[34] Functional Analysis and Purification of Enzymes for Carotenoid Biosynthesis Expressed in Photosynthetic Bacteria

By GLENN E. BARTLEY, ANETTE KUMLE, PETER BEYER, and PABLO A. SCOLNIK

Introduction

The bright yellow, orange, and red colors of carotenoids are due to absorption of light by the chromophore, a central array of conjugated double bonds. These double bonds are synthesized on the C_{40} skeleton of phytoene, a colorless precursor of the carotenoid biosynthesis pathway in both prokaryotes and eukaryotes which has a chromophore of only three conjugated double bonds. Further addition of double bonds to this chromophore results in increases of the absorption maxima that range from 7 to 35 nm.[1] Our laboratories are currently using combined genetic and biochemical approaches to study the structural and functional properties of carotenoid desaturases. The genetic approach is aimed at analyzing structure–function correlations through the complementation of microbial carotenoid mutants with homologous and heterologous genes that code for carotenoid desaturases. The biochemical approach consists of purifying carotenoid desaturases to homogeneity and, since carotenoids are highly nonpolar molecules, perfecting enzyme assays in nonaqueous phases.

Carotenoid desaturases are membrane-bound enzymes in both bacteria and plants.[2,3] To date, no *in vitro* system for testing enzymatic activity with purified protein has been described. However, the existence of enzymatically active reconstitution systems with partially purified enzymes[4] suggests that enzyme assays with purified carotenoid desaturases may be feasible. To this end, we have devised a procedure for the purification of phytoene desaturase from photosynthetic bacteria.

We have achieved functional expression of *Neurospora crassa*[5] and

[1] W. Vetter, G. Englert, N. Rigassi, and U. Schwieter, *in* "Carotenoids" (O. Isler, ed.), p. 189. Birkhaüser, Basel, 1971.
[2] G. E. Bartley and P. A. Scolnik, *J. Biol. Chem.* **264,** 13109 (1989).
[3] F. Lütke-Brinklhaus, B. Liedvogel, K. Kreuz, and H. Kleining, *Planta* **156,** 176 (1982).
[4] P. Beyer and H. Kleining, this series, Vol. 213 [8].
[5] G. E. Bartley, T. J. Schmidhauser, C. Yanofsky, and P. A. Scolnik, *J. Biol. Chem.* **265,** 16020 (1990).

Glycine max (soybean)[6] cDNAs coding for carotenoid desaturases in mutants of the photosynthetic bacterium *Rhodobacter capsulatus*. Thus, it is logical to expect functional expression in *R. capsulatus* of carotenoid desaturase cDNAs from other species. Also, it is possible that cDNAs coding for carotenoid enzymes other than desaturases could be expressed in *R. capsulatus* carotenoid mutants.

Phytoene desaturases from various organisms catalyze a different number of desaturations. *Rhodobacter capsulatus* phytoene desaturase (CrtI) converts phytoene to neurosporene, a three-step desaturation reaction.[7] *Neurospora crassa* Al-1 catalyzes six desaturations.[5] Soybean Pds1 converts phytoene to ζ-carotene, a two-step desaturation reaction.[6] Expression of *al-1* in a phytoene-accumulating strain of *R. capsulatus* leads to accumulations of lycopene and 3,4-dehydrolycopene.[5] Expression of *pds* in this bacterial strain results in the accumulation of ζ-carotene.[6] Lycopene, 3,4-dehydrolycopene, and ζ-carotene are not normally accumulated in *R. capsulatus*. Thus, expression of heterologous genes for phytoene desaturases in *R. capsulatus* mutants leads to the accumulation of carotenoids not normally found in this bacterium, illustrating the usefulness of this genetic complementation system in the characterization of phytoene desaturases from a variety of species.

In this chapter we present methods for the inducible expression of carotenoid biosynthesis genes in *R. capsulatus* and *Escherichia coli* along with novel procedures for the purification of phytoene desaturases.

