Isolation and Partial Characterization of an 18 kDa Carotenoid–Protein Complex from Carrot Roots

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A carotenoid–protein complex was isolated, purified, and partially characterized from carrot juice by detergent treatment followed by ammonium sulfate precipitation, gel filtration, and ion exchange chromatography. Results from SDS–urea–PAGE analysis indicated that the complex contained a major protein subunit with a molecular mass of 18 kDa. HPLC analysis of the pigments associated with this complex indicated that β-carotene, α-carotene, and lutein were the major carotenoids present along with small amounts of phytoene and ζ-carotene. Carrot chromoplast subfractionation and sucrose density centrifugation also resulted in a major carrot pigment fraction which was enriched in the 18 kDa protein. The 18 kDa protein-containing-carotenoid complex is the major complex associated with noncytosolic carrot pigments within the carrot chromoplasts. The relative concentrations of both 18 kDa protein and total carotenoids present in chromoplasts indicate that the carotenoid–protein complex is only associated with a small percentage of total carrot carotenoids. It is thus unlikely that the 18 kDa protein influences carotenoid bioavailability from carrots. This carotenoid–protein complex may function enzymatically in carotenoid synthesis.

Keywords: *Carotenoid–protein complex; carrot root; chromoplast; β-carotene*

INTRODUCTION

As early as 1883 Poulton (1885) suggested the existence of a carotenoid–protein complex in phytophagous larvae. Since then, efforts have been made to characterize carotenoid–protein complexes in different systems and to investigate their physiological functions. Carotenoid–protein complexes have been reported in invertebrates including the starfish (Clark et al., 1990; Zagalsky et al., 1989) and the lobster (Keen et al., 1991a,b), in photosynthetic systems including algae (Arsalane et al., 1992; Powls and Britton, 1976), higher plants (Irrgang et al., 1991; Markwell et al., 1992), and cyanobacteria (Bullerjahn and Sherman, 1986; Diverse-Pierluissi and Krogmen, 1988; Eagle et al., 1991; Masamoto et al., 1987; Reddy et al., 1989; Wariso et al., 1988), and in nonphotosynthetic chromoplasts (Cervantes-Cervantes et al., 1990; Hadjeb et al., 1988; Milicua et al., 1991; Dietz Bryant et al., 1992).

North Americans typically obtain over one-third of their vitamin A requirement from carotenoids, and carrots are the most commonly consumed carotenoid source in the American diet (Anon., 1989). However, carotenoid absorption from raw carrots is very limited (Rodriguez and Irwin, 1972), and it is not clear what factors specifically influence the bioavailability (BV) of carotenoids from this source. It has been suggested that the existence of carotenoid–protein complexes may affect the BV of carotenoids from carrots (Dietz Bryant et al., 1992). It also has been suggested that mild heating may result in increased absorption of carotenoids from carrots due to the denaturation of carotenoid–protein complexes (Dietz Bryant et al., 1992).

Carotenoid–protein complexes have been reported to be isolated from the carrot (Milicua et al., 1991; Dietz Bryant et al., 1992). Milicua and co-workers (Milicua et al., 1991) reported the isolation of a carotenoprotein containing mainly phytoene (77.8%) with a small percentage of α-carotene (AC) (2.3%) and β-carotene (BC) (3.5%) as well as other carotenoids. Dietz Bryant et al. (1992) isolated a specific AC- and BC-containing carotenoprotein with a protein subunit molecular mass of 54 kDa.

The objective of this study was to further isolate, characterize, and determine the subcellular location of a carotenoid–protein complex from carrot root.

MATERIALS AND METHODS

Crude Extract Preparation. Market fresh carrot roots (Bunny-Luv) were purchased from a local supermarket. Carrots (200 g) were peeled, washed with deionized water, grated, and further homogenized in a food processor at low speed for 1 min with addition of 100 mL of solution containing 2 mM EDTA and 5 mM ascorbic acid. The homogenate was filtered through four layers of cheesecloth. The filtrate was adjusted to a final concentration of 0.5% of Tween 20 (Sigma Chemical Co., St. Louis, MO) and extracted for 24 h with mild stirring in cold temperature (0–4 °C). The extract was centrifuged at 20000*g* for 40 min (precipitate discarded). To the supernatant was added ammonium sulfate to 35% of saturation, and the mixture was centrifuged at 10000*g* for 15 min. The precipitate was washed with 35% saturated ammonium sulfate, resolubilized in buffer (0.05 M Tris, 0.02 M NaCl, 1 mM EDTA, 0.01% Tween 20, pH 8.0), and centrifuged at 29000*g* for 30 min. The crude extract was obtained by filtering the supernatant through a Tuffryn membrane (0.2 μm, Gelman Sciences, Ann Arbor, MI).

