallised (1 → 4)-α-D-glucan oligomers was studied. It has previously been shown that debranched glycogen can be used as a model for amylopectin crystallisation (Gidley and Bulpin, 1987). Melting temperatures (assessed by d.s.c.) of crystallised debranched glycogen are similar to those for starch gelatinisation, and associated enthalpy values are similar if the relative double helix contents are taken into account (Cooke and Gidley, 1992). Debranched glycogen contains a broad range of chain lengths which can be fractionated by chromatography (Gidley and Bulpin, 1987).

A number of fractions were obtained by chromatography on Bio-Gel P-6 and analysed by HPAEC yielding the example oligomeric profiles shown in Fig. 8. Crystallisation of each fraction was achieved by storing 5% (w/w) solutions in water at 1°C for a minimum of 7 days. This resulted in significant crystallisation of, for example, samples profiled in Fig. 8*A–C* but not Fig. 8*D,E*. These two samples were crystallised by two freeze–thaw cycles (14 h at 18°C, 7 h at 20°C). In order to provide a comparison, d.s.c. traces were recorded for crystallised samples each prepared by two freeze–thaw cycles, as well as for isothermal crystallisation at 1°C. Typical traces are shown in Fig. 9 with peak temperatures presented in Table 2. In the temperature ranges characteristic for starch gelatinisation, it is seen that a relatively small change in DP has a marked effect on the melting temperature of crystallised (1 → 4)-α-D-glucan oligomers. A very approximate estimate is that, within the range studied, each additional glucose unit in a crystallised oligosaccharide results in a 3–6°C increase in melting temperature. Since this work was completed, Moates et al. (1997) have reported very similar melting temperatures for oligoglucan fractions derived from lintnerised starch crystallised at 1°C. The lower melting temperatures for freeze–thaw-treated systems (Table 2) is presumably due to reduced perfection of helix formation under these more undercooled conditions.

Table 2. Effect of (1 → 4)-α-D-glucan chain length on the melting temperatures of materials re-crystallised from 5% (w/w) aqueous solution as assessed by d.s.c. peak temperatures

HPAEC profile	DP peak	d.s.c. peak temperatures (°C)	
		1°C storage	Two freeze–thaw cycles
Fig. 8*B*	24	93.5	—
Fig. 8*C*	19	87.5	75.6
Not shown	17–18	80.0	76.6
Fig. 8*D*	15	—	60.1
Fig. 8*E*	12	—	49.2

Transformation of potato with alternative AS-SBE constructs

In an effort to obtain even greater reductions in SBE activity in tubers, and hence more marked changes in starch structure, transformations were carried out with alternative antisense-SBE constructs. These included the 2.3 kb potato SBE cDNA linked to a patatin type I promoter and a full-length (3.0 kb) potato SBE cDNA linked to a granule-bound starch synthase (GBSS) promoter. Analysis of tubers from resultant transgenic plants again revealed a range of decreased SBE activities, but none lower than previously achieved with the CaMV 35S–2.3 kb potato cDNA

