Isolation and Partial Characterization of α- and β-Carotene-Containing Carotenoprotein from Carrot (*Daucus carota* L.) Root Chromoplasts

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A simple procedure for the isolation and purification of carotenoproteins from carrot root (*Daucus carota* L.) chromoplasts was developed. The procedure involved isolation and lysis of the chromoplasts and gel filtration to yield one major protein complexed with α- and β-carotene. The native carotenoprotein complex has a molecular weight of over 2 million. Isoelectric focusing analysis indicated a single prominent protein with a p*I* of 3.8 and minor protein with p*I* of 3.6. SDS-PAGE analysis showed single protein subunits of MW 54 000. These data, along with other research papers, suggest the native protein consists of long polymeric chains of MW 54 000 protein subunits. HPLC analysis demonstrated that one molecule of α-carotene and two molecules of β-carotene were bound to each 54-kDa subunit. The same molar ratio of α- to β-carotene exists in the carrot. This work shows the existence of a specific α- and β-carotene binding protein complex in carrots.

INTRODUCTION

The metabolic implications of carotenoids in the human diet have become an important issue in today's nutritionally conscious society. In addition to their role as provitamin A, carotenoids may play an important role in body tissues as free-radical quenchers and antioxidants (Bendich and Olson, 1989; Krinsky, 1989). Despite their importance, there are still many unanswered questions concerning carotenoid stability and their bioavailability in foods. Knowledge of how carotenoids are bound in plant systems would shed light on the answers to these questions.

It has been established that carotenoprotein complexes occur in invertebrates (Zagalsky, 1976; Rivas et al., 1988; Zagalsky et al., 1989), in algae (Powls and Britton, 1976), and in cyanobacteria (Bullerjahn and Sherman, 1986; Russo, 1986; Masamato et al., 1987). Goodwin (1984) dedicates an entire chapter on carotenoproteins in the animal kingdom in his book *The Biochemistry of Carotenoids*, Vol. 2. It has also been speculated that carotenoids are present in plant cells in a bound form like the chlorophyll proteins (Ke, 1971; Anderson et al., 1978; Goodwin and Britton, 1988). In a review of early work, Ke (1971) reported carotenoproteins to be present in plant cells. However, very little chemical characterization of these complexes has been reported. In a very recent publication, Milicua et al. (1991) reported the presence of a carotenoprotein containing seven carotenoids (78% phytoene) from carrot. Green (1988) states that it remains unknown how carotenoids are complexed within plant cells. If these complexes do exist in higher plant tissues, they may play a role in the stability of carotenoids during thermal processing and the complexes may also affect the bioavailability of carotenoids in the diet.

The purpose of this study was to isolate, purify, and characterize carotenoprotein(s) from carrot root (*Daucus carota* L).

MATERIALS AND METHODS

Chromoplast Preparation. Carrot roots were obtained from a local food market and pureed in a food processor; the juice was squeezed through four layers of cheesecloth (approximately 800 mL of juice was obtained per 1.5 kg of carrots). After a low-speed centrifugation to remove larger pieces of tissue (650*g*, 5 min), the supernatant was centrifuged at 20000*g* for 30 min to sediment the chromoplasts. The pellet was resuspended in 0.05 M Tris buffer (pH 8) to form a thick paste (ca. 30 mL). The chromoplasts were disrupted using an Aminco (Urbana, IL) French pressure cell at 12 000 psi. The lysed chromoplasts were centrifuged at 29000*g* for 1 h, and the supernatant was collected (LC29).

Gel Filtration. Sepharose 6 (Sigma, St. Louis, MO) resin was packed into a HR 10/30 Pharmacia LKB (Piscataway, NJ) column as instructed by the manufacturer and equilibrated in a 0.5 M NaCl, 0.05 M Tris buffer (pH 8) at a flow rate of 0.25 mL/min. One milliliter of LC29 supernatant was loaded onto the Sepharose 6 column, and carotenoid and protein elution were monitored at 461 and 280 nm, respectively. Peaks containing both carotenoid and protein were pooled from several runs and concentrated using an Amicon (Danvers, MA) 8200 ultrafiltration cell with a molecular weight cutoff of 10 000. Molecular weight estimations of the proteins eluted were determined by running protein standards on the same column.

