

# Purification and Characterization of Sucrose Synthase from the Cotyledons of *Vicia faba* L.

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## ABSTRACT

Partial purification (approximately 270-fold) of sucrose synthase (EC 2.4.1.13) from developing cotyledons of *Vicia faba* L. cv Maris Bead was achieved by ammonium sulfate fractionation and hydrophobic, affinity, anion-exchange, and gel filtration chromatography. Further purification to homogeneity resulted from gel elution of single bands from native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was identified as a homotetramer with a total molecular mass of 360 kD and subunits of 92 to 93 kD. Antibodies were raised to both native and denatured protein. The identity of the polypeptide was confirmed in western blots using antibodies raised against soybean nodule sucrose synthase. The enzyme has a pH optimum of 6.4 (cleavage direction) and an isoelectric point of 5.5. The affinity of the enzyme for sucrose ( $K_m$ ) was estimated at 169 mM, and for UDP at 0.2 mM. With uridine diphosphate as the nucleoside diphosphate, the  $V_{max}$  is 4-fold higher than with adenosine diphosphate. Fructose acts as a competitive inhibitor with an inhibitor constant ( $K_i$ ) of 2.48 mM.

Seeds of faba bean (*Vicia faba* L.) store approximately 35% (dry weight) of their food reserves as starch and 36% (dry weight) as protein. Since sucrose is the major carbohydrate transported into developing cotyledons (23), sucrose hydrolysis represents the first metabolic step en route to starch biosynthesis. Sucrose cleavage is catalyzed either by SS<sup>1</sup> (UDP-D-glucose:D-fructose 2- $\alpha$ -glucosyltransferase, EC 2.4.1.13) or invertase ( $\beta$ -D-fructofuranoside fructohydrolase, EC 3.2.1.26). The invertases can be categorized as acidic or neutral/alkaline on the basis of their pH optima (1).

The first genetic evidence indicating an important role for SS in starch biosynthesis came from a study of the maize endosperm *sh* mutant (6). Here enzyme activity is reduced to about 10% of the normal endosperm, and starch content to 40% of the wild type. In maize, a total of five SS isozymes have been identified (19). Developing endosperm cells contain the two homotetramers (S1S1S1S1 and S2S2S2S2), whereas in young roots and shoots, the three heterotetramers are also present (5). Similarly, five isozymes have been detected in sorghum but, in contrast to maize, both SS genes are expressed simultaneously in the endosperm, leading

<sup>1</sup> Abbreviations: SS, sucrose synthase;  $V_o$ , void volume; pI, isoelectric point; V8, endoprotease Glu-C from *Staphylococcus aureus* V8; Arg-C, endoprotease Arg-C from mice glands.

to the additional presence of the heterotetramers in this tissue (7).

The work of de Fekete (9) indicates that SS rather than invertase catalyzes sucrose breakdown in developing *Vicia faba* cotyledons. Although Pridham et al. (16) partially purified the protein, SS has never been fully purified or characterized from the species.

Recent work has questioned the previous consensus that UDP is the principal nucleoside diphosphate in the sucrose cleavage reaction catalyzed by SS. In sycamore suspension cells and spinach leaves, ADP-specific SS has been reported (17). Previous work with relatively crude SS preparations of *V. faba* cotyledons (9) showed no ADP specificity, although activity with ADP is clearly dependent on the assay conditions employed (17). The purification and characterization of faba bean SS is reported in this article.

## MATERIALS AND METHODS

### Plant Material

Developing field beans (*Vicia faba* cv Maris Bead) were grown in field plots at a density of 45 plants m<sup>-2</sup>, and pods were harvested 40 to 50 d after anthesis. Previous experiments revealed the highest activity of SS at this stage of bean seed development.

