Intracellular Distribution of Tocopherol in Soybean Cotyledons

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ABSTRACT

Soaked soybean seeds were homogenized and fractionated into cellular particles, cytoplasmic solution, and storage particles resembling protein bodies. Tocopherol in seeds is distributed about 10, 15, and 60% respectively in these fractions. Tocopherol is also distributed to the cellular particles and the cytoplasmic solution of hypocotyls which contain no deposit lipids. A large part of the tocopherol in cotyledons is distributed to the storage particles. This consisted of more than 20% lipids. However, tocopherol seemed not to be always contained in the storage lipids, because the tocopherol content in the preparations was not proportional to the lipid content. Part of the tocopherol may be contained in the unit membranes which are distributed widely in the cell. Tocopherol content in cotyledons did not decrease by imbibition or germination, and no oxidizing enzymes for tocopherol were detected in the homogenate. Therefore, the low tocopherol content in the extracted oil from moistened soybeans may be a result of difficulty of contact of solvent with the site of tocopherol in cellular components owing to interference by moisture.

Tocopherol content in oil extracted from soybeans was reported to be correlated with the moisture content (1). That is, the tocopherol content in crude oil extracted from dried soybeans was larger than that from moistened soybeans. The tocopherol content of the crude oils from ordinary (13% moisture) and dried soybeans (1.9%) was 1.3 and 1.9 mg./g. crude oil, respectively, but the content decreased to 0.33 mg./g. crude oil when the soybeans were remoistened (18%). This phenomenon can be observed only in whole beans, not in crushed beans. To elucidate this phenomenon, determining the status of tocopherol in soybean cotyledon cells was considered necessary. Tocopherol is known to perform the role of antisterility vitamins in animals, and a number of articles have appeared regarding the functions of tocopherol in animal bodies. However, information on the possible role of tocopherol in plants is relatively scarce.

Tocopherol occurs in seeds, and it is generally considered that its role is mainly that of *in vivo* antioxidant of deposit lipids (2). But tocopherol is contained in all green tissues, which contain little or no lipids (3). Tocopherol increased in young leaves at the moment of flower initiation (4). At germination of *Pisum sativum,* tocopherol content increased during the first 3 days and then decreased in seedlings 7 days old (5). Tocopherol is interconvertible in young growing plants of maize, wheat, barley, and pea (6). This may indicate that tocopherol is intended for some unknown essential function in the life of cells and growth of embryos in addition to its possible role as *in vivo* antioxidant of deposit fat. In chicken liver, tocopherol is distributed in cellular particles such as mitochondria, microsomes, and others which are necessary for performance of life functions of the cell (7).

This paper deals with determination of the intracellular distribution of tocopherol in soybean cotyledons. The possible mechanisms which elucidate the fact that tocopherol content in extracted oil is related to moisture content of soybeans are discussed.

MATERIALS AND METHODS

Soybeans ("Tsurunoko"), harvested in Nagano and Gunma prefectures, were used; they are called "Nagano" and "Gunma" in this paper. Nagano was used unless otherwise indicated. Seeds of Nagano had 38.0% protein, 18.6% lipid, and 0.014% tocopherol (dry basis). Gunma had 38.4% protein, 20.5% lipid, and 0.019% tocopherol (dry basis).

The method of Emmerie and Engel (8) was used to estimate tocopherol content. Various minor constituents of soybeans such as carotenoid pigments and sterols, which interfere with estimation of tocopherol, were removed by Green's method (9).

Nitrogen was determined by the Kjeldahl method. Protein was calculated as N $\times$ 6.25.