Protein Analysis. Protein concentrations were assayed using bicinchoninic acid (Sigma kit TPRO-562). Amino acid analysis was conducted using methods described by Spitz (1973). Briefly, samples containing between 1 and 10 mg/mL protein were first hydrolyzed with reagent grade 6 M hydrochloric acid at 110 °C for 24 h. The hydrolysate was filtered through Whatman No. 42 filter paper. The amino acid composition of the filtrate was determined using a Beckman (Palo Alto, CA) amino acid analyzer, Model 119CL.

Detergents were removed from samples before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by precipitating the proteins with 2 volumes of ice-cold ethyl alcohol, incubating for 10 min at 0 °C, and centrifuging with an Eppendorf for 15 min in a cold room. The precipitate was then redissolved in 10% mercaptoethanol buffer, diluted 1:1, and boiled for 5 min. The prepared samples were loaded and run on polyacrylamide gels as described by Laemmli (1970). Marker proteins obtained from Sigma were run on each gel for calibration

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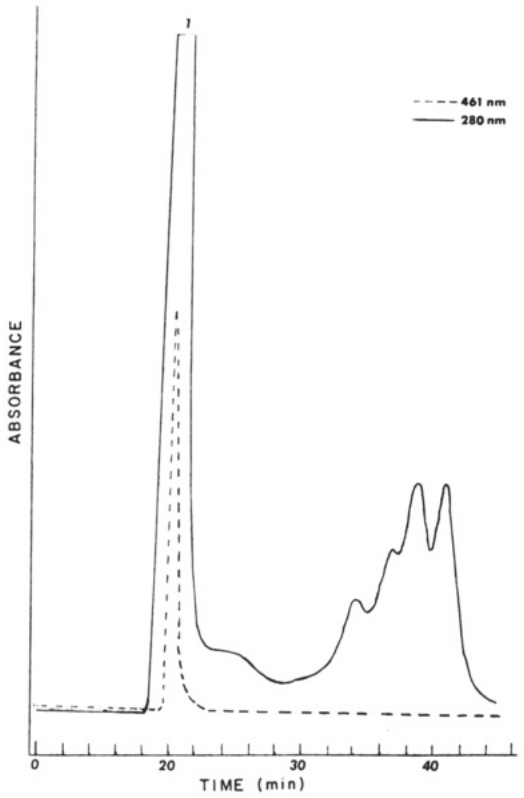

Figure 1. Chromatogram of lysed carrot chromoplasts using a Sepharose 6 column and monitoring at 280 and 461 nm. The running buffer was 0.5 M NaCl, 0.05 M Tris (pH 8).

of molecular weights. The gels were silver stained (Sigma kit AG-25) and photographed.

Isoelectric focusing (IEF) was performed on Iso Gel agarose plates (FMC, Rockland, ME) pH range 3–7. Samples were prepared, loaded, and run on a LKB (Bromma, Sweden) electrofocusing chamber, Model 2117. The gels were stained with Coomassie blue according to FMC instructions, dried, and photographed.

Pigment Analysis. The absorbance spectra of the carotenoproteins isolated, the pigments extracted from the carotenoproteins, and crystalline α- and β-carotene standards isolated from carrots (Sigma) were recorded on a Beckman DU 40 spectrophotometer. The standards were essentially pure, with only one peak being determined for each standard at expected retention times during HPLC analysis.

Pigments from samples were obtained for HPLC analysis by multiple extractions. Samples were first extracted with ethyl acetate (same volume as sample) and then dichloromethane (same volume as sample). If necessary, the sample was briefly centrifuged to obtain clear separation of the organic and aqueous phases. This sequence was repeated, and all organic extracts were pooled and evaporated under argon gas. The residue was redissolved in the solvent system used for the HPLC analysis. The extracted pigments were then analyzed using isocratic HPLC, on a TP silica C_{18} (Vydac, Hesperia, CA) stainless steel column (0.46 × 15 cm) with an Upchurch Uptight (Oak Harbor, WA) precolumn packed with ODS C_{18}. The solvents were of HPLC grade and were filtered, degassed, and used in a ratio of methanol/acetonitrile/distilled water (88:9:3). The chromatograms were monitored at 461 nm. The same α- and β-carotene standards as previously mentioned were run, and retention times were obtained for each standard. Standard curves were determined using peak area vs concentration.

