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Brassica napus Plastid and Mitochondrial Chaperonin-60 Proteins Contain Multiple Distinct Polypeptides[1]

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Plastid chaperonin-60 protein was purified to apparent homogeneity from *Brassica napus* using a novel protocol. The purified protein, which migrated as a single species by nondenaturing polyacrylamide gel electrophoresis, contained four polypeptides: three variants of $p60^{cpn60\alpha}$ and $p60^{cpn60\beta}$. Partial amino acid sequence determination demonstrated that each variant of $p60^{cpn60\alpha}$ is a distinct translation product. During this study, additional chaperonin-60 proteins were purified. These proteins, which were free from contaminating plastid chaperonin-60, were separated into at least two high molecular weight species that were resolved only by nondenaturing polyacrylamide gel electrophoresis. These proteins contained three 60-kD polypeptides. Two of these polypeptides were recognized by existing antisera, whereas the third was not. Partial amino acid sequence data revealed that each of these, including the immunologically distinct polypeptide, is a chaperonin-60 subunit of putative mitochondrial origin. The behavior of chaperonin-60 proteins during blue A Dyematrex chromatography suggests that this matrix may be generally useful for the identification of chaperonin-60 proteins.

Molecular chaperones are proteins required for the post-translational folding and assembly of many polypeptides into active conformations (Ellis and van der Vies, 1991; Gething and Sambrook, 1992). Chaperonins are sequence-related molecular chaperones found in prokaryotes and in the plastids and mitochondria of eukaryotes (Hemmingsen et al., 1988; Ellis and van der Vies, 1991). The oligomeric structures of most mitochondrial, plastid, and bacterial chaperonin-60 proteins are conserved, each appearing as a stack of two rings with 7-fold symmetry ($Cpn60_{14}$) (Hendrix, 1979; Pushkin et al., 1982; McMullin and Hallberg, 1988; Hutchinson et al., 1989; Prasad and Hallberg, 1989; Tsuprun et al., 1991). However, whereas bacterial and fungal mitochondrial $Cpn60_{14}$ proteins are composed of 14 identical subunits (Hemmingsen et al., 1988; Reading et al., 1989; Cheng et al., 1990), plastid $Cpn60_{14}$ contains two distinct polypeptides, $p60^{cpn60\alpha}$ and $p60^{cpn60\beta}$ (Hemmingsen and Ellis, 1986; Musgrove et al., 1987). The amino acid sequences of these are as divergent as each is from the bacterial homolog, GroEL (Martel et al., 1990). The precise subunit composition of plastid $Cpn60_{14}$ has not been determined: it may be restricted ($\alpha_7\beta_7$, α_{14}, β_{14}), or unrestricted ($\alpha_n\beta_{14-n}$). The structure of the plastid co-chaperonin (GroES homolog) is also distinct from that of the mitochondrial or bacterial co-chaperonins (Chandrasekhar et al., 1986; Lubben et al., 1990; Bertsch et al., 1992; Hartman et al., 1992). The structurally distinct plastid and bacterial chaperonin-60 proteins are also biochemically distinct. Plastid $Cpn60_{14}$ dissociates reversibly in vitro in the presence of concentrations of Mg^{2+}-ATP that have little effect on the structure of *Escherichia coli* $GroEL_{14}$ (Hemmingsen and Ellis, 1986; N. Lissin, personal communication).

The two distinct polypeptides of plastid $Cpn60_{14}$ may possess specialized functions. Evidence consistent with this was obtained from studies of *Brassica napus cpn60-α* and *cpn60-β* genes expressed in *E. coli* (Cloney et al., 1992a, 1992b). Although high levels of soluble $p60^{cpn60\alpha}$ accumulated, it was not efficiently assembled into tetradecameric chaperonin unless *cpn60-β* was co-expressed. In contrast, $p60^{cpn60\beta}$ was efficiently assembled in *E. coli* in the presence or absence of $p60^{cpn60\alpha}$. Thus, it appears that some function, required for the assembly of $p60^{cpn60\alpha}$, is contributed by $p60^{cpn60\beta}$ but not by GroEL.

Antibodies raised against synthetic oligopeptides with amino acid sequences derived from *B. napus cpn60-α* and *cpn60-β* cDNA clones recognized four putative chaperonin-60 polypeptides present in *B. napus* plastid stromal protein (Cloney et al., 1992b). Three of these were identified as α polypeptides and one as a β polypeptide (Cloney et al., 1992b). We wished to determine if the three α polypeptides result from posttranslational modification of a single gene product. To facilitate this and further in vitro studies, a protocol for purification of *B. napus* plastid $Cpn60_{14}$ has been developed.

MATERIALS AND METHODS

Purification of Chaperonin-60 Proteins

Brassica napus seeds (approximately 5×10^5) were sown in soil (2.5 $m^2 \times 3$ cm) and grown for 6 to 7 d at 22°C in near darkness. Seedlings were exposed to 42°C for 2.5 h immediately before use. All steps were carried out at 4°C. Seedlings cut at the soil surface were disrupted using a Polytron ho-

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Abbreviation: LS, low salt.

mogenizer in 10 L of an iso-osmotic isolation medium (0.35 M Suc, 25 mM Hepes, 2 mM EDTA, pH 7.6). This treatment produced a slurry in which little intact stem or leaf material was visible. This was filtered though four layers of cheesecloth and two layers of Miracloth. The filtrate was centrifuged (1,600*g*, 4 min). The pellet was resuspended in 1.2 L of a hypotonic lysis buffer (buffer A: 10 mM Tris-HCl, pH 7.3, 5 mM 2-mercaptoethanol) then centrifuged (15,000*g*, 10 min). The supernatant was retained and solid $(NH_4)_2SO_4$ was added to 30% saturation. The precipitated protein was removed by centrifugation (10,000*g*, 30 min), the supernatant was recovered, and solid $(NH_4)_2SO_4$ was added to 70% saturation. Precipitated protein was redissolved in 80 mL of buffer A and recentrifuged (28,000*g*, 30 min).