Expression in Photosynthetic Bacteria

Plasmid Vectors

The expression vectors used are based on high-level transcription provided by the promoter of the operon for nitrogen fixation structural genes *(nifHDK)*. This promoter is induced by low levels of fixed nitrogen in the medium, and it can be completely silenced by oxygen and ammonia. Intermediate levels of expression can be obtained by supplementing with amino acids. We currently work primarily with pNF3, a plasmid based on a ColE1 origin of replication and containing the *nif* promoter, leader sequence, and initiator codon (Fig. 1). Other plasmids based on this *nif* promoter have been described.[8] Prokaryotic genes or eukaryotic cDNAs

[6] G. E. Bartlet, P. V. Viitanen, Iris Pecker, Daniel Chamouitz, Joseph Hirschberg, and P. A. Scolnik, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 6532 (1991).
[7] G. E. Bartley and P. A. Scolnik, *J. Biol. Chem.* **264,** 13109 (1989).
[8] D. Pollock, C. E. Bauer, and P. A. Scolnik, *Gene* **65,** 269 (1988).

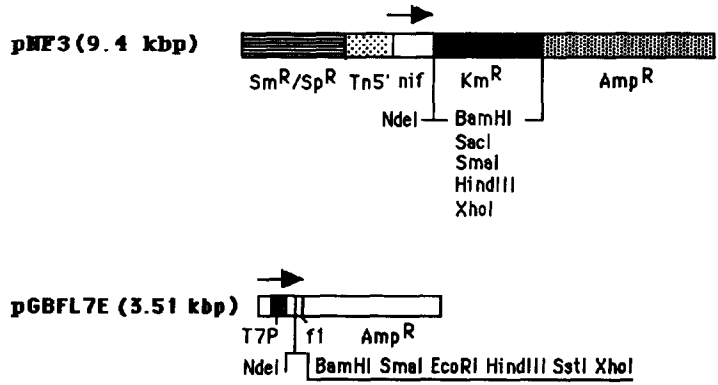

FIG. 1. *nif* and T7 expression vectors. The *nif* expression vector pNF3[8] is based on a pBR322 skeleton and carries resistance markers for streptomycin (Sm)/spectinomycin (Sp), and ampicillin (Amp). The *nif* segment carries the *R. capsulatus nifHDK* promoter and translation initiation region. The *Nde*I restriction site can be used for in-frame translational fusions. The remaining restriction sites in the polylinker region can be used for transcriptional fusion.

are cloned, preferably in-frame, into pNF3 in the orientation that places the coding region under *nif* control (Fig. 1). If an antibody is also desired, the gene or cDNA can also be cloned into an expression vector for overproduction of proteins in *E. coli*.[9] Our highest rate of success in this procedure has been achieved with vectors containing the T7 RNA polymerase promoter driving T7 gene 10 protein fusions.[10] We constructed a vector (pGBFL7E, Fig. 1) which contains an *Nde*I site at the T7 ϕ10 start codon. We use site-specific mutagenesis to obtain one or more segments of DNA with an in-frame *Nde*I site at the N-terminal initiator codon. These DNA fragments are simultaneously cloned in pGBFL7E and pNF3 for use in expression in both *E. coli* and *R. capsulatus*.

Growth and Characterization of Rhodobacter capsulatus Carotenoid Mutant Strains

To date, no efficient transformation system for *R. capsulatus* has been developed. Thus, we rely on conjugation for introducing plasmids into *R. capsulatus* carotenoid mutants. Efficient conjugations depend on using well-characterized donor and recipient cultures.

[9] E. Harlow and D. Lane, "Antibodies," pp. 88–91. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988.

[10] F. W. Studier, A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff, this series, Vol. 185, p. 60.

We use the minimal media RCV and RCVNF for growing *R. capsulatus* carotenoid mutants (modified from Ref. 11).

Stock Solutions

10% $(NH_4)_2SO_4$	50 g/500 ml
10% DL-Malic acid	50 g/500 ml
NaOH	30 g
Adjust pH to 6.8	
1% EDTA	1.00 g/100 ml
20% $MgSO_4 \cdot 7H_2O$	20.00 g/100 ml
7.5% $CaCl_2 \cdot 2H_2O$	7.50 g/100 ml
Thiamin hydrochloride	0.10 g/100 ml
0.5% $FeSO_4 \cdot 7H_2O$	1.25 g/250 ml

Trace Elements

$MnSO_4 \cdot H_2O$	0.3975 g
H_3BO_3	0.7000 g
$Cu(NO_3)_2 \cdot 3H_2O$	0.0100 g
$ZnSO_4 \cdot 7H_2O$	0.0600 g
$NaMOO_4 \cdot 2H_2O$	0.1875 g
Distilled H_2O	to 250 ml

