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Purification, characterization and physiological role of sucrose synthase in the pea seed coat (*Pisum sativum* L.)

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Abstract. The seed coat is a maternal organ which surrounds the embryo and is involved in the control of its nutrition. This study with pea (*Pisum sativum* L.) was conducted to understand more fully the sucrose/starch interconversions occurring in the seed coat. The concentrations of soluble sugars, the starch content, and the activities of the sucrose-metabolizing enzymes, sucrose synthase (Sus; EC 2.4.1.13), alkaline and soluble acid invertase (EC 3.2.1.26) and sucrose-phosphate synthase (SPS; EC 2.4.1.14) were compared at four developmental stages during seed filling. Among the four enzymes, only Sus activity was very high and strongly correlated with the starch concentration in the seed coat. Sucrose synthase catalyses the cleavage of sucrose in the presence of UDP into UDP-glucose and fructose. Sucrose synthase was purified from pea seed coats in a three-step protocol, consisting of diethylaminoethyl-Sephacel chromatography, gel filtration and affinity chromatography. The enzyme was characterized at the biochemical and molecular levels. Sucrose synthase exhibits biochemical properties which allow it to function in the direction of both sucrose cleavage and synthesis. The mass-action ratio of its four substrates was close to the theoretical equilibrium constant at the four developmental stages we studied. A labelling experiment on seed coats has shown that Sus activity is reversible in vivo and can produce 37% of neo-synthesized sucrose in the seed coat cells (minimum value). It is concluded that Sus could play a central role in the control of sucrose concentration in the seed coat cells in response to the demand for sucrose in the embryo during the development of the seed.

Key words: *Pisum* – Seed coat – Sucrose – Sucrose-metabolizing enzymes – Sucrose synthase – Starch

Introduction

At maturity, seeds of pea (*Pisum sativum* L.) contain approx. 50% starch (dry weight). Starch is synthesized from sucrose, which is the major form of assimilate transported from the photosynthetic organs (source organs) to all energy-consuming tissues (sink organs). Sucrose cleavage represents the first metabolic step of starch biosynthesis. It can be catalyzed either by sucrose synthase (Sus, UDP-glucose:D-fructose-2-glucosyl transferase, EC 2.4.1.13) or invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26). Sucrose synthase catalyzes the reversible interconversion of sucrose and UDP to UDP-glucose (UDPGlc) and fructose (Avigad 1982). Sucrose synthase can also function with other diphosphate nucleotides such as ADP or thymidine 5′-diphosphate (TDP; Nguyen-Quoc et al. 1990). In contrast, invertase irreversibly hydrolyzes sucrose into glucose and fructose. Invertase activity on a fresh-weight basis is very low in the pea embryo and does not change during seed filling (Edwards and ap Rees 1986a). The maximum catalytic activity of invertase is only comparable to the estimated in-vivo rate of sucrose cleavage, whereas that of Sus is considerably in excess of the rate of sucrose cleavage throughout development (Edwards and ap Rees 1986a). It is likely, therefore, that the contribution of Sus to the cleavage of sucrose in the pea embryo is very much greater than that of invertase. Sucrose synthase involvement in starch biosynthesis was first demonstrated in the maize endosperm *sh1* mutant (Chourey 1981). The loss of 90% of Sus activity in *sh1* endosperm resulted in starch deficiency in these mutants. The UDPGlc and the fructose produced by Sus can be

The sucrose synthase cDNA sequence appears in the EMBL data base under the accession number X98598

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Abbreviations: DEAE = diethylaminoethyl; *Ki* = inhibition constant; RT-PCR = reverse transcriptase-polymerase chain reaction; UDPGlc = UDP-glucose; Sus = sucrose synthase; SPS = sucrose-phosphate synthase

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fed into starch biosynthesis by UDPGlc pyrophosphorylase and fructokinase.

The pea embryo, formed by an embryonic axis and two cotyledons where starch accumulation takes place, develops inside the seed coat. The seed coat is a maternal organ where phloem unloading occurs, as no vascular connection exists between the embryo and the mother plant. Assimilates then migrate towards the embryo. Recent studies have demonstrated that the seed coat is not a passive cell layer in the unloading process model. In particular, evidence for sucrose-starch interconversion has been provided: a part of the unloaded sucrose is stored temporarily in the seed coat cells as starch, which could allow a buffering of the embryo's nutrition (Rochat and Boutin 1992). This indicates that sucrose must be neo-synthesized in the seed coat from the degradation of starch. Sucrose-phosphate synthase (SPS; UDPGlc:D-fructose-6-phosphate-αD-glucosyl transferase, EC 2.4.1.14) catalyses the synthesis of sucrose-phosphate from fructose-6-phosphate and UDPGlc. This enzyme is considered to be a rate-limiting enzyme of sucrose synthesis in leaves, but no data are available on SPS activity in seed coats. Moreover, it has been shown that Sus, the activity of which is reversible in vitro, can also synthesize sucrose in vivo in developing potato tubers, in heterotrophic *Chenopodium rubrum* cell-suspension cultures and in cotyledons of germinating *Ricinus communis* (Geigenberger and Stitt 1993). Both enzymes are therefore good candidates for sucrose synthesis in seed coats.

Our long-term aim is to understand the role of the seed coat in the nutrition of the embryo. This necessitates a better characterization of the sucrose/starch interconversions taking place in this organ. We firstly determined the activities of the sucrose-metabolizing enzymes in this organ; we focused on Sus, which appeared to have a very high activity in the seed coat. Sucrose synthase was purified, and characterized at the biochemical and molecular levels; the role of this enzyme in sucrose/starch interconversions was investigated.

Materials and methods

Plant material and bacterial strain. Garden pea (*Pisum sativum* L. cv. Finale; seeds kindly supplied by the Station de Génétique et d'Amélioration des Plantes, INRA, Versailles, France) plants were grown in pots filled with expanded clay and peat in a growth chamber with a day/night regime of 16/8 h at 20/14 °C. They were supplied daily with a complete nutrient solution (Lesaint and Coïc 1983). Seed coats were harvested at four developmental stages, when the seeds had reached fresh weights of 125–175 mg, 200–250 mg, 350–400 mg and 550–600 mg.