### Extraction and Purification of SS

Seeds (400 g total fresh weight), with their testas and embryonic axes removed, were extracted in ice-cold 200 mM Tris-HCl buffer (pH 8.5) containing 5 mM MgSO<sub>4</sub>, 5 mM 2-mercaptoethanol, and 2 mM PMSF in a prechilled blender (Atomix). Insoluble polyvinylpyrrolidone was included during the extraction (at 0.1% w/v). The homogenate was filtered through cheesecloth and re-extracted three times before centrifugation of the combined extracts at 10,000g (4°C) for 30 min. The supernatant was fractionated by the addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and the fraction that precipitated between 30 and 80% saturation was collected by centrifugation at 10,000g for 10 min. The precipitate was resuspended in 20 mM Tris-HCl buffer, pH 8.0, containing 5 mM MgSO<sub>4</sub>, 5 mM 2-mercaptoethanol, and 2 mM PMSF (buffer A), and was dialyzed against the same buffer overnight. Sufficient (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to make the solution 0.5 M with respect to the salt, and the sample was then applied to a Phenyl Sepharose column (Pharmacia LKB, UK) previously equilibrated with buffer A containing 0.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (buffer B).

Proteins bound to the column were eluted with a stepped gradient of buffer B and buffer A. SS activity was tightly bound and eluted at 100% of buffer A. The active fractions were dialyzed overnight against 50 mM Hepes-KOH (pH 8.5) containing 5 mM sucrose, 10 mM  $MgCl_2$ , and 5 mM 2-mercaptoethanol.

The dialysate was applied to a 5 mm  $\times$  100 mm phenyl boronate agarose-60 affinity column (Amicon, Stonehouse, UK), prewashed with 20 column volumes of 50 mM Hepes-KOH (pH 8.5) containing 200 mM sucrose, 10 mM  $MgCl_2$ , and 5 mM 2-mercaptoethanol, followed by further washes with 5 volumes of the same buffer containing only 5 mM sucrose. After unbound protein was eluted, SS activity was eluted with 0.1 M Tris-HCl, pH 8.5, containing 5 mM 2-mercaptoethanol (14). Active fractions were pooled, and following dialysis against 20 mM Tris-HCl (pH 7.2) containing 5 mM 2-mercaptoethanol, they were applied at 0.5 mL  $min^{-1}$  to an anion exchange column (Mono Q; Pharmacia LKB, UK) previously equilibrated with the same buffer. The protein was eluted with a KCl gradient (0–1 M) over 20 column volumes.

Active fractions were concentrated to about 1 mL, and 200- $\mu$ L volumes were applied at 0.2 mL  $min^{-1}$  to a Superose 6 gel filtration column (Pharmacia LKB) pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 100 mM KCl and 5 mM 2-mercaptoethanol. The column was calibrated with a mixture of blue dextran ( $V_0$ ), thyroglobulin ( $M_r$  669,000), apoferritin ( $M_r$  443,000),  $\beta$ -amylase ( $M_r$  200,000), BSA ( $M_r$  66,000), and carbonic anhydrase ( $M_r$  29,000). Highly purified SS preparations from this gel filtration column were used for kinetic studies. Active fractions were also dialyzed against 10 mM Tris-HCl (pH 7.2) containing 5 mM 2-mercaptoethanol and subjected to both denaturing SDS-PAGE and native PAGE.

### SDS-PAGE

SDS-PAGE was performed using a Bio-Rad mini-gel apparatus according to the method of Laemmli (11) and using 10% polyacrylamide. Gels were stained with 0.1% Coomassie brilliant blue R (Sigma) in methanol:acetic acid:water (45:10:45) and destained in methanol:acetic acid:water (30:5:65).

### Nondenaturing PAGE

Nondenaturing PAGE (SDS omitted) was performed essentially as described above with the following exceptions: (a) 100 mM sucrose was included in the gels to maintain the enzyme in its active form; (b) the pH of the resolving gel was reduced to 7.5; and (c) 7.5% polyacrylamide was used. Some samples were also electrophoresed on a 4.5 to 7.0% linear gradient nondenaturing polyacrylamide gel for 20 h at 4°C (4). To confirm the presence or absence of isozymes of SS, samples partially purified by  $(NH_4)_2SO_4$  fractionation only were subjected to two-dimensional electrophoresis for western blotting.