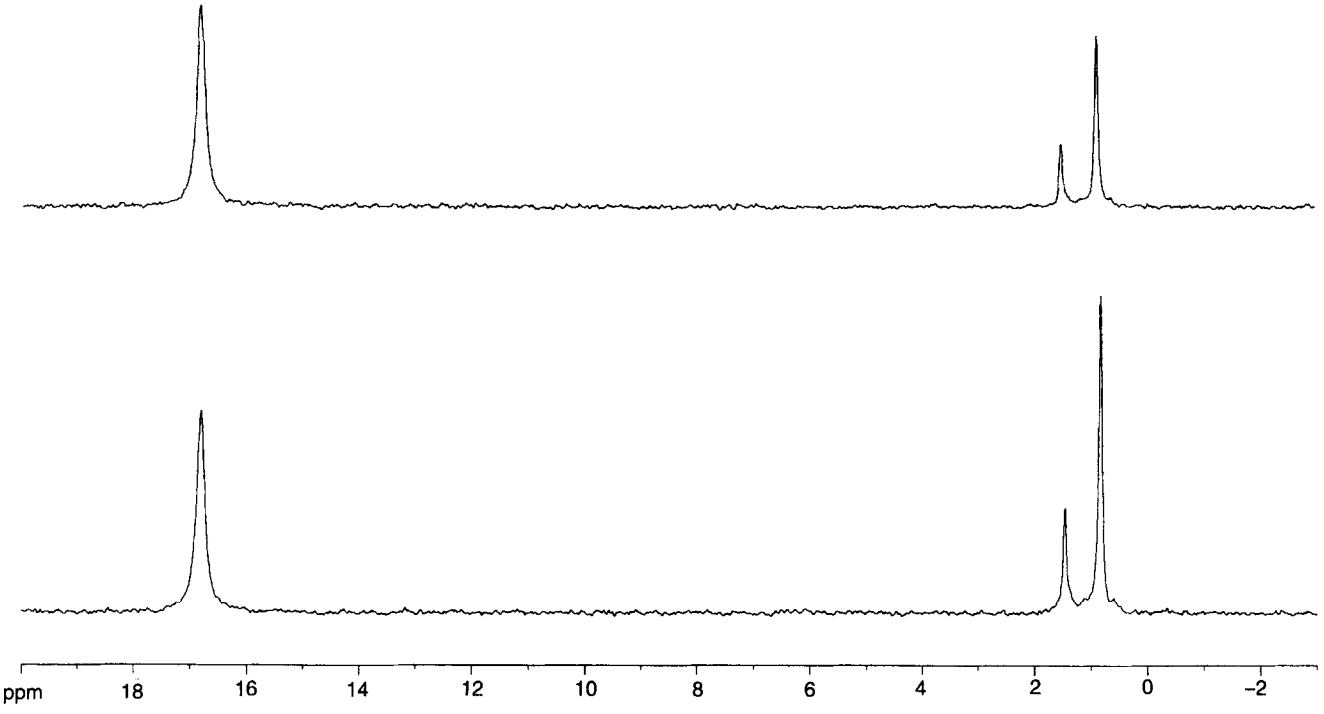

Fig. 3. ^{31}P n.m.r. spectra of starches from transgenic lines 32 (upper trace, P content 690 ppm) and 12 (lower trace, P content 1210 ppm) with a common integration standard of methylene diphosphonic acid (16.8 ppm). Signals at 0.85 and 1.48 ppm are assigned to starch-bound 6- and 3-phosphate, respectively (Mette Bay-Smidt et al., 1994).

construct. Likewise, analysis of starches from these plants revealed the same threshold relationship between altered physical properties and SBE activity. However, overall there was no greater alterations in starch properties compared to that previously observed (data not shown).

DISCUSSION

Antisense down-regulation of SBE shows phenotypic effects below a threshold level of 5% wild-type activity

Comparison of gelatinisation-related temperatures (d.s.c. peak in Fig. 5 and viscosity onset in Fig. 7) with residual tuber SBE activity shows a threshold level of *ca.* 0.75 u g^{-1} SBE below which alteration in starch properties can be detected. This corresponds to ~5% of average wild-type activity. Previous reports on the consequences of down-regulation of SBE activity in potato have found no change in contents of starch in tubers or amylose in starch with residual SBE activity in the 10%–20% range (Willmitzer et al., 1993), and no change in amylose content with "almost complete reduction" of branching enzyme activity (Müller-Röber and Kossmann, 1994). Also, in a recent study (Flipse et al., 1996) antisense inhibition of SBE

