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Biotin synthesis in higher plants: purification and characterization of *bioB* gene product equivalent from *Arabidopsis thaliana* overexpressed in *Escherichia coli* and its subcellular localization in pea leaf cells

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Abstract Biotin synthase catalyses the final step in the biotin biosynthetic pathway and is encoded by the *bioB* gene in *Escherichia coli*. To investigate the conversion of dethiobiotin to biotin in the plant kingdom, the cDNA encoding the *bioB* gene product equivalent from *Arabidopsis thaliana* was used to construct an *E. coli* overexpression strain. The purified *A. thaliana bioB* gene product is a homodimer (100 kDa) with a reddish color and has an absorbance spectrum characteristic of protein with [2Fe-2S] clusters. Its intracellular compartmentation in pea leaves discloses a unique polypeptide of 39 kDa within the matrix of mitochondria.

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Key words: Biotin biosynthesis; Biotin synthase; *Arabidopsis thaliana*; Intracellular compartmentation

1. Introduction

Biotin (vitamin H) has been known for about 30 years to be the prosthetic group of a small family of carboxylases which play essential roles in cell metabolism [1]. Biotin is synthesized by many microorganisms and plants [2–4]. Its biosynthesis has been widely investigated in bacteria especially in biotin mutants of *E. coli* K12 and *Bacillus sphaericus* [2,4,5]. In *E. coli* these enzymes have been characterized and are encoded in a gene cluster, which consists of five genes, *bioA*, *bioB*, *bioC*, *bioD*, and *bioF*. However, the last step, the insertion of a sulfur atom between C_6 and C_9 of dethiobiotin to form the thiophane ring of biotin is not yet very well established. Nevertheless, it has been established that this complex reaction, catalyzed by biotin synthase, the product of the *bioB* gene, required in addition three proteins such as flavodoxin, flavodoxin-NADP$^+$ reductase, and thiamine pyrophosphate-dependent protein [6–9]. On the other hand the potential sulfur donors are not yet characterized.

Our knowledge of the metabolism of biotin in higher plants is rather meagre. We reported previously the localization biotin in cells from green pea leaves [10]. The bulk of free biotin was shown to be associated with the cytosolic fraction, whereas protein-bound biotin was associated with the soluble fractions of chloroplast and mitochondria [10–12]. We also demonstrated that all the mechanisms for biotin synthesis established for bacteria can be extrapolated to the plant cells and that biotin synthase may proceed in two distinct steps involving 9-mercaptodethiobiotin as an intermediate [3].

To investigate further biotin biosynthesis in plants we characterized these enzymes. However, their low abundance in plant tissues led to severe difficulties in their purification. To face this problem, we decided to develop a functional complementation approach using the *bioB* mutant and a plant cDNA library, and we characterized a cDNA encoding *bioB* gene product from *A. thaliana* (GenBank accession number L34413) [13]. The plant sequence contained an N-terminal extension of 40 amino acids that is not found in the bacterial counterparts. The amino acid composition of this extension have many characteristics in common with transit peptides for targeting to the mitochondria or chloroplasts [14].

Here we describe the cloning of a cDNA encoding *bioB* gene product equivalent from *A. thaliana* into a pET expression vector and the overproduction of this enzyme in *E. coli*. Since enzymatic step catalysed by this protein is still unclear, we will refer to the product of the *A. thaliana bioB* gene product equivalent and not biotin synthase. Furthermore, we decided to follow the purification steps using immunodetection and this enabled a rapid preparation of large amounts of the recombinant protein. The recombinant plant enzyme has a reddish color, contains an [2Fe-2S] cluster, and is a homodimer. Finally, we reported the subcellular localization of the enzyme in the pea leaf cells and demonstrated that *bioB* gene product equivalent is located in the mitochondrial compartment as a 39 kDa polypeptide.