The cleared supernatant was applied to a Sephadex G-25 column (5.0 × 20 cm) equilibrated with buffer B (buffer A containing 15% glycerol). The desalted extract was cleared by centrifugation as before and applied to a Fractogel EMD DEAE 650 (S) (E. Merck, Gibbstown, NJ) ion-exchange column (2.5 × 16 cm) equilibrated with buffer B. The column was washed at 3 mL min^{-1} with 200 mL of buffer B and bound protein was eluted with a 0 to 0.6 M NaCl linear gradient in buffer B. SDS-PAGE immunoblot analysis was used to identify fractions containing chaperonin-60 protein. Activity assays were not employed since the quantities of chaperonin-60 protein required for in vitro polypeptide refolding (Herskowitz, 1987; Mendoza et al., 1991, 1992) were impractical. Optimum fractions were pooled and solid $(NH_4)_2SO_4$ was added to 65% saturation. Precipitated protein was recovered as before, desalted over Sephadex G-25 equilibrated with buffer B, centrifuged (10,000*g*, 10 min), and concentrated to 2 to 5 mL with Centriprep-10 centrifugal concentrators (Amicon).

The concentrated protein was applied at 0.5 mL min^{-1} to a blue A Dyematrex (Amicon) column (2.5 × 18 cm) equilibrated with buffer B. Fractions containing chaperonin-60 protein were detected, pooled, and precipitated as before. Precipitated protein was redissolved in 200 to 400 μL of buffer B, passed through a 0.22-μm filter, and applied to a Superose 6 HR10/30 column (Pharmacia LKB Biotechnology, Inc.) equilibrated with buffer C (buffer B with 100 mM NaCl). Molecular mass standards were carbonic anhydrase (29 kD), albumin (66 kD), alcohol dehydrogenase (150 kD), α-amylase (200 kD), apoferritin (443 kD), and thyroglobulin (669 kD) (Sigma). Fractions containing chaperonin-60 protein (approximately 840 kD) were pooled, diluted 2-fold with buffer B, and applied to a Fractogel EMD DEAE 650 (S) ion-exchange column (0.5 × 5.0 cm) equilibrated with buffer B. The column was washed (10 mL of buffer B, 0.5 mL min^{-1}) and bound protein was eluted as before. Fractions containing chaperonin-60 protein were pooled and concentrated with Centricon-100 centrifugal microconcentrators (Amicon). Purified chaperonin-60 protein was stored at less than 5.0 mg mL^{-1} at −20°C.

PAGE and Immunoblot Analysis

SDS-PAGE, nondenaturing PAGE, and immunoblot analysis were as described (Cloney et al., 1992b). Several different antisera were employed: (a) anti-α antiserum was raised against an α-specific synthetic oligopeptide as described (Cloney et al., 1992b); (b) anti-β antiserum was raised against a β-specific synthetic oligopeptide as described (Cloney et al., 1992b); (c) anti-α/β antiserum was generated by mixing equal volumes of the anti-α and the anti-β oligopeptide antisera; (d) anti-plastid Cpn60 antiserum was raised against purified *Pisum sativum* plastid $Cpn60_{14}$ as described (Hemmingsen and Ellis, 1986); (e) broad-specificity anti-Cpn60 antiserum was raised against hybrid chaperonin-60 species isolated from *Escherichia coli* cells that expressed a modified *B. napus cpn60-β* gene (Cloney et al., 1992b). A soluble protein extract was prepared from *E. coli* β cells as described (Cloney et al., 1992b) and applied to a Sephacryl S300 gel-filtration column (2.5 × 60 cm) equilibrated with buffer C. Fractions containing high M_r chaperonin-60 protein including both *E. coli* GroEL and plant $p60^{cpn60\beta}$ polypeptides were pooled, and approximately 1 mg of protein was subjected to nondenaturing PAGE. The gel was stained with Coomassie brilliant blue G-250, and the band corresponding to $Cpn60_{14}$ was excised. Protein recovered by electroelution in an ELUTRAP electro-separation chamber, as described by the manufacturer (Schleicher & Schuell), was used to generate a polyclonal anti-$Cpn60_{14}$ antiserum in rabbits. The antiserum was used without further treatment at a dilution of 1:1000.

Total protein was estimated as described (Bradford, 1976). Polypeptides in purified chaperonin-60 protein were estimated on silver-stained SDS gels by comparison to serial dilutions of *E. coli* GroEL that had been quantified by nitrogen determination (gift of N. Lissin). The recovery of chaperonin-60 polypeptides at each purification step was then estimated by comparing the signal intensities on SDS-PAGE immunoblots to those from the purified protein. Quantities of proteins in the crude stromal fraction were not determined since the methods employed did not allow reliable estimates from this source.