Escherichia coli (DH5, BRL, Life Technologies, Cergy Pontoise, France) was used as the host for cloning Sus cDNA.

Enzyme extraction. Sucrose synthase was purified from seed coats of seeds weighing 350–400 mg FW because Sus activity is very high at this stage of the development in the pea seed coat (Rochat and Boutin 1992). These organs (10 g FW) were ground at 4 °C in a mortar in 50 ml buffer A [50 mM Hepes-NaOH, pH 7.5, containing 10 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol (DTT), 10% (v/v) ethyleneglycol, 1 mM phenylmethylsulfonyl fluoride and 10 μM leupeptin]. Insoluble polyvinyl pyrrolidone was added at 0.1% (w/v). The homogenate was centrifuged three times for 20 min at 4 °C at 20000 · *g* before being filtered (0.45 μm). For enzyme assays, seed coats were homogenized in 50 mM Hepes-NaOH, (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT. The supernatant was desalted on a Sephadex-G25 column (Pharmacia, Orsay, France) equilibrated with the extraction buffer.

Sucrose synthase purification. Anion exchange chromatography. Firstly, the crude extract from seed coats was applied to a diethylaminoethyl (DEAE)-Sephacel column (14 cm long, 1.6 cm i.d.; Pharmacia) previously equilibrated with buffer A. The column was then washed with 200 ml of buffer A and proteins eluted with a 200-ml linear gradient of 0–0.4 M NaCl in the same buffer at a flow rate of 15 ml · h^{-1}. The fractions with the highest Sus activity were then pooled and concentrated in an ultrafiltration cell with an XM-50 membrane (Amicon; Epernon, France).

Gel filtration. Secondly, the sample was loaded at 5 ml · h^{-1} onto an AcA 22 column (65 cm long, 1.6 cm i.d.; Ultrogel; Sepracor, Villeneuve-la-Garenne, France) previously equilibrated with buffer B [50 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT] and eluted with the same buffer. The fractions with the highest Sus activity were pooled and concentrated in an Amicon ultrafiltration cell with an XM-50 membrane.

Affinity chromatography. Finally, the sample was loaded at a very low flow (2–3 ml · h^{-1}) onto a UDPGlucuronate agarose affinity column (Sigma, St Quentin Fallavier, France) of 1 ml previously equilibrated with buffer B. The column was washed with ten bed volumes and the proteins eluted at 10 ml · h^{-1} by a 20-ml linear gradient of 0–0.4 M KCl in buffer B. The fractions containing the highest Sus activity were pooled and stored at –80 °C after addition of 10% (v/v) glycerol. The enzyme was stored for several months at –80 °C without loss of activity.

Protein and enzyme assays. Sucrose synthase assays. Throughout the purification, Sus was assayed at 30 °C in the breakdown direction: 200 mM sucrose, 2 mM UDP were added to buffer C [50 mM Hepes-NaOH (pH 7.0), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT] in a final volume of 500 μl. The reaction was initiated with the extract and stopped after 15 min by a 2-min incubation at 100 °C. Blanks without UDP estimated invertase activity. The fructose produced was quantified by an enzymatic method using a commercially supplied kit (Boehringer Mannheim Meylan, France; Bergmeyer et al. 1974). On crude extracts, Sus was assayed by a radioactive method, previously described in Rochat and Boutin (1992), except that the reaction was conducted at optimum pH (9.4). To measure Sus activity in the synthesis direction, 20 mM fructose and 3 mM UDPGlc were added to 50 mM 2-(N-cyclohexylamino)ethanesulfonic acid (Ches)-NaOH, (pH 9.4). The reaction was initiated with the extract and carried out at 30 °C for 15 min and stopped by a 2-min incubation at 100 °C. The UDP produced was measured by an enzymatic method as described by Lunn and ap Rees (1990). To evaluate the pH-activity relationship, 2-(N-morpholino)ethanesulfonic acid (Mes; pH 5–7), Hepes (pH 7–8.5) and Ches (pH 8.5–10) were used at 50 mM in order to cover a large pH range. Sucrose synthase activity was determined in both directions. To measure kinetic parameters in the breakdown direction, Sus was assayed at 30 °C with a continuous spectrophotometric method previously described by Nguyen-Quoc et al. (1990). When the inhibition of Sus activity by fructose was tested, the formation of UDPGlc was recorded by a coupling-enzymatic method (Quick et al. 1989). To measure kinetic parameters in the sucrose synthesis direction, the formation of UDP was recorded as in Lunn and ap Rees (1990). To measure the equilibrium constant of Sus, pure enzyme was added to an equimolar incubation medium (5 mM, 10 mM or 20 mM) of UDP and sucrose or UDPGlc and fructose in 50 mM

Hepes-NaOH (pH 7), or in 50 mM Ches-NaOH (pH 9.4), respectively. The formation of fructose or sucrose was recorded from 1 to 40 h. The data were fitted to a model hyperbolic curve using STAT-ITCF software (ITCF, Boigneville, France) to evaluate the concentration of fructose or sucrose at the equilibrium. The concentrations of the three other substrates were deduced from this determination.

Sucrose-phosphate synthase assay. Sucrose-phosphate synthase was assayed as in Galtier et al. (1993), except that 50 mM sucrose was added to the reaction medium to limit the effect of Sus on the cleavage of radioactive sucrose produced by SPS.

Invertase assay. Alkaline invertase and soluble acid invertase were assayed at 30 °C for 30 and 60 min with 100 mM sucrose and, respectively, 20 mM Hepes-NaOH (pH 7.8) or 20 mM sodium acetate (pH 4.7). The reactions were stopped by a 1-min incubation at 100 °C. Blanks were boiled immediately. Fructose and glucose were measured as described above.

Protein assay. Protein content was measured by a Coomassie Blue binding assay (Bradford 1976) with bovine serum albumin (BSA) as a standard.