Super Salts

1% EDTA	20 ml
20% $MgSO_4 \cdot 7H_2O$	10 ml
7.5% $CaCl_2 \cdot 2H_2O$	10 ml
Trace elements	10 ml
0.5% $FeSO_4 \cdot 7H_2O$	24 ml
Thiamin	10 ml
Distilled H_2O	500 ml

RCV Medium

10% $(NH_4)_2SO_4$	10 ml
10% DL-malic acid	40 ml
Super salts	50 ml
0.64 *M* KPO_4	15 ml
Distilled H_2O	to 1000 ml
Adjust pH to 6.8	

RCVNF Medium. For RCVNF (nitrogen-free RCV), omit $(NH_4)_2SO_4$.

[11] B. C. Johansson and H. Gest, *J. Bacteriol.* **156,** 251 (1983).

Conjugation

Introduce the plasmid containing the carotenoid gene or cDNA into *E. coli* TEC5 cells either by transformation or electroporation.[12] For antibiotic selection in *E. coli* we use trimethoprim at 100 μg/ml or spectinomycin at 100 μg/ml. Grow recipient cells either phototrophically or heterotrophically. We keep our *R. capsulatus* strains as 1 ml 25% glycerol stocks frozen in cryovials at −70°. Add an entire cell stock to 25 ml RCV in a 50-ml flask. Grow at 35° with gentle (~50 rpm) shaking for 24–36 hr (some mutant strains may take up to 48 hr). For phototrophic growth, start a heterotrophic culture. Next day, transfer 1–2 ml to a 16.5 ml screw-capped tube (Pyrex No. 9826) and fill to the top with RCV. Place under illumination at 35° for 2–3 days.

A simple setup for growing cells phototrophically can be constructed with a 20-gallon home aquarium fitted with a heating and circulation pump and set at 35° in a cold room. Illumination is provided by 16 incandescent tubes (General Electric 40T8/F).

Grow the *E. coli* donor cells overnight at 37° in Luria broth (LB) containing either trimethoprim or spectinomycin. We generally start from a single colony or a loopful of frozen stock cells. In a 1.5-ml microcentrifuge tube mix 1 ml *R. capsulatus* and 0.4 ml *E. coli* cells. Pellet at 6500 rpm for 4 min at room temperature. Remove the supernatant and resuspend the pellet in 0.4 ml RCV. Centrifuge again, discard the supernatant, and resuspend in 0.2 ml RCV. With a micropipette, spot 10–20 drops of about 20 μl each onto the surface of a 10-day-old plate containing 25 ml RCV agar. Allow to dry partially at room temperature and then incubate at 35° overnight.

Remove plates from the incubator and overlay with 3 ml top RCV agar containing spectrinomycin, to give a final concentration of 10 μg/ml. Alternatively, cells can be resuspended in 0.5 ml RCV, concentrated by centrifugation, and spread directly on RCV plates containing 10 μg/ml spectinomycin. After the top agar hardens, place the plates in anaerobic jars (BBL Gas Pack 100 anaerobic system: No. 60626, Becton Dickinson and Co., Cockeysville, MD). Place the Gas Pack hydrogen and carbon dioxide generator envelope (No. 70304) and anaerobic indicator (No. 70504) in the jar and place the jar in the aquarium tank at 35° for 5–7 days.

Purify exconjugants from colonies by streaking in RCV plates contain-

[12] F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, "Current Protocols in Molecular Biology," Vol. 1, p. 181. Wiley (Interscience), New York, 1990.

ing spectinomycin. Prepare frozen stocks from single-colony overnight cultures.

Induction of nif Expression

Grow *R. capsulatus* cells containing the appropriate plasmid under heterotrophic conditions. Generally, we grow two 25-ml cultures under the conditions described above. When cultures reach stationary phase, harvest by centrifugation in sterile tubes, resuspend in 1.5 ml RCVNF, and add 1 ml each to two screw-capped tubes. Top off one tube with RCV and the other with RCVNF, both containing spectinomycin at 10 μg/ml. Incubate in the aquarium tank at 35° for 3–4 days. Pressure buildup, owing to hydrogen production by the nitrogenase reaction, should be evident at this point in the tube containing RCVNF. Harvest cells by centrifugation and process for carotenoid extraction (see below) or freeze at −70°.