RESULTS AND DISCUSSION

Figure 1 shows the elution pattern of the lysed carrot chromoplasts from the gel filtration column. Although several protein peaks elute, only peak 1 contained carotenoids as indicated by the absorbance at 461 nm. This peak essentially contained all of the carotenoid eluted from the gel filtration column.

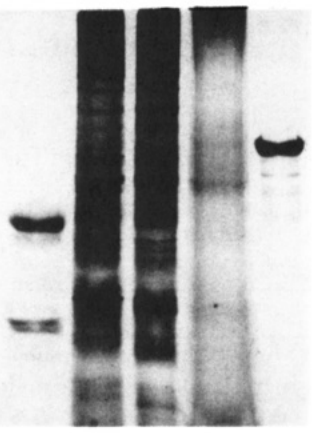

Figure 2. SDS–PAGE analysis of the isolation products of carotenoprotein from carrot chromoplasts. (Lane 1) Molecular weight standards (egg albumin, 45 000; carbonic anhydrase, 29 000); (lane 2) French pressed carrot chromoplasts; (lane 3) carrot chromoplast pellet (29000*g*); (lane 4) peak 1 from gel filtration column; (lane 5) molecular weight standard (bovine albumin, 66 000).

The molecular weight was determined for the native protein eluting in peak 1 of the Sepharose 6 column. The protein complex eluted at approximately the same time as the standard blue dextran, indicating a molecular weight of around 2 000 000. This molecular weight was within the column's exclusion limit as suggested by the manufacturer (5 000 000). Carotenoproteins have been isolated from invertebrates, ranging from 50 000 to 1 000 000 (Zagalsky, 1976; Zagalsky et al., 1989).

SDS–PAGE analysis of the lysed carrot chromoplasts (before and after centrifugation at 29000*g*) and peak 1 from the Sepharose 6 column is shown in Figure 2. Approximately the same protein profile was seen for the lysed carrot chromoplast before and after centrifugation. This indicates that the proteins were solubilized in the Tris buffer. However, a dramatic difference was seen in the protein profile before and after gel filtration (lanes 3 and 4). Peak 1 from the Sepharose column essentially contained one predominant protein band around 54 kDa. It is known that hydrocarbon carotenoids in carrot chromoplasts are incorporated in their crystalline form into actively growing helical "ribbons" of lipoproteins (Frey-Wyssling and Schweigler, 1965). It is possible that these long helical ribbons are formed from several subunits of protein of the size reported here (54 kDa). Zagalsky et al. (1989) reported a large carotenoprotein of ca. 500 kDa with a predominant glycoprotein subunit of 6 kDa to be present in the skin of starfish. The presence of lipids and carbohydrates in the isolated carotenoprotein in the current work is under investigation.

Figure 3 shows the migration of the carotenoprotein complex derived from peak 1 (eluted from the Sepharose 6 column) on an agarose gel (pH 3–7). The prominent protein present has a p*I* of 3.8. A smaller band of protein with a p*I* of 3.6 was also present. Several studies have reported carotenoproteins with an acidic nature. Shone et al. (1978), Garate et al. (1986), and Rivas et al. (1987) found carotenoproteins from invertebrates to have p*I* values of 4.5, 3.55, and 4.56, respectively. Dainese et al. (1990) found certain chlorophyll proteins from spinach to have light harvesting complexes with p*I* values of around 4.