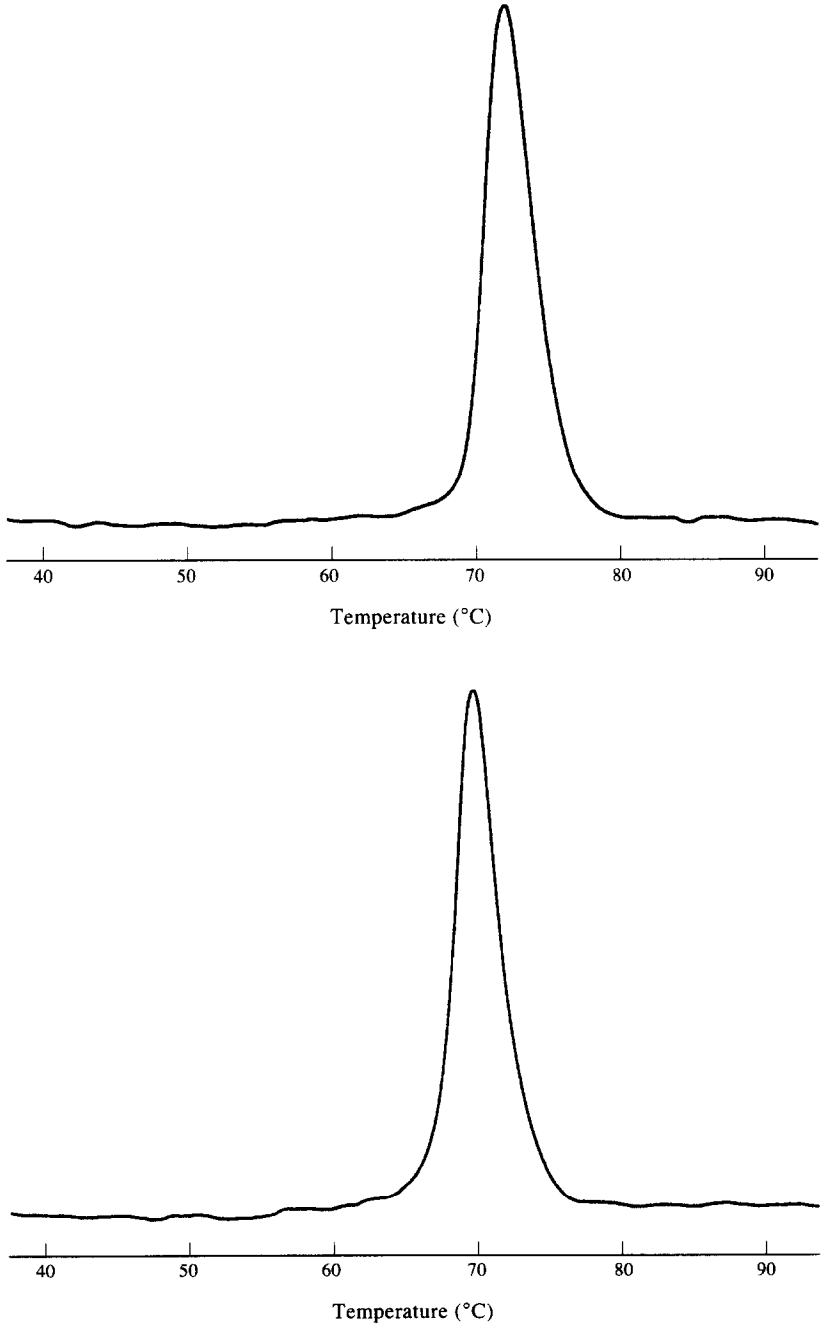

Fig. 4. D.s.c. heating traces for starches from transgenic lines exhibiting (upper trace) low SBE activity (0.3 u g^{-1} tuber) and (lower trace) high SBE activity (12.5 u g^{-1} tuber).