Amino Acid Sequence Analysis

Purified chaperonin-60 protein was subjected to SDS-PAGE and blotted to nitrocellulose as described (Cloney et al., 1992b). After staining with Ponceau S, bands corresponding to each p60 polypeptide were excised and submitted in parallel to in situ digestion (Aebersold et al., 1987) with lysyl endopeptidase (Boehringer Mannheim). The resulting peptide mixtures were individually separated by narrow-bore reverse-phase HPLC using a Hewlett-Packard 1090 chromatograph equipped with a diode array detector. Optimum fractions from these chromatograms were chosen based on differential UV *A* at 210, 277, and 292 nm. Matrix-assisted laser desorption mass spectrometry of these fractions was performed on a Finnigan Lasermat time-of-flight mass spectrometer (Finnigan Mat Corp., San Jose, CA) prior to automated Edman degradation. Details of strategies for the selection of peptide fractions and their microsequencing have been described previously (Lane et al., 1991).

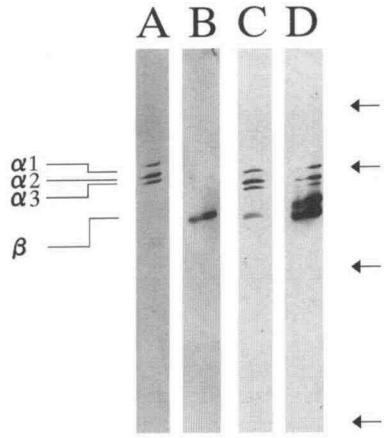

Figure 1. Identification of putative chaperonin-60 polypeptides from *B. napus*. Extracts of a crude protein were subjected to SDS-PAGE and immunoblot analysis with anti-α (A), anti-β (B), anti-plastid Cpn60 (C), and the broad-specificity anti-Cpn60 (D) antisera. Plastid chaperonin-60 polypeptides α1, α2, α3, and β are indicated. Arrows indicate the positions of migration of mol wt markers: rabbit muscle phosphorylase b (97,400), BSA (66,200), hen egg white ovalbumin (45,000), and bovine carbonic anhydrase (21,500).

RESULTS

Purification of Chaperonin-60 Proteins

P. sativum plastid $Cpn60_{14}$ contains two polypeptides with slightly different electrophoretic mobilities: $p60^{cpn60\alpha}$ and the more rapidly migrating polypeptide $p60^{cpn60\beta}$ (Hemmingsen and Ellis, 1986). Amino-terminal amino acid sequences of the *P. sativum* polypeptides were determined (Musgrove et al., 1987). Comparison of these sequences with the sequences predicted from two *B. napus* chaperonin-60 cDNA clones indicated that the cDNAs encoded a $p60^{cpn60\alpha}$ and a $p60^{cpn60\beta}$ polypeptide. A crude soluble protein extract from *B. napus* was analyzed by SDS-PAGE immunoblot analysis using several different antisera (see "Materials and Methods"). The anti-α antiserum recognized three bands, referred to as α1, α2, and α3 (Fig. 1A). The anti-β antiserum recognized one band, referred to as β (Fig. 1B). The anti-α/β and anti-plastid Cpn60 antisera both recognized each of these four bands (Fig. 1C, the anti-plastid Cpn60 antiserum result is shown). The broad specificity anti-Cpn60 antiserum recognized these four bands plus an additional band that migrated between α3 and β (Fig. 1D).

A protocol for the purification of *P. sativum* plastid $Cpn60_{14}$ has been reported (Hemmingsen and Ellis, 1986), but those procedures failed to separate *B. napus* plastid $Cpn60_{14}$ from Rubisco, the most abundant stromal protein. Therefore, a novel protocol was developed. *B. napus* seedlings grown under low-light conditions were used in this study, since the specific content of plastid $Cpn60_{14}$ was similar to that of seedlings grown under normal light conditions, whereas the content of Rubisco was much lower. This method (see "Materials and Methods") chosen for production of a crude soluble protein extract resulted in a modest enrichment for plastid proteins. It also resulted in a low initial yield of protein from the plant material; however, the presence of oxidized phenolic compounds was avoided.

Proteins in a crude soluble extract were subjected to ion-exchange chromatography. Protein eluting between 0 and 0.6 M NaCl was analyzed on SDS-PAGE immunoblots with the broad specificity anti-Cpn60 antiserum. Two distinct sets of immunologically reactive 60-kD polypeptides were observed (Fig. 2). One set, with the pattern expected for *B. napus* plastid $Cpn60_{14}$ (α1, α2, α3, and β), eluted with a peak at approximately 320 mM NaCl. In addition, two immunoreactive polypeptides of approximately 60 kD eluted with a peak at approximately 270 mM NaCl. The more slowly migrating of these corresponded to the polypeptide from the crude extract that migrated between α3 and β (Fig. 1D). The other polypeptide migrated in the same position as the plastid β polypeptide (Fig. 1B).

After ion-exchange chromatography, fractions containing the plastid chaperonin-60 polypeptides were pooled. A separate pool was made of the putative chaperonin polypeptides that eluted at lower salt concentrations (LS chaperonin-60 polypeptides: LS1 and LS2, Fig. 2). The subsequent purification steps were similar for both pools of proteins and included dye-ligand, gel-permeation, and ion-exchange chromatography. The approximate yields and purification of the chaperonin-60 polypeptides from each step were estimated as described (see "Materials and Methods") and are summarized in Tables I and II. Plastid $Cpn60_{14}$ was estimated to be 17 times more abundant than LS chaperonin-60 protein in the crude soluble extract, and the yield of plastid $Cpn60_{14}$ was significantly higher. Aliquots of protein recovered from

