Lutein Dispersed in Acetone Aqueous Solution; Spectroscopic Analysis and Electron Microscope Observation[†]

Shigeaki TAKAGI, Shinsuke NAKANO, Keiichi KAMEYAMA*
and Toshio TAKAGI*

*Department of Agricultural Chemistry, Faculty of Agriculture,
Okayama University, Okayama 700, Japan*

**Institute for Protein Research, Osaka University,
Suita, Osaka 565, Japan*

Received November 25, 1986

The sizes and shapes of lutein aggregates in acetone aqueous solution were examined by spectroscopic analyses and electron microscopic observation, since lutein dispersed into acetone aqueous solution and provided a much simpler system than others. This system is suitable for basic research on lutein aggregates. The lutein aggregate gave maximum molar ellipticity in acetone concentrations from 35 to 45% aqueous solution. When the acetone concentration increased, CD spectra were reversed in 45% concentration from a negative exciton chirality to the positive. Since the aggregates were most slender in these acetone concentrations, it was found that the length of the lutein aggregate was closely related to the molar ellipticity. The apparent widths of the aggregates became large with increases of the acetone concentration after the reversion of CD spectra, where the molar ellipticities were diminished. These results are similar to those for the lutein aggregates in dilute surfactant solution, and this system is clearer and simpler for electron microscopic observation than those in surfactant solutions.

In previous papers,^{1~4)} we reported the following facts concerning lutein, a major carotenoid in green leaves, dispersed in surfactant and protein aqueous solution. 1) Lutein dispersed in various aqueous solutions loses the fine structure of its absorption spectrum, and its absorption maximum shifts to shorter wavelength by 60 nm. The spectrum, furthermore, shows an appreciable low absorbance. These phenomena indicate a formation of lutein aggregates larger than the wavelengths of visible light.⁵⁾ 2) The lutein aggregates acquired a strong circular dichroism (CD) of split type, whose spectra showed negative exciton chirality with a peak at about 376 nm and a trough at about 408 nm. The above results indicate that two transition moments of different lutein molecules in near position are not in the same place and occupy a definite space orientation not parallel with each other.

Many chromophores of lutein molecules associate side-by-side and slightly twisted in relation to each other to form "chiral card packed aggregates".⁴⁾ 3) Bradley *et al.*⁶⁾ suggested that a helical polyene aggregate with ten chromophores in a pitch showed a CD spectrum of split type which was similar to those observed for lutein in aqueous systems.⁴⁾ 4) The size of the aggregates measured by both ultrafiltration⁷⁾ and electron microscopy in surfactant solutions⁸⁾ was over 0.4 μ m long.

In this study, lutein aggregates were studied in acetone aqueous solution by spectroscopic measurements and by observation with transmission electron microscopy. The results were similar to those for lutein dispersed in other aqueous solutions.⁸⁾ This dispersion system was found to be much simpler than other systems, and was suitable for basic research on lutein aggregates and also for further electron mi-

[†] Biochemical Studies on Carotenoids, Part XVII. For Part XVI, see ref. 8.

croscopic observation of lutein aggregates.

MATERIALS AND METHODS

Lutein and its aggregate. Crystalline lutein was prepared from spinach leaves,⁹⁾ and dissolved in ethanol to obtain a stock solution. Lutein aggregate was prepared as in the previous paper,¹⁾ in which 0.2 ml of lutein ethanol solution was added very slowly to 5.3 ml of acetone aqueous solution with mild stirring at room temperature.

Spectrophotometric measurements. Absorption and CD spectra were measured with a spectrophotometer (Shimadzu MPS 5000) and a spectropolarimeter (JASCO J-500 A), respectively, at room temperature.

Transmission electron microscope. The aggregate was precipitated from the dispersion by centrifugation at $220,000 \times g$ for 2 hr to obtain a concentrated suspension of aggregate. A droplet of the suspension was placed on a carbon coated grid and air dried in a dark place. These samples on the grids were further dried under high vacuum and shadowed with Pt-Pd with a vacuum evaporator Model JEE-4X. Another droplet of the suspension on the grid was negatively stained by uranyl acetate. A droplet of 1% aqueous solution of uranyl acetate was also placed over the lutein aggregates on the grid, and removed with filter paper one or ten minutes later. An electron microscope, JEOL Model JEM 100C, was used at an accelerating voltage of 80 kV.

RESULTS AND DISCUSSION

Absorption and CD spectra of lutein aggregate dispersed in acetone aqueous solution

Lutein disperses in water containing acetone at a concentration lower than 40% and forms molecular aggregates. Absorption spectra of the lutein aggregates in acetone aqueous solution were blue-shifted by about 60 nm at $\lambda_{\max}$ and gave a simple pattern with a single peak (Fig. 1A). When the concentration of acetone was higher than 50%, the lutein aggregates showed the usual resonance absorption spectrum of conjugated polyenes, because lutein dissolves as a monomer. The CD pattern of the aggregate in water containing acetone at a concentration lower than 40% showed similar to but stronger CD intensities than those in surfactant solutions^{4,7)} (Fig. 1B). Since lutein shows no CD at a concentration of acetone higher than 55%, the aggregate is assumed to be dissociated into monomers. In this dis-

FIG. 1. Absorption and CD Spectra of Lutein Aggregate Dispersed in Acetone Aqueous Solution.