Electrophoresis and staining. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, 7.5% acrylamide, was performed according to the method of Laemmli (1970), at room temperature for 4–6 h at 80 V. The relative molecular mass (M_r) of Sus subunits was determined with standards (Sigma) covering the 45- to 205-kDa range. Native-PAGE, 7% acrylamide, was performed at 4 °C for 4–6 h at 80 V as in Laemmli (1970) but SDS was ommited. Native M_r determination was obtained by a 4–15% gradient gel (Bio-Rad, Ivry sur Seine, France) with egg albumin, BSA and urease as standards (Sigma), covering the 45- to 545-kDa range. Native isoelectric focusing, 5% acrylamide, 3% ampholines (pH 4–6.5; Pharmacia), was performed at 4 °C overnight at 50 V. The isoelectric point of Sus was determined with standards (Bio-Rad), covering the unit range 3.6–6.6. Proteins were stained with Coomassie Brilliant Blue R-250.

Antisera production and immunotechniques. Antisera were produced in rabbits from gel-purified Sus. Preparation of polyclonal antibodies was done as reported in Suzuki et al. (1994).

The gel-slab-separated protein bands were analysed by Western blot on a nitrocellulose sheet (0.45 μm; Schleicher and Schuell, Céra Labo, Equevilly, France) after electrophoretic transfer in 25 mM Tris-HCl (pH 8.3) containing 192 mM glycine and 20% (v/v) methanol (24 V for 12 h at 4 °C). Blots were incubated with antibodies (1:500 dilution with Tris-buffered saline) and antigen-antibody complexes were detected with goat anti-rabbit immunoglobulin conjugated with peroxidase (1:800).

The amounts of Sus protein were determined by the immunorocket technique of Laurell (1966) on 1% agarose gels in 160 mM Tris-HCl, 800 mM glycine (pH 8.6) containing antibodies (100 μl for 15 ml gel), run for 20 h at 4 °C, 70 V. The gels were then rinsed for 2 h in 9% (w/v) NaCl, 0.05% (v/v) Triton X-100 and for 15 min in water. Proteins were stained with Coomassie Brilliant Blue R-250. The purified Sus was used as standard for absolute determination.

Isolation of cDNA and sequencing. The Poly(A)$^+$RNA was isolated from total mRNA of seed coats on oligo (dT) cellulose columns (Pharmacia). Sucrose synthase cDNA was obtained by rapid amplification of 3′cDNA ends (3′RACE system; BRL) using reverse transcriptase-polymerase chain reaction (RT-PCR) on poly(A)$^+$RNA with a gene-specific primer and an universal adapter primer (poly-T sequence). The gene-specific primer was an oligonucleotide of 20 bases (TCGTCTCAGATGAACCGTGT) in a very homologous region of the Sus genes of *Vicia faba*, *Vigna radiata*, *Daucus carota* and *Solanum tuberosum*, situated 700 bp upstream of the 3′RNA end. The 3′RACE products were cloned in pAMP1 (BRL). The cDNA obtained was cloned in phage M13 and sequenced by the dideoxysequencing method (Sanger et al. 1977; Sequenase Version 2.0 kit; USB, Amersham, Les Ulis, France).

Preparation of DNA and Southern blot hybridization. The DNA was isolated from cotyledons of developing pea seeds according to a micro-scale procedure (Dellaporta et al. 1983). The DNA was digested overnight by restriction enzyme (*Eco*RI, *Bam*HI or *Hind*III) in the presence of BSA (0.03% w/v), RNase (1.5 $\mu g \cdot \mu l^{-1}$) and spermidine (7 mM). The DNA restriction fragments were separated on a 0.8% agarose gel, blotted in 10 × saline sodium citrate (SSC; 1.5 M NaCl, 0.15 M Na_3-citrate, pH 8.0) on Gene Screen nylon membrane (Nen Research Products, Les Ulis, France) according to the Southern technique (Maniatis et al. 1982). The membrane was then rinsed in 10 × SSC and heated at 90 °C for 2h. The hybridization was carried out at 68 °C with Quickhyb hybridization solution kit (Stratagene, Ozyme, Montigny le Bretonneux, France). A probe was synthesized from Sus cDNA by random priming with [^{32}P] dCTP (Prime-it RmT Random Primer Labelling kit; Stratagene). The membrane was exposed to Kodak XAR film at –80 °C.

Preparation of RNA and Northern blot hybridization. Total RNA was extracted from seed coats as in Verwoerd et al. (1989) and separated on a 1.5% agarose gel. The RNA was blotted in 10 × SSC on nylon membrane, positively charged (Boehringer Mannheim) according to the Northern technique (Maniatis et al. 1982). The membrane was then rinsed in 10 × SSC and nucleic acids were fixed by UV crosslinking (Stratalinker, Stratagene). The hybridization was performed at 50 °C with DIGeasy Hyb (Boehringer Mannheim). The digoxygenin (DIG)-labelled DNA probe was generated according to the method of random primed labelling with DIG-11-dUTP (DIG DNA labelling kit; Boehringer Mannheim). Detection was carried out using the chemiluminescent substrate CDP-Star (Boehringer Mannheim). Procedures were performed according to the manufacturer's instructions. Membranes were exposed to Kodak XAR film at room temperature for about 15 min.

Labelling experiments and analysis of radioactive extracts. Seed coats were collected from seeds of 440–470 mg FW. Twenty seed coats were incubated at room temperature on a shaker in 100-ml Erlenmeyer glass flasks containing 50 ml incubation medium [25 mM Mes-KOH (pH 6.25), 5 mM glucose, 5 mM fructose, 390 mM mannitol]. After a 20-min pre-incubation in unlabelled medium, radiolabelled compounds were added ([^{14}C]glucose at 32 kBq.μmol^{-1} or both [^{14}C]glucose and [^{14}C]fructose at 16 kBq.μmol^{-1} each). The seed coats were then incubated for 10 min in labelled medium. The seed coats were then rinsed in water at 4 °C for 5 min, gently dried with tissue paper and frozen in liquid nitrogen.