RESULTS

Intracellular Distribution of Tocopherol

In Soybean Seeds. Soaked seeds were homogenized and fractionated as shown in Diagram 1. Supernatant A is considered to be the whole cytoplasm of the cotyledon cells. This solution was diluted with water to make 0.25*M* sucrose and then centrifuged at 20,000 $\times$ *g* for 30 min. Much precipitate was obtained (precipitate B). This seems to be particles resembling protein bodies (also referred to herein as PB particles), which are the stored source of nutrition in soybean cotyledons. Supernatant C corresponds to the whole cytoplasm. At this centrifugation, only a trace of "cream" layer was separated at the top of the tube, and deposit lipids seemed to be contained in precipitate B. Supernatant C was centrifuged at 100,000 $\times$ *g* for 60 min. to separate precipitate F (containing cellular particles) and supernatant G (which corresponds to the cytoplasmic solution). A thin cream layer was separated (fraction H). Supernatant E is a washing of precipitate B and the solution was fractionated like supernatant C.

The tocopherol content of these fractions is shown in Table I. Tocopherol was distributed in all fractions; however, cellular particles (F and I) contained less than 10% of the total amount. Cytoplasmic solution (G and J) contained about 15% of the total. About 10% of the tocopherol was found in the cream layer. Most of the tocopherol was distributed to the PB particles; i.e., fractions B, D, and L. These particulate preparations may be contaminated with mitochondria, but the contribution of mitochondria may not be so large in this fraction, which was composed of 30% of lipids (see Table IV). The T/P ratio of the cellular particles was also high.

In Hypocotyls. Hypocotyls from 5-day-old seedlings were homogenized and fractionated as shown in Diagram 2. Supernatant M is the whole cytoplasm of the hypocotyl cells. Precipitate N consists of cellular particles: mitochondria, microsomes, and others. Supernatant O is the cytoplasmic solution. No cream layer was obtained. Tocopherol was distributed equally in both the

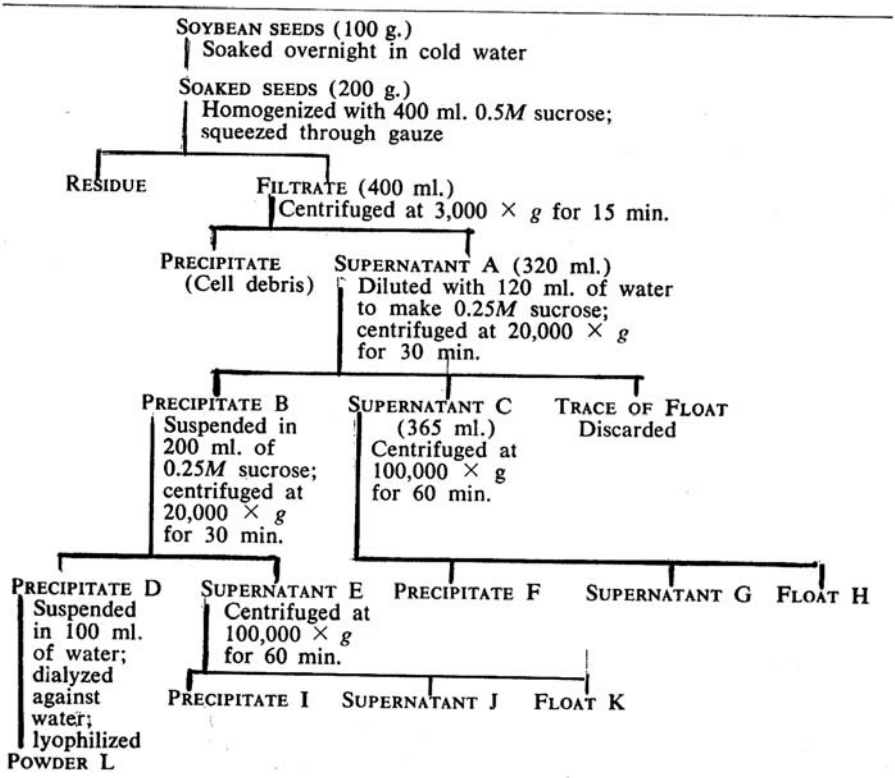

DIAGRAM 1
FRACTIONATION OF SOYBEAN HOMOGENATE

TABLE I
INTRACELLULAR DISTRIBUTION OF TOCOPHEROL IN SOYBEAN CELLS[a]