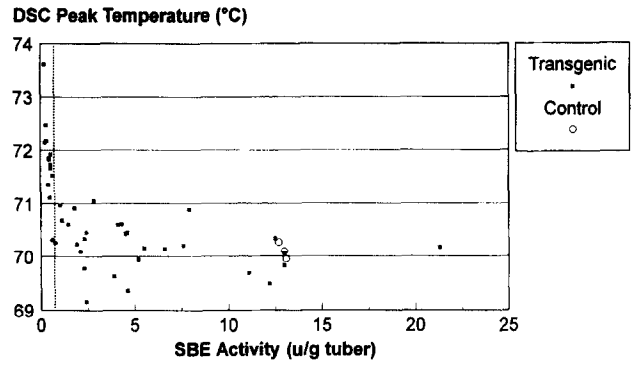

Fig. 5. Relationship between starch d.s.c. gelatinisation peak temperatures and SBE activity for a range of transgenic potato lines. The vertical dashed line represents the threshold SBE activity of 0.75 u g^{-1} tuber, below which alterations in viscosity behaviour were observed.

activity in the amylose-free potato mutant also failed to produce any detectable changes in degree of starch branching and only minor alterations in starch physical properties were observed. There is clearly a large excess capacity of SBE in potato, the repression of most of which has no discernible effect on starch composition or properties. Attempts to obtain further reductions in SBE activity, and hence greater modification to starch structure, by transformation with vectors containing tuber-specific promoters (patatin/GBSS I) in combination with either partial or full-length antisense potato SBE cDNAs were not successful. Potato SBE shows sequence homology to the SBE isoform B family (Burton et al., 1995). Unlike the A isoform, there are no reported mutants of B isoform SBEs, consistent with the finding that large reductions in potato SBE activity lead to no noticeable phenotype. In contrast, mutations in isoform A SBEs cause major changes in starch composition (Martin and Smith, 1995). In pea this results in a reduction of up to 50% in starch content and an increase in amylose from ~30 to ~70%; in maize a reduction of up to 20% in starch content and an increase in amylose from ~25 to ~50% is found. In the latter case (amylose extender) only a partial reduction in isoform A activity (Boyer and Preiss, 1978) is required.

Physical properties are a more sensitive probe of phenotypic change than molecular analysis

Due to the nature of the enzyme activity, the logical approach to assessing the impact of SBE down-regulation is to measure the level of branching within starch polysaccharides. We have taken two approaches to this measurement. In one, the percentage of α-(1 → 4)-glucan which is branched infrequently enough to result in an iodine complex with an absorption maximum at 620–630 nm is determined ('% amylose'). In the second, polymers not precipitated by thymol ('amylopectin') are debranched with isoamylase and the resulting unit chain length profile determined using high-performance anion-exchange chromatography. This provides an indirect measure of the degree of branching via an accurate description of branch lengths, but provides no information on the pattern of branching. In neither case were any significant differences detected between controls and transgenic lines (e.g. Fig. 2).