Seed coats were extracted sequentially at 4 °C in 96%, 80% and 50% (v/v) ethanol and water for 1 h. The extracts were combined and separated into neutral, acidic and basic fractions on cation- and anion-exchange resins. The neutral fraction was dried in a SpeedVac (Savant, Bioblock Scientific, Strasbourg, France), then resuspended in water. Sucrose, glucose and fructose were separated by HPLC on an Aminex HPX42C column (Bio-Rad). The radioactivity of each sugar was counted by liquid scintillation (Betamatic IV; Kontron, Montigny le Bretonneux, France). To isolate glucosyl and fructosyl residues from sucrose, an aliquot of sucrose was incubated overnight at room temperature with 2 U of invertase and reapplied to the HPLC column; the amount of label in glucose and fructose was determined by liquid scintillation counting.

Metabolite analysis. Sucrose, glucose, fructose and starch were extracted and assayed as in Rochat et al. (1995). Both UDP and UDPGlc were extracted and assayed as in Quick et al. (1989). Recovery assay for UDPGlc was done and calculated to be 94%.

Table 1. Metabolite contents and mass-action ratios for Sus in the seed coats of developing pea seeds at four developmental stages during seed filling. Data are the means ± SE of three separate samples of seed coats collected from seeds of 125–175 mg FW (SC150), 200–250 mg FW (SC225), 350–400 mg FW (SC375), 550–600 mg FW (SC575)

Developmental stage	Starch	Sucrose	Glucose	Fructose	UDP	UDPGlc	[Fructose] [UDPGlc]
	(mg · (seed coat)$^{-1}$)	(μmol · (g FW)$^{-1}$)			(nmol · (g FW)$^{-1}$)		[Sucrose] [UDP]
SC150	2.3 ± 0.2	140 ± 5	2.4 ± 0.3	2.5 ± 0.1	104 ± 17	331 ± 27	0.06
SC225	3.0 ± 0.4	161 ± 9	2.6 ± 0.5	1.8 ± 0.1	260 ± 16	442 ± 39	0.02
SC375	3.4 ± 0.2	148 ± 2	1.0 ± 0.1	2.3 ± 0.1	214 ± 28	527 ± 14	0.04
SC575	1.3 ± 0.2	95 ± 5	0.8 ± 0.1	1.9 ± 0.2	272 ± 60	496 ± 79	0.04

Table 2. Activities of sucrose-metabolizing enzymes in the seed coats of developing pea seeds at four developmental stages during seed filling. Values in parentheses are the amount of Sus protein in mg · (g FW)$^{-1}$, determined by the immunorocket technique (see *Materials and methods*). Data are the means ± SE of three separate samples of seed coats collected from seeds of 125–175 mg FW (SC150), 200–250 mg FW (SC225), 350–400 mg FW (SC375), and 550–600 mg FW (SC575)

Developmental stage	Sus	SPS	Alkaline invertase	Soluble acid invertase
	(μmol · min^{-1} · (g FW)$^{-1}$)			
SC150	2.67 ± 0.23 (0.50)	0.49 ± 0.09	0.47 ± 0.09	0.09 ±0.04
SC225	2.83 ± 0.10 (0.50)	0.45 ± 0.09	0.46 ± 0.07	0.06 ± 0.02
SC375	2.50 ± 0.25 (0.45)	0.44 ± 0.03	0.47 ± 0.08	0.03 ± 0.01
SC575	1.40 ± 0.22 (0.38)	0.37 ± 0.04	0.27 ± 0.04	0.03 ± 0.01

Results

Sucrose/starch metabolism in the seed coat of the developing pea seed. Sucrose, glucose, fructose concentrations and starch content were determined in seed coats of developing pea seeds at four developmental stages during seed filling (Table 1). Seed coats were collected from seeds of 125–175 mg FW, 200–250 mg FW, 350–400 mg FW and 550–600 mg FW. Sucrose was the major soluble sugar and had a high concentration in the seed coat. Glucose and fructose concentrations remained low throughout development of the seed. Starch was stored in the seed coat cells very early in the pea seed development (Table 1). This temporary reserve was mobilized throughout development, probably to the benefit of the embryo: in seeds of 550–600 mg FW, the seed coats contained much reduced starch. It follows that sucrose has to be cleaved and synthesized in this organ, and the next objective was therefore to identify the enzymes responsible for this metabolism.

Sucrose synthase and alkaline invertase are both enzymes that are located in the cytoplasm and can potentially cleave sucrose in this compartment. In the seed coat, Sus activity was higher than that of alkaline invertase (Table 2); moreover, the former was highly correlated to starch concentration in the seed coat, whereas alkaline invertase activity was low and did not change (Fig. 1). Soluble acid invertase, localized in the vacuole, had a very low activity throughout seed development (Table 2). Sucrose-phosphate synthase catalyses the synthesis of sucrose. Its activity was determined in the seed coat at the four developmental stages (Table 2): SPS activity was lower than Sus activity and decreased slightly during seed filling. We focused our study on Sus which appeared to be the major enzyme of sucrose metabolism in the seed coat.

Purification of sucrose synthase. Sucrose synthase was purified 20-fold from pea seed coats in a three-step protocol. A typical purification is shown in Table 3. Because of its high capacity, DEAE-Sephacel in the first step allowed direct chromatography of the crude homogenate without a previous protein precipitation

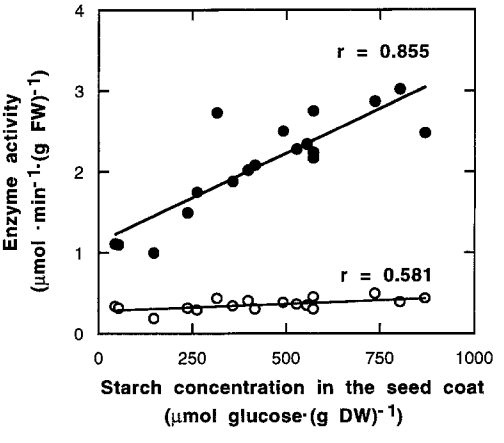

Fig. 1. Correlation between the starch concentration in the seed coat and activities of Sus (●) and alkaline invertase (○) in pea seed coats during seed filling

Table 3. Typical purification of pea seed coat Sus. For this experiment, 10 g of seed coats was collected from seeds of 350–400 mg FW

Fraction	Total activity μmol · min^{-1}	Total protein (mg)	Specific activity (μmol·min^{-1} ·(mg protein)$^{-1}$)	Yield (%)	Purification (fold)
Crude extract	24.33	81.7	0.30	100	
DEAE-Sephacel	16.30	19.4	0.84	67	2.8
AcA 22	13.08	6.1	2.14	54	7.1
UDPGlucuronate agarose	10.17	1.7	5.98	38	20.0

step. Then the gel filtration and the affinity step increased Sus specific activity.

