Occurrence of Lipolytic Acyl-hydrolase in Higher Plants and Its Subcellular Distribution in Potato Tubers

Hideyuki※ MATSUDA and Osamu HIRAYAMA※

高等植物における脂質分解酵素の存在とその馬鈴薯塊茎中における細胞内分布

松 田 英 幸※・平 山 修※

INTRODUCTION

In recent years, it has become apparent that glyco-and phospholipids mainly exist in the membranes of cells and of the organelles in cells, and play extremely important roles in the physiological functions of cells. Galactolipase (EC. 3. 1. 1. 26) or lipolytic acyl-hydrolase which catalyze the hydrolysis of the membrane lipids have been purified from some higher plant tissues such as tubers,[1-4] leaves[5-7] and seeds[8,9] and characterized. However, occurrence of these enzymes in higher plants remained obscure because these enzymes are generally unstable and low in activity.

On the other hand, the authors reported that a lipolytic acyl-hydrolase purified from potato tubers exhibits the hydrolyzing activities for galacto- and phospholipids with a single active site.[3] The subcellular distribution of such a enzyme is very interesting. However, it is widely recognized that the nature of subcellular structure in plant tissues is very fragile.[10] Galliard also described that the membrane structures are readily disrupted by the degradative actions of endogenous lipolytic enzymes during the fractionation of organelles in potato tubers.[11] Therefore, the subcellular distribution of lipolytic acyl-hydrolase in the tubers is not yet fully examined.

In the present paper, occurrence of the enzyme in higher plants and the subcellular distribution of the enzyme in potato tubers were investigated.

MATERIALS and METHODS

Plant materials. The tubers and the leaves of potato (*Solanum tuberosum* L. cv. Benimaru, Danshaku and Norin No. 1), and the leaves of bean (*Phaseolus vulgaris* L. cv. kinugasa, Kinugasa, Edogawa, Satsukimidori, Hotaka, etc.) were grown on the farm of Kurodane- Shimane University. Rice grains (*Oryza sativa*, L. Japonica cv. Koshihikari) were obtained from Central Agricultural Experiment Station of Shimane Prefecture. Soybean (*Glycine Max* M.) and spinach (*Spinacia Oleracea* L.) were also grown on the same farm. Sweet potato, cabbage and carrots were obtained from local markets.

Substrates. Monogalactosyldiacylglycerol was prepared from spinach leaf lipids.[12] Phospha-

※ Laboratory of Food Chemistry, Department of Agricultural Chemistry.

tidylcholine was purified from egg lipids.[13]

Enzyme assay. Galactolipase and phospholipase activities were determined according to the methods described previously.[3]

Gel filtration. A sephadex G-100 microcolumn (0.9×22 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0) was used, and applied enzyme was eluted with the same buffer.[3] The molecular weight of the enzyme was estimated by the method described previously.[8]

Enzyme extraction. The extraction of the enzyme from potato tubers was performed by the method descrived previously.[3] The enzymes were also extracted from bean leaves,[7] potato leaves,[14] and rice grains[8] according to the methods described in REFERECES. Protein was determined by the Lowry method modified by Hartree.[15]

Subcellular distribution. The subcellular distribution of the enzyme in potato tubers was examined by according to the method described by Ohad *et al.*[16] and Wardale and Galliard[11] except for the addition of diisopropyl fulorophosphate. Potato tubers were washed, peeled and gently homogenized with 0.01 M phosphate buffer (pH 6.5, 0.4 M sucrose and 2 mM cysteine) in the presence or absence of the inhibitor (1 mM). The homogenate was filtered through four layers of nylon clothes and centrifuged at 300×g for 10 min. The resulting precipitate was rehomogenized and centrifuged under the same conditions, and the precipitate was used as the starch fraction. The supernatants were pooled and then fractionated by differential centrifugation. The fractions of 40,000×g-precipitate (rich in mitochondria), 105,000×g-precipitate (rich in microsome) and 105,000 × g-supernatant (soluble fraction) were obtained.

On the other hand, a part of the resulting starch fraction was rehomogenized and sonicated, and then centrifuged at 3,000×g for 20 min. The supernatant was further centrifuged at 60,000×g for 40 min, the precipitate was used as a membrane fraction.