Acetone concentration: 1) 30; 2) 40; 3) 45; 4) 50; 5) 75%. Lutein concentration, $7.4 \mu\text{M}$.

sociated form, the $\lambda_{\max}$ of both absorption and CD shift to longer wavelengths than those of the aggregate. Absorption (383 nm) and CD maxima (371 nm) in 10% acetone were shifted by 2 or 3 nm and by about 5 nm for each 10% increase of acetone concentration, respectively. Furthermore, both the extinction coefficient (ϵ) and molar ellipticity ($[\theta]$) were varied by acetone concentrations (Figs. 2 and 3 curve A). The extinction coefficient of the aggregate, whose absorption maximum ranges from 383 nm to 390 nm, is almost constant at concentrations of acetone lower than 35%, but decreases at concentrations of acetone higher than 40% (Fig. 2). In these higher concentrations of acetone, absorption spectra were similar to that of lutein dissolved in organic solvents or solubilized into some surfactant micelles⁴⁾ (Fig. 1). In CD spectrum from 370 to 383 nm, lutein gave a maximal $[\theta]$ at 35% acetone concentration with an abrupt decrease of $[\theta]$ both at concentrations of acetone lower than 20% and higher than 45% acetone (Fig. 3A). The reversion phenomena of the CD pattern⁴⁾ were also observed at acetone con-

FIG. 2. Variation of ϵ of Lutein Aggregates Dispersed in Acetone Aqueous Solution.

Lutein concentration, $7.4 \mu\text{M}$.

FIG. 3. Variation of Molar Ellipticity of Lutein Aggregates and Its Recovery through $0.5 \mu\text{m}$ Millipore Filters in Various Acetone Concentrations.

Lutein concentration, $7.4 \mu\text{M}$. Arrow indicates a reversion point of the CD pattern. (A), molar ellipticity at λ_{max} ; (B), lutein recovery in ultrafiltration.

concentrations from 38 to 45%. It was found that lutein molecules easily aggregated also in acetone aqueous solution. Dispersion systems composed of acetone are so simple that this may be suitable for physicochemical studies and for electron microscopic observation of the lutein aggregates. The aggregates began to dissociate in an increase of acetone concentration to smaller aggregates and finally "dissolve" into acetone as lutein monomers. In acetone concentrations from 38 to 45%, which were critical concentrations between the dispersion and the dissociation of lutein, a weak reversion of the CD pattern was seen, and this phenomenon was the same as that seen in SDS solution. This fact indicates that lutein molecules in the aggregate is pulled apart by acetone molecules acting as a modulator, and the reversion is considered to be a specific phenomenon in the process of the dissociation

of the lutein aggregate.

Electron micrograph of lutein aggregate

Lutein disperses in water containing acetone at a concentration lower than 45% and forms a molecular aggregate. The sizes of the lutein aggregates dispersed in SDS⁴⁾ and DTAB solution⁷⁾ have already been measured by ultrafiltration, in which the size depended on surfactant concentration and reached $1.2 \mu\text{m}$. The apparent sizes of lutein aggregates in acetone aqueous solution were also measured with a Millipore filter (Teflon) of $0.5 \mu\text{m}$ pore size (Fig. 3 curve B). The lutein aggregate hardly pass through this filter up to 35% acetone concentration while the aggregate easily passed through at concentrations of acetone higher than 40%. As reported previously,⁷⁾ this phenomenon occurs when the lutein aggregate dissociates in high acetone concentrations. In this dissociation process of the aggregates, a reversion of the CD spectrum pattern was seen (Fig. 3).

In the next experiment, the lutein aggregates in water containing acetone at concentrations lower than 50% were observed by transmission electron microscope. Samples for electron microscope were prepared by ultracentrifugation. The recoveries of the aggregate preparation, which were obtained from ratios of absorbances of supernatants before and after the centrifugation, were more than 90% when acetone concentrations were 20~40%. Electron micrographs of lutein aggregates shadowed by Pt-Pd or negatively stained by uranium acetate were shown in Figs. 4 and 5, respectively. At the top of each figure, both absorption and CD spectra of lutein dispersed in acetone aqueous solutions of the same concentration are shown. The aggregates in water containing acetone at a concentration lower than 30% were small, with various shapes, and became long and slender with an increase of acetone concentration (Fig. 4). During the transformation of the image at concentrations of acetone lower than 30%, the width of the aggregate became slightly broader. When the acetone concentration was over

FIG. 4. Electron Micrograph of Lutein Aggregates in Acetone Aqueous Solution (1).