To determine if there were interactions between the protein and carotenoid present in peak 1, spectra were

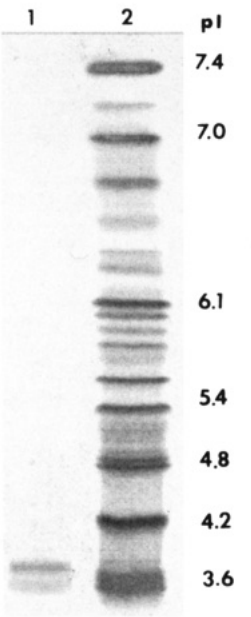

Figure 3. IEF agarose gel analysis of carotenoproteins eluted from peak 1 of gel filtration column. (Lane 1) Carotenoprotein; (lane 2) standard IEF reference mix (FMC).

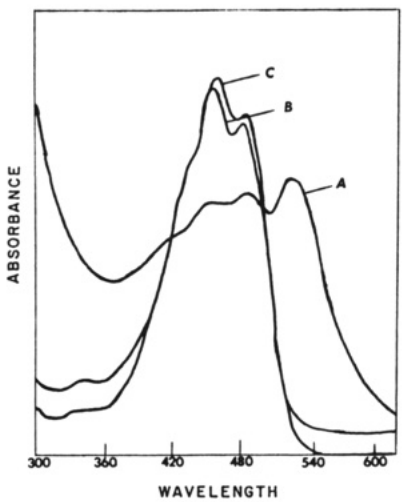

Figure 4. Comparison of absorption spectra. (A) Spectra of carotenoprotein eluted from peak 1 of gel filtration column suspended in 0.05 M Tris buffer, pH 8. (B) Pigments extracted from carotenoprotein in methylene chloride. (C) α- and β-carotene mixed standards in methylene chloride.

taken of the carotenoprotein complex, the pigments were extracted from the complex, and a mix of α- and β-carotene standards of approximately the same concentration was found in the complex (Figure 4). The spectra of the extracted pigments from the carotenoprotein complex match very closely with the spectra of the mixed α- and β-carotene standards. The aqueous carotenoprotein complex, however, was red-shifted, and it matched the spectra of β-carotene protein complexes described by Ke (1971) for crude spinach extracts. Powls and Britton (1976) noted a red shift in a carotenoprotein isolated from algae. Although the exact reason for this red shift is not known, it is believed to be caused by the protein interaction with the conjugated double-bond chain of the carotenoid (Ke, 1971). These interactions lower the absorption energy of the carotenoid, and its entire spectrum is shifted to a longer wavelength.

HPLC analysis confirmed α- and β-carotene to be the carotenoids present in the carotenoprotein complex (Figure 5). The lysed carrot chromoplast loaded onto the

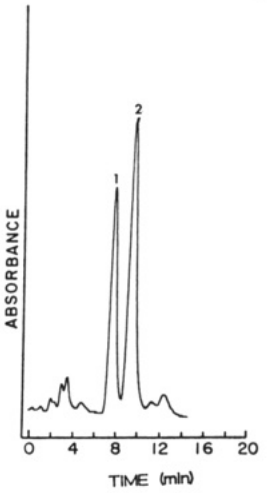

Figure 5. Reversed-phase HPLC analysis (methanol/acetonitrile/distilled water, 88:9:3, at 461 nm) of an organic extract of the carrot chromoplast protein eluted in peak 1 of gel filtration column. (Peak 1) α-Carotene; (peak 2) β-carotene.

Table I. Amino Acid Analysis[a] of Carotenoprotein Isolated from Carrot Chromoplast (MW 54 000)

	isolation 1		isolation 2	
amino acid	residues[2]	mol %[3]	residues	mol %
hydrophobic				
alanine	57.8	10.1	58.8	11.5
valine	36.8	6.4	27.0	5.3
leucine	50.8	8.9	39.7	7.8
isoleucine	27.1	4.7	19.0	3.7
proline	28.7	5.0	24.8	4.9
methionine	8.7	1.5	4.7	0.9
phenylalanine	24.9	4.3	18.9	3.7
tryptophan		–[d]		–
polar uncharged				
glycine	54.4	9.5	47.8	9.4
serine	41.8	7.3	38.4	7.5
threonine	32.8	5.7	27.5	5.4
cystine		–		–
tyrosine	14.9	2.6	12.6	2.5
asparagine		–		–
glutamine		–		–
negatively charged				
aspartic acid	59.5	10.4	65.9	12.9
glutamic acid	63.4	11.1	70.6	13.9
positively charged				
lysine	36.9	6.4	28.4	5.6
arginine	23.5	4.1	17.6	3.4
histidine	10.7	1.9	7.9	1.5

[a] Amino acid analysis performed as described by Spitz (1973). [b] Residues = approximate number of residues/protein subunit. [c] Mol % = residues/(MW/total residues). [d] Not detected.