Incubation of tuber tissues and the homogenate and analysis of lipids. Potatotubers were washed, peeled and cut into small cubes, about 3 mm thick. The cubes (1 g) and the homogenates obtained from the cubes (each 1 g) under the same conditions as that for the fractionation were incubated in 0.01 M phosphate buffer (pH 6.5, 0.4 M sucrose, 2 mM cysteine) at 30°C for 60 min. After incubation, lipids were extracted with chloroform-methanol (2 : 1, v/v) and subjected to lipid analysis by thin-layer chromatography.[17]

RESULTS

Occurrence of galactolipase and phospholipase activities. Homogenates from various plant tissues were assayed for both galactolipase and phospholipase activities. As summarized in Table I, both enzymic activities exist in many plant tissues. Especially, three varieties of potato tubers showed high activities among the plant tissues tested. The leaves of potato, bean and soybean as well as rice grains exhibited considerable activities, whereas spinach leaves, cabbage, sweet potato and carrot roots showed little or no activities under the present assay conditions. Porcine pancreas had a respectable phospholipase activity together with galactolipase activity which remarkably decreased by delipidation treatment. Snake venom of *Habu* and the crude extract from *Rhizopus delmer*, which have the strong activities of phospholipase and lipase, respectively, had no or hardly galactolipase activity under the present conditions.

Degradation and its inhibition of membrane lipids of potato tubers. Since potato tubers

Table I. Occurrence of Galactolipase and Phospholipase Activities in Various Higher Plant and Animal Extracts.

Enzyme source			Total activity (U*/100 g tissues)	
			Galactolipase	Phospholipase
Potato (*Solanum tuberosum* L.)				
cultivar	Benimaru	leaves	5.6	0.9
		tubers	345.0	379.0
	Norin No. 1	leaves	4.7	0.8
		tubers	325.4	384.6
	Danshaku	leaves	5.4	0.9
		tubers	298.7	342.3
Bean (*Phaseolus vulgaris* L.)				
cultivar	Kurodane-kinugasa	leaves	38.2	13.0
	Kinugasa	leaves	29.4	11.0
	Hotaka	leaves	13.2	8.7
	Shaku-gosun	leaves	36.4	16.8
	Master peace	leaves	26.5	9.3
	Edogawa	leaves	25.7	9.1
	Kintoki	leaves	6.9	2.9
	Hon-kintoki	leaves	1.3	0.5
	Kurosando	leaves	8.3	2.3
Soybean (Glycine max M.)		leaves	7.6	2.7
Spinach leaves			0.2	0.1
Cabbage			not detectable	not detectable
Rice grains (Oryza sativa, L. Japanica)				
cultivar Koshihikari			29.3	18.1
Sweet potato			not detectable	not detectable
Carrot root			not detectable	not detectable
Porcine pancreas (non-delipidated extract)			7.3	14.5
(dilipidated extract)			0.1	16.3
Snake venom of Habu (*Trimeresurus flavoviridis*)			not detectable	1.2 (U/mg protein)
Rhizopus delmar			not detectable	not detectable

The enzymes of potato tubers, potato and bean leaves, and rice grains were assayed by the standard methods for each enzyme described in REFERECES. The other enzymes were assayed by the standard methods for galactolipase or phospholipase activities of potato tubers (see MATERIALS and METHODS). * One unit, 1 μmol freefatty acid released/min • 35°C.

involve the very high activities of lipolytic enzymes such as lipolytic acyl-hydrolase and lipoxygenase,[18] it appears that membrane structures are considerably disrupted by the degradative action of such lipolytic enzymes during the preparation of the subcellular fractions. In practice, as demonstrated in Fig. 1 and Table II, the incubation of the potato tuber homogenate caused a large loss of the lipids by the action of endogenous lipolytic enzymes (Fig. 1, C), although the incubation of potato tuber tissue itself did not result in the degradation of its membrane lipids (Fig. 1, B). When diisopropyl fluorophosphate, a strong inhibitor of the enzyme in potato tubers,[3] was added to the homogenate, the degradation

Table II. Hydrolysis of Potato Tuber Lipids under Various Conditions by Endogenous Lipolytic Enzymes.

Conditions	Hydrolysis rates (%)*	
	Glycolipids	Phospholipids
Incubation (30°C)		
for 60 min in tissues	1	2
in homogenate	84	87
in homogenate with 1 mM DFP	9	11

* Hydrolysis rates were calculated from the contents of sugar (for glycolipids) and phosphorus (for phospholipids) in each extracted lipids.
DFP, Diisopropyl fluorophosphate.

of the lipids remarkably retarded (Fig. 1, D and Table II). Even by the incubation at 0°C, similar lipid degradation was observed in the homogenate.

