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The amino acid sequence previously attributed to a protein kinase or a TCP1-related molecular chaperone and co-purified with phytochrome is a β-glucosidase

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Abstract

A 60 kDa protein (P60) co-purified with phytochrome was identified as avenacosidase, a β-glucosidase which is part of the defense system of *Avena sativa*. An antiserum raised against P60 was used to isolate a cDNA clone coding for the complete amino acid sequence of P60. The cDNA-derived amino acid sequence contained the partial sequences described before for a protein kinase [(1989) Planta 178, 199–206] and for a TCP1-related molecular chaperone [(1993) Nature 363, 644–647] co-purified with phytochrome. We conclude that these activities were related to minor contaminants and that only sequences of avenacosidase had been obtained.

Key words: *Avena sativa*; Avenacosidase; β-Glucosidase aggregate; Phytochrome

1. Introduction

A 60 kDa protein (P60) co-purified with phytochrome has attracted the attention of plant physiologists for some time. We became interested in this protein (P60) when Grimm [1] observed increased solubility of the Pr-form of phytochrome in the presence of this protein. He proposed a possible interaction of P60 with phytochrome and found that crude preparations of P60 had protein kinase activity [2]. The amino-terminal sequence of this presumed protein kinase was ALESA/GKL/QVPW. Mummert et al. [3] found the same amino-terminal sequence (ALESA/GKL/Q with more variation after the seventh residue) in a protein complex which co-purified with the plant photoreceptor phytochrome. The complex was considered as a TCP1-related molecular chaperone for the following reasons: it cross-reacted with monoclonal antibodies raised against mouse TCP1, and it was able to refold denatured phytochrome in an ATP-dependent manner. The authors assumed that the protein might be a chaperone involved in the biogenesis of phytochrome. We report here the cloning of the corresponding cDNA and characterization of the protein as avenacosidase, a β-glucosidase probably involved in the defense mechanism of oat [4,5].

2. Materials and methods

P60 was isolated from 3.5-day-old etiolated oat seedlings (*Avena sativa* L. cv. Pirol). The standard method of phytochrome isolation [6] was followed until the step of hydroxyapatite chromatography. For further purification, P60-containing fractions were chromatographed on Biogel A1.5 in 20 mM potassium phosphate buffer. SDS-PAGE was performed according to Laemmli [7] using 10% gels. Proteins were blotted onto nitrocellulose (0.2 μm pore size) according to [8]. Isoelectric focussing was done in 5% polyacrylamide slab gels as described by Redpath [9]. The proteins were then transferred to glassfiber sheets. Microsequencing was performed according to [10]. The P60 band of denaturating PAGE (see Fig. 1) was cut out of the gel and directly treated with pepsin. The resulting peptides were separated by reverse-phase HPLC (Hewlett Packard 1090, Vydac C-18 column, 1 × 250 mm). The separated fractions were applied to a protein sequencer (HP G 1005 A). Fractions containing pure P60 after Biogel chromatography were used for immunization of rabbits. A λgt11 expression library of oat cDNA was screened with the polyclonal antiserum. Partial clones obtained were then used for preparation of digoxygenin-labeled DNA probes. Re-screening of the library resulted in a cDNA clone coding for P60; it contained the amino-terminal and internal sequences of P60 determined by Edman degradation.

3. Results and discussion

P60 elutes from the hydroxyapatite column only some-

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Abbreviations: TCP1, t-complex polypeptide-1; P60, 60 kDa protein with β-glucosidase activity; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; K, kilodalton; cpn60, 60 kDa plant chloroplast chaperonin; GroEL, 60 kDa prokaryotic cytosol chaperone.

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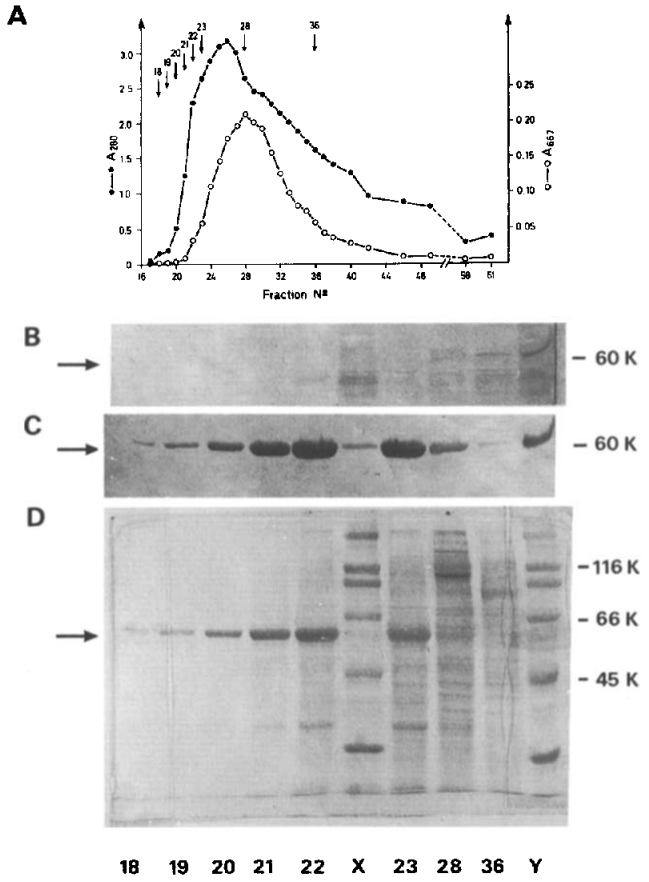