The purity of Sus was tested: firstly, only one sharp peak was observed at 165 mM KCl in the last step of purification. Only one polypeptide was revealed by Coomassie Brilliant Blue coloration on a non-denaturing gel (Fig. 2). Antisera raised against maize Sus (kindly supplied by Dr. A. Lecharny, Université Paris-Sud, France) cross-reacted specifically with the polypeptide (Fig. 2). Our own antibodies raised against gel-purified pea Sus also specifically recognized this protein (Fig. 2). The purified Sus was found to be free from contamination by invertase, UDPGlc pyrophosphorylase and SPS activities. This preparation of Sus was used for biochemical characterization.

Biochemical characterization of the pea seed-coat sucrose synthase. The relative molecular mass (M_r) of the polypeptide was determined. The purified enzyme appeared to be a homotetramer. On SDS-PAGE, one band was revealed at 94 kDa, which corresponds to a protein at around 380 kDa on a non-denaturing gel (data not shown).

The enzyme exhibited an optimum pH of 6.5–7 in the direction of sucrose cleavage and 9.5 in the direction of sucrose synthesis (Fig. 3). Sucrose synthase activity is totally reversible in vitro: maximal activity was identical in both the cleavage and synthesis directions. Moreover, at the cytoplasmic pH (around 7.2; Roberts et al. 1980), pea Sus activity in the direction of sucrose synthesis represented 70% of Sus potential activity in the direction of sucrose cleavage (Fig. 3): a contribution of Sus to sucrose synthesis cannot be excluded. Physiologically, Sus activity will clearly depend on the substrates available in the cytoplasm. The isoelectric point of the enzyme was estimated to be 5.4 by isoelectric focusing (IEF) techniques (data not shown).

The kinetic parameters of Sus were determined at pH 7.0 in the sucrose cleavage direction for sucrose, UDP and ADP, and at pH 9.4 in the sucrose synthesis direction for fructose and UDPGlc. In each case, saturation curves were typically hyperbolic. The K_m and V_{max} values were estimated from the Michaelis-Menten equation (Table 4).

Fructose is a competitive inhibitor of the cleavage of sucrose by Sus. Dixon plots gave an estimated inhibition constant value (*Ki*) of 12 mM for fructose at sucrose concentrations of 25, 50, 100 and 200 mM (data not shown).

As Sus activity is totally reversible in vitro, its equilibrium constant was determined experimentally. It is defined as the ratio [fructose][UDPGlc]/[sucrose][UDP], when the reaction is at equilibrium. It was dependent on pH. At pH 7.0, it was estimated to be 0.15; at pH 9.4, it was estimated to be 0.004. These values

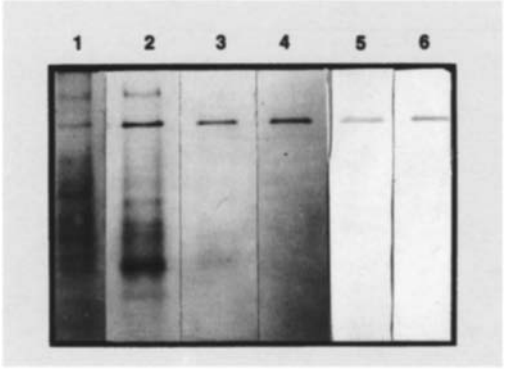

Fig. 2. Purification and identification of Sus protein. Non-denaturing 7% polyacrylamide gel stained with Coomassie Brilliant Blue R-250 showing protein pattern of the seed-coat crude extract (*lane 1*), after DEAE chromatography (*lane 2*), after gel filtration (*lane 3*) and after affinity chromatography (*lane 4*); immunoblots of purified Sus probed with maize antibodies (*lane 5*) and pea antibodies (*lane 6*)

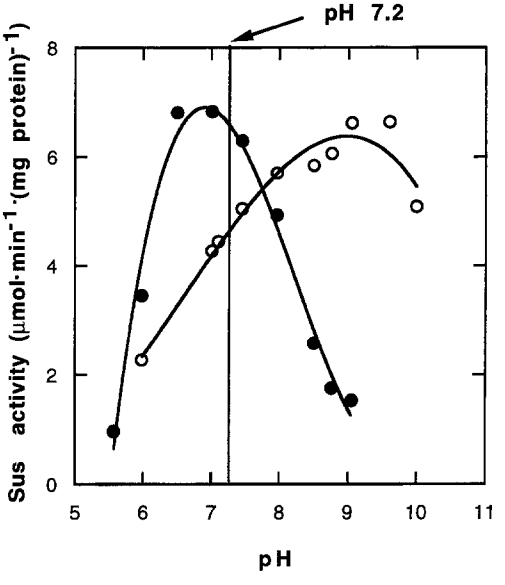

Fig. 3. Influence of pH on pea seed-coat Sus activity in the sucrose synthesis (○) and sucrose cleavage (●) direction. Each point is the mean of three measurements

Table 4. Kinetics parameters of Sus from pea seed coats. Kinetic parameters for sucrose were obtained with UDP as second substrate. In the cleavage direction, the assays were performed at pH 7.0 with 200 mM sucrose or 2 mM UDP and different concentrations of the substrate of interest. In the synthesis direction, the assays were performed at pH 9.4 with 20 mM fructose or 4 mM UDPGlc and different concentrations of the substrate of interest. Data are the means ± SE of three separate determinations

Substrates	K_m (mM)	V_{max} ($\mu mol \cdot min^{-1} \cdot (mg\ protein)^{-1}$)
Sucrose	33 ± 5	9.5 ± 1.0
UDP	0.025 ± 0.001	7.6 ± 1.0
ADP	0.508 ± 0.130	1.5 ± 0.2
Fructose	7.2 ± 0.3	4.4 ± 0.1
UDPGlc	0.158 ± 0.009	3.5 ± 0.1

show that the equilibrium is strongly displaced towards the sucrose synthesis reaction at both pH values.