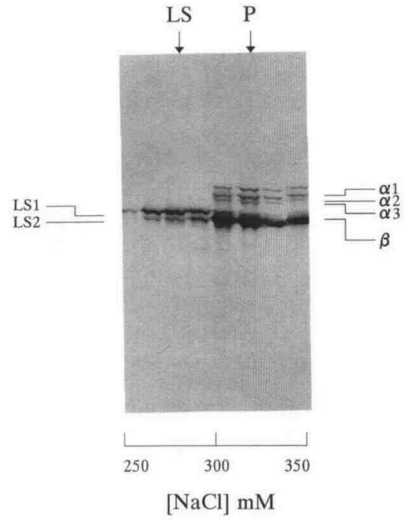

Figure 2. Separation of *B. napus* plastid and LS chaperonin proteins by ion-exchange chromatography. Protein recovered in every second fraction, at the indicated NaCl concentrations, was analyzed on SDS-PAGE immunoblots using the broad-specificity anti-Cpn60 antiserum. Fractions containing peak quantities of each of two distinct sets of 60-kD polypeptides are indicated by arrows and labeled LS (low salt) and P (plastid). Plastid chaperonin-60 polypeptides, α1, α2, α3, and β, and putative chaperonin-60 polypeptides, LS1 and LS2, are indicated.

Table I. *Purification of plastid Cpn60$_{14}$ protein*

Stage	Total Protein	Plastid Cpn60$_{14}$ Protein[a]	Recovery Final	Purification
	mg	*mg*	%[a]	*fold*
Ammonium sulfate fraction	684	5.1	ND[b]	ND
Ion-exchange chromatography	63	4.6	90	11
Dye-ligand chromatography	9.3	3.7	72	55
Gel-permeation chromatography	3.1	2.7	58	120
Ion-exchange chromatography	2.4	2.4	52	130

[a] Quantitation as in "Materials and Methods." [b] ND, Not determined.

each step were analyzed for purity by SDS-PAGE and silver staining (Fig. 3).

Characterization of *B. napus* Plastid Cpn60$_{14}$

Relative to the fraction obtained by ammonium sulfate precipitation, plastid Cpn60$_{14}$ was finally purified approximately 130-fold with a yield estimated to be 52% (Table I). Four polypeptides were present in the purified protein: α1, α2, α3, and β (Fig. 4A, right lane). These polypeptides were recognized by both the broad-specificity anti-Cpn60 antiserum (Fig. 4B, lane 3) and the anti-α/β antiserum (Fig. 4C, lane 3). Crude extracts from two different preparations are shown in lanes 1 of Figure 4, B and C. The extract in Figure 4C shows the more complex pattern of immunoreactive Cpn60 polypeptides that was sometimes observed. The additional bands, however, were eliminated in the course of the purification, and the final purified plastid Cpn60$_{14}$ protein in all cases comprised four Cpn60 polypeptides by SDS-PAGE (see Fig. 4C, lane 3). The purified plastid Cpn60$_{14}$ was subjected to nondenaturing PAGE immunoblot analysis using the broad-specificity anti-Cpn60 antiserum (Fig. 5A, lane 3) and the anti-α/β antiserum (Fig. 5B, lane 3). In each case, a single high-M_r species was recognized.

Previously, an *E. coli* strain was transformed with a *B. napus cpn-60α* cDNA clone that had been modified to remove the nucleotides encoding the predicted transit peptide (Cloney et al., 1992b). This *E. coli* strain accumulated a novel polypeptide that co-migrated with the α2 polypeptide of *B. napus* (Cloney et al., 1992b). We have isolated a second distinct cDNA clone that also produced a product in *E. coli* that co-migrated with the *B. napus* α2 polypeptide (our unpublished data). It remained possible that the *B. napus* α1 and α3 polypeptides resulted from posttranslational modification of α2. Therefore, the amino acid sequences were determined for several proteolytic peptides resulting from endoproteinase Lys-C digestions of α1, α2, and α3. To minimize the effort required to identify sequence differences, proteolytic fractions of similar retention and UV absorbance ratios were screened for molecular mass differences by matrix-assisted laser desorption mass analysis prior to microsequencing. As expected based on the *E. coli* expression study, the determined amino acid sequence of an α2 fragment (Fig. 6, α2 fragment I) was identical to the sequence predicted from the α2 cDNA (GenBank accession No. M35599). Comparison of the α2 fragment I sequence to that of an α1 fragment (Fig. 6, α1 fragment I) revealed a single amino acid difference (Ala→ Ser). The amino acid sequence of a second fragment from α1 (Fig. 6, α1 fragment II) differed at one position from the predicted sequence for the corresponding region of α2 (Ser→Phe). Thus, α1 and α2 are distinct translation products. The sequence of a third fragment from α1 (Fig. 6, α1 fragment III) was identical to the predicted sequence of the corresponding region of α2 but differed at two sites (LysLeu→ThrPhe) from the sequence of a fragment from α3 (Fig. 6, α3 fragment III). Thus, α3 is a third distinct translation product. Therefore, at least two distinct transcripts in *B. napus* encode α2 polypeptides with two other transcripts encoding α1 and α3 polypeptides.

Characterization of LS Chaperonin-60 Protein

Relative to the fraction obtained after ammonium sulfate precipitation, the LS putative chaperonin-60 protein was finally purified more than 1500-fold with an estimated yield of 7% (Fig. 3B, Table II). Silver staining revealed that these

Table II. *Purification of LS Cpn60$_{14}$ protein*

Stage	Total Protein	LS Cpn60$_{14}$ Protein[a]	Recovery	Purification
	mg	*mg*	%[a]	*fold*
Ammonium sulfate fraction	684	0.298	ND[b]	ND
Ion-exchange chromatography	169	0.268	90	3.6
Dye ligand chromatography	15	0.062	21	8.3
Gel-permeation chromatography	0.04	0.034	12	1440
Ion-exchange chromatography	0.02	0.020	6.7	1580

[a] Quantitation as in "Materials and Methods." [b] ND, Not determined.