Fig. 1. Co-purification of P60 and phytochrome by elution from hydroxyapatite. (A) Elution diagramm of total protein (determined by A_{280}) and phytochrome (determined by A_{667}). Arrows indicate fractions analyzed by SDS-PAGE. (D) Coomassie-stained gel; X, Y = marker proteins; (B,C) Immunoblot of the gel corresponding to D, but X = crude extract and Y = pellet of ammonium sulfate fractionation, using polyclonal antiserum anti-GroEL (B) and anti-P60 (C), respectively. The chaperone cpn60 was only detected in fraction 36 (B).

what prior to phytochrome (Fig. 1). Mummert et al. [3] used the 'phytochrome pool' (corresponding to fractions 23–26; Fig. 1) as starting material for preparation of their protein; further purification was traced according to the presence of cpn60 which we detected in fraction 36 (Fig. 1B). For isolation of the protein kinase, the 'phytochrome pool' was also used [2]. We preferred here to start with earlier fractions (21,22) for further purification of P60 by rechromatography on Biogel A 1.5. Purified P60 did not interact with phytochrome in any way, and it did not show any protein kinase activity. Purified P60 is separated into 5–6 distinct species with isoelectric points between 5 and 6. These characteristics are identical to those of the protein described by Mummert et al. [3]. We found the same amino-terminal sequence ALESAKQVKPWQV in all bands obtained by isoelectric focussing.

An antiserum was raised against purified P60; it was used for isolation of a cDNA clone coding for P60 (see section 2). The cDNA sequence revealed an open reading frame coding for a protein of 519 amino acid residues (Fig. 2A). This sequence contained the amino-terminal sequence of P60 which was also described for the protein kinase [2] and for the presumed chaperone [3]. Furthermore it contained the internal sequences determined for P60 (underlined in Fig. 2A) and also those described by Mummert et al. [3] (see Fig. 2B).

Sequence comparison against the EMBL Nucleotide Sequence Database revealed a significant homology of the sequence with glucosidases. The highest score (58% identity) was obtained with the sequence of a β-glucosidase from *Zea mays* [7]. Efforts to find any similarity between P60 and TCP1-related chaperones failed (Fig. 2B,C). This holds true even for recently published TCP1-related sequences from oat [8].

We tested glycosidase activity of purified P60. As shown in Table 1, P60 does indeed have β-glucosidase activity. Almost no hydrolysis was found with α-glycosides (Table 1). In summary, there is no doubt that the bulk of the protein complex co-purified with phytochrome is actually a β-glucosidase.

A glucosidase with the size of P60 occurring in oat is the avenacosidase [4,5]. It is an essential part of a preformed defense system of oat: avenacosides are hydrolyzed to 26-desgluco-avenacosides immediately after damage of the plant tissue. Only the desgluco-derivatives exhibit fungicidal activity. The identity of P60 with avenacosidase was demonstrated in two ways. (i) Avenacosidase isolated by Nisius [4,5] gave the same signal as P60 with our antiserum (Fig. 3A, lane d); (ii) the antiserum prepared by Nisius [4,5] against avenacosidase reacted in an identical manner as our antiserum with the corresponding fractions (Fig. 3B, lanes a–d).

It appears rather unlikely that different proteins (protein kinase, chaperone, glucosidase) share long stretches of identical amino acid residues. We consider multiple enzyme activity of P60 unlikely: we tested for, but did not find, any protein kinase or chaperone activities in purified P60. Due to the abundance of P60, sequences of this protein instead of those of the desired enzymes were apparently determined previously [2,3]. This possibility is based on several observations: (i) Grimm et al. [2] and Mummert et al. [3] started with the phytochrome fraction proper of the hydroxyapatite column. This contains

Table 1

Substrate	nkat/mg protein
p-Nitrophenyl-β-glucoside	45.3
p-Nitrophenyl-β-galactoside	28.8
p-Nitrophenyl-α-glucoside	< 0.1
p-Nitrophenyl-α-galactoside	< 0.1

Enzyme activity of isolated P60 Substrates (1.2 mM) were incubated with protein P60 (7 $\mu g \cdot ml^{-1}$) at pH 5.4 and 34°C for 15 min. The reaction was stopped with 0.1 M Na_2CO_3 and free *p*-nitrophenol determined as $A_{414\,nm}-A_{550\,nm}$.

