Developmental patterns of free and protein-bound biotin during maturation and germination of seeds of *Pisum sativum*: characterization of a novel seed-specific biotinylated protein

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Mature dry pea seeds contain three major biotinylated proteins. Two of these of subunit molecular mass about 75 kDa and 200 kDa are associated with 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) and acetyl-CoA carboxylase activities (EC 6.4.1.2) respectively. The third does not exhibit any of the biotin-dependent carboxylase activities found in higher organisms and represents the major part of the total protein-bound biotin in the seeds. This novel protein has been purified from a whole pea seed extract. Because in SDS/polyacrylamide gels the protein migrates with an apparent molecular mass of about 65 kDa, it is referred to as SBP65, for 65 kDa seed biotinylated protein. The molecular mass of native SBP65 is greater than 400 kDa, suggesting that the native protein assumes a polymeric structure, resulting from the association of six to eight identical subunits. The results of CNBr cleavage experiments suggest that biotin is covalently bound to the protein. The stoichiometry is 1 mol of biotin per 1 mol of 65 kDa polypeptide. The temporal and spatial pattern of

expression of SBP65 is described. SBP65 is specifically expressed in the seeds, being absent from leaf, root, stem, pod and flower tissues of pea plants. The level of SBP65 increases dramatically during seed development. The protein is not detectable in very young seeds. Its accumulation pattern parallels that for storage proteins, being maximally expressed in the mature dry seeds. SBP65 disappears at a very high rate during seed germination. The level of free biotin has also been evaluated for various organs of pea plants. In all proliferating tissues examined (young developing seeds, leaf, root, stem, pod and flower tissues), free biotin is in excess of protein-bound biotin. Only in the mature dry seeds is protein-bound biotin (i.e. that bound to SBP65) in excess of free biotin. These temporal expression patterns, and the strict organ specificity for expression of SBP65, are discussed with regard to the possibility that in plants, as in mammals, biotin plays a specialized role in cell growth and differentiation.

INTRODUCTION

In all organisms biotin is an essential cofactor for a small number of enzymes involved in CO₂ transfer during carboxylation reactions [1]. Although the existence of this vitamin is well recognized in plants, little is known about its biosynthesis and function. It is known that animal cells contain four biotinylated enzymes, acetyl-CoA carboxylase (ACC; EC 6.4.1.2), 3-methylcrotonyl-CoA carboxylase (MCC; EC 6.4.1.4), propionyl-CoA carboxylase (PCC; EC 6.4.1.3) and pyruvate carboxylase (PC; EC 6.4.1.1). They play central roles in a variety of metabolic and catabolic processes, supporting essential cellular housekeeping functions. ACC, which catalyses the ATP-dependent carboxylation of acetyl-CoA, is recognized as the regulatory enzyme of lipogenesis. MCC catalyses the conversion of 3-methylcrotonyl-CoA into 3-methylglutaconyl-CoA, a key reaction in the degradation pathway of leucine. PCC is a key enzyme in the catabolic pathway of odd-chain fatty acids, isoleucine, threonine, methionine and valine. PC has an anaplerotic role in the formation of oxaloacetate [2].

It is now established that plant cells contain the four biotinylated enzymes found in animals [3]. The most extensively studied biotin carboxylase is ACC, because of its obvious function in membrane biogenesis, and because the enzyme isolated from vegetative tissues and developing seeds of monocotyledonous

plants is the target of the very potent cyclohexanedione and aryloxyphenoxypropionate herbicides [4–7]. There is evidence suggesting that biotin and biotin-containing proteins might play specialized roles in regulation of plant development. Thus ACC is required for both growth of vegetative tissues and synthesis of storage lipids in developing seeds [8,9]. MCC was found to increase rapidly during pea leaf development [10]. Also, during development of carrot somatic embryos, a 50-fold increase in the level of a 62 kDa biotinylated polypeptide has been observed, as embryogenic cell clusters developed into torpedo embryos [11]. Attention has been focused recently on the existence of free biotin in plant cells. A study conducted with pea leaves showed the existence of a free biotin pool in the cytosolic compartment, accounting for about 90 % of the total (free plus protein-bound) biotin. It has been argued that this pool might control the expression of genes encoding biotinylated carboxylases and/or enzymes involved in biotin synthesis [12]. The role of biotin in plants is also best illustrated by the discovery of a mutation that causes defective embryo development in *Arabidopsis thaliana* and requires the vitamin at a critical stage of embryogenesis. Thus no biotin was detectable in the arrested embryos of the mutant, and mutant embryos were specifically rescued when grown in the presence of biotin [13,14].

As a means of understanding the function of this vitamin in developing and germinating seeds, we have analysed the content

Abbreviations used: ACC, acetyl-coA carboxylase (EC 6.4.1.2.); DTT, dithiothreitol; MCC, 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4); PBST, PBS containing 0.1 % (v/v) Tween 20; PC, pyruvate carboxylase (EC 6.4.1.1.); PCC, propionyl-CoA carboxylase (EC 6.4.1.3); PEG, poly(ethylene glycol); SBP65, the major biotinylated protein in mature dry pea seeds.