Sepharose 6 column had 0.523 mmol of α-carotene/mmol of protein (assuming a molecular weight of 54 000) and 1.44 mmol of β-carotene/mmol of protein. Pigment protein ratios of peak 1 eluting off the Sepharose 6 column were 0.875 mmol of α-carotene/mmol of protein and 2.15 mmol of β-carotene/mmol of protein. An increase in the pigment protein ratios for the sample loaded to the peak eluted was expected since the gel filtration column removed some unbound protein. These millimolar ratios round off to one molecule of α-carotene and 2 molecules of β-carotene per subunit of protein. Powls and Britton (1976) found a 1:1 ratio of violaxanthin to protein in a carotenoprotein in algae.

The amino acid analysis of the carotenoproteins from peak 1 of the sepharose 6 column is reported in Table I. Amino acid analyses from two separate isolations of two batches of carrots are very similar, demonstrating that the isolation procedure is reproducible.

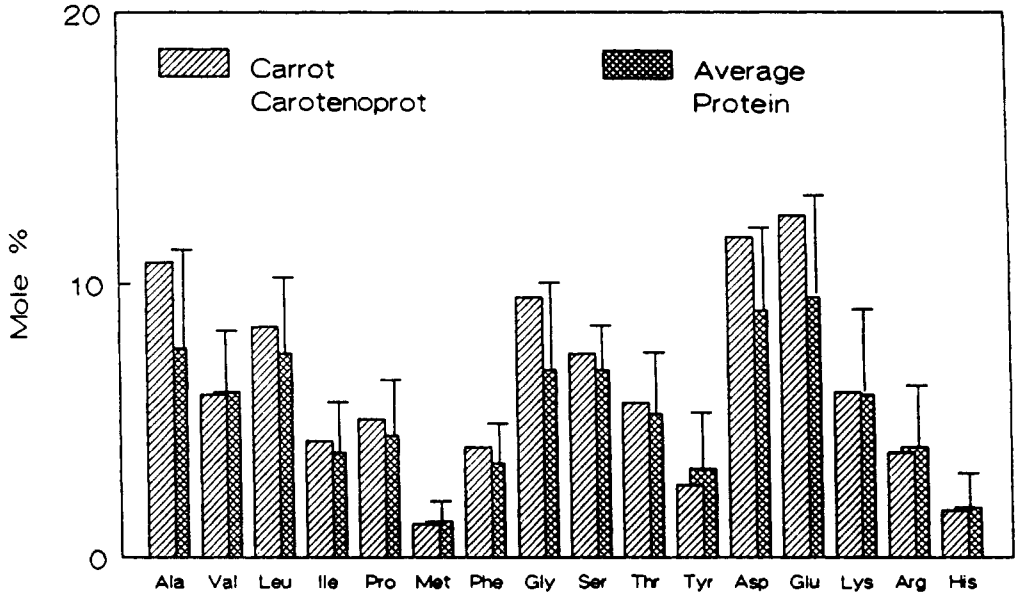

Figure 6. Comparison of amino acid composition of isolated carotenoprotein form carrot chromoplast to the average protein calculated by Smith (1966).

In 1966, Smith calculated an "average pattern" for amino acids that occur in proteins by comparing 80 different proteins from varied sources. Zagalsky (1976) surveyed 13 carotenoprotein complexes in invertebrates and compared them to this "average protein". Zagalsky found that carotenoproteins from the invertebrates were high in acidic amino acids (particularly aspartic acid), serine, threonine, and proline and low in leucine. Figure 6 is a comparison of the carotenoprotein from carrot chromoplasts to this average protein. The isolated carotenoprotein from carrot chromoplasts was relatively high in alanine, glycine, tyrosine, aspartic acid, and glutamic acid; slightly above average in leucine, serine, and proline; and below average in threonine.

