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Localization of Lipoxygenase in Germinating Soybeans

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Abstract

The subcellular distribution of lipoxygenase in germinating soybean seeds (*Glycine max* [L.] AmSoy) was investigated by using differential centrifugation and sucrose density gradient fractionation. Most of lipoxygenase -1 and -2/3 activities was present in the supernatant fraction after differential centrifugation of homogenates prepared from three-day-old seedlings; only 1.5% of lipoxygenase activity remained in particulate fractions. The results of a sucrose density gradient fractionation (three-day-old) showed that the lipoxygenase activity coincided with acid phosphatase at the densities of 1.19, 1.23, 1.25 g/cm^3, even though most of lipoxygenase and acid phosphatase activities appeared in supernatant fractions. There was no indication that mitochondria contained any lipoxygenase activity, and it does not appear that glyoxysomes and ER contained any lipoxygenase activity either.

Introduction

Lipoxygenase (E.C.1.13.11.12; linoleate: oxygen oxidoreductase) is a dioxygenase containing nonheme iron. It catalyzes the oxidation of polyunsaturated fatty acids having a *cis, cis* 1,4-pentadiene system to produce conjugated hydroperoxydiene derivative through the insertion of molecular oxygen into the substrate. These primary products are converted into secondary products, such as alcohols, aldehydes, ketones, and epoxy compounds by enzymatic and nonenzymatic processes, which are responsible for the development of off-flavors during the processing of certain plant food-stuffs, particularly soybeans[1,2,3]

Seeds of soybean (*Glycine max* [L.] Merr.) contain significant amounts of lipoxygenase-1 and -2 (1.4 and 2.8 *mg/g* dry weight, respectively)[4]. The properties of lipoxygenase and the methods for its purification have been studied extensively, and the existence of multiple lipoxygenase in soybeans is also well-established[5,6,7]. However, the distribution and physiological function of lipoxygenase in plants are far from clear.

For the better understanding of action, distribution and physiological function of lipoxygenase in plants, the study of the exact cellular and subcellular localization of lipoxygenase has been emphasized. Unfortunately, evidence for the subcellular localization of lipoxygenase is insufficient. Douillard and Bergeron[8] found that lipoxygenase activity was distributed in chloroplast stroma of young pea shoots, and Wardale and Galliard[9] reported that lipoxygenase in pea root was localized in the lysosomal fractions. In this study, differential centrifugation and density gradient fractionation techniques were incoporated to investigate the subcellular distribution of lipoxygenase in germinating soybean seeds.

Materials and Method

Materials

Soybean seeds (*Glycine max* [L.]. Merr. Variety 1984 AmSoy) were obtained from the Department of Agronomy, Iowa State University, Ames, Iowa, U.S.A.

Seed Germination

Soybean seeds were soaked in running water for 1 hour and germinated on vermiculite at 28±1°C in the dark.

Tissue Extraction

The grinding medium contained 13% sucrose, 150*mM* tricine buffer (pH 7.5), 1*mM* EDTA and 0.1% BSA. Germinated seeds were homogenized in a waring blender for 3 min. Homogenates were filtered through 2 layers of cheese cloth and centrifuged at 270×*g* for 10 min. The supernatant (Sl) was saved and used for differential centrifugation and sucrose density gradient fractionation studies.

Differential Centrifugation

Conditions of centrifugation for the separation of

subcellular fractions[10] were as follows: whole broken cells were removed by a 10 min centrifugation at 1,200 x *g*, A chloroplast fraction (P1) was obtained by centrifugation at 4,000 x *g* for 10 min, and an additional 10 min centrifugation at 8,000 x *g* removed chloroplastic debris (P2). Mitochondria (P3) was obtained after 20 min at 15,000 x *g*, followed by mitochondrial debris after 20 min at 25,000 x *g* (P4). A final centrifugation at 105,000*g* for 60 min yielded a microsomal fraction (P5) and supernatant cytosol (S2). Pellets were quickly frozen, washed with grinding buffer twice, and ground with a mortar and pestle for 3 min in 4 *ml* of grinding buffer containing 0.1% Triton-X 100. The mixture was centrifuged at 20,000 x *g* for 30 min and the pellets discarded. The supernatant was collected and assayed for lipoxygenase activity.