Molecular characterization of Sus. A partial cDNA was obtained by RT-PCR from seed coat mRNA. It contained 800 bp, 620 bp from the open reading frame and the complete 3′ untranslated region (180 bp). Pea Sus is closely related to *Vicia faba* Sus with 95% identity at the level of the nucleotide sequence and 96% identity at the level of the deduced amino-acid sequence (data not shown). On the 165-amino-acid deduced sequence, we detected one supplementary residue (lysine) and only two significant changes in the sequence. Moreover, the 3′ untranslated region of the pea mRNA was very homologous to those of *Vicia faba* mRNA with only microheterogeneities (6% mismatch in their sequence alignment). Sucrose synthase genes of maize, wheat and potato are organized in a two-gene family (Werr et al. 1985; McCarty et al. 1986; Maraña et al. 1988; Fu and Park 1995). Westerns blots of extracts from different organs of the pea plant revealed a single band whatever the technique used (IEF, 4–15% acrylamide gradient gel). To investigate whether there is only a single gene in pea, a Southern blot analysis was performed (data not shown). The data obtained seem to indicate that there is only one gene in pea. Low-stringency hybridization and washing conditions gave no clear evidence for more than one gene, but we cannot completely exclude the existence of a distantly related gene.

Expression of the Sus gene in seed coats during seed filling. The steady-state levels of Sus mRNA in seed coats at the four developmental stages were also determined (Fig. 4). They were found to be closely related to the levels of the activity and quantity of Sus (Table 2), suggesting that transcription may be the key regulatory step in the developmental expression of Sus.

Physiological role of Sus in the seed coat of the developing pea seed. Sucrose synthase from pea seed coats has biochemical properties which allow it to work in either direction in vivo. We investigated the possible involvement of Sus in sucrose synthesis from starch degradation in the seed coat.

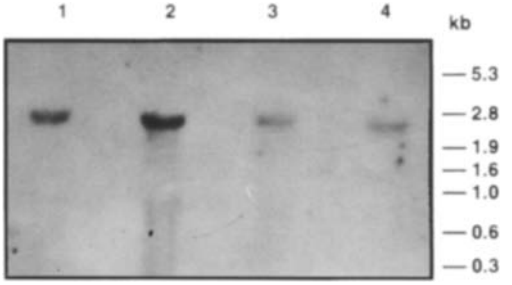

Fig. 4. Northern blot of total RNA from the seed coats of developing pea seeds at four developmental stages during seed filling. *Lane 1*, 125–175 mg FW seeds; *lane 2*, 200–250 mg FW seeds; *lane 3*, 350–400 mg FW seeds; *lane 4*, 550–600 mg FW seeds (20 µg RNA loaded per track and probed with digoxygenin-labelled partial Sus cDNA)

Firstly, we measured the in-vivo contents of sucrose, fructose, UDPGlc and UDP in the seed coats at the four developmental stages we studied (Table 1). Assuming that these compounds are homogeneously distributed in the seed coat tissue and not compartmentalized in the cell, we calculated a mass-action ratio for the four developmental stages (Table 1). Whatever the developmental stage, the estimated mass-action ratio was very close to the theoretical equilibrium (0.15 at pH 7.0), although slightly lower (Table 1).

As this first approach relied on the hypothesis of a homogeneous distribution of sugars, we needed to further investigate the relative contribution of Sus and SPS in the synthesis of sucrose by a labelling experiment as in Geigenberger and Stitt (1993). Phosphoglucose isomerase catalyses an equilibrium between glucose-6-phosphate and fructose-6-phosphate (Geigenberger and Stitt 1993). Sucrose synthesized by SPS will be symmetrically labelled on its glucosyl and fructosyl moieties whether [^{14}C]glucose or [^{14}C]fructose is supplied. On the other hand, if sucrose is synthesized by Sus, the fructosyl moiety will be derived from the free fructose pool: it will be labelled from [^{14}C]fructose, but not from [^{14}C]glucose, whereas the glucosyl moiety will be labelled from both hexoses. The intramolecular labelling of sucrose from [^{14}C]glucose and unlabelled fructose, or that from [^{14}C]glucose and [^{14}C]fructose, were compared to determine the relative contributions of Sus and SPS to sucrose synthesis. The seed coats were incubated for 10 min with 5 mM glucose and 5 mM fructose. We used a very short period of incubation to limit the possible appearance of labelling in fructose from labelled glucose. Moreover, we used glucose and fructose concentrations very close to the endogenous concentrations: in this experiment, it was important not to strongly modify the endogenous concentrations in order to compare Sus and SPS contributions to sucrose synthesis in-vivo conditions. Indeed, glucose and fructose concentrations in the seed coat after incubation increased only by 10% (data not shown). The sucrose concentration within the seed coat decreased by 15% during the treatment. Sugar concentrations, however, remained very close to those found in vivo. Table 5 presents the incorporation of ^{14}C in sucrose, starch, glucose, fructose, amino acids and organic acids,

Table 5. Incorporation of ^{14}C in different metabolites and labelling of glucose and fructose moieties of sucrose after supplying [^{14}C]fructose and/or [^{14}C]glucose for 10 min to seed coats collected from seeds of 440–470 mg FW. The total amount of radioactivity was the same for the two experimental conditions: 8 MBq in 50 ml. Data are the means ± SE of four different samples. The significance of the results was tested: *, **, ***, the mean values between the two treatments differ significantly at the 5%, 1% and 0.1% level of probability, respectively; ns: the mean values do not differ significantly between the two treatments