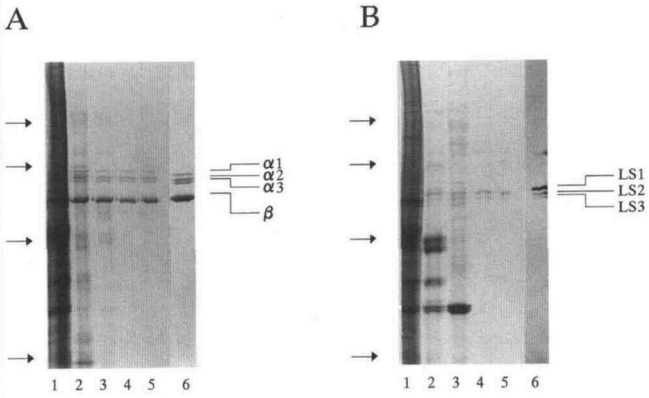

Figure 3. Purification of *B. napus* plastid $Cpn60_{14}$ and LS chaperonin-60 proteins. Protein recovered from each of the five successive chromatography steps indicated in Table I were analyzed for the purities of plastid $Cpn60_{14}$ (A) and LS chaperonin-60 protein (B) on SDS-PAGE silver-stained gels (lanes 1–5). An increased loading of the protein recovered from the final purification step is shown in lane 6. Plastid Cpn60 polypeptides, $\alpha 1$, $\alpha 2$, $\alpha 3$, and β, and putative chaperonin-60 polypeptides, LS1, LS2, and LS3, are indicated. Arrows indicate the positions of migration of mol wt markers; rabbit muscle phosphorylase b (97,400), BSA (66,200), hen egg white ovalbumin (45,000), and bovine carbonic anhydrase (21,500).

fractions were not completely free of contaminating proteins (Fig. 4A, left lane). Immunoblot analysis with the broad-specificity anti-Cpn60 antiserum confirmed the presence of two putative chaperonin-60 polypeptides, LS1 and LS2 (Fig. 4B, lane 2), neither of which was recognized by the anti-α/β antiserum (Fig. 4C, lane 2). Thus, these polypeptides are components of chaperonin-60 protein that are immunologically distinct from the plastid protein. An additional immunologically unrelated polypeptide with an M_r of approximately 60,000 (LS3) was also present at a level that was visible by silver staining (Fig. 4A, left lane). Since LS3 copurified with LS1 and LS2 in each of the chromatography steps (not shown), it seemed likely that it was an immunologically unrelated chaperonin-60 polypeptide.

The broad-specificity anti-Cpn60 antiserum recognized purified LS chaperonin-60 protein as a predominant high-M_r species resolved by nondenaturing PAGE (Fig. 5A, lane 2). A second species, which migrated slightly faster under these gel conditions, was also faintly visible with this antiserum. The relative abundance of the two species varied between preparations. Nondenaturing PAGE immunoblot analysis of purified LS chaperonin-60 protein from an independent preparation revealed two distinct bands of approximately equal intensity (Fig. 5A, inset, lane 2). Neither of these high-M_r species was recognized by the anti-α/β antiserum (Fig. 5B, lane 2).

Peptides resulting from endoproteinase Lys-C digestion of each of LS1, LS2, and LS3 were subjected to amino acid sequence determination. Alignment of these sequences to the corresponding sequences predicted from published chaperonin-60 genes permitted the identification of LS1, LS2, and LS3 as chaperonin-60 polypeptides (not shown). In each case the determined amino acid sequences were most similar to known plant mitochondrial chaperonin sequences.

Sequence data were obtained for corresponding fragments of LS1 and LS2. From these it is clear that LS1 and LS2 are distinct translation products, since they differ at 3 of 21 positions (Fig. 7A). The amino acid sequence of the LS1 fragment is identical to amino acid residues 434 to 456 of the predicted *Arabidopsis thaliana* mitochondrial chaperonin-60 polypeptide (Prasad and Stewart, 1992) (GenBank accession No. Z11546), whereas the sequence of the corresponding LS2 fragment is identical to that of the predicted *Zea mays* mito-

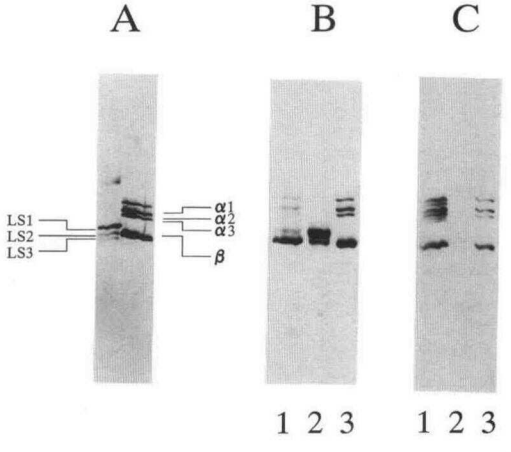

Figure 4. Immunological comparison of chaperonin-60 polypeptides present in purified *B. napus*. A, Purified plastid $Cpn60_{14}$ (right lane) and LS chaperonin-60 protein (left lane) were subjected to SDS-PAGE and the polypeptides present were visualized by silver staining; the polypeptides are labeled $\alpha 1$, $\alpha 2$, $\alpha 3$, and β and LS1, LS2, and LS3. B and C, Crude soluble protein (lane 1), purified LS chaperonin-60 (lane 2), and purified plastid $Cpn60_{14}$ (lane 3) proteins were subjected to SDS-PAGE immunoblot analysis using the broad-specificity anti-Cpn60 (B) and the anti-α/β antisera (C).