Gel Filtration Chromatography. The crude extract was first purified by a gel filtration column (HR 10/30 column, Pharmacia LKB Biotechnology, Piscataway, NJ, packed with Superose 6 resin, Sigma) at a flow rate of 1.0 mL/min. The elution buffer used was the same as above. The fractions having absorbance at both 460 (by carotenoids) and 280 nm (by proteins) were collected and protein profiles determined by SDS–urea–PAGE using DTT (0.2 M) as a reducing agent.

Anion Exchange Chromatography. The gel filtration fraction containing the carotenoid–protein complex was further applied to an anion exchange column (Mono Q column, Pharmacia, Alameda, CA) at the flow rate of 0.5 mL/min. The

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buffers used were 0.05 M Tris, 0.02 (low salt)–1.0 M (high salt) NaCl, 1 mM EDTA, and 0.01% Tween 20 (pH 8.0). Fractions with both 280 and 460 nm absorbance were collected. All fractions were concentrated by ultrafiltration (Amicon, Beverly, MA, MWCO 12K) and protein profiles monitored by SDS–urea–PAGE.

Protein Analysis. The purification efficiency was determined by using SDS–urea–PAGE with urea (4 M). The gel was electrophoresed according to the methods of Laemmli (1970) and Peter and Thornber (1991). After electrophoresis, the gel was silver-stained, and molecular weights of the protein subunits were estimated by standard protein markers. Protein concentrations were measured by BCA assay (Sigma kit TPRO-562).

Carotenoid Determination. An internal standard, ethyl β-apo-8′-carotenoate, was added to the samples to quantitate the amount of AC and BC. Carotenoids were extracted by adding 2 volumes of absolute ethanol and 4 volumes of hexane to 1 volume of sample with 20 μL of internal standard in ethanol. After three hexane extractions, the hexane layers were combined and dried under argon gas. The dried samples were reconstituted with methylene chloride and applied to a reversed-phase HPLC system using a Vydac (Hesperia, CA) TP 201 stainless steel column with C_{18} packing material. The solvents were of HPLC grade and were filtered, degassed, and used in a ratio of methanol/acetonitrile/distilled water of 88:9:3. The chromatograms were monitored at 461 nm. The concentrations of AC and BC in samples were calculated from calibration curves generated from peak area ratios of BC or AC to an internal standard.

The carotenoid profile and the absorbance spectra of individual carotenoids in the sample were obtained by using a Waters (Milford, MA) 991 photodiode array detector (PDA). The carotenoids were identified by comparing the spectra obtained with those of known carotenoid standards.

Phospholipid Analysis. The procedure of phospholipid extraction from samples is based on that of Blight and Dyer (1959). The lipid extract was transferred to a clean vial, evaporated, redissolved using HPLC mobile phase of acetonitrile/methanol/85% H_3PO_4/H_2O (97.8:1:1:0.2), and applied to a HPLC system (Model 600E, Waters) using a Supelco LC-18 (Bellefonte, PA) analytical column for phospholipid analysis.

Wavelength Scans. The possible bathochromic shift was determined by comparing the absorbance spectrum of the purified anion exchange fraction with that of its hexane-extracted sample. The sample was scanned from 250 to 700 nm in a DU-40 spectrophotometer (Beckman Instrument, Inc., Irvine, CA). Deionized water and hexane were used as blanks for the anion exchange fraction and hexane extract, respectively.

Microsequencing and Amino Acid Analysis. The purified 18 kDa protein band from electrophoresis was blotted onto a PVDF membrane basically according to the procedure of Matsudaira (1987) except that a SDS–urea–PAGE gel (4 M sequanal grade urea, Pierce, Rockford, IL) was used. The blotted 18 kDa protein band was cut and sent to the University of Illinois Biotechnology Center for microsequencing and amino acid analysis.