only a small portion of P60 (together with other contaminating proteins) whereas the bulk of P60 elutes prior to phytochrome. Our starting material for further chromatography therefore already had a higher degree of purity than that of the other authors. (ii) The further purification steps of Mummert et al. [3] were traced with the anti-chloroplast cpn60 antiserum. This reacted mainly with the 800 kDa contaminant of their preparation and cross-reacted with the desired 600 kDa protein only weakly. The purest preparation presented in [3] still contained both the 800 kDa and the 600 kDa protein. (iii) The amino-terminal sequence, which is more diver-

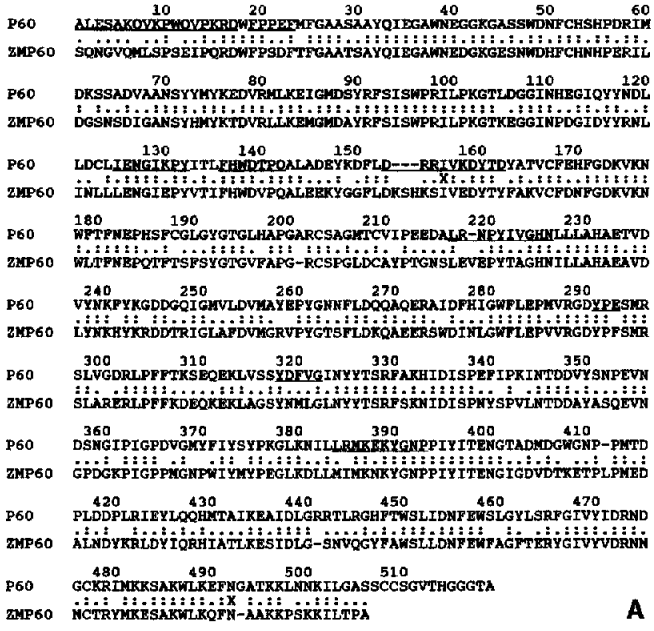

Fig. 2. Amino acid sequence of P60 and alignment with several sequences. (A) Complete sequence of P60 as deduced from cDNA sequence. Partial sequences determined by microsequencing are underlined. Alignment with β-glucosidase from maize [11]. (B) The internal sequence used by Mummert et al. [3] in order to demonstrate similarity with TCP1 chaperones shows striking homology with the glucosidase P60. (C) Segment comparison scores of P60 with TCP1 chaperones and with β-glucosidase from maize. The scores (given in SD units) were calculated by the RELATE Program [13]. Scores >5 SD units indicate significant homology. The complete sequence of P60 does not show any similarity with TCP1 chaperones but a highly significant homology with the β-glucosidase.

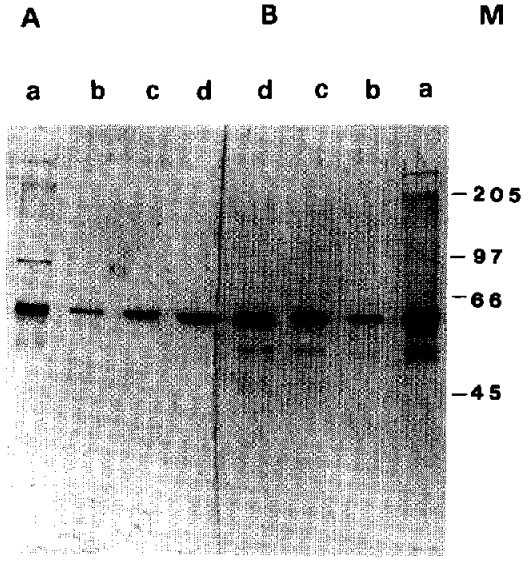

Fig. 3. Western blot analysis of different preparations to show identity of P60 and avenacosidase (β-glucosidase described by Nisius [4,5]), using anti-P60 antiserum (A) or anti-avenacosidase antiserum prepared by Nisius [4,5] (B). Lanes: a, pellet of ammonium sulfate precipitation; b, etioplast preparation; c, hydroxyapatite eluate containing P60; d, avenacosidase preparation from Nisius. M, marker proteins: 205, 97, 66, 45 kDa.

gent in [2] and [3] than in ours, also points to a higher purity of our preparation. (iv) P60 aggregates apparently tend to retain other proteins under the conditions of 'native' gel electrophoresis. Remarkably, only a particular P60 aggregate (450 kDa) showed protein kinase activity although other aggregates were present. It is quite possible that other aggregates (e.g. 600 kDa) retain other (e.g. chaperone) activities. (v) We found that short incubation with trypsin removed contaminating proteins (e.g. phytochrome, protein kinase) from P60 aggregates without measurable attack of the aggregate itself. Glucosidase activity was retained as the only activity after this treatment. We therefore conclude that the glucosidase, as the core protein of the complex, co-purified with phytochrome.

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