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of free and protein-bound biotin of pea seeds at various developmental stages. A detailed comparative study was also carried out with various organs of pea plants (leaves, roots, stems, flowers and pods). The general finding is that in mature dry seeds the level of bound biotin is higher than that of free biotin, owing to the existence of a seed-specific biotinylated 65 kDa polypeptide which we called SBP65. This protein does not support *in vitro* any of the four biotin-dependent carboxylase activities present in higher organisms. This contrasts with the situation observed in all developing organs, including young developing seeds and pea plants, where the free vitamin is present in higher amounts than that bound to proteins. The possible implications of these findings are discussed.

MATERIALS AND METHODS

Reagents

ATP, acetyl-CoA, 3-methylcrotonyl-CoA, D-biotin, biotin-labelled β -galactosidase, biotin-labelled molecular-mass markers, anti-biotin peroxidase-conjugated antibodies, horseradish peroxidase-conjugated streptavidin, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) and 4-chloro-1-naphthol were from Sigma. $\text{NaH}^{14}\text{CO}_3$ (53.1 mCi/mmol) and D-[carbonyl- ^{14}C]biotin (56.6 mCi/mmol) were from Amersham. All other chemicals were of analytical grade. Solutions were made in ultrapure water (Millipore) and filtered with 0.2 μm filters.

Plant material and samples for developmental studies

Mature dry pea seeds (*Pisum sativum*, cv. Douce Provence) used in this study are referred to as S0 seeds. They had a mean fresh weight of 250 mg and a diameter of about 6–7 mm. Plants were grown from these seeds in soil under a 12 h photoperiod of white light from fluorescent tubes (10–40 $\mu\text{E} \cdot \text{ml}^{-2} \cdot \text{s}^{-1}$) at 20 °C. They were watered every day with tap water. Different organs (leaves, roots, stems, flowers, pods and seeds) were harvested at different times, and stored at –75 °C until use. No germination was visible at day 1 after planting. The mean fresh weight of the 1-day-old seeds was 475 mg. Germination occurred between 36 h (0% of seed germination) and 48 h (approx. 90% of seed germination) after sowing. The mean fresh weight of the 2-day-old seeds was 528 mg. At day 2 and day 3, mean fresh weights of the seedlings (minus cotyledons) were 62 and 120 mg respectively. Flowering started at day 25. During the initial period of seed formation seed samples showed heterogeneity in size. S36(1), S36(2) and S36(3) refer to seeds collected from pods at day 36. Mean fresh weights per seed were 7, 32 and 144 mg respectively. Samples sizes were approx. 1.5, 2.5 and 3.5 mm diameter, respectively. S41, S52 and S76 refer to seeds collected at day 41, 52 and 76. Mean fresh weights per seed were 300, 500 and 210 mg respectively. The size of these three samples was approx. 6–7 mm diameter. At day 76, seeds were fully matured and desiccated.

Preparation of extracts for development studies

Plant materials (1–5 g) were frozen in liquid nitrogen and finely ground using a mortar and pestle. The powder was homogenized in 2 vol of buffer A [50 mM Hepes, pH 8.0, 10% (v/v) glycerol, 1 mM EDTA, 5 mM dithiothreitol (DTT), 1 mM phenylmethanesulphonyl fluoride, 1 mM benzamide/HCl, 5 mM 6-aminohexanoic acid], followed by centrifugation (40000 g,

30 min). The supernatant comprised the crude extract which was processed as follows. (i) Portions of the extract were used for the determination of total (free plus protein-bound) biotin. (ii) To 1 ml of crude extract, 4 ml of cold acetone (–20 °C) was added, and the mixture left to stand for 30 min to precipitate proteins. After centrifugation (3000 g, 10 min), the supernatant was evaporated to dryness under a stream of nitrogen, and 1 ml of PBS containing 0.1% (v/v) Tween 20 (PBST) was added. This solution was used for quantification of free biotin. Controls, in which D-[^{14}C]biotin was added to the extracts before the addition of acetone, established that the radioactivity was quantitatively recovered from the supernatant fraction. Also, it was verified that the supernatants were depleted of protein-bound biotin. (iii) Crystalline $(\text{NH}_4)_2\text{SO}_4$ was added to each crude extract with stirring until 50% (w/v) saturation was achieved. The mixture was stirred for 30 min at 4 °C, and then centrifuged (40000 g, 20 min). The pellet was resuspended in 2 vol. of buffer A, and the solution was used for biotin carboxylase activity measurements and for quantification of protein-bound biotin. For SDS/PAGE analyses, 1 vol. of SDS sample buffer [10 mM Tris/HCl, pH 6.8, 2.5% (w/v) SDS, 15% (v/v) 2-mercaptoethanol, 30% glycerol, 0.06% (w/v) Bromophenol Blue] was added to 2 vol. of each sample and heated to 100 °C for 5 min.