Isolation and Subfractionation of Carrot Chromoplasts. A modified method of Liedvogel et al. (1978) was used for the carrot chromoplast isolation and subcellular fractionation. Carrot roots (100 g) were peeled, ground, and homogenized by adding 200 mL of grinding buffer (0.3 M mannitol, 0.05 M Tris, 3 mM EDTA, 2 mM ascorbic acid, pH 8.0). The homogenate was filtered through four layers of cheesecloth and centrifuged at 500*g* for 15 min (pellet discarded). The supernatant was further centrifuged at 4000*g* for 20 min. The pellet was resuspended in 20% sucrose (0.05 M Tris, 20% sucrose, pH 8.0) and layered onto a 35% sucrose layer. The sample was centrifuged at 60000*g* for 2 h, and the colored layer between 20 and 35% sucrose was removed, diluted with grinding buffer, and centrifuged at 25000*g* for 20 min. The pellet was washed with grinding buffer and pelleted again. The resulting carrot chromoplasts were resuspended again in grinding buffer, lysed by French press at 7000 psi, layered on the top of a 10% sucrose layer, and centrifuged at 60000*g* for 2 h. The supernatant was removed and concentrated by ultrafiltration for further analysis. A colored layer in the interface was taken, diluted with distilled H_2O (1:1 v/v), and centrifuged at 60000*g* for 1 h. The pellet was washed and pelleted for further analysis.

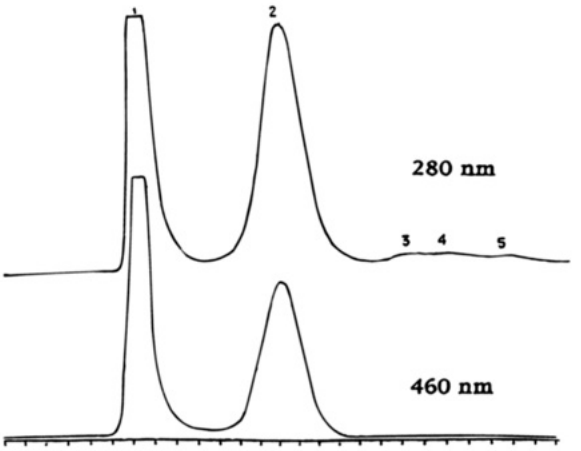

Figure 1. Gel filtration chromatograms of crude extract, monitored at both 280 and 460 nm.

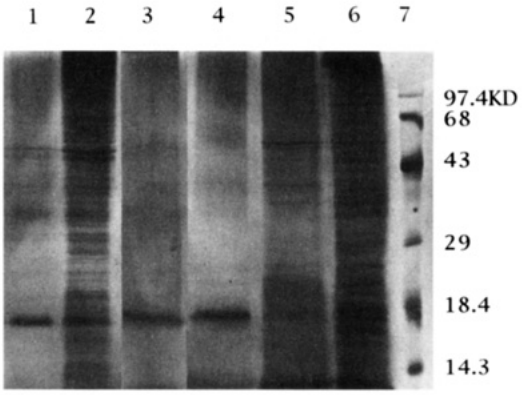

Figure 2. SDS–urea–PAGE of the isolation and purification of the 18 kDa protein-containing carotenoid complex: lane 1, subfraction of carrot chromoplasts from sucrose gradient ultracentrifugation; lane 2, carrot chromoplasts; lane 3, anion exchange fraction 3 from gel filtration fraction 1; lane 4, gel filtration fraction 1; lane 5, detergent-treated crude extract; lane 6, carrot juice; lane 7, molecular weight markers.

RESULTS AND DISCUSSION

It was repeatedly shown by HPLC chromatograms that the detergent extraction of the carrot juice resulted in a crude extract which was lutein-enriched and contained substantial quantities of AC and BC.

Gel filtration chromatographic purification of the crude extract resulted in two major fractions with absorbance at both 280 and at 460 nm, as shown in Figure 1. SDS–urea–PAGE analysis indicated that gel filtration fraction 1 predominantly contained a carotenoid–protein complex with an 18 kDa molecular mass subunit (Figure 2, lane 4). Molecular mass estimation using gel filtration showed that the native carotenoid–protein complex had a molecular mass of about 2000 kDa. Gel filtration fraction 2 contained many protein bands including a band with a molecular mass of 54 kDa (gel not shown). This is the same molecular mass of the carotenoprotein reported by us earlier (Dietz Bryant et al., 1992). The estimation of the apparent molecular mass of the native protein in gel filtration fraction 2 was 250 kDa.