### Preparation of Antisera

Initially, active fractions from gel filtration chromatography were run on 7.5% nondenaturing gels and a section of the

gel was stained with Coomassie brilliant blue R to identify the major protein band. The adjacent nonstained region of the gel was excised and eluted at 4°C for 48 h in 5 gel volumes of water. The aqueous extract was lyophilized, redissolved in a small volume of water, and assayed for SS activity to confirm the identity of the protein. A parallel aliquot was subjected to SDS-PAGE. An additional lyophilized preparation was redissolved in 1 mL of Tris-buffered saline (10 mM Tris/HCl, 10 mM borate [pH 7.3], and 0.9% NaCl) and divided into three aliquots, each containing approximately 50  $\mu$ g of protein. An equal volume of complete Freund's adjuvant was mixed with one sample before injecting, intramuscularly, into a New Zealand White rabbit. Two booster injections with the addition of an equal volume of incomplete Freund's adjuvant were given 20 and 41 d later. Serum was collected 11 d after the final injection.

Antisera raised against denatured SS protein were prepared in a similar way to those against the native protein. Serum was centrifuged at 16,000g for 30 min, diluted 10-fold with water, and partially purified by the addition of an equal volume of saturated  $(NH_4)_2SO_4$  followed by gentle stirring overnight at 4°C. After centrifugation at 10,000g for 30 min, the resulting pellets were resuspended in PBS. The antibody solutions were dialyzed against PBS overnight and stored at  $-80^\circ C$ .

### Protein Blotting

Western blots using polyclonal antibodies raised against denatured SS from soybean nodules, and both native and denatured SS from *Vicia faba* cotyledons were carried out according to instructions issued by Biorad (UK). Blots were incubated with the antibodies (1:2,000 to 1:10,000 dilution with Tris-buffered saline), and antigen-antibody complexes were detected using goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (1:8,000). The chromogenic substrate for alkaline phosphatase used for detection was bromochloroindoyl phosphate/nitro blue tetrazolium.

### Protein Sequencing

Purified SS was subjected to SDS-PAGE using the improved method of Yuen et al. (24) to give higher yields for sequencing. The protein was electroblotted on to Problot membrane (Applied Biosystems, UK), stained with amido black, and sequenced on an Applied Biosystems model 477A sequencer (12). The  $NH_2$  terminus of the protein was blocked, necessitating the use of proteases to cleave the protein to obtain a partial sequence. Both V8 and Arg-C proteases were used according to the method of Cleveland et al. (8).

### Enzyme Assay

Throughout the purification, SS activity was assayed in the cleavage direction (18). Additionally, sucrose cleavage activity with nucleoside diphosphates other than UDP was determined using a stopped assay system. The 1-mL reaction mixture contained buffer (either 20 mM Tris/HCl or 20 mM Hepes/KOH, both at pH 7.0), 200 mM sucrose, 10  $\mu$ L of purified faba bean SS, and nucleosides in the range from

**Table I.** Purification of Faba Bean Cotyledon Sucrose Synthase

Fraction	Total Activity	Total Protein	Specific Activity	Yield	Purification
	units $\mu\text{mol min}^{-1}$	mg	units $\text{mg}^{-1} \text{protein}$	%	fold
Crude	679	22,263	0.031	100	
30–80% $(\text{NH}_4)_2\text{SO}_4$	586	18,042	0.032	86.3	1.1
Phenyl sepharose	337	408.3	0.83	49.6	27
Phenyl boronate agarose-60	232	124.5	1.86	34.2	61
Mono-Q	114	19.53	5.83	16.8	191
Superose-6	74	8.89	8.34	10.9	273

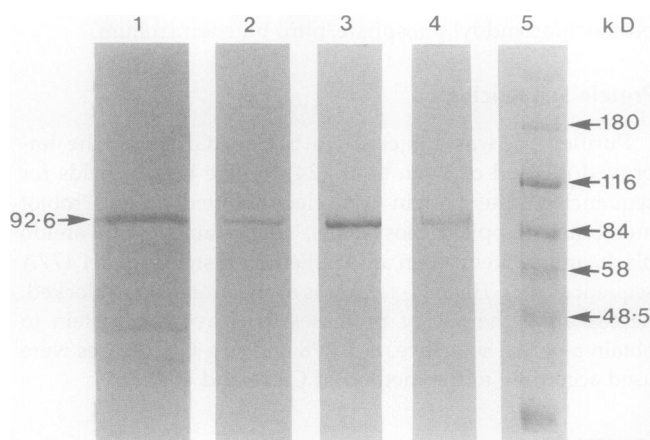
0.025 to 4.0 mM (all buffered at pH 7.0). The reaction was stopped after 3, 6, or 9 min by heating in boiling water. Fructose released was determined using an autoanalyzer system based on the method of Bergmeyer and Bernt (2, 3). Boiled enzyme extracts treated in the same way were used as controls. The unit of enzyme activity is defined as  $1 \mu\text{mol} \cdot \text{min}^{-1}$ .