Density Gradient Fractionation

For the isolation of total organelle population, supernatant from tissue extraction was layered directly onto a sucrose gradient. The gradient consisted of a 3 *ml* cushion of 60% (*w/w*) sucrose, a 30 *ml* linear gradient from 13 to 60% (*w/w*) sucrose which was prepared with Hoefer gradient maker (San Francisco, CA), and 7 *ml* of sample in ultra-clear centrifuge tube. All sucrose solutions contained 1*mM* EDTA (pH 7.5). The gradients were centrifuged at 21,000 *rpm* for 4 hours in a Beckman L5-65 preparative ultracentrifuge with a Spinco SW 27 rotor following which 1.0 *ml* fractions were collected from the top of the tube.

Analysis

Lipoxygenase activity was determined polarographically using a YSI Oxygen Monitor model 53 equipped with a Clark electrode. The substrate solution was prepared with 50*mM* borax buffer (pH 9.0) and 50*mM* sodium phosphate buffer (pH 6.8) for lipoxygenase-1 and 2/3, respectively, giving a final concentration of 2.57*mM* linoleic acid[11]. Sucrose concentrations were determined by using a Bausch and Lomb refractometer at room temperature.

Marker enzymes for density gradient fractionation were assayed spectrophotometrically. Cytochrome oxidase, a marker enzyme of mitochondria, was assayed by the method of Wharton and Tzagoloff[12], except that a trace amount of sodium hyposulfite was used to reduce the cytochrome C. NADH dependent cytochrome c reductase was used as a marker enzyme for ER. The reaction was run at 37°C, and the readings were taken against a blank cell containing the same amount of enzyme solution and all reagents except NADH[13]. Catalase activity, as a marker enzyme for the glyoxysomes, was measured at room temperature by monitoring the decrease of absorbance at 240 *nm* in a reaction mixture containing 500*mM* sodium phosphate buffer (pH 7.5), 12.5*mM* H_2O_2, and enzyme solution[14]. Acid phosphatase activity, as a marker enzyme of the lysosomal fraction, was measured by monitoring the increase of absorbance at 420 nm. A standard curve was prepared by using a dilute solution of p-nitrophenol. The final concentration of the solution used was: 100*mM* sodium acetate buffer (pH 5.6), 5*mM* p-nitrophenylphosphate, and enzyme solution. After 10 min incubation at room temperature, the reaction was terminated by the addition of 0.02N NaOH.

Result and Discussion

Differential Centrifugation

After three days of seedling growth, most of the organelles were observed in the cotyledon tissues. This period was, therefore, chosen for the studies on subcellular localization of lipoxygenases. Most of lipoxygenase-1 and -2/3 activities was present in the supernatnat fraction after differential centrifugation of homogenates prepared from the three-day-old seedlings; only 1.5% of the lipoxygenase activity remained in particulate fractions (Table 1). It remained unclear at the moment whether this small activity was associated with mitochondria and microsomes or the result of contamination during centrifugation and subfractionation steps. Furthermore, the lipoxygenase activity in partculate fractions before and after the addition of 0.1% Triton-X 100 did not show any significant change, indicating lipoxygenase is not a membrane-bound enzyme, and the presence of lipoxygenase activity in mitochondrial and microsomal fractions might be a contaminant rather than being associated with the particles.

Previous works on the localization of lipoxygenase activity by differential centrifugation from soybean seed homogenates had shown that 100% of lipoxygenase activity remained in the supernatant fraction[15,16]. It was claimed that the activity of lipoxygenase could not be sedimented, possibly because of the destructive action of lipoxygenase on subcellular membrane, producing hydroperoxides which affect membrane structure[16].