Anion exchange purification of gel filtration fraction 1 resulted in the separation of three fractions (Figure

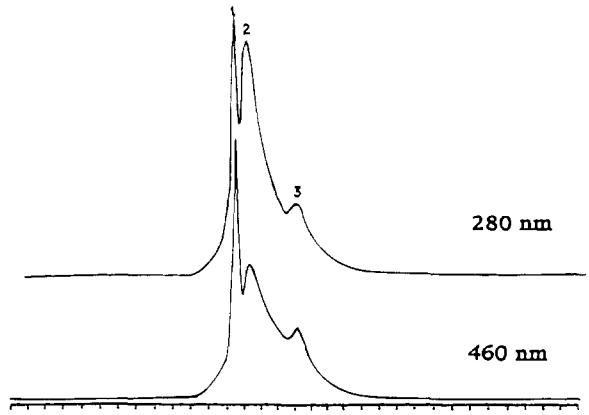

Figure 3. Anion exchange chromatograms of gel filtration fraction 1, monitored at both 280 and 460 nm.

Table 1. BC/AC Molar Ratios, BC/Lutein Molar Ratios, and Protein/(AC + BC + Lutein) Molar Ratios of Anion Exchange Fractions from Gel Filtration Fraction 1

	BC/AC[a] mol/mol	BC/lutein mol/mol	protein[b]/(AC + BC + lutein), mol/mol
fraction 1	2.10 ± 0.25	16.7 ± 0.12	0.31 ± 0.02
fraction 2	2.13 ± 0.21	12.5 ± 0.10	0.31 ± 0.01
fraction 3	2.27 ± 0.27	1.4 ± 0.13	0.73 ± 0.01

[a] The BC/AC molar ratio in carrot juice is 2.04 ± 0.05. [b] Assume the molecular mass is 18 kDa.

3). It was shown that addition of 0.01% Tween 20 to the anion exchange buffer was critical. Without addition of Tween 20 in the buffer, most of the sample remained on the column, while higher percentages of Tween 20 (0.05%) did not result in efficient separation. Fractions 1–3 of anion exchange were collected, concentrated by ultrafiltration, and applied to SDS–urea–PAGE for protein analysis and HPLC for carotenoid determination.

SDS–urea–PAGE showed that anion exchange fractions 1 and 2 contained almost no protein (gel not shown). Fraction 3 contained essentially one protein band with a molecular mass of 18 kDa (Figure 2, lane 3). It was occasionally noticed that gels of fraction 3 had bands with molecular masses that were multiples of 18 kDa. It is possible that these minor bands are polymers of the 18 kDa subunit, due to incomplete dissociation of a polymeric complex. This view was supported by the fact that the sample treated with a reducing agent, BME, as compared to that treated with DTT, favored the formation of the polymer bands of the 18 kDa band. Several methods were attempted to dissociate the protein complex. It was found that ethanol precipitation worked efficiently, which resulted in a darker 18 kDa band and clearer background. The time course was also important. Freshly prepared sample was easily dissociated, while samples stored in the refrigerator for several weeks were difficult to efficiently dissociate into the smallest subunits.

Carotenoid molar ratios and protein/carotenoid molar ratios (we assume a molecular mass of 18 kDa) of anion exchange fractions 1–3 are shown in Table 1. Fractions 1 and 2 did not show the significant increase of the relative amount of lutein compared to BC and AC, while fraction 3 contained a significantly higher percentage of lutein, as compared to that found in carrot juice. The BC/AC molar ratio in all three fractions from anion exchange after gel filtration was around 2, which is similar to that of carrot juice (2.04). PDA determination of the carotenoid profile of anion exchange fraction 3