Acetone concentration, 30%. A, Pt-Pd shadowing; B, negative staining with uranyl acetate. Spectra at the top of the photographs indicate that this lutein aggregate gives a simple absorption spectrum (dotted line) and an ordinary CD pattern of negative exciton chirality (solid line). Black arrow under the spectra indicates $\lambda_{\max}$ of absorption spectrum.

35%, the aggregate became longer and much broader than those seen at concentrations of acetone lower than 30% (Fig. 5). Through all these experiments, the shape and the size of the lutein aggregates, which were separately shad-

owed by Pt-Pd and negatively stained by uranyl acetate, were almost the same. Lengths, widths, and heights of these lutein aggregates are shown in Table I. From ratios of width to length of the aggregate, it is found that the

FIG. 5. Electron Micrograph of Lutein Aggregates in Acetone Aqueous Solution (2).

Acetone concentration, 45%. A, Pt-Pd shadowing; B, negative staining with uranyl acetate. Spectra at the top of the photographs indicate that this lutein aggregate gives a simple absorption spectrum (dotted line) but a reversed CD pattern of positive exciton chirality (solid line). Black arrow under the spectra indicates $\lambda_{\max}$ of absorption spectrum.

aggregates formed in acetone concentrations from 30 to 35% are the most slender. Since the largest molar ellipticity was obtained in such acetone concentrations (Fig. 3A), it was apparent that the slender configurations

of the aggregates gave high CD intensities. In ultrafiltration, lutein aggregates passed through $0.5\ \mu\text{m}$ pores at concentrations of acetone higher than 40%, while the lengths of the images in electron micrographs of

TABLE I. LENGTH, WIDTH AND HEIGHT OF LUTEIN AGGREGATE DISPERSED IN ACETONE AQUEOUS SOLUTION OF VARIOUS CONCENTRATION

Acetate concentration	Length (μm)	Width (μm)	Height ^a ($\mu\text{m} \times 10^2$)
0	0.071 ± 0.013	0.026 ± 0.0055	—
10	0.087 ± 0.016	0.023 ± 0.0047	0.84 ± 0.14
20	0.163 ± 0.016	0.036 ± 0.005	0.83 ± 0.16
25	0.304 ± 0.060	0.045 ± 0.012	1.09 ± 0.16
30	0.650 ± 0.11	0.077 ± 0.022	1.24 ± 0.17
35	0.739 ± 0.15	0.110 ± 0.024	1.16 ± 0.10
40	1.230 ± 0.20	0.240 ± 0.051	1.64 ± 0.29
45	1.160 ± 0.17^b	0.334 ± 0.046^b	2.08 ± 0.46
48	2.090 ± 0.31^b	1.510 ± 0.42^b	2.98 ± 0.37
50	4.430 ± 0.70^b	6.070 ± 0.81^b	6.05 ± 1.10

^a Height was estimated from the angle and length of shadow.

^b Length was decided to be parallel to the direction of lines on surface.

the aggregate were found to be over $1 \mu\text{m}$. This phenomena in ultrafiltration is considered to be a disintegration of the aggregate in high concentrations of acetone. This fact shows that an interaction of the lutein molecules in aggregate is weakened by the action of acetone above 40% concentration, and the disintegration of the aggregate is closely related to a disappearance of the chiral configuration (Fig. 3A).

The results and discussion above mentioned

agreed well with the deduced facts^{4,7)} that lutein associated side-by-side in surfactant solution to form a large slender helical aggregate. Since no helical structure is observed in these electron micrographs, and furthermore, widths of the image are too much large for a simple aggregate, it is necessary to further examine whether the images are true single structures of lutein aggregates or not.

Acknowledgment. The authors wish to thank Dr. Seiji Ouchi of the Faculty of Agriculture, Kinki University, and Dr. Tomonori Shiraishi of the Faculty of Agriculture, Okayama University, for their valuable advice in the electron microscopic observation.

REFERENCES

- 1) S. Takagi, M. Shiroishi and T. Takagi, *Agric. Biol. Chem.*, **44**, 2111 (1980).
- 2) S. Takagi, M. Shiroishi and T. Takagi, *Agric. Biol. Chem.*, **45**, 1159 (1981).
- 3) S. Takagi, K. Takeda and T. Takagi, *Agric. Biol. Chem.*, **46**, 399 (1982).
- 4) S. Takagi, K. Takeda, K. Kameyama and T. Takagi, *Agric. Biol. Chem.*, **46**, 2035 (1982).
- 5) L. N. Dysens, *Biochim. Biophys. Acta*, **18**, 1 (1956).
- 6) D. B. Bradley, I. Tinoco, Jr. and R. W. Woody, *Biopolymers*, **1**, 239 (1963).
- 7) S. Takagi, K. Takeda and M. Shiroishi, *Agric. Biol. Chem.*, **46**, 2217 (1982).
- 8) S. Takagi and T. Takagi, *Sci. Rep. Fac. Agr. Okayama Univ.*, **65**, 47 (1985).
- 9) S. Takagi, *Sci. Rep. Fac. Agr. Okayama Univ.*, **44**, 44 (1974).