Despite the inability to detect significant changes in

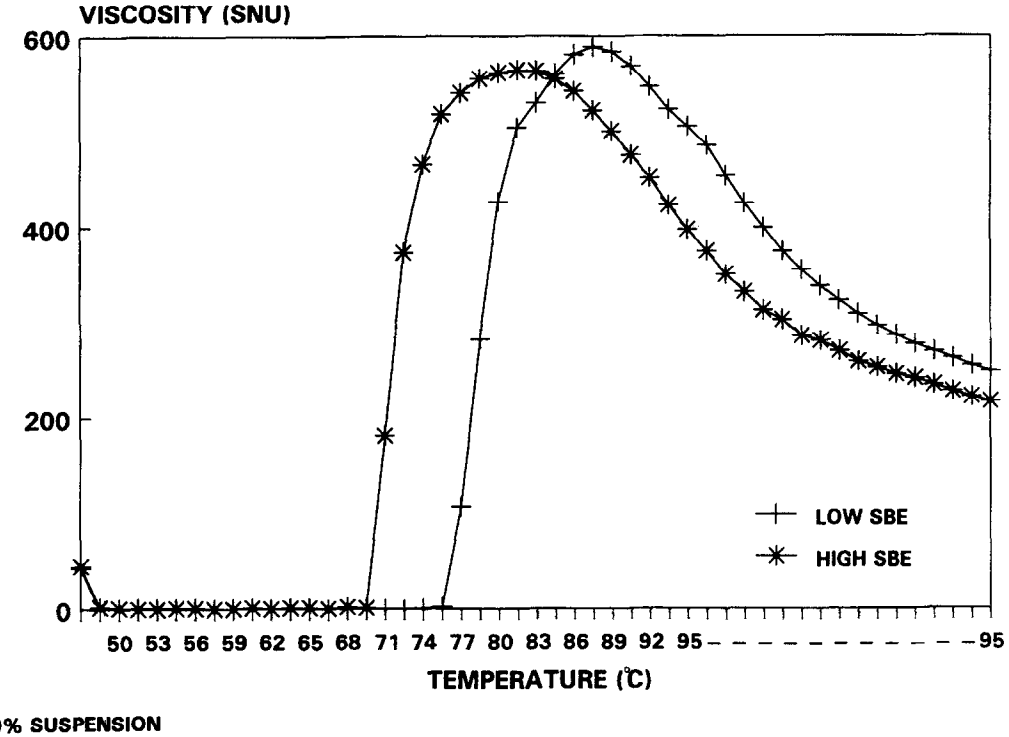

Fig. 6. Viscosity development (in instrumental stirring number units [SNUs]) on heating for starches from transgenic lines with low (0.3 u g^{-1} tuber) and high (12.5 u g^{-1} tuber) SBE activities.

glucan branching, there was a clear correspondence between tubers showing the lowest residual SBE activities and starches with elevated temperatures associated with gelatinisation phenomena. Although temperature differences are relatively small (<10°C) they are both significant and reproducible. D.s.c. has also been independently suggested as a useful screening method for potato genotypes (Kim et al., 1995). The reason why probes of gelatinisation provide a better phenotypic screen than molecular analysis may be due to the co-operative nature of gelatinisation. Upon heating a collection of starch granules, each granule is observed (typically using polarised light) to show loss of long-range order (e.g. birefringence) over a temperature range of 1–2°C, whereas the range between granules spans typically 10°C. Once triggered, each granule is envisaged to undergo a cooperative loss of order with all subsequent structural changes following in a defined fashion (Atwell et al., 1988). The temperature at which gelatinisation events are observed could thus be due to a small percentage of material responsible for initiation of the process. In this case bulk analyses, such as iodine binding or debranching may not detect subtle differences which affect gelatinisation temperatures.

Possible molecular origins for observed phenotypes

An unexpected consequence of SBE down-regulation is an increase in starch-bound phosphorous content (Kossmann, 1994) which we have shown to be due to an equal elevation of both 3- and 6-linked phosphate groups. It is, however, questionable whether this effect is a direct consequence of modification of SBE levels, as Abel et al. (1996) have described elevated phosphorous contents for potato starches derived from lines with down-regulated levels of each of a range of starch biosynthetic activities.

At the bulk molecular level, this is the only feature which has so far been shown to correlate with SBE activity in down-regulation experiments. Is this related to the observed elevation of gelatinisation temperature? It is interesting that

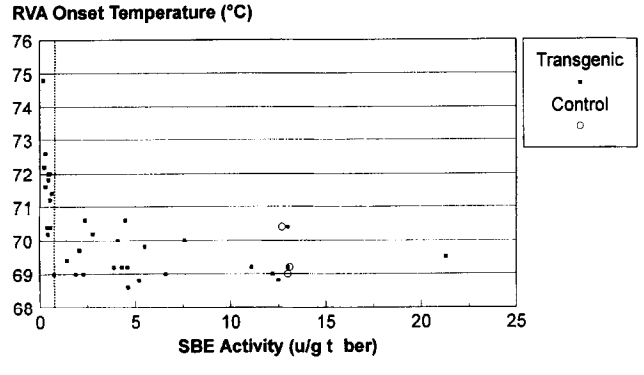

Fig. 7. Relationship between viscosity onset temperatures for 10% (w/v) starch in water and SBE activities for a range of trangenic potato lines. The vertical dashed line represents the threshold SBE activity of 0.75 u g^{-1} tuber, below which changes in d.s.c. properties were observed.