FRACTION		PROTEIN	TOCOPHEROL	T/P[b]
		g.	γ	
A	Cell homogenate	15.47	6,620	428
B	Protein body[c]	4.47	4,100	917
C		11.47	960	84
D	Washed protein body[c]	3.29	2,610	793
E		....	....	....
F	Cellular particles	1.14	320	281
G	Cytoplasmic solution	10.13	600	59
H	Trace of cream layer	....	340	....
I	Cellular particles	....	60	....
J	Cytoplasmic solution	1.49	350	235
K	Trace of cream layer	....	370	....
L	Dried protein body[c]	2.66	2,920	1,098

[a] Fractionated as shown in diagram 1.
[b] Tocopherol, γ/g. protein.
[c] Particles resembling protein bodies.

cellular particles and supernatant (Table II). The T/P ratio of the cellular particles was comparatively high.

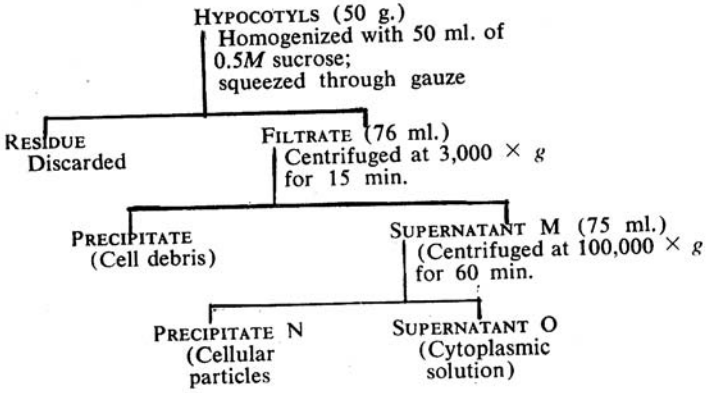

DIAGRAM 2
FRACTIONATION OF SOYBEAN HYPOCOTYL HOMOGENATE

TABLE II
INTRACELLULAR DISTRIBUTION OF TOCOPHEROL IN SOYBEAN HYPOCOTYL CELLS[a]

	FRACTION	PROTEIN	TOCOPHEROL	RATIO: T/P[b]
		g.	γ	
M	Cytoplasm	1.01	(170)[c]	168
N	Cellular particles	0.10	70	700
O	Cytoplasmic solution	0.90	100	111

[a] Fractionated as shown in diagram 2.
[b] γ Tocopherol/g. protein.
[c] Obtained by calculation.

Tocopherol Content of Cotyledons during Imbibition and Germination

Soybeans were soaked in cold water and germinated in vermiculite at 25°C. in the dark. The tocopherol content of cotyledons and hypocotyls was determined (Table III). Total tocopherol showed almost no change during soaking or germination.

Test for Oxidation of Tocopherol by Enzymes

The homogenate of soybeans was added to a tocopherol suspension, $10^{-2}M$, emulsified with sucrose fatty acid ester in 0.1*M* phosphate buffer, pH 6.0, or 0.1*M* phosphate buffer, pH 7.6. Oxygen absorption was tested at 37°C. with a Gilson differential respirometer, but no oxygen uptake was observed.

PB Particles

Precipitate B in Diagram 1 seemed to be so-called protein bodies. Analysis of the preparations is shown in Table IV. Precipitate B was washed, dialyzed, and lyophilized (No. 1). In Gunma the amount of precipitate B (No. 3) was very little, but much "float" was obtained. When the float was washed with water and centrifuged at 20,000 × *g* for 30 min., all components were precipitated (No. 4).