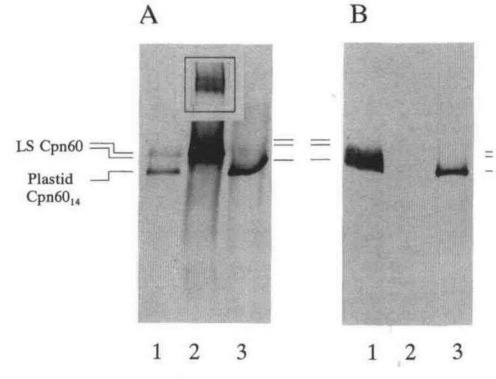

Figure 5. Identification of high-M_r chaperonin-60 species purified from *B. napus*. Crude soluble protein (lanes 1), purified LS chaperonin-60 protein (lanes 2), and purified plastid $Cpn60_{14}$ protein (lanes 3) were subjected to nondenaturing PAGE immunoblot analysis using the broad-specificity anti-Cpn60 antiserum (A) and the anti-α/β antiserum (B). The positions of migration of plastid $Cpn60_{14}$ and two bands that were resolved from the LS chaperonin-60 protein are indicated. An aliquot of LS chaperonin-60 protein from a different preparation resolved by nondenaturing PAGE is shown as an inset to panel A, lane 2.

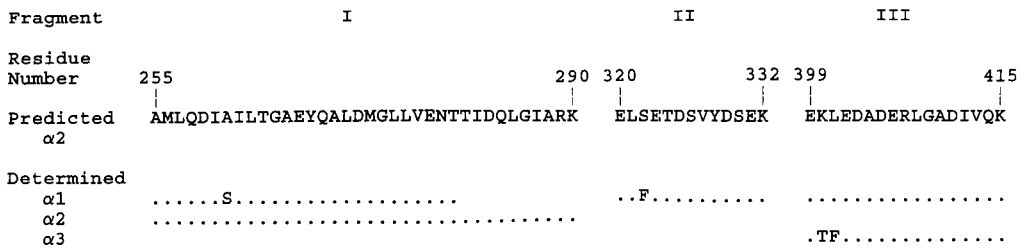

Figure 6. Amino acid sequence analysis of proteolytic fragments of α1, α2, and α3. The amino acid sequences of three proteolytic fragments of α2 (I, II, and III), predicted from a *B. napus cpn-60α* cDNA, are shown. Amino acid residues are numbered from the amino terminus of the mature cpn60α polypeptide. The corresponding amino acid sequences that were determined for α1, α2, and α3 are shown. A period (.) indicates each amino acid residue confirmed to be identical to the predicted sequence, and residues that were different from the predicted sequence are shown.

chondrial polypeptide (Prasad and Stewart, 1992) (GenBank accession No. Z11547) (Fig. 7B). A second fragment of LS2 is also more similar in sequence to the published *Z. mays* sequence than to that of *A. thaliana*. The amino acid sequence of the LS3 fragment is distinct from the predicted sequences of either the *A. thaliana* or the *Z. mays* mitochondrial chaperonin-60 polypeptides (Fig. 7B).

Dye-Ligand Chromatography

The plastid and mitochondrial $Cpn60_{14}$ proteins behaved similarly when subjected to chromatography over blue A Dyematrex (Fig. 8). In each case, the first protein recovered from the column included highly purified chaperonin-60. Plastid $Cpn60_{14}$ from tobacco and $GroEL_{14}$ from *E. coli* also