2. Materials and methods

2.1. Materials

Enzymes and cofactors were obtained from Boeringer-Mannheim, France. Synthetic oligonucleotides were from Bioprobe Systems. The pET11a and pET9 used to construct the expression vectors were from Novagen. Chromatographic columns were from Pharmacia Chemicals except for the EMD DEAE 650 (M) ion-exchange column which was from Merck.

2.2. Plant material and bacterial strains

Pisum sativum cv Douce Provence was grown as described previously [10]. DNA manipulation was performed in *E. coli* DH5α cells (Gibco-BRL). Competent *E. coli* BL21(DE3) strain (Novagen) prepared according to the method of Dower et al. [15] was transformed with a pET11a/9 hybrid construction.

2.3. Preparation of pea leaf crude extract

Pea leaves (2 g) were ground in liquid nitrogen. The powder was then homogenized with 10 ml of 50 mM HEPES-NaOH (pH 8.0), 1 mM EDTA, 5 mM DTT, 0.1% (w/v) BSA, 5 mM 6-aminohexanoic

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Abbreviations: DTT, 1,4-dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PVP-25, polyvinylpyrrolidone (M_r 25000); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid

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acid and 1 mM benzamidine-HCl. After centrifugation at 48 000×*g* for 30 min, the supernatant comprised the crude extract. All procedures were carried out at 4°C.

2.4. Preparation and fractionation of pea leaf protoplasts

Pea leaf protoplasts were purified from young leaves (10–15 g, 10 days old) according to Baldet et al. [10]. Organelles (chloroplasts and mitochondria) and cytosolic fractions were obtained as previously described [10].

2.5. Preparation of purified chloroplasts and mitochondria

Chloroplasts from young pea leaves (9 days old) were purified using Percoll gradients as described by Mourioux and Douce [16]. Intact chloroplasts were lysed [12] and the suspension was centrifuged (72 000×*g*, 20 min): the pellet and the supernatant comprised the chloroplast membranes (envelope membranes and thylakoids) and the soluble fraction (stroma), respectively. Mitochondria were purified from pea leaves (12 days old) using self-generating Percoll gradients as described by Douce et al. [17]. Intact mitochondria were lysed [11] and the suspension was centrifuged (100 000×*g*, 20 min): the pellet and the supernatant comprised the mitochondrial membranes and the soluble fraction (matrix), respectively.

2.6. Enzyme assays, protein, and spectroscopic determinations

The activities of each compartment marker enzyme, glyceraldehyde-3-phosphate dehydrogenase for chloroplast, fumarase for mitochondria and pyrophosphatase: fructose-6-phosphate-1-phosphotransferase for cytosol, were assayed as described by Baldet et al. [10].

Protein was measured by the method of Bradford [18] using Bio-Rad protein assay reagent (Bio-Rad Laboratories) with γ-globulin as a standard. Absorption spectra were recorded at 25°C in 1 cm optical path length quartz cuvettes (1 ml) using a Uvikon 860 (Kontron) spectrophotometer.

2.7. Preparation of antibodies specific to bioB gene product equivalent from A. thaliana

Peptide synthesis and rabbit antiserum were prepared by Neosystem Laboratory, Strasbourg, France. Antibodies were raised against the following synthetic peptide G-P-R-N-D-W-S-R-D-E-I-K-S-V-Y coupled to ovalbumin and deduced from the cDNA encoding *A. thaliana bioB* gene product previously described (at position 43–57) [13]. Antibodies were purified according to the method described by Saint Blancard et al. [19] and then by affinity chromatography as described by Rolland et al. [20]. Dilution to (1/30 000)th of this antibody solution allowed detection of 1 ng of the synthetic peptide. A second preparation of antiserum raised against the recombinant protein was performed as described above. Dilution to (1/10 000)th of this purified antibody allowed detection of 1 ng of the purified recombinant protein.