Protein determination, activity measurements and electrophoresis

Protein was quantified by the method of Bradford [15] with BSA as the standard. Biotin carboxylase activities were measured as the incorporation of radioactivity from $\text{NaH}^{14}\text{CO}_3$ into an acid-stable product [10,11]. Assays contained 50 mM Hepes, pH 8.0, 2.5 mM MgCl_2 , 1 mM ATP, 2 mM DTT, 10 mM $\text{NaH}^{14}\text{CO}_3$ (1 mCi/mmol), 20 mM KCl, 0.4 mM appropriate substrate (acetyl-CoA, 3-methylcrotonyl-CoA, propionyl-CoA or pyruvate) and 1–150 μg of protein sample, in a final volume of 200 μl . Incubations were for 15 min at 30 °C. One unit of enzyme activity is equivalent to the incorporation of 1 nmol of $^{14}\text{CO}_2$ into acid-stable product in 1 min at 30 °C. SDS/PAGE was conducted with a PhastSystem (Pharmacia) in preformed gels (PhastGel Pharmacia) containing 12.5% (w/v) acrylamide. Proteins were electrotransferred from the gel on to nitrocellulose (Bio-Rad), using a PhastTransfer device (Pharmacia), as recommended by the manufacturer. Blots were incubated for 1 h at 25 °C in PBS containing 3% (w/v) BSA, then for 1 h with peroxidase-conjugated streptavidin (250 ng/ml) in PBST, followed by three washes with PBST. Biotinylated polypeptides were revealed by adding peroxidase/substrate solution (1 μl of 30% H_2O_2 /ml and 0.5 mg of 4-chloro-1-naphthol/ml).

Streptavidin-binding assays for biotin

Since streptavidin binds both free and protein-bound biotin [16], two types of assays were used.

Direct solid-phase biotin assay for protein-bound biotin

Microtitre e.l.i.s.a. plates (Greiner) were incubated for 3 h at 25 °C with various amounts of sample (0.01–10 μg of protein per well) in 100 μl of PBS, washed four times with PBST, and each well received 50 ng of peroxidase-conjugated streptavidin in 100 μl of PBST. After incubation for 1 h at 25 °C, wells were washed four times with PBST, and each received a 100 μl peroxidase/substrate solution [0.1 μl of 3% H_2O_2 and 100 μg of 2,2'-azino[bis-(3-ethylbenzothiazoline-6-sulphonic acid)] in 100 mM citrate phosphate, pH 4.0. Colour development was quantified with an EL-312 microplate reader (Biotek Instruments),

using a 405 nm filter. Standard curves were generated from control wells coated with known amounts (1–20 ng) of biotin-labelled β -galactosidase.

Indirect solid-phase biotin assay for free and protein-bound biotin

Control experiments showed that free D-biotin cannot bind to the wells of the microtitre plates. Free biotin was quantified by a modification of standard procedures [13,17]. E.l.i.s.a. plates were incubated for 3 h at 25 °C with a fixed amount of biotin-labelled β -galactosidase (usually 100 ng per well) in 100 μ l of PBS, and then washed four times with PBST. To generate a standard curve, serial dilutions (0.002–80 ng) of D-biotin (final volume 100 μ l in PBST) were prepared in Eppendorf centrifuge tubes. They were mixed with 100 μ l of PBST containing a fixed amount (usually 20 ng) of peroxidase-conjugated streptavidin, followed by incubation for 1 h at 25 °C. This yielded a series of mixtures containing various amounts of free and biotin-bound peroxidase-conjugated streptavidin. Portions (100 μ l) of these mixtures were then transferred to each biotinylated β -galactosidase-coated well of the plates. After incubation for 1 h at 25 °C, plates were processed as for the direct solid-phase biotin assay, i.e. they were washed four times with PBST and, after addition of the peroxidase/substrate solution, A_{405} was measured as above. Because only free peroxidase-conjugated streptavidin will bind to biotinylated β -galactosidase, and because exchange of biotin from preformed biotin–streptavidin complexes is extremely slow [16], this yielded a titration curve, A_{405} versus [D-biotin], such that (i) the lower the [biotin] the higher the A_{405} and (ii) at high [biotin] the A_{405} was close to the background values. The point at which the slope undergoes a sharp increase indicates the equivalent point, i.e. the minimal amount of biotin required to saturate the fixed amount of conjugated streptavidin used in the assay. The concentration of free D-biotin at the equivalence point was not dependent on the duration of the incubation with peroxidase substrates, although prolonged incubations increased the sensitivity. Furthermore, the concentration of free D-biotin at the equivalence point was dependent on the concentration of conjugated streptavidin, but not on that of biotin-labelled β -galactosidase in the assays. The sensitivity was of the order of 2 pg of biotin.