When the experiment was repeated using soybeans

Table 1. Localization of lipoxygenase activity in fractions obtained from three-day-old seedlings of soybean by differential centrifugation

Fractions	activity	*(unit/ml)*	% in homogenate	
	Lipoxygenase-1	Lipoxygenase-2/3	Lipoxygenase-1	Lipoxygenase-2/3
S1	118.70	164.80	100	100
P1	—	—	—	—
P2	—	—	—	—
P3	—	0.39	—	0.23
P4	0.82	0.97	0.69	0.59
P5	0.91	1.30	0.77	0.79
S2	90.51	160.30	76.25	97.27

at early and late stages of seedling growth, little change was observed.

Sucrose Density Gradient Fractionation

Subcellular organelles of a tissue preparation on sucrose density gradients were separated as determined by their protein and lipid composition and lastly, as influenced by their bound water content. After centrifugation, the gradient was first characterized by its sucrose concentration and lipoxygenase profile. Sucrose in each fraction was measured manually with a refractometer and converted to density at 20°C. The selection of marker enzyme for each organelle depended upon organelle specificity and ease of assay. Catalase, NADH-dependent cytochrome c reductase, cytochrome oxidase and acid phosphatase were used as marker enzymes for glyoxysomes, ER, mitochondria, and lysosomal fractions, respectively.

The results of a sucrose linear gradient fractionation (three-day-old seedlings) showed that the lipoxygenase-1 activity coincided with acid phosphatase at the densities of 1.19, 1.23, 1.25 *g/cm³* (Fig. 1), even though most of lipoxygenase and acid phosphatase activities appeared in supernatant fractions. The appearance of the acid phosphatase activity at the high densities could be accounted for in several ways. Possibly, it might be a trapping artifact, or it was derived from protein bodies with different densities due to different degrees of hydration and hydrolysis.

Evidences had been accumulating that the protein bodies were transformed into lysosomes in which their reserves are mobilized during germination and seedling growth. Cytochemical staining[17] and histochemical observation[18] showed that protein bodies in mung bean cotyledon were rich in acid phosphatase, a marker of lytic activity in cells. Biochemical analysis also revealed that protein bodies contained protease, α-glucosidase, phosphatase, and an esterase in pea seeds and mung beans[18,19].

Fig. 1 also showed that the NADH-cytochrome c reductase activity was present in the soluble fraction, and a peak at density of 1.09 *g/cm³* demonstrating that some leakage of marker enzyme from ER may have occurred. Cytochrome oxidase activity showed two broad peaks at the densities of 1.13 and 1.16 *g/cm³*. The peak at lighter

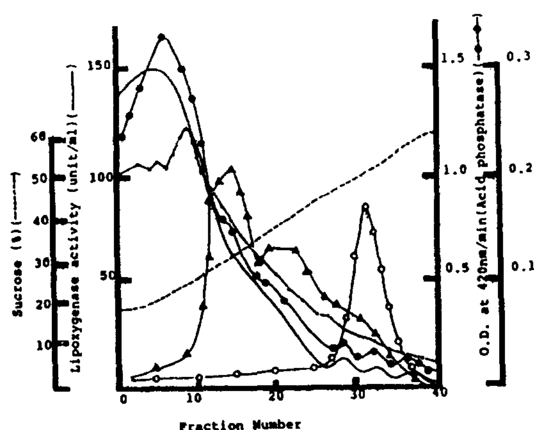

Fig. 1. Distribution of marker enzyme activities of homogenates from three-day-old seedings on a linear sucrose gradient (13-60%)

O.D. at 550nm/min (Cytochrome oxidase) (—▲—▲—)
O.D. at 240 nm/min (Catalase) (—○—○—)
O.D. at 550nm/min (NADH:cytochrome c reductase) (—●—●—)

density might be due to broken mitochondria or the evidence of nonspecific enzymes. The peak of catalase was somewhat symmetrical, which refleced the occurrence of homogeneous glyoxysomes present after three days of seedling growth. From these observations, there was no indication that mitochondria contained any lipoxygenase activity, and ER contained any lipoxygenase activity either.