Carotenoid Extraction and Analysis

Resuspend cells in 2–5 ml of distilled water, save 50 μl for protein assay, and pellet the rest by centrifugation for 10 min at room temperature in glass screw-cup test tube. Extract the pellet with diethyl ether/methanol (50:50, v/v) until all pigment has been extracted (usually 3 times). Transfer the solvent to screw-capped glass tubes and dry under a stream of nitrogen. Resuspend the pigment in the solvent of choice for thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), or saponification (see below).

Saponification removes bacteriochlorophyll from the sample. However, if one desires to obtain a rapid qualitative characterization of the main carotenoids accumulated, TLC can be used without saponification because bacteriochlorophyll is retained at the origin of the chromatogram. Also, methods are being developed for analyzing both bacteriochlorophyll and carotenoids in the same sample using HPLC. At the present time, however, we prefer to use saponification for quantitative analysis of carotenoids.

To saponify a sample, resuspend the pigments in 2 ml of 100% ethanol. Add 0.2 ml of 60% KOH (w/v). Mix and incubate overnight at room temperature under nitrogen. Add 2 ml diethyl ether and 0.3 ml distilled water and shake under N_2 for a few seconds. Allow for full separation of phases. Transfer the top phase to a glass screw-capped tube. Repeat the extraction steps until no more pigment can be seen in the upper phase. If working with a colorless compound such as phytoene, repeat the extraction

steps 5 times. Dry the combined top phases under nitrogen purge and resuspend pigments in the solvent of choice.

Overexpression in *Escherichia coli* and Generation of Antibodies

The following protocol is currently used in our laboratory for expression of foreign genes in *E. coli* using the bacteriophage T7 RNA polymerase. A complete description of the system, including other bacterial strains and plasmids, can be found in Ref. 10.

Prepare competent cells of the *E. coli* strain BL21(DE3) by standard procedures.[12] This strain contains a gene encoding the phage T7 DNA polymerase gene under control of a *lacUV5* promoter.[10] Transform *E. coli* BL21(DE3) cells with the plasmid containing the carotenoid gene under control of the T7 promoter. Dilutions of the plasmid DNA must be made to ensure a reasonable number of colonies. Expression of foreign proteins can be toxic to *E. coli,* and this toxicity can result in instability of the plasmid carrying the foreign gene. Stability of the plasmid should be checked by plating cells onto LB, LB containing 100 μg/ml ampicillin (Amp), LB plus 1 m*M* of the *lacUV5* inducer isopropyl-β-D-thiogalactopyranoside (IPTG), and LB plus Amp and IPTG. Cells containing an active T7 promoter will not grow in the presence of the inducer IPTG. Thus, if the plasmid expressing the target DNA is not lost at a high frequency, very few colonies (fewer than 0.1–1% of the LB controls) will form on plates containing both Amp and IPTG.[10]

Grow overnight liquid cultures from a single colony and prepare stocks by adding glycerol to 25% and freezing at −70°. Inoculate two tubes containing 5 ml of LB plus Amp (100 μg/ml) and grow overnight at 37°, shaking at 250 rpm. Use the entire 5-ml overnight cultures to inoculate two 100 LB plus Amp cultures. Grow with shaking (250 rpm) at 37° until the OD_{600} reaches 1.2. Pellet one 1-ml aliquot from each culture at 16,000 *g*, 4°, for 1 min in microcentrifuge tubes. Discard supernatants and save pellets, which are uninduced controls. Add 400 μl of 100 m*M* IPTG to one of the cultures and continue growing both cultures for 3–4 hr. Harvest cells by centrifugation at 10,000 *g* at 4° for 10 min. Discard the supernatant and freeze the pellet at −70° until ready for preparation of protein.

When ready to extract protein, thoroughly resuspend cells in resuspension buffer [50 m*M* Tris-HCl pH 7.8, 2 m*M* dithiothreitol (DTT), 5 m*M* EDTA, 2 m*M* benzamidine hydrochloride, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10% (v/v glycerol]. Transfer cells to microcentrifuge tubes. Add 0.25 ml lysis buffer (resuspension buffer plus 0.6% Triton X-100 and 0.2 mg/ml lysozyme) and incubate at 4° for 30 min. Sonicate on ice with seven 30-sec pulses spaced by 15-sec pauses. Centrifuge tubes in

a microcentrifuge for 15 min at 4°. Save the supernatant. Resuspend the pellet in 1× protein sample buffer [50 m*M* Tris-HCl, pH 7.00, 2 m*M* EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) mercaptoethanol, 10% glycerol, and 0.025% bromphenol blue].