TABLE III

CHANGES OF TOCOPHEROL CONTENT IN SOYBEAN SEEDLINGS DURING GERMINATION
(Amount in 15 grains)

SAMPLE	FRESH WEIGHT	PROTEIN	TOCOPHEROL	RATIO: T/P[a]
	g.	g.	γ	
Dry soybeans	6.2	2.24	1,190	531
Soaked seeds[b]	13.3	2.22	1,250	563
Seedlings[c]:				
1 day old	11.3	2.17	1,160	535
Seedlings:				
2 days old: Cotyledons	13.1	2.18	1,120	514
Hypocotyls[d]	0.4 (5 mm.)	0.065	54	831
3 days old: Cotyledons	12.6	2.20	1,270	577
Hypocotyls[d]	1.2 (25 mm.)	0.079	43	544
5 days old: Cotyledons	12.0	2.01	1,080	537
Hypocotyls[d]	5.4 (80 mm.)	0.20	76	380

[a] γ Tocopherol/g. protein.
[b] Soaked overnight in cold water.
[c] Germinated in vermiculite at 25°C. in the dark.
[d] Values in parentheses show length of hypocotyls.

TABLE IV

ANALYSIS OF PARTICLES RESEMBLING PROTEIN BODIES[a]

PREPARATION No.	PROTEIN	LIPID	TOCOPHEROL
	%	%	%
1	38	29	0.042
2	56	44	0.051
3	42	23	0.020
4	37	60	0.023

[a] Values are shown as percentage on dry basis. 1 and 2 were prepared from Nagano, 3 and 4 were prepared from Gunma. When Gunma was used and the supernatant (fraction A in diagram 1) was centrifuged at 20,000 × *g* for 30 min., a little amount of precipitate (fraction B in diagram 1) and much float were obtained. The precipitate and the float were lyophilized as preparations 3 and 4, respectively.

The protein content of the preparations (except No. 2) was about 40%, but lipid and tocopherol contents were rather low. Preparation 2 was like No. 1, except that the cotyledons were homogenized under milder conditions. This preparation seemed to be close to the intact protein bodies and had high contents of proteins, lipids, and tocopherol. Comparing these preparations, tocopherol contents seemed not to be proportional to the deposit lipids.

Fractionation of Components. PB particles were fractionated as shown in Diagram 3. Analysis of the particles is shown in Table V.

Proteins in precipitate B′, which corresponds to precipitate B, were solubilized with 10% sodium chloride solution. Most of the particles were broken, and the proteins appeared in supernatant Q. Only about half of the tocopherol was found in the microsomal fraction (precipitate S). Much

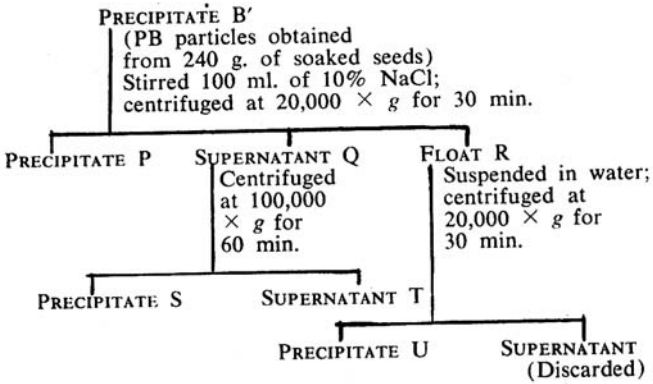

DIAGRAM 3
FRACTIONATION OF COMPONENTS OF PARTICLES
RESEMBLING PROTEIN BODIES

TABLE V
DISTRIBUTION OF TOCOPHEROL IN PB PARTICLES[a]

FRACTION	PROTEIN	TOCOPHEROL	FRACTION		PROTEIN	TOCOPHEROL
	%	%			%	%
B′ PB particles[b]	100 (4.7 g.)	100 (2,740 γ)	R		....	55
			S	Microsomes	6	14
P	5	14	T	Storage proteins	66	17
Q	74	30	U	Insoluble fraction	25	48

[a] Fractionated as shown in diagram 3.
[b] Fraction B in diagram 1.

tocopherol was found in the insoluble fraction, precipitate U. This fraction floated on 10% sodium chloride solution (float R) and precipitated in water. When float R was washed and centrifuged, the supernatant was clear and seemed not to contain any substances. Precipitate U was considered to contain most of the storage lipids and also biological unit membranes in the cotyledons. (Unit membranes are made of lipoproteins; typical membranes have been reported in the protein bodies from wheat (10).)