To quantify free biotin in the various plant extracts, the same protocol was used except that the serial dilutions of free D-biotin used to construct the calibration curve were replaced by serial dilutions in PBST of the supernatants derived from the acetone-treated plant extracts. Free biotin content in these samples was then calculated from the equivalence points, with reference to the calibration curve, obtained under the same conditions. In principle, protein-bound biotin could also be quantified by this assay from the precipitate of the acetone-treated samples. Yet, attempts to solubilize the acetone pellet from the various extracts under conditions compatible with preservation of streptavidin-binding activity (e.g. in the absence of denaturing agents such as SDS) produced unreliable results. The same limitations were encountered when proteins in the crude extracts were precipitated with trichloroacetic acid. The unprocessed crude extracts and the corresponding acetone supernatants were used therefore for total (free plus protein-bound) and free biotin determinations respectively. Whenever possible, the level of protein-bound biotin was then calculated from the difference between these two quantities.

Purification of SBP65 from mature pea seeds

All purification steps were carried out at 4 °C. Chromatographic

steps were performed with a Pharmacia f.p.l.c. system. Frozen (–75 °C) mature pea seeds (100 g) were finely ground in a Waring blender. To the powder 500 ml of buffer A was added, and the mixture was homogenized with a Polytron homogenizer. After centrifugation (15000 g, 30 min), the resulting supernatant (17 g of protein) was brought to 200 g/l $(\text{NH}_4)_2\text{SO}_4$, stirred for 30 min at 4 °C, allowed to stand at this temperature for 2 h, and then centrifuged (15000 g, 30 min). The pellet (4 g of protein) was resuspended in 200 ml of buffer A. This protein extract was brought to 50 g/l poly(ethylene glycol) (PEG) 6000, stirred for 20 min at 4 °C, and centrifuged (15000 g, 30 min). The derived supernatant was brought to 200 g/l PEG 6000, stirred for 20 min at 4 °C, and centrifuged as above. The sticky brown-coloured pellet was resuspended in 15 ml of buffer A with an Ultra-Turrax homogenizer; then the suspension was clarified by centrifugation (50000 g, 30 min), and the supernatant (0.75 g protein, 12 ml) filtered on a 0.2 μ m filter. At this step, biotinylated proteins had been purified about 22-fold with a 60 % recovery. This extract was subjected to monomeric-avidin affinity chromatography [10, 18–20]. Sample was loaded (flow rate 0.1 ml/min) on the column (1.5 cm $\times$ 20 cm) equilibrated in buffer B (20 mM Hepes, pH 8.0, 10 % glycerol, 0.5 M KCl, 1 mM EDTA, 1 mM benzamidine/HCl, 5 mM 6-aminohexanoic acid). After the column had been washed with 350 ml of buffer B (flow rate 0.2 ml/min), bound proteins (0.8 mg) were eluted with 50 ml of buffer B containing 2 mM D-biotin. The yield for protein-bound biotin was in the range 20–30 %. Eluted fractions were dialysed against buffer B containing 50 mM KCl, concentrated to 6 ml with Macrosep-10 tubes (Filtron) and applied to a Mono Q HR5/5 column (0.5 cm $\times$ 5 cm; Pharmacia) equilibrated in buffer B containing 50 mM KCl. SBP65 was recovered in the non-absorbed fraction. After the column had been washed with 20 ml of buffer B containing 0.1 M KCl, adsorbed proteins, supporting ACC and MCC activities, were eluted with 10 ml of buffer B containing 0.3 M KCl (flow rate 0.5 ml/min; fraction size 0.5 ml). Molecular-mass determinations were effected by gel filtration on a Sephacryl S-300 HR column (2.6 cm $\times$ 35 cm, 180 ml; Pharmacia). Protein samples were allowed to react with CNBr as described [21].

Preparation of antiserum

Purified SBP65 (800 μ g) was subjected to SDS/PAGE. After electroelution from the gel with 25 mM Tris/192 mM glycine, pH 9.0, containing 0.1 % SDS, the protein was dialysed against 50 mM Tris/HCl, pH 7.8. Antibodies directed against the electroeluted SBP65 (anti-SBP65) were raised in a rabbit by standard protocols. For the preparation of affinity-purified antibodies, SBP65 (30 μ g) was subjected to SDS/PAGE and electrotransferred on to nitrocellulose. A thin strip corresponding to SBP65 was excised. After being washed with PBS containing 3 % BSA, the strip was incubated with a 20-fold dilution of the anti-SBP65 serum, overnight at 4 °C. After three washes with PBST, specific antibodies were eluted by a quick (30 s) wash of the strip with 1 ml of 0.2 M glycine/HCl, pH 2.2, and then immediately neutralized by adding 170 μ l of 1 M Tris/HCl, pH 8.8 [22].