When this experiment was repeated by using one-day old and five-day-old seedlings, no lipoxygenase activity was localized in any particulate fractions (Fig. 2 and 3). However, five-day-old seedlings apparently showed two peaks of acid phosphatase activity at the densities of 1.19 and 1.25 g/cm^3, indicating that this enzyme is trapped or localized in protein bodies.

From all these observations, it became quite evident that the particles with which lipoxygenase and acid phosphatase activity localized on a density gradient are protein bodies which had different internal matricies. Similar results were observed by Vernooy-Gerritsen et al[4] in soybean tissues. They reported the distribution of lipoxygenase in protein bodies of vascular bundles by using indirect immunogold labelling method.

The metabolic and physiological function of lipoxygenase in soybean is still far from clear based on this

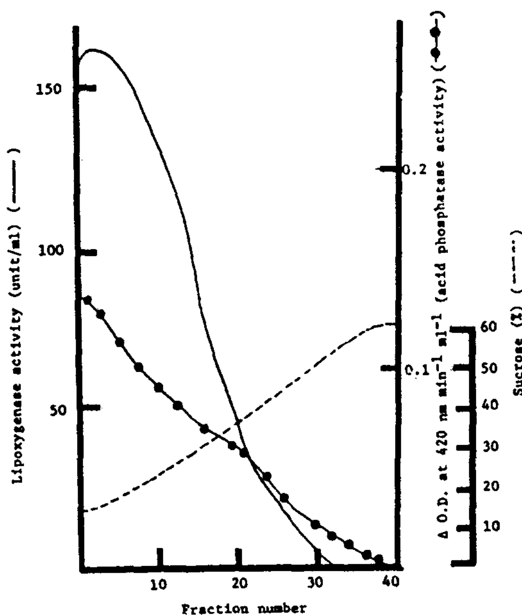

Fig. 2. Distribution of lipoxygenase and acid phosphatase activities of homogenates from one-day-old seedlings on a sucrose gradient (13-60%)

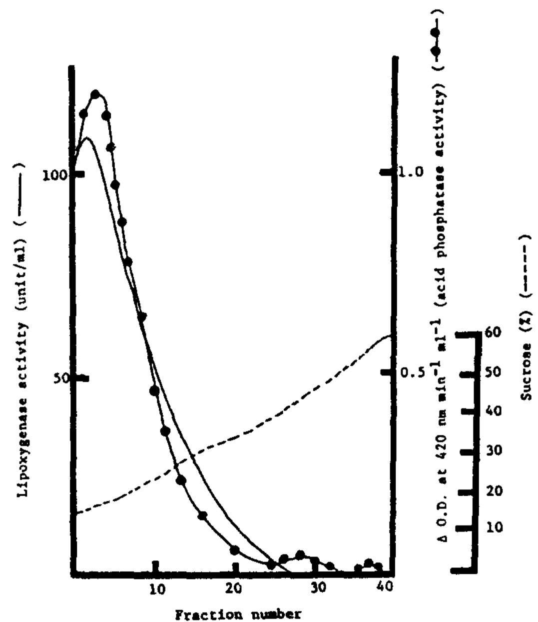

Fig. 3. Distribution of lipoxygenase and acid phosphatase activities of homogenates from five-day-old seedlings on a sucrose gradient (13-60%)

observation. Lipid bodies were hypothesized for the lipoxygenase location, since enzyme and its substrate usually are closely associated with each other. From the observations of its distribution in cytosol, it is proposed that the function of lipoxygenase might lie in the dioxygenation of fatty acids to make their transport from lipid bodies to glyoxysomes possible[4]. The distribution of lipoxygenase in protein bodies of three-day-old seedlings is considered as a stage in the digestion of lipoxygenase orginating from the cytosol, which may be explained by the finding that the protein bodies are the intracellular sites at which the digestion of cytoplasmic structure occurs[17,18].