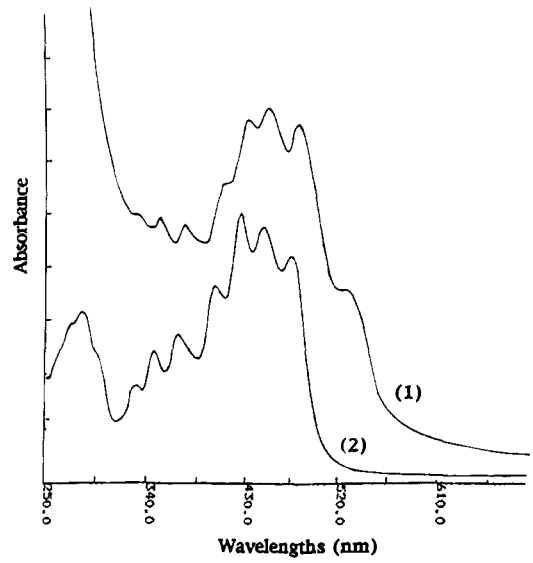

Figure 4. Spectra of anion exchange fraction 3 (1) and its hexane-extracted sample (2).

showed that fraction 3 contained BC, AC, lutein, phytoene, and ζ-carotene. These results, together with that from SDS–urea–PAGE, indicated that the detergent extraction of the carrot juice followed by gel filtration and anion exchange resulted in a purified carotenoid–protein complex fraction (anion exchange fraction 3) which contained the 18 kDa protein subunit and BC, AC, lutein, pytoene, and ζ-carotene, with BC, AC, and lutein predominating (the approximate molar ratios of BC, AC, and lutein is 2:1:1).

The carotenoid profile of gel filtration fraction 2 (a molecular mass of 250 kDa, as estimated in gel filtration) showed that the fraction contained mainly phytoene, phytofluene, ζ-carotene, AC, and small amounts of BC and lutein. This profile of carotenoids is consistent with the result of Milicua et al. (1991), who reported the isolation of a carotenoprotein with the carotenoid profile of mostly phytoene as well as phytofluene, ζ-carotene, AC, BC, lutein, and a carotenoid epoxide. It is possible that the carotenoprotein isolated by Milicua et al. (1991) may be a cytosol fraction of the carrot chromoplasts which may be enzymatically involved in one or more earlier stages of carotenoid biosynthesis. Further studies are needed to assess the possible enzymatic function of this fraction.

The spectra of the pure carotenoid–protein complex fraction (anion exchange fraction 3) and its hexane extract are shown in Figure 4. The carotenoid–protein complex shows a bathochromic shift of about 12 nm compared to hexane-extracted free carotenoids from the complex. The shift may be due to refractive index effects associated with dissolution of the carotenoids in the lipids or protein components (Zagalsky et al., 1989).

Several attempts of N-terminal amino acid sequencing of the 18 kDa protein were unsuccessful, suggesting that the protein was N-terminally blocked. It is not clear if it is naturally blocked, which is very likely because 80–90% of eucaryotic proteins are N-terminally blocked (Brown 1970), or whether the blockage is due to experimental treatment.

The results of amino acid analysis of the 18 kDa protein are listed in Table 2. The results indicate that the protein contains a relatively high proportion of hydrophobic amino acids (50.6%), with neutral amino acid glycine predominating. The polar uncharged amino acid serine is also in high proportion (11.19%). This

Table 2. Amino Acid Composition of the 18 kDa Subunit

	amino acid	18 kDa (mol %)	54 kDa (mol %)
hydrophobic	Gly	21.36	9.45
	Val	3.78	5.85
	Ala	5.88	10.80
	Leu	8.01	8.35
	Ile	3.75	4.20
	Phe	3.49	4.00
	Pro	2.21	4.95
	Trp		
	Met	2.16	1.20
polar	Ser	11.19	7.40
	Thr	3.73	5.55
	Tyr	3.37	2.55
	Cys		
	Lys	6.22	6.00
	Arg	4.86	3.75
	His	1.84	1.70
	Glx	10.20	12.50
	Asx	7.93	11.65

amino acid profile is different from that of a cytosolic 54 kDa-containing protein–carotenoid complex isolated in our laboratory previously from carrot root chromoplasts (Dietz Bryant et al., 1992). The amino acid profile found for the 54 kDa protein is also listed in Table 2. That protein contains relatively high levels of alanine (10.8%) and glycine (9.45%). The comparison of amino acid profiles between 18 and 54 kDa proteins clearly indicates that they are different proteins.