Run aliquots of both pellet and supernatant in SDS-PAGE gels. Most enzymes for carotenoid biosynthesis are expected to be present in the pellet. For antibody production, sections of SDS–polyacrylamide gels containing the expressed protein can be cut, ground, and, after mixing with Freund's adjuvant, inoculated into New Zealand White rabbits.[9]

Purification of CrtI

Most of the enzymes of the carotenoid biosynthesis pathway are bound to membranes. This, plus the relatively low abundance of some of these proteins, makes purification in an active form rather difficult. Overexpression in photosynthetic bacteria could greatly facilitate this process. We are currently developing general methods for the purification of phytoene desaturases from overproducing strains of photosynthetic bacteria. The following section summarizes our current purification protocols.

Plasmid Construction

To obtain an *R. capsulatus* strain overproducing phytoene desaturase, we used site-directed mutagenesis to place an *NdeI* site at the initiator ATG codon of *crtI*. The mutagenized gene was used to construct an in-frame translation fusion into pNF3. The resulting plasmid (pGBN4.2) was transferred from *E. coli* into *R. capsulatus* BPY69 by conjugation. This *R. capsulatus* strain contains a point mutation in *crtI*, the gene encoding phytoene desaturase.[7]

Purification

All separation steps are carried out at 4°. A 200-ml bacterial culture [*R. capsulatus* strain BPY69 (pGBN4.2)] induced for *nif* is harvested by centrifugation at 17,000 *g* for 30 min. After resuspension in buffer A (100 m*M* Tris-HCl, pH 7.2, 10 m*M* $MgCl_2$, and 2 m*M* dithioerythritol), the cells are disrupted by use of a French pressure cell at 84,000 MPa (12,000 psi). The protein content at this stage is usually 5.5 mg/ml. Nondisrupted material and crude debris are removed by centrifugation at 62,000 *g* for 15 min, and a membrane pellet is obtained by subsequent centrifugation at 187,000 *g* for 60 min. The membrane pellet is sonicated on ice at low energy in 5 ml of buffer A. The membrane suspension (4.1 mg/ml protein) is then adjusted to a detergent concentration (CHAPS, 3-[(3-

cholamidopropyl)dimethylammonio]-1-propanesulfonate} of 300 m*M*, corresponding to about 30-fold the critical micellar concentration (CMC). The suspension is flushed with nitrogen and stirred for 40 min on ice. Nonsolubilized material is removed by centrifugation for 60 min at 187,000 *g*. The clear red supernatant (2.6 mg/ml) is then used for further purification.

A 5-ml aliquot of the supernatant is subjected to size-exclusion chromatography using Ultrogel AcA 22 (gel bed 3 × 45 cm). The column is equilibrated with CHAPS starting buffer (buffer A containing CHAPS at a 20 m*M* concentration, 2 times the CMC). During development (flow rate 0.5 ml/min), some red-colored material elutes in the void volume and two additional red bands are resolved. The corresponding spectra indicate the separation of native light-harvesting complex (B800–850) at higher molecular mass from the respective denatured complex and/or solubilized bacteriochlorophyll, pheophytin, and carotenoids (Fig. 2). The CrtI protein is detected in dot blots using the anti-CrtI antibodies (see above). As can be seen in Fig. 2, the desaturase elutes between these two red bands and thus could be easily localized. The two positive fractions (11 and 12, 10 ml each) show only faint color and are pooled (protein content 0.04 mg/ml).

Any residual amounts of colored protein complexes and lipid/pigment-containing mixed detergent micelles are removed by a batch procedure employing hydroxyapatite (HAP type III, Sigma). The material is equilibrated to 2 times the CMC, washing 3 times (5 ml each) with CHAPS starting buffer. After adsorbing the positive fractions from size-exclusion chromatography with HAP, nonbinding material is removed by two additional washings with CHAPS starting buffer; resuspensions are carried out by sonication at low energy. The bound protein is eluted with 5 ml CHAPS starting buffer containing 0.2 *M* Na_3PO_4 (pH 7.5). This step is repeated once.