2.8. Construction of bioB gene product expression vector

The vector chosen for the overproduction of the recombinant protein was a hybrid of pET9 and pET11a constructed by cloning the fragment *Acc*I-*Eco*RI (containing the gene for kanamycin resistance) from pET9 into the pET11a vector linearized by *Eco*RI and *Acc*I digestions [21]. The hybrid pET11a/9-BS plasmid coding for *bioB* gene product was constructed via mutagenesis by PCR using the pYes CBS1 *bioB* gene product cDNA as a template [13]. The PCR fragment corresponding to the *bioB* gene product was obtained using the two oligonucleotides: P1 (5′-AAAGCTGGAGCTCATATGATGCTTGTTCG-3′) which introduced the ATG translation-initiation codon (underlined) corresponding to the first Met residue of the protein [13]; P2 (5′-AATTCGGATCCATTAGTGAGAAGCGGAAGC-3′) which is complementary to the 3′ end of the cDNA and introduced a *Bam*HI restriction site 1 bp after the stop codon. The PCR fragment was subcloned into the pET11a/9 vector, the final construct is referred to pET11a/9 CBS1. The DNA insert was sequenced on both strand [22] to ensure that no mutation was introduced during the course of the PCR amplification.

2.9. Immunological assay for the detection of recombinant bioB gene product

Since no activity assay was sensitive enough to follow the protein along the several chromatography steps, we used a colorimetric enzyme assay coupled with an immunological reaction using pure antibodies specific to the synthetic peptide. Microtiter ELISA plates (Greiner) were incubated 3 h at 25°C with 10–20 μl per well of the collected fractions during the chromatography steps, added to 100 μl TBST (Tris-buffered saline containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% (v/v) Tween 20). The plate washed was then incubated with the pure antibodies at a 1/20 000 dilution for 18 h at 4°C. Then the plate was washed and incubated with goat anti-rabbit IgG horseradish peroxidase conjugated at a dilution 1/10 000 in TBST for 1 h at 25°C. Finally, wells were washed and each received a 100 μl peroxidase substrate solution (0.1 μl 3% H_2O_2 and 100 μg 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) in 100 mM citrate phosphate (pH 4.0). Plates were read in an EL-312 microplate reader (Biotek Instruments), using a 405 nm filter.

2.10. Expression and purification of recombinant bioB gene product

E. coli BL21(DE3) cells were cotransformed with pET11a/9 CBS1 and pBkat37 (containing the GroE*SL* and streptomycin resistance genes, personal communication, Dr. J. Pierrard, Rhône-Poulenc Cie, Lyon, France). Expression of the recombinant protein was induced by adding 1 mM IPTG and the culture was maintained at 20°C for 18 h. All subsequent procedures were carried out at 4°C. Harvested cells were lysed in buffer A: 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, supplemented with 5 mM 6-aminohexanoic acid, 1 mM benzamidine-HCl and 1 mM PMSF, by sonication and the lysate was centrifuged at 28 000×*g* for 20 min. The cell-free extract obtained (658 mg of protein starting from a 1 l cell culture) was subjected to a cut 20–80% of $(NH_4)_2SO_4$. The resulting pellet was desalted by passage through a Sephadex G25 (M) column equilibrated in the buffer A. This solution (484 mg protein, 12 ml) was applied onto a Fractogel EMD DEAE 650 (M) column. After loading and washing the column with 200 ml of buffer A, proteins were eluted with a 500 ml linear gradient from 0 to 0.3 M NaCl in buffer A. The *bioB* gene product immunologically detected, was eluted as a single peak at 180 mM, concentrated by $(NH_4)_2SO_4$ precipitation (80% saturation) and resuspended in buffer A. The sample (55 mg protein) was applied onto a Hiload 16/10 Q Sepharose HP. The column was washed with 50 ml buffer A and proteins were eluted with 100 ml linear gradient from 0 to 0.3 M KCl. The peak of *bioB* gene product eluted at 150 mM KCl was concentrated as above. The sample (36 mg protein) was applied to a Superdex 200 column equilibrated in buffer B: 20 mM KPi (pH 6.8), 1 mM EDTA, 1 mM DTT supplemented with 150 mM NaCl. The peak of *bioB* gene product concentrated by filtration using filtron microsep-30 system (10.5 mg), was applied to a Hydroxyapatite Bio-Gel HTP (Bio-Rad) column equilibrated with buffer B. The peak of pure *bioB* gene product was eluted at 25–30% of the step gradient (20 to 300 mM KPi) that is 100 mM KPi.