#### Determination of pI

The pI of SS was determined on a Rotofor apparatus (Biorad) using Ampholines (Pharmacia LKB, UK) in the pH range of 3.5 to 10. To confirm the pI, the active fractions were collected and refocused.

#### Protein Assay

Protein concentrations were determined using the dye-binding Biorad method with BSA as the standard (0–100  $\mu\text{g}$ ).



**Figure 1.** Identification (10% SDS-PAGE gel) of the SS subunit (92.6 kD). Lane 1, Enzymically active protein fraction recovered from Superose-6. Lane 2, Western blot of crude faba bean extract using polyclonal antibodies raised against soybean nodule SS (1/2000 dilution). Lane 3, Denaturing gel of polypeptide purified following elution from SDS-PAGE. Lane 4, Denaturing gel of active SS protein eluted from native-PAGE. Lane 5, Prestained markers (SDS-7B Sigma).

## RESULTS

#### Purification of Enzyme

The protocol developed resulted in a 270-fold purification of SS (Table I). The purified preparation contained one major polypeptide ( $M_r$  92,600) and additional minor polypeptides on SDS-PAGE (Fig. 1, lane 1). The major polypeptide reacted strongly in western blots with antisera raised against SS from soybean nodules (Fig. 1, lane 2). This polypeptide was gel purified to homogeneity from a denaturing gel (Fig. 1, lane 3). A denaturing gel of enzymically active protein eluted from native PAGE also revealed a single polypeptide ( $M_r$  92,600) (Fig. 1, lane 4). Antisera raised against gel-purified native faba bean SS also cross-reacted specifically with the polypeptide (data not shown).

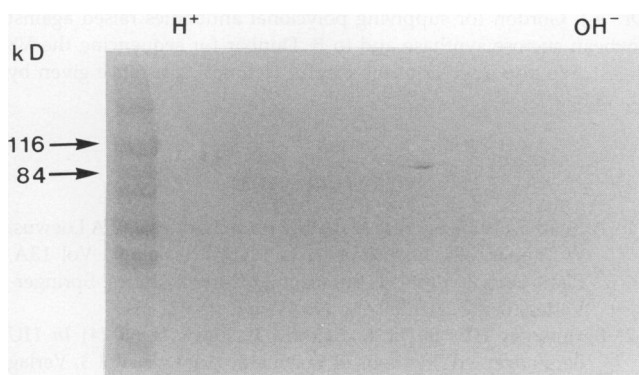
Both V8 and Arg-C proteases were tested as a means of producing peptide fragments from the 92.6-kD polypeptide for amino acid sequencing, but only V8 provided a product (78 kD) in sufficient quantity (data not shown). A sequence of 13 amino acid residues was obtained that showed substantial homology (about 50%) with potato SS (20) (Fig. 2). All the evidence therefore indicates that the protein purified is SS.

#### Determination of Relative mol wt

The mean mol wt of SS was calculated at  $353,000 \pm 19,000$  following gel filtration on Superose-6 (data not shown). The protein therefore appears to be a homotetramer. This is confirmed by the two-dimensional western blot shown in Figure 3. Several blots were prepared with a range of protein loadings, but in every case, only one cross-reacting polypeptide was observed.

AMINO ACID POSITION	130	140	150
POTATO	N F V L E L D F E P F T A S F P K P T L T		
	:	:	:
BEAN	L D F E P F S A G G L - G V		

**Figure 2.** Internal amino acid sequence (13 residues in total) of bean SS (V8 digest) showing homology with potato SS.