References

1. Mustakas, G.C., Albrecht, W.J., McGhee, J.E., Black, L.T., Bookwalter, G.N. and Griffin, E.L.: *J. Am. Oil Chem. Soc.*, **46**, 623 (1969)
2. Kon, S., Wagner, J.R., Guadagni, D.F. and Horvat, R.J.: *J. Food Sci.*, **35**, 343 (1970)
3. Ashraf, H-R. L. and Snyder, H.E.: *J. Food Sci.*, **46**, 1201 (1981)
4. Vernooy-Gerritsen, M., Leunissen, J.L. M., Veldink, G.A. and Vliegenthart, J.F. G.: *Plant Physiol.*, **76**,

1070 (1984)
5. Christopher, J.P., Pistorius, E.K. and Axelrod, B.: *Biochim. Biophys. Acta,* **284,** 54 (1972)
6. Yamamoto, A., Yasumoto, K. and Mitsuda, H.: *Agric. Biol. Chem.,* **34,** 1169 (1970)
7. Verhue, W.M. and Francke, A.: *Biochim. Biophys. Acta,* **284,** 43 (1972)
8. Douillard, R. and Bergeron, E.: *Plant Sci. Lett.,* **22,** 263 (1981)
9. Wardale, D.A. and Galliard, T.: *Phytochem.,* **16,** 333 (1977)
10. Grossman, S., Ben-Aziz, A., Ascarelli, I. and Budowski, P.: *Phytochem.,* **11,** 509 (1972)
11. Rackis, J.J., Honig, D.H., Sessa, D.J. and Moser, H.A.: *Cereal Chem.,* **49,** 586 (1972)
12. Wharton, D.C. and Tzagoloff, A.: *Methods Enzymol.,* **10,** 245 (1967)
13. Bowles, D.J. and Kauss, H.: *Biochim, Biophys. Acta,* **443,** 360 (1976)
14. Lück, H.: *Methods of enzymatic analysis,* Academic Press, New York, p.885 (1965)
15. Vernooy-Gerritsen, M., Bos, A.L. M. and Veldink, G.A.: *Plant Physiol.,* **73,** 262 (1983)
16. Wardale, D.A. and Galliard, T.: *Phytochem.,* **14,** 2323 (1975)
17. Herman, E.M., Baumgartner, B. and Chrispeels, M.J.: *Eur. J. Cell Biol.,* **24,** 226 (1981)
18. Harris, N. and Chrispeels, M.J.: *Plant Physiol.,* **56,** 292 (1975)
19. Matile, P.H.: *Z. Pflanzenphysiol.,* **58,** 365 (1968)

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발아중인 대두콩에서의 Lipoxygenase의 局在

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발아중인 대두콩에서의 lipoxygenase의 세포내 위치를 차별 원심 분리법과 밀도 구배법을 이용하여 규명하였다. 차별 원심 분리법을 사용하였을때, lipoxygenase 활성은 거의 supernatant fraction에서 나타났으며, 1.5%의 활성만이 particulate fractions에서 나타났다. 밀도 구배법에서는 lipoxygenase와 acid phosphatase의 활성이 1.19, 1.23, 1.25g/cm^3의 높은 밀도들에서 일치하였으며, 이것은 발아중 가수분해되는 단백질체내에 이 효소가 국재되어지는 것으로 사료되어진다. 다른 세포내 소기관인 미토콘드리아, ER, 그리고 glyoxysomes에 lipoxygenase가 존재한다는 증거는 보이지 않았다.