The phosphate concentration in the 10-ml eluate from HAP is lowered to 20 m*M* by 10-fold dilution with CHAPS starting buffer. An ultrafiltration Diaflow Cell equipped with a hydrophilic filter, YM 30 (Amicon, Danvers, MA), is used to concentrated mixed micelles without concentrating free detergent micelles and phosphate. Ultrafiltration starting with 100 ml proceeds to a final volume of 10 ml. This concentrate is then applied to an anion-exchange fast protein liquid chromatography (FPLC) column, Mono Q HR 5/5 (Pharmacia Piscataway, NJ). The nonbinding material is eluted with CHAPS starting buffer.

As can be seen in Fig. 3, the CrtI protein elutes essentially quantitatively with the nonbinding fraction. Developing with a linear NaCl gradient (0–1 *M* NaCl in CHAPS starting buffer, same flow rate) elutes the

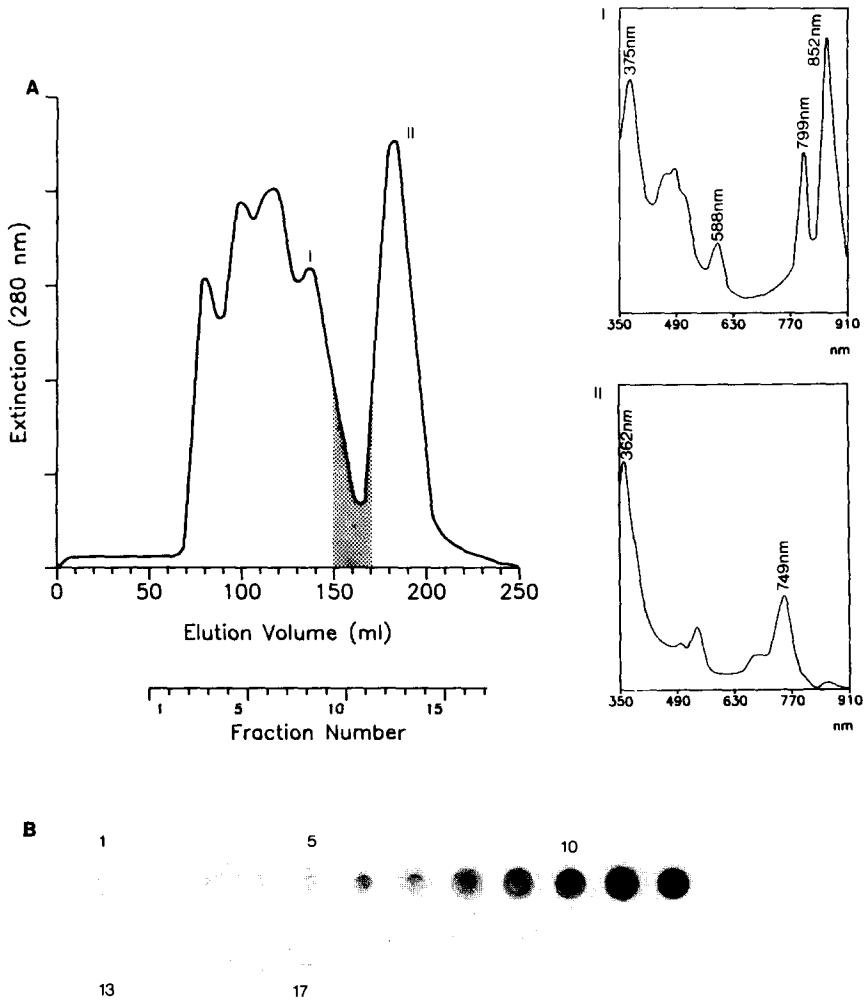

FIG. 2. Size-exclusion chromatography of solubilized membrane proteins. (A) Elution profile. The shaded area indicates the position of the CrtI protein. Insets show the spectra of peaks I and II. (B) Detection of the CrtI protein by immunoblotting, employing an alkaline phosphatase label.[7]