DISCUSSION

As shown in Table I, tocopherol was distributed in all fractions. Tocopherol was always found in the cytoplasmic particles and cytoplasmic solution as the usual constituents of the cell. The hypocotyl cells also contained tocopherol, both in the cellular particles and the cytoplasmic solution which contained little or no deposit lipids (Table II). Tocopherol must have some unknown essential function(s) in the cell, because it is always found in the cellular particles, which are the essential biological components of the cells. On the other hand, it is generally considered that the role of tocopherol in seeds is mainly that of *in vivo* antioxidant of deposit fat (2), because tocopherol occurs in all seeds and the content is very high compared with that of other tissues which contain no deposit proteins and lipids.

About 60% of the tocopherol in soybeans was contained in precipitate B (Table I), which was insoluble in 0.25*M* sucrose. This preparation was similar to the protein bodies described for a variety of seeds (11). Protein bodies have been found in many kinds of seeds, such as peanuts (12-14), maize endosperm (15), cottonseed (16), wheat endosperm (10,17,18), peas (19), and soybeans (20). Protein bodies of soybeans were reported to be prepared thus: ground in cottonseed oil and fractionated by centrifugation in cottonseed oil and carbon tetrachloride mixture, with adjustment of densities (20). However, such particles prepared by the classic method for isolating particles have not been reported to contain lipids. Particles prepared from peanuts (14) and wheat endosperm (10), ground with glycerol, were reported to contain lipids. Particles we obtained from soybeans contained more than 20% of lipids. Most of the lipids in the cotyledons appeared to be concentrated in this fraction. Deposit lipids of cottonseeds have been reported to be contained in the protein bodies (11,16). Yatsu and Altschul (16) found densely staining spots in the so-called protein bodies and concluded that lipid inclusions exist within the protein particles of the cottonseed cotyledons. They also noted lipid inclusions in the matrix of the desiccated cytoplasm between the bodies. However, the tocopherol content of our PB particles seemed not to be proportional to the lipid content (Table IV), especially in Gunma. When PB particles were stirred in 10% sodium chloride solution, storage proteins were solubilized and the insoluble preparations contained most of the tocopherol. Most of the storage proteins of soybeans called glycinin should be solubilized in 10% sodium chloride solution. Therefore, precipitate U may be composed of some deposit lipids and membranes. The association of storage proteins with membrane structure has been suggested (11).

There may be some question that the PB particles may be a fat-protein complex formed as an artifact by homogenization as in milk (21). However, in the preparation of PB particles of soybeans, complex formation seems not to contribute much. With more gentle grinding, some more intact particles were prepared which contained much more proteins, lipids, and tocopherol (Table IV, preparation 2).

Total tocopherol content of soybean cotyledons did not decrease (Table III) when the determination was applied directly to the homogenate. Though it was reported that in some seeds the amount of tocopherol in extracted oil decreased at the earliest stage of germination (22), no decrease was observed in direct determination. No oxidation enzymes were detected for tocopherol. Therefore, the lower tocopherol content of the extracted oil from moist soybeans than from dry soybeans is deduced to be a result of difficulty of contact of the solvent with the site of tocopherol in the moist cell. When part of the tocopherol is contained in the membrane structures made of lipoproteins, moisture may prevent contact of the solvent.

From these results, it was concluded that tocopherol in seeds is distributed widely in cells, especially in the storage particles as well as in cellular particles. It should be recalled that these particles contain membrane structures. Tocopherol may be concerned with some unknown essential functions

in the cells, perhaps in the membrane structures, in addition to a possible role as *in vivo* antioxidant for deposit lipids.

Acknowledgment

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