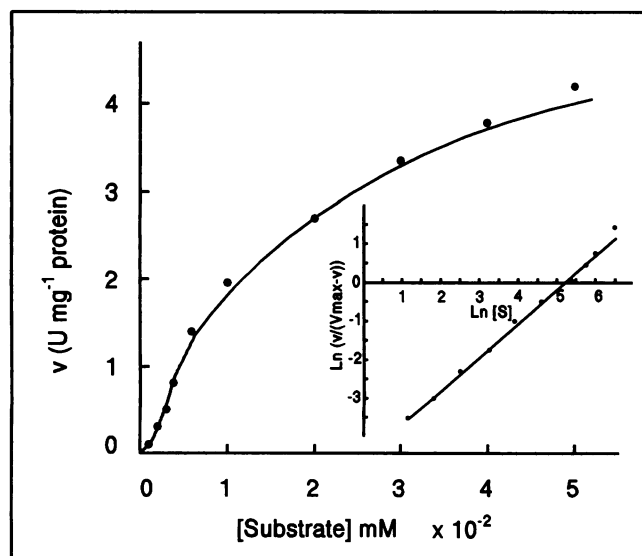


**Figure 3.** Two-dimensional western blot of bean SS using first-dimension ampholines ranging from pH 3 to 10 and second-dimension run on 10% SDS-PAGE. Antibodies used were raised against bean SS (native protein), dilution 1:10,000.

### Enzyme Kinetics

The enzyme has a pH optimum of 6.4 (Tris-HCl buffer) in the cleavage direction, but there was less than a 5% decrease in activity between pH 6.2 and 6.6 (data not shown). The pI was calculated at 5.4 to 5.5 (data not shown).

The  $K_m$  value for sucrose was estimated from the Michaelis-Menten equation to be  $169 \text{ mM} \pm 26 \text{ mM}$ . However, neither the  $V_{max}$  nor the  $K_m$  for sucrose with UDP as a nucleoside diphosphate could be determined accurately because complete saturation did not occur, even with 500 mM sucrose (Fig. 4). Similar kinetic properties for sucrose saturation have been reported for maize kernel SS by Su and Preiss (21). They offered the hypothesis that this is due to different quaternary structural forms of the enzyme in solution and,



**Figure 4.** Sucrose saturation curve of purified faba bean SS (inset: Hill plot [ $n = 1.1$ ]) (sucrose concentration 3–500 mM).

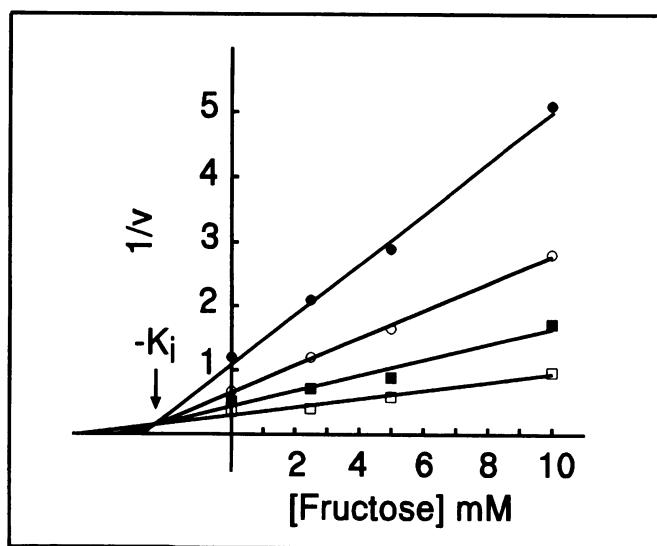
to our knowledge, this is the only explanation offered so far. The sucrose saturation curve was sigmoidal rather than hyperbolic with a Hill coefficient ( $n = 1.1$ ) (Fig. 4).

Standard Michaelis-Menten type kinetics were observed for UDP ( $V_{max}$  [UDP]  $1.29 \pm 0.03 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein). Of the four nucleoside diphosphates tested (UDP, ADP, CDP, and GDP [0.025–4 mM]), SS activity was only detected with UDP and ADP. In agreement with the findings of Pozueta-Romero et al. (17), the reaction with ADP as substrate was strongly inhibited by Tris-HCl buffer (70–80% reduction in rate), whereas with UDP, the reaction was only slightly affected (<1% reduction). With HEPES buffer, the  $K_m$  for UDP was  $0.212 \pm 0.004 \text{ mM}$  and the  $V_{max}$  was  $2.03 \pm 0.12 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein. With Tris buffer, the  $K_m$  for UDP was  $0.149 \pm 0.003 \text{ mM}$  and the  $V_{max}$  was  $1.72 \pm 0.11 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein. With HEPES buffer and ADP as the nucleoside diphosphate, the  $K_m$  for ADP was identical to UDP, but the  $V_{max}$  was only  $0.496 \pm 0.027 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein (25% of UDP). No values could be obtained for ADP in the presence of Tris buffer due to the high level of inhibition.