D-[¹⁴C]Biotin binding and exchange

Assays contained 50 mM Hepes, pH 8.0, 1 mM DTT, 1.6 μ M D-[¹⁴C]biotin (56.6 mCi/mmol) and sample (10–200 μ g of protein), in a total volume of 15 μ l. Assays were incubated at 25 °C for 30 min, and then portions (9 μ l) of reaction mixtures were spotted on glass-fibre filters. After eight washing steps in 5 % trichloroacetic acid, the filters were dried, and the trichloroacetic-

Figure 1 Biotin content of pea seeds

(a) Titration curves for biotin in mature dry pea seeds. A crude extract (1.5 seeds/ml; 72 mg of protein/ml) was prepared as described in the Materials and methods section. Serial dilutions of the unprocessed crude extract in PBST were analysed for total (free plus protein-bound) biotin (●) by the indirect solid-phase biotin assay as described in the Materials and methods section. The equivalence point was obtained at 1.7 μ l of unprocessed extract. To 1 ml of this extract 4 ml of cold (-20°C) acetone was added. After centrifugation, the supernatant was saved, evaporated to dryness and resuspended in 1 ml of PBST. Serial dilutions in PBST of this acetone-derived extract were analysed for free biotin (○) by the indirect solid-phase biotin assay. The equivalence point was obtained at 9.4 μ l of extract. For the calibration curve constructed as described in the Materials and methods section, the equivalence point was obtained at 71 pg of free *D*-biotin, allowing us to calculate amounts of 5 ng of free biotin and 28 ng of total biotin per S0 seed. These corresponded mostly to cotyledons; contributions from the seed coats and embryonic axes were negligible. (b) Evaluation at different times after sowing of the protein-bound (●) and free (○) biotin content of total seeds (day 0 and day 1) and cotyledons (days 2, 3, 5 and 7). Results are expressed on a per cotyledon basis. Total and free biotin were quantified as in (a). Bound biotin was calculated as the difference between the amounts of these two quantities. Radicle emergence occurred between 36 h and 48 h after sowing (arrow). (c) Evaluation of the protein-bound (■) and free (▨) biotin content during pea seed development expressed on a per mg of protein basis. For nomenclature of seed samples, see the Materials and methods section. Free biotin was quantified as in (a). Protein-bound biotin was estimated by using the direct solid-phase biotin assay.

acid-precipitable radioactivity was measured by liquid-scintillation counting.

RESULTS

Non-covalent protein-bound biotin in extracts from mature dry pea seeds

Biotin-containing proteins from plant extracts have usually been

analysed on nitrocellulose filters of blotted gels, in a system using streptavidin as a specific reagent, analogous to Western blotting [10,11,23]. Presumably, only those proteins containing the covalently attached prosthetic group are detected by this method [23]. To quantify the bound biotin content of pea seeds, it was therefore of importance to investigate whether these seeds contained proteins involved in non-covalent binding of the vitamin. When an unprocessed crude extract from S0 seeds was

Figure 2 Requirement for biotin in early plant growth

(a) Evaluation of ACC activity in pea leaves. (b) Evaluation of MCC activity in pea roots.

Figure 3 Distribution of SBP65 in different compartments of mature dry pea seeds

(a) Coomassie Blue R250 staining of a homogeneous SDS/polyacrylamide gel containing 12.5% acrylamide loaded with total protein extracts from seed coat (lane 2, 1 μ g), embryonic axis (lane 3, 4 μ g) and cotyledons (lane 4, 4 μ g) of mature dry pea seeds. Molecular-mass markers (lane 1) are indicated in kDa. (b) Nitrocellulose blot of a duplicate of (a) probed with peroxidase-conjugated streptavidin. Lane 1, cotyledon extract (40 μ g); lane 2, embryonic axis extract (40 μ g); lane 3, seed coat extract (10 μ g). Molecular-mass biotinylated markers (lane 4) are indicated in kDa.

Table 1 Purification procedure for pea SBP65

SBP65 was purified starting from 100 g of mature dry pea seeds. Protein was determined by the method of Bradford [15] with BSA as standard. Bound biotin was measured by using the direct solid-phase biotin assay described in the Materials and methods section.

Purification stage	Total protein (mg)	Total biotin (μ g)
Crude extract of soluble proteins	17 000	10
PEG 6000 homogenate	750	6.4
Monomeric-avidin Sepharose pool	0.8	2
Mono Q HR5/5 pool (salt-eluted fraction)	0.15	0.2
Mono Q HR5/5 pool (flow-through fraction)	0.6	2

incubated with D-[14 C]biotin, no radioactivity was recovered in a form precipitable by trichloroacetic acid. Under the same conditions, binding of D-[14 C]biotin to streptavidin could be demonstrated. Therefore, unlike egg yolk and egg white [24], mature dry pea seeds do not seem to contain significant amounts of proteins involved in non-covalent binding of biotin.

Free and protein-bound biotin in developing, mature and germinating pea seeds

Figure 1(a) shows that mature dry seeds contain a 5-fold higher amount of protein-bound biotin than free biotin. Respective amounts of about 24 ng and 5 ng of vitamin per seed were calculated, corresponding to about 100 pg of protein-bound biotin and 20 pg of free biotin per mg fresh weight. These values were within the range of total biotin levels reported for various feedstuffs of plant origin [25] and for seeds of *A. thaliana* [13].