	Seed coats incubated in:				*t*
	[^{14}C]glucose + unlabelled fructose		[^{14}C]glucose + [^{14}C]fructose		
Rate of ^{14}C uptake ($Bq \cdot (g\ FW)^{-1} \cdot h^{-1}$)	9281	± 166	8817	± 219	ns
Rate of ^{14}C incorporation in: ($Bq \cdot (g\ FW)^{-1} \cdot h^{-1}$)					
Glucose	2888	± 192	1522	± 92	***
Fructose	990	± 14	1499	± 159	**
Sucrose	382	± 28	488	± 14	*
Starch	812	± 49	509	± 38	**
Amino acids	656	± 19	598	± 56	ns
Organic acids	2033	± 13	1831	± 80	*
Distribution of ^{14}C in sucrose (glucose/fructose)	1.59 ±	0.06	0.96 ±	0.05	**

and the distribution of the labelling of glucose and fructose moieties in sucrose. Total uptake of ^{14}C was not different between the two sets of experiments. Sucrose and starch were both synthezised during the incubation. Labelling of sucrose was more rapid when equally labelled fructose and glucose were provided as substrates than when only glucose was labelled. As the total amount of radioactivity was the same for the two experimental conditions, this indicates that Sus must contribute to sucrose synthesis. Conversely starch was labelled more rapidly when only glucose was labelled. The fructosyl moiety of sucrose contained 1.6-fold less label than its glucosyl moiety of sucrose when only glucose was labelled. When both hexoses were labelled, the glucosyl and fructosyl moieties of sucrose contained comparable amounts of label. There was recycling of label from [^{14}C]glucose into the cold endogenous pool of fructose, although the seed coats were incubated for a very short time. Nevertheless, the difference in label of fructose between the two sets of experiment stayed very significant.

The contribution of Sus to sucrose synthesis during the experiment can be estimated from the analysis of the intramolecular labelling pattern in sucrose as mentioned above. The relative contribution of Sus was estimated to be 37% by the formula:

$$\%\ \text{Sus} = \left(1 - \frac{^{14}C \text{ in glucosyl moiety of sucrose}}{^{14}C \text{ in fructosyl moiety of sucrose}}\right) \times 100$$

This is an underestimate due to the appearance of label in the free fructose pool. But it clearly demonstrates that Sus can synthesize a significant proportion of sucrose in the seed coat.

Discussion

Biochemical properties of pure sucrose synthase. The enzyme was purified to homogeneity from pea seed coats collected from seeds of 350–400 mg FW by a three-step protocol (DEAE-Sephacel chromatography, gel filtration and affinity chromatography). It resulted in a yield of 38%. The purification factor we obtained (20) is comparable to those found for Sus from soybean nodules (23; Morell and Copeland 1985), maize leaves and kernels (36 and 50, respectively; Nguyen-Quoc et al. 1990), and wheat leaves (20–40; Larsen et al. 1985), but it is lower than that found for Sus from *Vicia faba* cotyledons (273; Ross and Davies 1992). The low purification factor we obtained was due to the abundance of this enzyme in pea seed coats. Taking into account recovery and purification yield, Sus can be estimated to make up 5% of the total extracted protein at the stage used (seeds of 350–400 mg FW). This value was confirmed by the quantification of Sus protein by the immunorocket technique (4.6% of the total extracted proteins, data not shown). We obtained a specific activity for purified pea Sus of 6 $\mu mol \cdot min^{-1}$.(mg protein)$^{-1}$. This is comparable to the value found for Sus from *Vicia faba* cotyledons (8.3; Ross and Davies 1992), but it is lower than those obtained for Sus from maize kernels (15.1; Nguyen-Quoc et al. 1990) and soybean nodules (15.1; Morell and Copeland 1985).

The M_r estimated by PAGE was found to be 380 kDa for the native enzyme and 94 kDa for the subunit, which confirmed the tetrameric configuration of Sus. These values are similar to those found for wheat leaves and germs (Larsen et al. 1985), soybean nodules (Morell and Copeland 1985), maize leaves and kernels (Nguyen-Quoc et al. 1990), and *Vicia faba* cotyledons (Ross and Davies 1992).

Sucrose synthase of pea seed coat followed typical Michaelis-Menten kinetics for sucrose, UDP, ADP, fructose and UDPGlc. Michaelis-Menten kinetics are displayed by Sus in other species, although sigmoidal kinetics have been reported for the enzyme from *Vicia faba* cotyledons (Ross and Davies 1992). Pea seed-coat Sus has a high affinity for UDP. The K_m for UDP (25 μM) is among the lowest *Km* values ever reported for Sus (5–800 μM). The K_m values for ADP and UDP were found to be similar for enzymes from some material: *Vicia faba* cotyledons (Ross and Davies 1992), peach fruit (Moriguchi and Yamaki 1988). However, for pea seed-coat Sus, the K_m for ADP is 20-fold higher than the K_m for UDP. It is clear that UDP is the preferred substrate in terms of relative velocities: the V_{max} with ADP was five-fold lower than that with UDP.

In the cleavage reaction ADP, could act as the glucosyl acceptor but is much less effective than UDP. Pea seed-coat Sus has a high K_m for sucrose (33 mM), which, however, is still among the lowest K_m values reported in the literature (17–169 mM).

Number of Sus genes in pea. Southern blot analysis of pea genomic DNA revealed only one Sus gene with the

probe used. Monocotyledons have two isoforms of Sus. This has been shown in particular for maize (Echt and Chourey 1985) and wheat (Larsen et al. 1985). It is rather unclear for dicotyledons: two genes have been found in *Arabidopsis thaliana* (Chopra et al. 1992; Martin et al. 1993) and recently, two cDNAs have been identified in potato (Fu and Park 1995). In legumes, only one cDNA has been sequenced in *Vigna radiata* (Arai et al. 1992) and *Vicia faba* (Heim et al. 1993). The pea 3′ untranslated region is very homologous to those of *Vicia faba* mRNA: this indicates that these genes are very closely related. In *Arabidopsis thaliana*, the two genes are very different, with only 80% identity (Martin et al. 1993). These authors report that on a Southern blot with a probe prepared with only one of the two cDNAs, the second gene is nearly undetectable. We cannot therefore exclude the existence of a second gene in pea, very different from the first one.