Kim et al. (1995) found a significant correlation ($r = 0.80$) between phosphorous content and d.s.c. peak temperature. Analysis for the same genotypes grown in different years showed significant variation in phosphorous content and led Kim et al. (1995) to suggest that environmental factors were important determinants of phosphorous contents.

Phosphate groups (both 3- and 6-linked) are located in the amylopectin component, mostly in longer chains and remote from branch points (Takeda and Hizukuri, 1982). The implication is that phosphate groups are present within regions which are likely to contain the partially crystalline double helical structures characteristic of starch granules. Intuitively it would be envisaged that this would result in both a de-stabilisation of double helical and crystalline order and a greater tendency to absorb moisture. The trigger which controls gelatinisation temperatures will involve some combination of enhanced mobility in non-ordered regions (caused by water uptake) and/or low melting temperatures in ordered regions. In both cases, elevated starch-bound phosphate would be predicted to result in a decreased gelatinisation temperature. We therefore propose that the correlation between phosphorous content and gelatinisation temperature observed here and by Kim et al. (1995) is coincidental and not a direct cause and effect relationship.

One of the factors which may determine gelatinisation temperatures is the length of double helices. It has been suggested (Gidley et al., 1995) that the consequence of longer double helical α-(1 → 4)-glucan structures is to increase the melting temperature without major effects on transition enthalpies (per gram). The high (140–160°C) melting temperature for double-helical amylose and the relative gelatinisation temperatures of normal and amylose-extender maize (70 *versus* 90°C) due to longer amylopectin branches in the latter can be explained on this basis. In order to estimate quantitatively the relationship between amylopectin branch length and gelatinisation temperature, oligosaccharides with defined chain lengths have been studied as model materials. Results suggest that a single additional glucose residue available for double helix formation corresponds to 3–6°C difference in melting temperature (Table 2). This is in line with recently reported data for the effect of chain length on melting temperature in fractions obtained from lintnerised potato starch granules (Moates et al., 1997). By implication, similarly small differences in amylopectin double helix lengths could underlie the observed gelatinisation temperature variation encountered in this study.

Although analysis of amylopectin branch lengths, as in Fig. 2, would detect changes significant enough to cause large differences in gelatinisation behaviour (e.g. SBE isoform A mutants in pea and maize), the method does not directly provide information on functional double helix lengths. The latter are usually assessed by either enzyme or acid digestion (Jane and Robyt, 1984, Morrison et al., 1993, Gidley et al., 1995), but the accuracy of these methods is insufficient to detect differences of the order of a single glucose unit. For the same amylopectin branch profile, it is

possible that molecular architecture (i.e. distribution of branch points) differs sufficiently for double helix lengths in the granule to be different. However, we consider it more likely that the observed phenotypic differences at low SBE activities are due to subtle differences in double helix lengths (or their non-ordered environment) at regions within granules which trigger the gelatinisation process. It is possible that starches generated in this study may be of utility in further defining the nature of the gelatinisation trigger.

In conclusion, we have used antisense RNA technology to generate a population of transgenic potato plants in which