A considerable amount of the initial protein-bound biotin disappeared during early stages of germination. At day 2, which coincides with radicle emergence, the level of bound biotin was of the order of 20% of that in the mature dry seeds. After day 5, this level was below detection (Figure 1b). Figure 1(c) shows the data obtained from seed samples collected at various development stages. On a per mg of protein basis, the level of free biotin was high in the young seeds, and then decreased during development. In contrast, the protein-bound biotin level was low in the young seeds, and then rose sharply during late stages of seed development and onset of desiccation, being maximal in the mature dry seeds.

Content of free and total biotin in various organs of pea plants

Biotin contents were determined for various organs of pea plants collected from 4 to 40 days after sowing. All extracts from leaves, roots, stems, pods and flowers yielded the same results as those obtained for the young pea seeds, i.e. although free biotin could be easily detected with the indirect solid-phase biotin assay, the amount of protein-bound biotin was almost negligible compared with that of free biotin. At day 39, free biotin levels of 11, 8.5, 7.5, 2 and 1 ng/mg of protein were calculated for the leaves, roots, stems, pods and flowers respectively. These levels varied during plant growth. For example, at day 5 and 25 the free biotin content was 1 and 9 ng/mg of protein for the leaves and 3 and 10 ng/mg of protein for the roots. This is in agreement with reported values of 1.2 ng/mg of protein and 0.2 ng/mg of protein

Figure 4 Purification of SBP65 by chromatography on Mono Q HR5/5

Biotinylated proteins were purified as described in the Materials and methods section, from a crude extract of mature dry pea seeds. Biotinylated fractions eluted from the monomeric-avidin affinity column were pooled (0.8 mg of protein), dialysed against buffer B containing 50 mM KCl, and loaded on a Mono Q HR5/5 column equilibrated in the same buffer. The column was washed with 20 ml of buffer B containing 0.1 M KCl, and then eluted with 10 ml of buffer B containing 0.3 M KCl. Fractions of 0.5 ml were collected, and assayed for protein-bound biotin (a, ○), affinity-purified anti-SBP65 antibody reactivity (a, ●), ACC activity (b, ●), MCC activity (b, ○) and protein (c, ●); the KCl concentration is shown (—). (d) Coomassie Blue R250 staining of an SDS/polyacrylamide gel containing 12.5% acrylamide loaded with pooled flow-through fractions 3–8 from the Mono Q column (lanes 2 and 5, 1.2 μg of protein), and pooled fractions 43–48 eluted from this column in the presence of 0.3 M KCl (lanes 3 and 6, 0.5 μg of protein). The proteins loaded on the Mono Q column are shown (lane 4, 0.8 μg of protein). Molecular-mass markers (lanes 1 and 7) are indicated in kDa.

for the free and protein-bound biotin content of 10-day-old pea leaves respectively [12].

Although the protein-bound biotin content was low, extracts from leaves, roots, stems, pods and flowers contained significant levels of ACC and MCC activities. Those for PCC and PC were close to background values. ACC activity increased strongly in the leaves from day 3 to day 8, and then declined in older tissue (Figure 2a), similarly to previous observations with maize leaves [26]. The same pattern of behaviour was observed for MCC in the roots (Figure 2b). These data suggest that there is a strong demand for biotin in the early stages of plant growth.

Characterization of biotinylated proteins from mature dry pea seeds

Seed coats, cotyledons and embryonic axes were excised from mature dry seeds, and extracts were prepared as described in the Materials and methods section. Figure 3 shows that a major biotinylated polypeptide of molecular mass 65 ± 2 kDa was present in both cotyledons and embryonic axis. This protein is referred to as SBP65, for 65 kDa seed biotinylated protein. On a

per mg of protein basis, the level of SBP65 was nearly the same in both seed compartments. As the total protein content of cotyledons is much higher than that of embryonic axis, the greater proportion of SBP65 is in the cotyledons. In contrast, SBP65 was not detected in the seed coat (Figure 3).

SBP65 was purified from mature dry pea seeds by a two-step chromatographic procedure (Table 1). Proteins specifically eluted from the monomeric-avidin Sepharose column were separated into two groups after chromatography on Mono Q HR5/5 (Figure 4). One group, which did not absorb to this column, comprised the major part of the loaded biotinylated proteins (Figure 4a) and contained SBP65 (Figure 4d). This fraction exhibited no biotin carboxylase activity, using any of the four acceptor substrates, acetyl-CoA, 3-methylcrotonyl-CoA (Figure 4b), propionyl-CoA and pyruvate (not shown). Another group (the salt-eluted fraction) supported MCC and ACC activities (Figure 4b). Again PCC and PC activities were not detectable. SDS/PAGE analysis of this fraction disclosed the presence of four major polypeptides (Figure 4d). The molecular masses of the three fast-migrating polypeptides were 75 kDa, 65 kDa and 50 kDa (Figure 4d). That of the slowly migrating species was