Subcellular distribution of the enzyme. On the bases of these results described above, the fractionation of organelles in potato tubers was carried out in the presence and in the absence of the inhibitor. The subcellular distributions of the enzymic activities and protein in the tubers are summarized in Table III. In the absence of the inhibitor, 85% of total protein existed in the supernatant fraction, while the addition of the inhibitor lowered the proportion of protein in the fraction to 55% of total protein, indicating that the disruption of subcellular structures is markedly suppressed by the inhibitor. In the presence of the inhibitor, however, galactolipase activity was mostly found in the supernatant fraction (87% of total activity), as was it in the absence of the inhibitor. A similar distribution was also observed for phospholipase activity in the tubers.

Gel filtration of supernatant and mitochondrial fractions. The supernatant fraction obtained by the fractionation in the presence of the inhibitor was applied to a Sephadex G-100 microcolumn (0.9×22 cm)as described in MATERIALS and METHODS. As Fig. 2 shows, galactolipase and phospho-

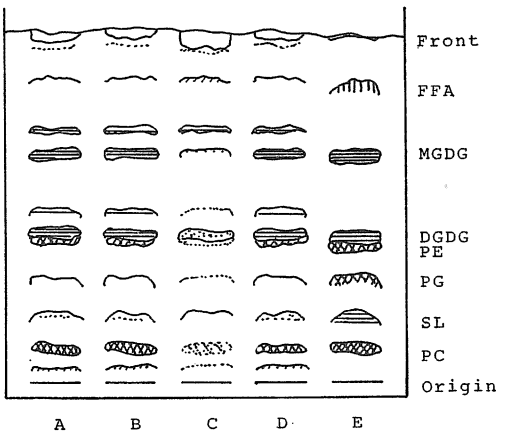

Fig. 1. Thin-layer Chromatography of Lipids Extracted from Potato Tuber Tissues and Their Homogenates Incubated.

Thin-layer chromatography was carried out as described in MATERIALS and METHODS. α-Naphthol-H_2SO_4 and 50% H_2SO_4 were used as color reagents.

A, Lipids extracted from potato tuber tissue.
B, Lipids extracted from the tuber tissue incubated (60 min, 30°C).
C, Lipids extracted from potato tuber homogenate incubated (60 min, 30°C)
D, Lipids extracted from the same homogenate incubated (60 min, 30°C) in the presence of 1mM diisopropyl fluorophosphate.
E, Standard Lipids. FFA, free fatty acid ; MGDG, monogalactosyldiacylglycerol ; DGDG, digalactosyldiacylglycerol ; SL, sulfoquinovosyl diacylglycerol ; PE, phosphatidylethanolamine ; PC, phosphatidylcholine ; PG, phosphatidylglycerol.

Table III. Subcellular Distribution of Protein and Galactlipase Activity in Potato Tubers.

Fractions	Distribution (%)			
	Without DFP		With DFP	
	Protein	Galactolipase activity	Protein	Galactolipase activity
Starch fraction	2.8	—	8	—
Membrane fraction	0.2	0.1	10	3
Mitochondria fraction	8	2	23	6
Microsome fraction	4	3	7	4
Supernatant fraction	85	95	52	87

DFP, Diisopropyl fluorophosphate.

lipase activities in the fraction were eluted as the same single peak. The elution volume of the peak corresponded to that of a globular protein with molecular weight of about 132,000. The similar result was also obtained in the enzyme from the mitochondrial fraction. The molecular weights differed from that of a purified enzyme of potato tubers (MW=70,000).[3]

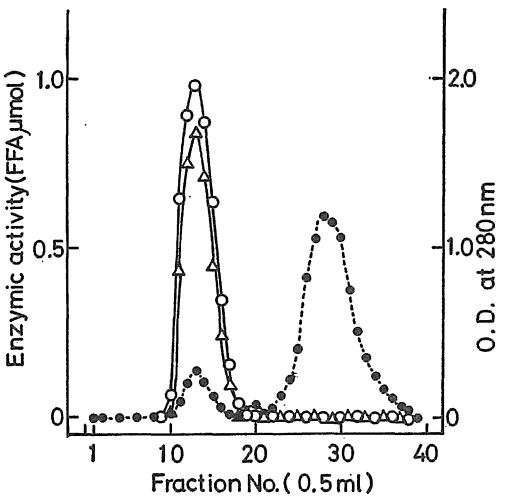

Fig. 2. Gel Filtration of Supernatant Fraction on a Sephadex G-100 Microcolumn.