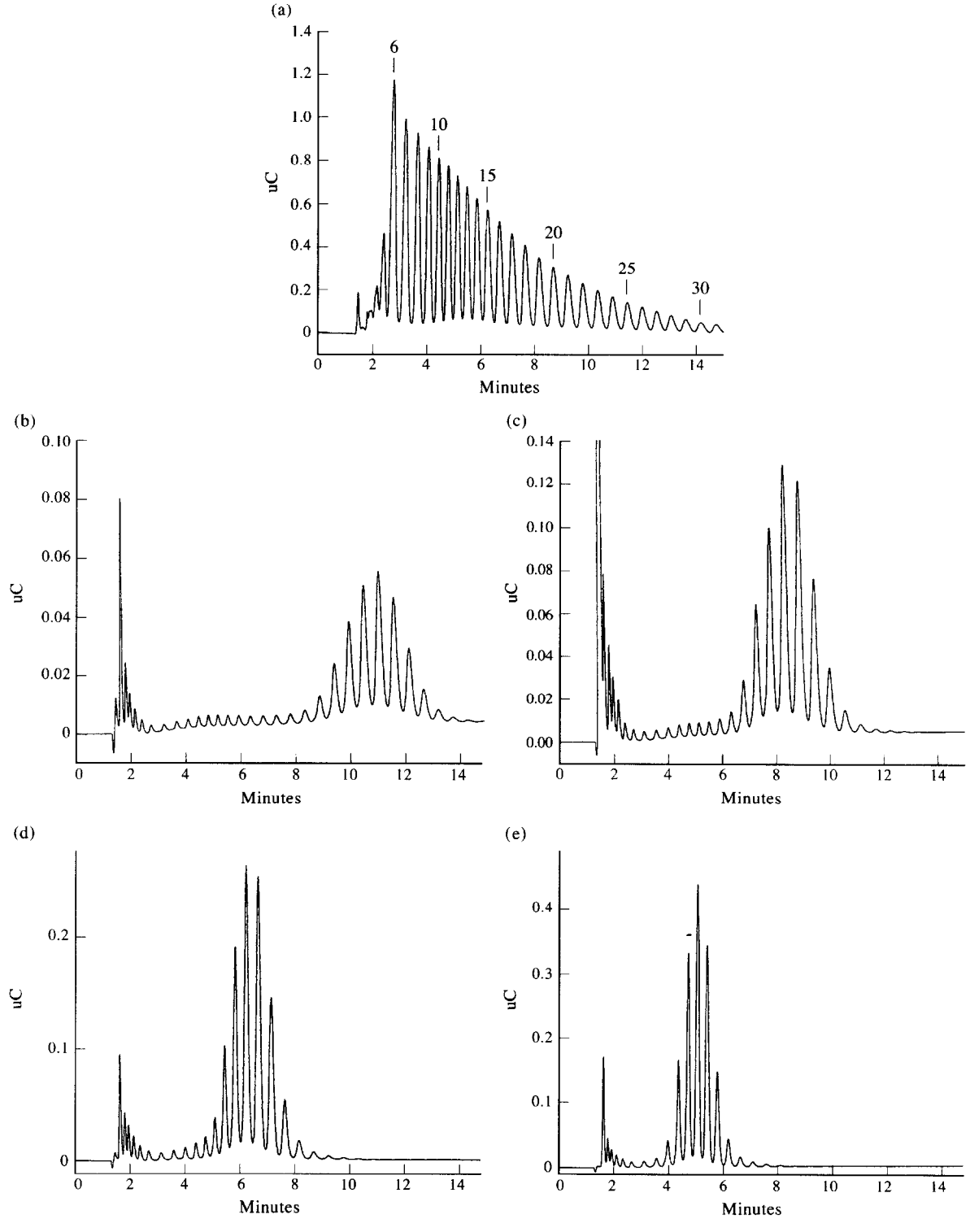

Fig. 8. Chain length profiles for (*a*) debranched glycogen and (*b–e*) fractions derived from chromatography on Bio-Gel P-6. Degrees of polymerisation of highest peaks are: (*a*) 6, (*b*) 24, (*c*) 19, (*d*) 15 and (*e*) 12.