Figure 5 Evaluation of SBP65 in pea seeds and cotyledons during germination

(a) Coomassie Blue R250 staining of a homogeneous SDS/polyacrylamide gel containing 12.5% acrylamide loaded with total protein extracts from mature seeds (S0) (lane 2), seeds collected at day 1 (lane 3) and cotyledons collected at day 3 (lane 4), 5 (lane 5) and 7 (lane 6) after sowing. An amount of 4 μ g of protein was loaded in each lane. Molecular-mass markers (lane 1) are indicated in kDa. (b) Nitrocellulose blot of a duplicate of (a) probed with peroxidase-conjugated streptavidin. Lanes 1, 2, 3, 4 and 5 correspond respectively to lanes 6, 5, 4, 3 and 2 in (a). An amount of 16 μ g of protein was loaded in each lane. Molecular-mass biotinylated markers (lane 6) are indicated in kDa. (c) Evaluation of total protein (■), protein-bound biotin (○) and anti-SBP65 antibody reactivity (●) in seeds (day zero and 1) and cotyledons (day 2, 3, 5, 7 and 8) at different times after sowing. Radicle emergence is indicated by the arrow. Results are expressed as percentage of corresponding S0 values. (d) Evaluation of ACC (▨) and MCC activities (■).

estimated to be of the order of 200 kDa on SDS/PAGE carried out in gels of higher resolution than that of Figure 4, containing a 7–20% polyacrylamide gradient. Of these four polypeptides, a Western blot analysis with peroxidase-conjugated streptavidin revealed that three (the 65 kDa, 75 kDa and 200 kDa proteins) were biotinylated (not shown). The 65 kDa polypeptide corresponded presumably to contaminating SBP65, which would account for the slight reactivity of the anti-SBP65 antibodies with the salt-eluted fraction (Figure 4a). The 75 kDa biotinylated protein and the 50 kDa non-biotinylated protein could be assigned to the two non-equivalent subunits of MCC. Indeed, this latter enzyme was purified to homogeneity from pea leaves with similar molecular-mass subunits [10]. The 200 kDa polypeptide corresponded presumably to ACC, as this enzyme was purified from pea embryos with similar molecular mass [27]. We note that the purification protocol used by Bettey et al. [27] included a fractionation step on DEAE-Sepharose, and that only the adsorbed proteins were analysed further for purification. Presumably, SBP65 was not detected in the above cited experiments, because the protein adsorbs to neither Mono Q HR5/5 (Figure 4) nor DEAE-TSK (Merck) (not shown). The fact that the 200 kDa and 75 kDa polypeptides were almost not detected in crude extracts (Figure 3) confirmed that SBP65 is the major biotinylated protein in mature dry pea seeds. The purification procedure yielded about 600 μ g of SBP65, starting from 100 g of mature dry pea seeds (Table 1).

Biochemical properties of SBP65

An acid hydrolysate [12] of SBP65, but not a native sample of this protein, supported *bioB105* bacterial growth (this strain requires added biotin for growth [28]), suggesting that biotin was covalently bound to the protein. This finding was further supported by the following observations. (i) SBP65 was specifically detected on nitrocellulose filters of blotted SDS/polyacrylamide gels with peroxidase-conjugated streptavidin (Figure 3); (ii) there was no binding or exchange of biotin after incubation of extensively dialysed SBP65 with D-[14 C]biotin in the temperature range 25–70 °C, and in the pH range 4–9; and (iii) SBP65 reacted positively and specifically in e.l.i.s.a. conducted with the anti-biotin peroxidase-conjugated antibodies. When SBP65 was allowed to react with CNBr (which cleaves polypeptide chains on the carboxyl side of methionine residues) and the resulting mixture assayed for protein-bound biotin, the reacted sample contained less than 0.5% of the biotin present in the native SBP65 control. An indirect solid-phase biotin assay showed that biotin was released in the supernatant when the treated SBP65 was precipitated by cold acetone. The same behaviour was observed with a sample of biotin-carboxylase mixture, as obtained from the Mono Q column. As the consensus sequence for covalent binding of biotin to biotin carboxylases is Met-Lys-Met, where the vitamin is attached to the lysine residue [2,29], these results suggest a similar mode for binding of biotin

Figure 6 Evaluation of SBP65 during seed formation and maturation

Crude extracts were prepared from various seed samples as described in the Materials and methods section. See the Materials and methods section for nomenclature for samples. **(a)** Evaluation of total protein. **(b)** Evaluation of anti-SBP65 antibody reactivity. **(c)** Coomassie Blue R250 staining of a homogeneous SDS/polyacrylamide gel containing 12.5% acrylamide loaded with 4 μ g of total protein extracts from mature dry seeds (S0) (lane 2), and S36(3) (lane 3), S41 (lane 4), S52 (lane 5) and S76 (lane 6) seeds collected from pods. Molecular-mass markers (lane 1) are indicated in kDa. **(d)** Nitrocellulose blot of a duplicate of **(c)** probed with peroxidase-conjugated streptavidin. An amount of 16 μ g of protein was loaded in each lane. Lanes 1–5 correspond to S76, S52, S41, S36(3) and S0 seed samples respectively. Molecular-mass biotinylated markers (lane 6) are indicated in kDa. **(e)** Evaluation of ACC activity on a per seed (▨) or on a per mg of protein basis (■). **(f)** Evaluation of MCC activity on a per seed (▨) or on a per mg of protein basis (■).

to SBP65. The biotin content of SBP65, as determined using the indirect solid-phase biotin assay, yielded a stoichiometry of 0.95 ± 0.15 mol of biotin per 1 mol of 65 kDa polypeptide. The native molecular mass of SBP65 was estimated by gel filtration on Sephacryl S-300 HR to be 450 ± 60 kDa. This indicates that the native protein assumes a polymeric structure, resulting from the association of six to eight identical subunits.