2.11. Electrophoresis and immunoblotting

SDS-PAGE was performed in slab gels containing a 12% (w/v) acrylamide. The conditions for gel preparation, sample solubilization, electrophoresis and gel staining were detailed by Chua [23]. Polypeptides were also transferred electrophoretically onto nitrocellulose sheets (Bio-Rad) as described by Towbin et al. [24]. Identification of the *bioB* gene product polypeptide was performed using rabbit polyclonal antibodies raised against the recombinant protein followed by detection with goat horseradish peroxidase-coupled anti-rabbit IgG (Bio-Rad) using 4-chloro-1-naphthol as substrate.

3. Results and discussion

3.1. Purification of bioB gene product from A. thaliana overproduced in E. coli

In order to express the *bioB* gene product from *A. thaliana* in *E. coli* a synthetic gene encoding the protein was obtained by PCR and cloned in the hybrid vector pET11a/9 CBS1 as described in Section 2. Efficient expression of the recombinant protein was achieved in *E. coli* BL21(DE3) strain, which contains the T7 polymerase system but is deficient in two major proteases [21]. To achieve a correct folding of the protein in the presence of associated overexpressed chaperone (see Section 2) and to facilitate the insertion of the iron-sulfur cluster, the induction was carried out at a lower temperature (20°C).

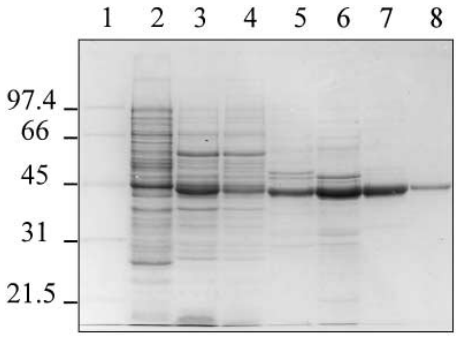


Fig. 1. SDS-PAGE analysis of *bioB* gene product equivalent from *A. thaliana* at various steps of purification from overproducing *E. coli* cells. Polypeptides were separated by SDS/PAGE 10% (w/v) acrylamide and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular-mass markers (kDa). Lane 2, soluble protein extract from *E. coli* BL21(DE3) cells prior induction by 1 mM IPTG (75 μg). Lane 3, soluble protein extract from *E. coli* BL21(DE3) after an 18 h induction by 1 mM IPTG at 20°C (50 μg). Lane 4, $(NH_4)_2SO_4$ fraction (40 μg). Lane 5, EMD DEAE 650 (M) pool (10 μg). Lane 6, Hiload pool (10 μg). Lane 7, Superdex 200 pool (10 μg). Lane 8, Hydroxyapatite pool (5 μg).

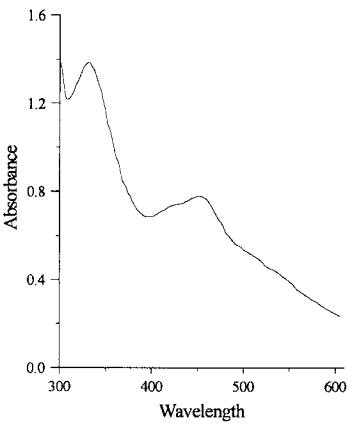


Fig. 2. UV-visible absorbance spectrum of 110 kDa protein. The absorption spectrum of the 110 kDa protein (6 mg/ml) in buffer C is shown in its native state.

In these conditions, the polypeptide accounted for approx. 2% of the bacterial soluble proteins. It was assigned to *A. thaliana bioB* gene product equivalent since polyclonal antibodies raised against the synthetic peptide reacted only with a M_r 44 000 polypeptide in a preparation of total *E. coli* BL21(DE3) proteins separated by SDS-PAGE and transferred to nitrocellulose (data not shown).