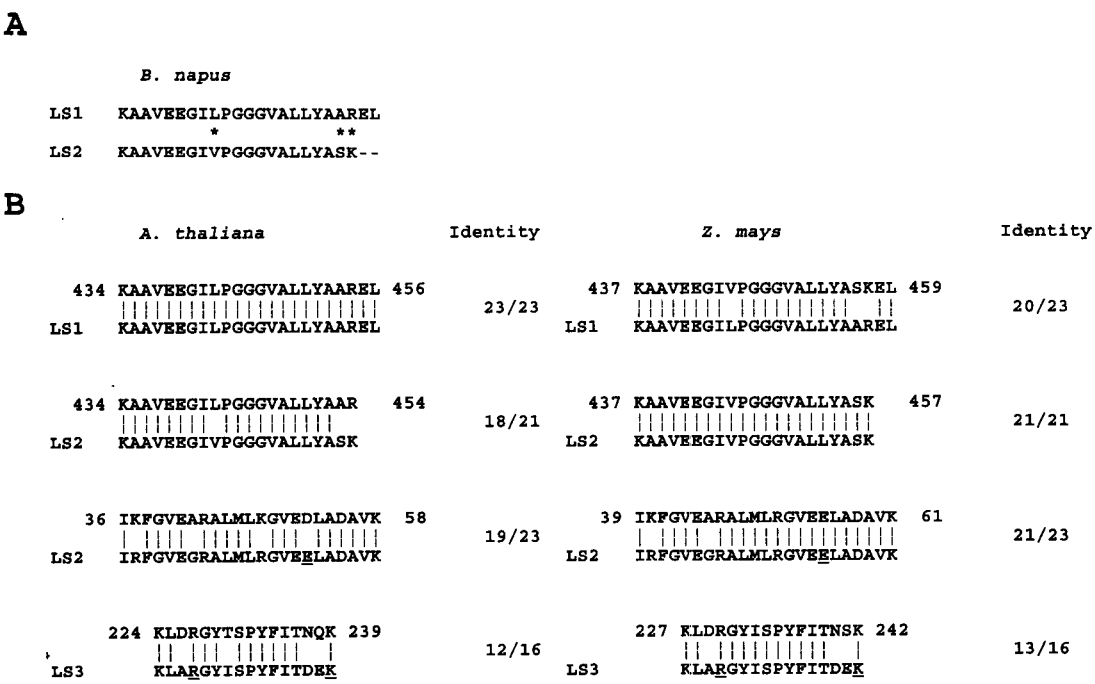

Figure 7. Comparison of partial amino acid sequences of LS1, LS2, and LS3 to predicted sequences of plant mitochondrial chaperonin-60 polypeptides. A, The determined amino acid sequence of a proteolytic fragment of LS1 is aligned with that of the corresponding fragment from LS2. Nonidentical residues are indicated by asterisks (*). B, The determined amino acid sequences of proteolytic fragments of LS1, LS2, and LS3 are aligned with the sequences predicted from published *A. thaliana* and *Z. mays* cDNA (GenBank accession Nos. Z11546 and Z11547). Amino acid residues identical to the predicted sequences are indicated by vertical lines. Amino acid residues determined with a probable or reasonable confidence level as opposed to a high confidence level are underlined. The amino acid positions, numbered from the amino terminus of the predicted chaperonin-60 polypeptide, are indicated. The numbers of identical amino acid residues are expressed as fractions of the fragment lengths.

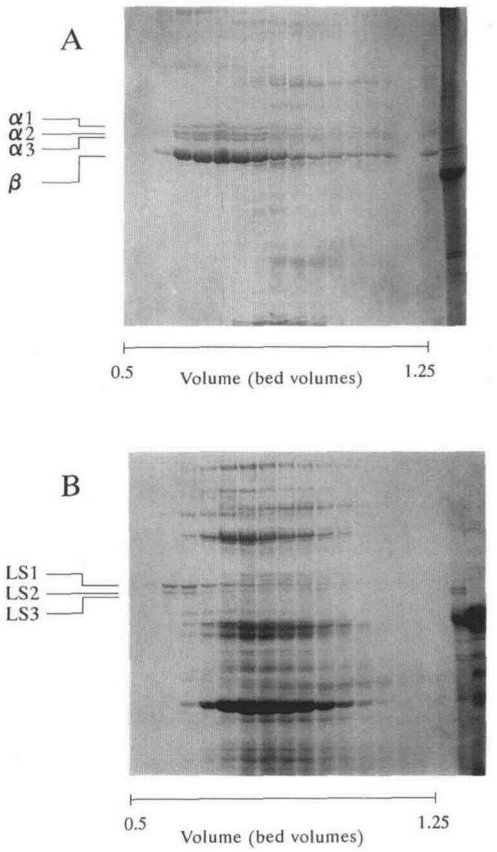

Figure 8. Blue A Dyematrex chromatography of chaperonin-60 proteins. Fractions pooled after ion-exchange chromatography to contain plastid (A) or mitochondrial (B) chaperonin-60 proteins were subjected to chromatography over blue A Dyematrex. Protein recovered between the indicated bed volumes were analyzed for chaperonin-60 polypeptides by SDS-PAGE silver staining and immunoblotting with the broad-specificity anti-Cpn60 antiserum (not shown). Polypeptides α1, α2, α3, and β (A) and LS1, LS2, and LS3 (B) are indicated. An aliquot of the proteins pooled after ion-exchange chromatography was loaded in the second lane from the right and crude stromal protein was loaded in the lane on the extreme right as references.

exhibited this behavior (H.B. Wu, personal communication). Thus, blue A Dyematrex chromatography may be useful for identifying chaperonin-60 proteins from other sources.

It was noted that the recovery of chaperonin-60 proteins from blue A Dyematrex chromatography was subject to variation. In an independent experiment, fractions were pooled after ion-exchange chromatography to produce a sample that included *B. napus* plastid and mitochondrial $Cpn60_{14}$ proteins. Approximately 2- to 3-fold more protein was present in this sample, and it included proteins from several fractions that were excluded from the previous experiments.

A proportion of the Rubisco from this sample did not bind to the column, indicating that the column was overloaded, at least for this protein (Fig. 9A, fractions with high A_{280}). Furthermore, plastid and mitochondrial chaperonin-60 polypeptides were detected in the fractions between 0.5 and 3.0 bed volumes. From this large volume we infer that a proportion of the chaperonin-60 proteins displayed stronger interactions with this column than in the previous experiment, in which chaperonin-60 polypeptides were recovered between 0.50 and 1.25 bed volumes (Fig. 8). Proteins recovered between 1.75 and 3.0 bed volumes were therefore pooled and passed over a regenerated blue A Dyematrex column. The chaperonin-60 proteins were recovered between 0.50 and 1.25 bed volumes from this regenerated column (Fig. 9B). It seems likely that the chaperonin-60 proteins recovered in the late fractions from the first blue A Dyematrex column had interacted, not directly with column matrix, but rather with other proteins present in the sample. Thus, this chromatography step should be monitored to ensure maximum recovery of chaperonin-60 protein.