HPLC analysis of phospholipids indicated that the crude extract and the anion exchange fractions contained only trace amounts of phospholipids. This suggests that the carotenoid–protein complex is not associated with substantial amounts of phospholipids.

It has been shown that the carrot chromoplast is a crystallous type in which most of the carotenoids are in a crystal form (called carotene bodies) surrounded by a number of thickened membranes (Ben-Shaul et al., 1968). It has also been suggested that the crystalline chromoplasts in the carrot root result from overproduction of carotene, which is then stored in sheets of lipoprotein. Thus, carotene bodies are thought to be lipochromoprotein sheets with a laminated structure in which carotene molecules are inserted (Frey-Wyssling and Schwegler, 1965). Early work (Straus, 1954) suggested that carotene was associated with nucleoproteins in the chromoplasts of carrot.

SDS–urea–PAGE analysis of carrot chromoplasts and their subfractions showed the presence of an 18 kDa protein band with other minor protein bands (Figure 2, lane 2). After subfractionation of the chromoplasts, the pigment fraction contains predominantly the 18 kDa protein with a few minor protein bands (Figure 2, lane 1). This suggests that the 18 kDa protein subunit-containing complex is associated with the pigment fraction of the chromoplasts. The carotenoid profile of the chromoplast subfraction was similar to that of the carrot juice and the carrot chromoplast. The supernatant fraction from chromoplast subfractionation contained several other protein bands, including the band with a molecular mass of 54 kDa, suggesting that the 54 kDa protein we previously reported (Dietz Bryant et al., 1992) may exist in the cytosol of the chromoplast. The results also indicated that the carotene bodies, as isolated according to the method of Ben-Shaul et al. (1968), mainly contain crystalline carotenoids without substantial amounts of carotenoid–protein complex (data not shown).

It has been suggested that carotenoid–protein complexes may contribute to the decreased BV of carotenoids from carrots (Dietz Bryant et al., 1992). In the current study, we found that the 18 kDa protein-containing carotenoid complex was the major complex associated with the pigments of the chromoplasts. Evaluation of the relative concentrations of carotenoids and the 18 kDa protein in carrot chromoplasts indicated that the carotenoid–protein complex only contributes a small percentage of total carotenoids in carrots (about 5–10%). Thus, it is unlikely that the carotenoid–protein complex is substantially involved in carotenoid BV from carrots. The 18 kDa protein may function, at least in part, as an enzyme involved in the later stages of the carotenoid biosynthesis, possibly in AC, BC, or lutein biosynthesis.

The support for the enzymatic function of 18 kDa protein-containing complex is provided by our observation that after 4 weeks of refrigerator storage, carotenoid analysis of the purified carotenoid–protein fraction showed that the BC/AC molar ratio decreased from 2.3 to 1.9 and BC/lutein area ratio increased from 1.4 to 2.0. The results also showed that the carotenoid–protein complex contained AC, BC, lutein, phytoene, and ζ-carotene, with BC, AC, and lutein predominating. Considering the pathway of carotenoid biosynthesis, particularly the pathway of lutein biosynthesis via hydroxylation of AC, it is suggested that the 18 kDa protein-containing carotenoid complex may be involved enzymatically in the later stages of the carotenoid biosynthesis, mainly AC, BC, and lutein biosynthesis from phytoene.

In conclusion, a carotenoid–protein complex with the subunit molecular mass of 18 kDa was isolated, partially purified, and characterized from carrot root. This complex is associated with carotenoid pigments, especially BC, AC, and lutein, and is the major protein-containing carotenoid complex within the carrot chromoplasts. The previously isolated 54 kDa carotenoid–protein complex (Dietz Bryant et al., 1992) is found in the cytosol of carrot chromoplasts The 18 kDa protein-containing complex is unlikely to influence carotenoid BV from carrots, but it may function enzymatically in carotenoid biosynthesis.

ABBREVIATIONS USED

AC, α-carotene; BC, β-carotene; BME, β-mercaptoethanol; BV, bioavailability; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; kDa, kilodalton; MWCO, molecular weight cutoff; PAGE, polyacrylamide gel electrophoresis; PDA, photodiode array detector; SDS, sodium dodecyl sulfate.

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