Free ammonia was found in the current amino acid analysis, suggesting that some of the aspartic and glutamic acids are in the amide form in situ. The high amounts of aspartic and glutamic acids are consistent with the approximate p*I* of 3.8 determined for the isolated carotenoprotein.

Calculations to predict which amino acids favor or break conformational structures have been made from proteins with known amino acid conformations (Levitt, 1978). According to these calculations the isolated carotenoprotein's amino acid analysis favors α-helical formations due to the large number of alanine, leucine, glutamic acid, and aspartic acid residues. Several β-sheets are unlikely because of high levels of glutamic acid and aspartic acid. A fairly high number of β-bends or reverse turns are favored because of the high concentration of glycine, serine, aspartic acid, and proline (Levitt, 1978). A more precise prediction of conformation can be undertaken once amino acid sequencing is completed.

Zagalsky's (1976) suggested conformation of invertebrate carotenoproteins was that they were composed mainly of random coils and β-conformations with a large number of β-bends or reverse turns. α-Helices were considered unlikely, due to the high content of helix-breaking amino acids. A complete amino acid sequence has been determined for a carotenoprotein isolated from lobsters (Keen et al., 1991). The protein was found to be similar to the retinol-binding protein superfamily. Each of these proteins is an antiparallel eight-stranded β-barrel of unusual topology, where each strand is connected to its nearest neighbor. The hydrophobic ligand is bound between the two orthogonal sheets forming the cone-shaped barrel. The sequence identity between members of this superfamily is low, but all members have at least two consensus sequences of 13 and 11 residues, separated by 80 residues.

A comparison of the amino acid content of the carotenoproteins isolated from carrots to the reported amino acid content of carotenoproteins from invertebrates suggests that their conformations are not the same. However, since the sequence identity between members of the retinol-binding superfamily is low, there is still a possibility that the carrot carotenoprotein belongs to this family. Further work on the sequence of the reported carotenoprotein from the carrot should evaluate whether the same consensus sequences of 13 and 11 residues are present, which should suggest membership into this superfamily and would help elucidate the mechanism of binding to the ligands.

Although the existence of carotenoproteins in higher plant cells has been reported, the evidence presented was based on crude extracts from chloroplasts (Ke, 1971; Goodwin and Britton, 1988) or chromoplasts (Milicua et al., 1991). The carotenoprotein isolated from carrot (Milicua et al., 1991) was reported to contain 77.8% phytoene, 6.8% phytofluene, 7.8% carotene, 3.5% β-carotene, 2.3% α-carotene, 1.2% lutein, and 0.5% carotenoid epoxide and to have a estimated molecular weight of 290 000. Neither amino acid composition nor p*I* was given. Clearly, however, that carotenoprotein from whole chromoplasts is a different entity from the carotenoprotein reported here. Milicua et al. (1991) found only 5.8% of total associated carotenoids to be α- and β-carotene. The 2 000 000 molecular weight protein reported in this paper was found to essentially bind only α- and β-carotene. Therefore, in this work, we have isolated and partially purified a carotenoprotein from higher plant cells. Specifically, an α- and β-carotene binding protein has been isolated from carrot root chromoplasts.

The development of this procedure opens new avenues of research. The sequence of the amino acids present in the carotenoprotein complex must be achieved to allow for more definite analysis of the protein's conformation. The information gained from the isolation of carotenoproteins in carrot root may be applied to the isolation of

carotenoproteins from other plant systems. For example, preliminary results from this laboratory suggest the presence of a lutein-containing carotenoprotein in spinach leaves. Bioavailability and thermoprocessing studies of carotenoproteins would further determine their impact in the human diet.

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Registry No. α-Carotene, 7488-99-5; β-carotene, 7235-40-7.