DISCUSSION

We have purified *B. napus* plastid Cpn60 to apparent homogeneity using a novel protocol. This protocol will also provide sufficient quantities of $Cpn60_{14}$ to perform in vitro studies of its structure and function. Four polypeptides, α1, α2, α3, and β, co-purified and migrated as a single high-M_r species by nondenaturing PAGE and thus may be subunits of a single plastid $Cpn60_{14}$ molecular species. By direct amino acid sequence determination we have found that each of the three α-type polypeptides is a distinct translation product. Thus, cDNA clones corresponding to α1 and α3 remain to be described. The presence of multiple distinct α-type polypeptides may reflect the amphidiploid nature of *B. napus*; however, we have observed the presence of two α-type polypeptides in the diploid *A. thaliana* (our unpublished observation).

In the course of this study other chaperonin-60 proteins, immunologically distinct from the plastid protein, were purified. These additional chaperonin proteins, although not purified to homogeneity, were free from contaminating plastid chaperonin-60 protein. In these fractions, two high-M_r species were resolved by nondenaturing PAGE and two 60-kD polypeptides were resolved by SDS-PAGE. These two polypeptides were recognized only by a subset of the antisera that was developed against plastid chaperonin-60 protein.

Partial amino acid sequence analysis revealed that LS1 and LS2 are chaperonin-60 polypeptides. Further, these determined sequences are most similar to plant mitochondrial chaperonin-60 sequences predicted from nucleotide sequence data. Thus, LS1 and LS2 are two chaperonin-60 polypeptides of putative mitochondrial origin that have similar reactivity to the antisera used in this study but have distinct amino acid sequences. The identity of the determined partial sequence of LS1 to the published *A. thaliana* sequence and of LS2 to the published Z. *mays* sequence suggests that LS1 and LS2 are encoded by paralogous genes, genes present at distinct loci, that are present in both dicot and monocot plants.

A third 60-kD polypeptide co-purified with LS1 and LS2 but was not recognized by any of our anti-chaperonin-60 antisera. Amino acid sequence data confirm that this immunologically distinct polypeptide is a chaperonin-60 polypeptide, and indicate that it is also of putative mitochondrial origin. It remains to be determined if a homolog exists in monocot plants.

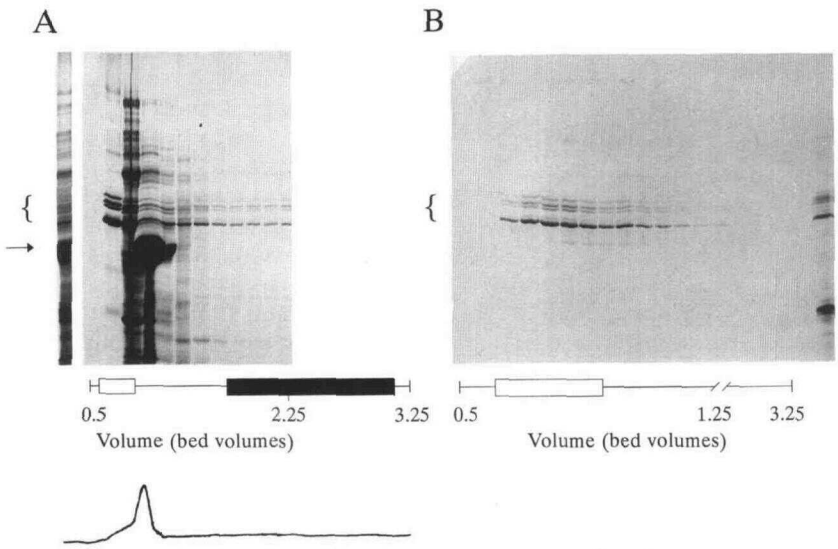

Figure 9. Analysis of the behavior of chaperonin-60 protein on blue A Dyematrex. Fractions pooled after ion-exchange chromatography to contain both mitochondrial and plastid chaperonin-60 protein were subjected to chromatography over blue A Dyematrex. Protein recovered in fractions between the indicated bed volumes was analyzed for chaperonin-60 polypeptides by SDS-PAGE silver staining (A, the silver-stained gel shown includes fractions from 0.5–2.25 bed volumes) and immunoblotting with the broad-specificity anti-Cpn60 antiserum (not shown). The A_{280} profile is shown below the gel and peak quantities of chaperonin-60 polypeptides are indicated by an open bar on the volume scale. An aliquot of the protein applied to the column was resolved by SDS-PAGE and is shown on the left as a reference. The position of migration of the Rubisco large subunit is indicated by an arrow and those of the chaperonin-60 polypeptides are indicated by a bracket. Protein recovered in fractions between 1.75 and 3.0 bed volumes (indicated by the solid black bar on the volume scale) were pooled and subjected to chromatography over a regenerated blue A Dyematrex column. Protein recovered from the second column, between the indicated bed volumes, was analyzed for chaperonin-60 polypeptides by SDS-PAGE silver staining (B) and immunoblotting with the broad-specificity anti-Cpn60 antiserum (not shown). The open bar on the volume scale indicates fractions equivalent to those indicated in A. An aliquot of the proteins applied to this column was loaded in the far-right lane as a reference.

The distributions of LS1, LS2, and LS3 in seedlings have not been determined and further study is required to determine the subunit composition(s) of the putative mitochondrial chaperonin-60 proteins. Mitochondrial chaperonin-60 proteins from diverse species have been reported to be composed of a single subunit type (McMullin and Hallberg, 1988; Hutchinson et al., 1989; Prasad and Hallberg, 1989; Reading et al., 1989; Cheng et al., 1990), although partial amino acid sequence data indicate that moth (*Heliothis virescens*) mitochondrial chaperonin-60 contains two distinct polypeptides that may co-assemble to form a hetero-heptameric protein (Miller et al., 1990). Thus, evidence from moth and now from higher plants suggests that mitochondrial chaperonin proteins may be more complex than previously thought.

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