Developmental patterns of SBP65 expression in pea seeds

Because SBP65 appears to correspond to the major biotinylated protein in pea seeds, one might expect similar developmental patterns for the expression of this protein and for that of total protein-bound biotin. In agreement with this expectation, there was a rapid fall for SBP65 from seeds and cotyledons in early

stages of germination (Figure 5b), which was even more rapid than that of total extractable proteins (Figure 5c). It is known that, in *Pisum sativum*, mobilization of protein reserves from the cotyledons commences just after radicle emergence [30]. SBP65 was absent in the initial period of seed development, and then accumulated considerably in later stages. This accumulation paralleled that of storage proteins (Figures 6a, 6b, and 6c).

Clearly, the development patterns of ACC and MCC activities differed from that of SBP65 (Figure 5d and Figures 6e and 6f). In the developing seeds both ACC and MCC appeared to be constitutively expressed, being present at all developmental stages. Also, the Western-blot analyses of Figures 5(b) and 6(d) confirmed that the two biotin-dependent carboxylases were only present in much smaller amounts than SBP65 in the final period of seed maturation.

Organ-specific expression of SBP65

Leaves, roots, stems, pods and flowers were harvested from plants from day 4 to 40 after sowing, and crude extracts were prepared as described in the Materials and methods section. In contrast with the results obtained with the mature seeds, SBP65 was not detectable in these extracts, after either incubation of nitrocellulose filters of blotted SDS/polyacrylamide gels with peroxidase-conjugated streptavidin or by conducting e.l.i.s.a. with the anti-SBP65 antibodies. The expression of SBP65 is therefore confined exclusively to the seeds.

DISCUSSION

A major rationale of this work was to characterize the free and protein-bound biotin contents of pea seeds, and to investigate whether the temporal expression of these contents can be used to define developmental stages during seed formation and germination. Ion-exchange chromatography on Mono Q HR5/5 allows one to separate the biotinylated proteins of mature dry pea seeds into two classes. The first one is not adsorbed by this column, and is associated with a single protein which we have called SBP65. This protein is seed specific, being absent from leaves, roots, stems, pods and flowers. No other biotin-containing protein, exhibiting such a strict organ-specific expression, has yet been reported in plants. A second class of proteins is retained by the Mono Q column, and is mainly associated with MCC and ACC activities. In sharp contrast with SBP65, these two biotinylated enzymes do not exhibit specific organ expression, being detected at all developmental stages in all tissues examined.

We have analysed the relative levels of free and protein-bound biotin in various organs of pea plants. For all proliferating tissues, including the young developing seeds, free biotin is in excess of protein-bound biotin. Presumably, in such tissues free biotin plays a specialized role in growth, and biotinylation of the biotin-dependent carboxylases can only be achieved with an excess of the free vitamin. Studies with mammalian cells suggested that biotin might be involved in regulation of diverse processes such as DNA transcription and replication, cell growth and differentiation, highlighting functions for the vitamin other than as a prosthetic group for the biotinylated carboxylases [2]. In marked contrast, only for the mature dry pea seeds is the level of bound biotin greater than that of free biotin, owing to the accumulation of SBP65.

In considering possible roles for SBP65 in seed development, it is plausible that kinetic competition between different protein acceptors for biotin may divert the normal flux of the vitamin from the housekeeping biotinylated carboxylases to SBP65. In

favour of this hypothesis, the results of CNBr cleavage experiments suggest a similar mode for binding of biotin to the pea seed biotinylated carboxylases and to SBP65. Also, SBP65 might constitute a storage form of the vitamin, reserved for the embryo to start its growth during the germination process. It is worth noting in this context the very fast decrease in the level of SBP65 after seed planting. Other possibilities are that SBP65 supports some as yet unidentified enzyme activity, or that it has a regulatory function at the level of gene expression. As the sequences of several biotinylated proteins are now available [31–34], more definitive evidence about the function of SBP65 can be acquired by cloning and determination of the nucleotide sequence of the gene for this protein. An interesting parallel to our observations with the pea seeds is found in a recent study showing the accumulation of a 62 kDa biotin-containing polypeptide during developing of carrot somatic embryos [11]. If the temporal pattern of expression of major biotinylated polypeptides in various seeds were similar, further characterization of pea SBP65 may provide valuable molecular insights for understanding the role of biotin and protein biotinylation in seed development and germination.

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