A four-step protocol was devised to purify *bioB* gene product from *A. thaliana* overproduced in *E. coli* cells (see Section 2). The procedure consisted in two anion-exchange chromatographies followed by gel filtration and finally a hydroxyapatite from which the protein was eluted as a single peak. Thus, 3.6 mg of pure *bioB* gene product equivalent was obtained from 1 l of cell culture. The estimation of the molecular mass by several methods, including native gel electrophoresis, gave an average value of 100 kDa. On the other hand, on SDS-PAGE the protein migrated as a single 44 kDa polypeptide (Fig. 1) suggesting, therefore, that the native protein is a dimer composed of two identical subunits.

The pure native 100 kDa protein has a reddish color and its UV-visible absorbance spectrum (Fig. 2) shows characteristic peaks for iron-sulfur proteins which contain [2Fe-2S] clusters [25–27]. The protein absorbance at 275 nm gives an extinction coefficient of 2.93×10^4, based on the monomer molecular mass. The extinction coefficients deduced from the spectrum at 330, 420, 448 and 540 nm, are respectively 1.04×10^4, 5.54×10^3, 5.75×10^3, and 3.19×10^3 M^{-1} cm^{-1}. These parameters for the *bioB* gene product from *A. thaliana* are similar to those defined for the *E. coli* protein [8]. Most proteins containing [2Fe-2S] clusters have extinction coefficients in the 400–450 nm region of $(8–10)\times10^3$ M^{-1} cm^{-1}/cluster (with iron in the +2 oxidation state) [28]. Thus, like for the bacterial protein, the values in this region of the spectrum for the native *bioB* gene product (i.e. for the homodimer) are somewhat lower, but suggest the presence of one [2Fe-2S] cluster per monomer.

3.2. *Intracellular localization of bioB gene product equivalent in pea leaf cells*

To determine the distribution of *bioB* gene product equivalent within the different compartments of pea leaf cells we carried out a large scale purification of intact protoplasts as well as chloroplasts and mitochondria. Protoplasts, isolated by enzymatic digestion from young pea leaves (10 days), were fractionated by gentle rupture through a fine nylon mesh followed by centrifugation to yield a cytosolic (supernatant) and an organelle fraction (pellet). The purity of these two fractions were assayed by measuring selected subcellular marker enzyme activities which were pyrophosphate-fructose-6-phosphate 1-phosphotransferase for cytosol, NADP-glyceraldehyde-3-phosphate dehydrogenase for chloroplasts and fumarase for mitochondria. Most of the chloroplast (83%) and mitochondrial (87%) marker activities were recovered in the

Table 1
Subcellular distribution of marker enzyme activities in pea leaf protoplasts

	Total extract	Distribution		
		Supernatant	Pellet	
mg protein/10^6 cells	1.2	0.32	0.91	
Enzyme activity	nmol/min/mg protein	% total activity		Recovery
PFP	23.6	63 ± 8	31 ± 6	94 ± 14
NADP-GAPDH	304	7 ± 3	83 ± 2	90 ± 5
Fumarase	5.8	3 ± 1	87 ± 5	90 ± 6

Activities of marker enzymes to check cross-contamination were assayed in broken pea leaf protoplasts, organelles (pellet) and cytosol (supernatant). The marker enzymes were as follows: cytosol, pyrophosphate-fructose-6-phosphate 1-phosphotransferase (PFP); chloroplasts, NADP glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH); mitochondria, fumarase. The data presented for distribution in pellet and supernatant and for recovery are expressed in percentage of total activity recovered, and are the mean values obtained from four different experimental determinations ± S.D.