Fructose (10 mM) inhibited sucrose cleavage by 74% (inhibition was competitive). A Dixon plot provided an estimated  $K_i$  value of 2.48 mM at sucrose concentrations of 25, 50, 100, and 200 mM (Fig. 5).

### DISCUSSION

The purification to homogeneity of SS from *V. faba* cotyledons showed that the enzyme has a molecular mass of 360 kD and is composed of four subunits of 92 to 93 kD. The tetrameric structure of the native protein is similar to that observed with mung bean seedlings (10), rice grains (15), maize kernels (21), soybean nodules (14), and peach fruit (13). In these instances, the molecular mass of the protein ranges between 360 and 400 kD, with the molecular masses



**Figure 5.** Dixon plot showing competitive inhibition of SS by fructose: ●, 25 mM; ○, 50 mM; ■, 100 mM; and □, 200 mM Sucrose ( $v$  [units  $\text{mg}^{-1}$  protein]).

of identical subunits ranging between 87 and 100 kD. As with soybean nodules (14), *V. faba* cotyledons contain only one (detectable) form of the enzyme. Polyclonal antibodies raised against SS from whole kernels of wild-type maize (kindly supplied by Dr. Karen Koch, University of Florida) showed no specific cross-reaction on western blots with *V. faba* SS protein, unlike those raised against soybean SS, which reacted specifically with the 92-kD polypeptide subunit. The maize antibody does, however, recognize potato SS (18).

The amino acid sequence data, although only for a small portion of the faba bean SS protein, show distinct homology with potato tuber SS, which itself has a 75% overall identity with maize SS (20). Additionally, the polyclonal antibodies raised against the faba bean SS protein detect a single polypeptide (90 kD) from a crude tuber extract on a western blot (data not shown).

Recent work (17) has suggested that ADP rather than UDP is the principal nucleoside diphosphate utilized in the SS reaction. Previous work with relatively crude extracts of *V. faba* cotyledons demonstrated that the activity of SS with ADP was only 16% of that with UDP (9). This has essentially been confirmed in the present study using purified enzyme. Although significant inhibition of the faba bean enzyme with Tris buffer and ADP confirms the results of Pozueta-Romero et al. (17) with spinach leaves and sycamore cell suspensions, the faba bean enzyme is unable to utilize other nucleoside diphosphates as effectively as UDP, even when Tris is replaced by Hepes. The bean enzyme is certainly not ADP specific. The data do not, therefore, agree with the hypothesis that ADP is the principal substrate for faba bean SS, at least as far as maximum catalytic activity is concerned. Physiologically, the proportion of SS activity driven by ADP and/or UDP in vivo will clearly depend on the concentration of the nucleoside diphosphates in the cytosol. It will also depend on whether or not ADP activity is suppressed by the presence of UDP. According to Pozueta-Romero et al. (17), this is not the case. The hypothesis has not been tested for the purified faba bean enzyme. It should be noted that the kinetic data obtained for faba bean with UDP and ADP are similar to those reported for peach (13).

Fructose acts as a competitive inhibitor of faba bean SS with respect to sucrose (as shown previously with *Helianthus tuberosus* [22]). We have calculated (unpublished data) that during bean seed development, the concentration of fructose in cotyledons (on a whole tissue basis) decreases from about 7.5 mM 30 d after anthesis to about 2 mM 20 d later (at the time of maximum SS activity). At the sucrose concentrations prevailing in the tissue at the same time, we calculate that SS activity (cleavage direction) may be inhibited between 70 and 30% (assuming that sucrose, fructose, and SS are within the same cellular compartment). Fructose-specific hexokinases, known to be present in a range of tissues, including developing *V. faba* cotyledons (9, A. Gardner and H.V. Davies, unpublished data), may therefore play an important role in regulating SS activity in vivo.

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