Physiological role of Sus in the pea seed coat. Phloem unloading occurs in the seed coat cells, where sucrose/starch interconversions take place and starch is temporarily stored. Sucrose synthase activity was closely associated with starch concentration in the seed coat (Fig. 1), unlike alkaline invertase activity. This strongly suggests that Sus is the major sucrose-cleaving enzyme involved in starch synthesis. Our results are consistent with the data obtained with pea embryos by Edwards and ap Rees (1986a). Moreover, in the seed coat cells, starch is degraded throughout seed development, probably to the benefit of the growing embryo. We have shown that Sus can also participate in the synthesis of sucrose. First, the comparison of the calculated mass-action ratio with the theoretical equilibrium is compatible with a role of Sus in both sucrose synthesis and cleavage. However the validity of this comparison relies on the hypothesis of a homogeneous distribution of sugars within the cells. Both UDP and UDPGlc are mainly restricted to the cytosol (Dancer et al. 1990). Sucrose and fructose may be located in the vacuole, but the calculation will be affected only if these two sugars are distributed in very different ways. No data are available on the cellular localization of sugars in the pea seed coat. This localization seems to be dependent on organs and species studied (Gerhardt and Heldt 1984; Heineke et al. 1994). The very low soluble acid invertase activity (Table 2) is actually circumstantial evidence against a specific localization of hexoses in the vacuole. We therefore show, directly by a labelling experiment, that Sus activity can be reversible in vivo. This has also been clearly demonstrated in developing potato tubers, in heterotrophic *Chenopodium rubrum* cell-suspension cultures and in cotyledons of germinating *Ricinus communis* (Geigenberger and Stitt 1993). Although it is clear that Sus can produce a significant part of neo-synthesized sucrose in the seed coat, we obtained only a minimum value of Sus contribution (37%) and cannot compare it to the contribution by SPS to sucrose synthesis. Indeed, even for a 10-min incubation with [^{14}C]glucose, fructose became labelled (Table 5). This labelling provides evidence for rapid sucrose turnover, as already observed by Wendler et al. (1990) for sugar cane cell-suspension cultures. Sucrose synthesized via SPS is symmetrically labelled and its cleavage via Sus will therefore liberate labelled fructose. Sucrose synthase could play a key role in the balance between sucrose and starch, in response to the embryo's need for sucrose. In a seed of 150 mg FW containing 2 mg of starch, the demand for sucrose by the young embryo is low: sucrose is cleaved by Sus to synthesize starch in the seed coat cells; phloem unloading is therefore maintained at a high rate. In a seed of 550 mg FW containing 50 mg of starch, the embryo's need for sucrose increases: starch is then degraded and sucrose is synthesized in the seed coat. The sucrose concentration could be regulated very accurately by the reversible reaction of Sus together with SPS in response to the embryo's demand for sucrose. Moreover, the labelling experiment has shown that sucrose and starch were synthesized concurrently during the incubation. This experiment was done on seeds of 440–470 mg FW when the starch concentration in the seed coat was decreasing. These data confirmed previous experiments of [^{14}C]sucrose loading into pea pods still attached to the mother plant (Rochat and Boutin 1992; Rochat et al. 1995). Whatever the stage of the seeds, about 10% of sucrose was always incorporated into starch in the seed coat cells. This suggests that the starch pool in the seed coat turns over rapidly and acts as a buffer between phloem unloading and sucrose supply to the embryo. In addition, this experiment indicates that the seed coat is an organ where a rapid turnover of the sucrose pool occurs. This seems to be a widespread phenomenon in plants (Geigenberger and Stitt 1993). With its reversible reaction, Sus could play a central role in these mechanisms together with SPS. In contrast to Sus, alkaline invertase is irreversible and has a low K_m for sucrose (7–15 mM; Avigad 1982). The cleavage of sucrose by alkaline invertase would require sophisticated mechanisms of regulation to prevent the accumulation of glucose and fructose. There is no evidence for such a regulation (Stitt and Steup 1985).

Fructokinase and UDPGlc pyrophosphorylase utilize, respectively, fructose and UDPGlc produced by Sus. These activities could render the Sus reaction irreversible in vivo and drive carbon into starch. Both enzyme activities are indeed high in the seed coat: during the stage of development we studied, they covered, respectively, a range between 22–40 $\mu mol \cdot min^{-1} \cdot (mg\ FW)^{-1}$ and 0.2–0.6 $\mu mol \cdot min^{-1} \cdot (mg\ FW)^{-1}$. In our labelling experiment, in conditions very close to the in-vivo conditions, both sucrose and starch are nevertheless synthesized concurrently and the Sus reaction is reversible. This has also been observed in developing potato tubers with a high UDPGlc pyrophosphorylase activity (Geigenberger and Stitt 1993). That seems to indicate that both enzymes are less active in vivo than expected by in-vitro measurements. First, UDPGlc pyrophosphorylase is a reversible enzyme and also thought to be close to its equilibrium in vivo in pea cotyledons (Edwards and ap Rees 1986b). The fructokinase reaction is irreversible, but is strongly inhibited by fructose concentrations above 0.25 mM (Copeland

et al. 1978). At a very low fructose concentration (2 mM), this activity is reduced by 50%. In developing potato tubers, it is thought to be a limiting factor in the utilisation of sucrose for biosynthesis processes (Viola 1996). Fructose also acts as a competitive inhibitor of pea Sus with respect to sucrose with a *Ki* of 12 mM. Fructose inhibition of fructokinase and of Sus in the sucrose cleavage direction could provide a mechanism to coordinate the use of fructose with the rate of sucrose breakdown via Sus.

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