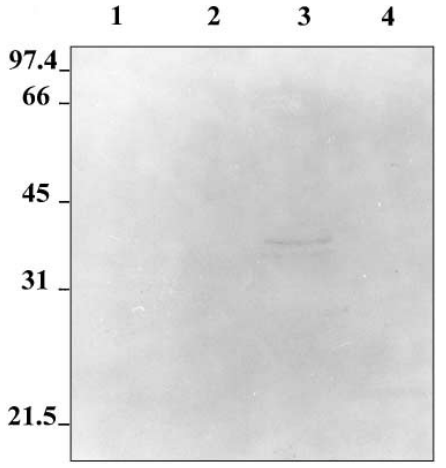


Fig. 3. Western blot analysis using polyclonal antibodies raised against recombinant *bioB* gene product from *A. thaliana*. Lane 1, crude extract from pea leaf (100 μg); lane 2, Percoll-purified pea chloroplasts (100 μg); lane 3, Percoll-purified pea mitochondria (100 μg); lane 4, cytosol from pea leaf protoplasts (100 μg). Position of molecular markers, in kDa, is given on the left.

pellet (Table 1). Since it is difficult to rupture the small protoplasts quantitatively without affecting chloroplast integrity, we performed a gentle rupture of the protoplasts in order to prevent a massive contamination of the cytosolic fraction with stromal proteins. For that reason 31% of the total cytosolic marker activity was in the pellet containing intact protoplasts. The supernatant obtained after differential centrifugation of lysed protoplasts contained a substantial part of cytosolic marker enzymes (63% of the total activity) and slightly contaminated by chloroplast and mitochondrial enzymes. At last, the distribution of the *bioB* gene product within the organelle pellet was achieved by purification of chloroplasts and mitochondria on Percoll gradients. In both cases cytosolic contamination of these organelles was negligible (not shown).

To investigate the distribution of the *bioB* gene product in plant cells, proteins from pea leaf crude extract, purified chloroplasts, mitochondria and cytosol were subjected to SDS-PAGE and the separated polypeptides were transferred to nitrocellulose sheets. Fig. 3 indicates that the purified antibody raised against the recombinant *bioB* gene product from *A. thaliana* reacted with a single 39 kDa polypeptide exclusively located in the soluble fraction of mitochondria. No other band was revealed in the cytosolic and chloroplast compartments. This result is the first direct clue that the *bioB* cDNA clone that we have isolated from *A. thaliana* encodes a protein targeted in mitochondria. The difference in molecular masses between the polypeptide encoded by the *bioB* cDNA (44 kDa) and the protein revealed by Western blotting in the pea leaf mitochondria (39 kDa) suggests that the *bioB* gene product is synthesized as a precursor and is imported within the mitochondria to yield its mature form. It is interesting to note that similarly in *E. coli* the *bioB* gene product corresponds to a 39 kDa polypeptide. Analysis of the 41 amino acid extension in the N-terminal had been suggested to represent a mitochondrial transit peptide [14]. Our result is in complete accordance with this prediction. At last, the absence of detection of the 39 kDa polypeptide in the crude extract is obviously due to the very low abundance of this protein in plant cells and this could be compared with the biotin ligase which has been proposed to represent about 1×10^{-6} of the total pea leaf cells (Tissot and Alban, personal communication).

3.3. Discussion

These results together indicate that the enzyme catalysing the last step of biotin synthesis is confined in the matrix space of plant mitochondria and suggest that the enzymatic machinery involved in the assembly of all the constitutive parts of the biotin molecule occurs in this cell organelle. If this holds true, such a localization raises the problem of biotin transport through the inner mitochondrial membrane and its distribution in the cytosol (containing an important pool of free biotin, [10]) and plastids. These results also raise the question of the origin of pimelic acid. However, Wada et al. [29] have demonstrated that AcylCarrier Protein present in mitochondria is involved in the de novo synthesis of fatty acids in plant mitochondria. It is possible, therefore, that one of the functions of this pathway is production of pimelic acid as pimeloyl-ACP. Finally these results also raise the problem of the source of the sulfur atom for the conversion of dethiobiotin to biotin.

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