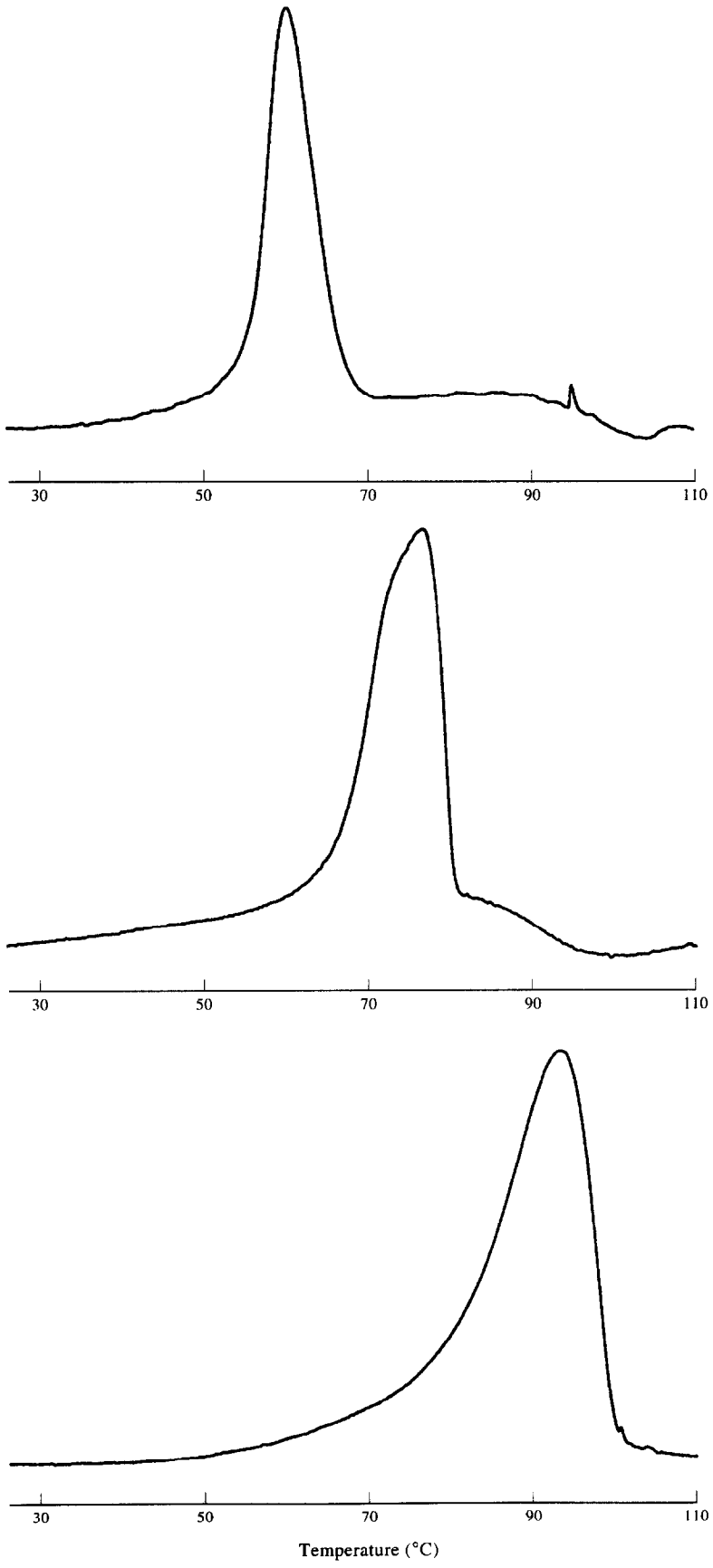

Fig. 9. D.s.c. traces obtained on heating oligoglucans precipitated from fractionated debranched glycogen corresponding to chain length profiles shown in Fig. 8*b* (upper trace), Fig. 8*c* (middle trace) and Fig. 8*d* (lower trace). Precipitates were obtained after storage at 1°C for 6 days (upper trace) or following two freeze–thaw cycles (lower two traces).

tuber SBE activity has been reduced by between 5 and 98%. Surprisingly, almost complete reduction in SBE activity did not result in alteration of amylose content and produced relatively limited changes in the physical properties of the starches. It may be that the level of enzyme repression is still not sufficent to limit synthesis of branched glucan or that there is another enzyme activity present which is able to branch linear glucan chains and which could possibly be overexpressed to 'compensate' for the reduction in SBE activity. A further possibility is that SBE activity, as measured by the indirect phosphorylase-stimulation assay, does not reflect the in vivo situation and does not truly represent the total 'branching activity' present within the cell. Further studies are in progress to attempt to unravel this enigma.

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