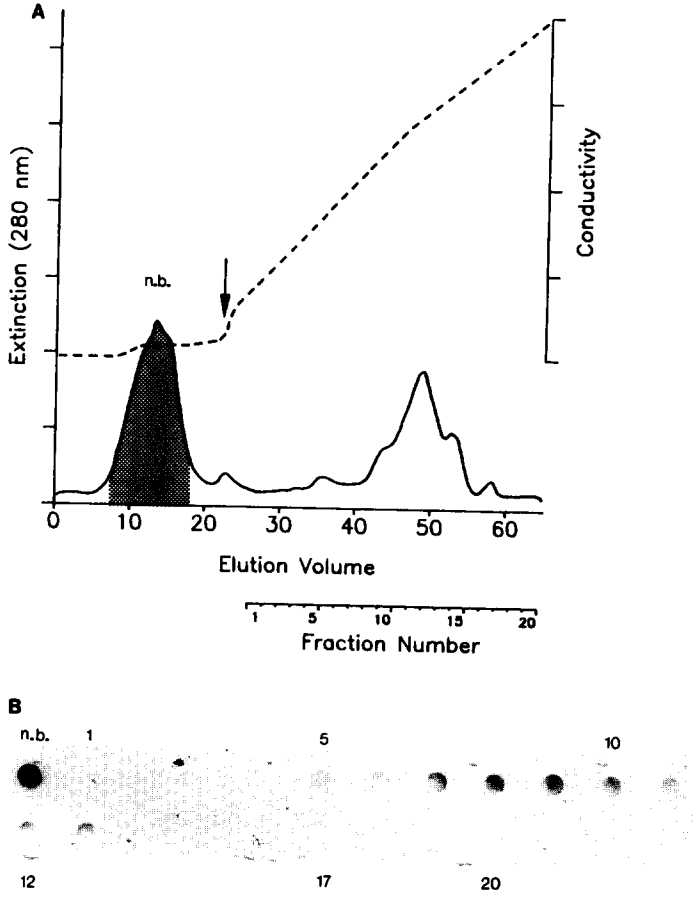

FIG. 3. Final anion-exchange chromatography of the CrtI protein. (A) Elution profile. The shaded area indicates the position of the protein with the nonbinding fraction (n.b.). The conductivity detector response indicates the course of the gradient. The arrow marks the start of the NaCl gradient (0–1 *M*). (B) Detection of the CrtI gene product in immunoblots, stained as in Fig. 2.

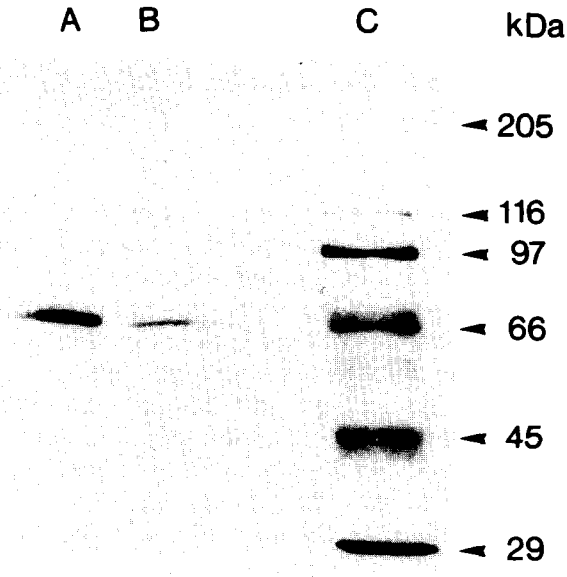

FIG. 4. SDS–polyacrylamide gel electrophoresis of the purified CrtI protein. Lanes A and B, purified CrtI protein at two different concentrations; lane C, marker proteins. The protein bands were visualized by silver staining.

binding proteins; small amounts of bound CrtI protein are also detected in fractions 5–13 in dot blots. The purity of the protein in the nonbinding fraction is judged using SDS–polyacrylamide gel electrophoresis according to Neville.[13] Figure 4 shows the purified desaturase. In some cases copurification of some faint bands of lower molecular weight proteins may occur. These bands also give a positive signal with the anti-CrtI antibody in immunoblots and are probably the result of either premature termination during overexpression or some degree of proteolytic cleavage. The purified protein seems to be homodimerically associated as judged by apparent molecular mass on gel filtration; its enzymatic characterization is currently underway.

[13] D. M. Neville, Jr., *J. Biol. Chem.* **246**, 6328 (1971).