The supernatant fraction obtained from the homogenate of potato tubers by differential centrifugation was subjected to gel filtration on a Sephadex G-100 microcolumn (0.9×22 cm) as described in MATERIALS and METHODS. Fractions of 0.5 ml portions were collected and subjected to enzyme assay.

○—○, galactolipase activity ; △—△, phospholipase activity ; ●—●, optical desnsity at 280 nm.

DISCUSSION

Galactolipase and phospholipase activities were detected in the homogenates from many plant tissues. Several species of potato tubers and bean leaves showed a higher enzymic activity than other plant tissues tested. It is known that these plant tissues contain glycolipids at relatively higher concentrations than those of other plant tissues.[19] Therefore, it seems likely that the amounts of the enzymic activities are generally related to the lipid content and compositions in the tissues.

Potato tubers exhibited the highest hydrolyzing activity among higher plant tissues. The examination on subcellular distribution of the lipolytic enzyme in potato tubers is very difficult, because of the desructive action of the enzyme on the subcellular membranes during fractionation. The degradation of the membrane lipids were, however, remarkably retarded by the addition of the diisopropyl fluorophosphate (Fig. 1 and Table II). Thus, the subcellular fractionations in the presence and absence of the inhibitor were compared. In both cases, most of the enzyme activity were found in the soluble fraction. These results seem to indicate that the enzyme is not located in the ordinary particle fractions. If the

enzyme which has a strong lipid hydrolyzing activity exists in the soluble fraction in the tubers, it probably causes the breakdown of the membrane lipids associated with the membrane structures within the cells[11] even in the tissue itself. But the incubation of the tissue did not cause the degradation of the lipids (Fig. 1). It is well known that the nature of large vacuoles in plant cells is very fragile[10], so that the contents of the fragile organelles are frequently mixed with a soluble fraction. Therefore, it is possible that most of the above enzyme are present in these organelles and make its apperence in the soluble fraction due to the disruption of the organelles during the fractionation.

A similr suggestion has been repoted on a lipolytic enzyme in cauliflower floret tissues.[18] Galliard and Matthew reported on a subcellular fractionation of the tuber of Desivee variety having low level of a lipolytic enzyme activity.[19] They found that a large portion of the enzyme activity was present in the soluble fraction although a considerable activity was also found in the particle fraction.

The lipolytic enzyme in leaves of bean and potato have been reported to be distributed in the chloroplast fraction.[5—7] It seems likely that the enzyme has some difference in the physiological roles or functions in each plant tissue.

The molecular weights of the enzymes in the supernatant and mitochondria fractions were estimated be to 132,000, which differed from that of the purified enzyme.[3] It is possible that the enzymes exist as a dimer of a purified enzyme (MW=70,000) in the fractions.

The relation among the two enzymes in the fractions and a purified enzyme remains to be solved.

ACKNOWLEDGEMENT

The authors are indebted to Laboratories of Olericulture and Plant Pathology, Faculty of Agriculture, Shimane University, as well as Central Agricultural Experiment Station of Shimane Prefecture, for their kind assistances for plant culture or supplying of samples.

SUMMARY

Lipolytic acyl-hydrolase existed in potato tubers, the leaves of potato, bean, and soybean as well as rice grains with a relatively high activity, but spinach leaves, cabbage, sweet potato, and carrot root showd little or no activity under the present enzyme assay conditions.

During the incubation of the homogenate of potato tubers, the membrane lipids were rapidly hydrolyzed by endogenous lipolytic enzymes. When diisopropyl fluorophosphate (1 mM) was added to the homogenate, the degradation of the membrane lipids was retarded. The subcellular fractionation in the presence and absence of the inhibitor showed that the enzyme was mostly present in the supernatant fraction (about 90% of total activity). But, with other evidence it is possible that the enzyme occurs in some fragile organelles such as vacuoles and was mixed with the soluble fraction by the disruption of the organelles.

The molecular weight of the enzyme in the supernatant and mitochondria fractions was estimated to be 132,000, which differed from that of the purified enzyme (70,000) described previously.

REFERENCES

1) T. GALLIARD and S. DENNIS, *Phytochemistry*, **13**, 1731 (1974).
2) E. P. HASSON and G. G. LATIES, *Plant Physiol.*, **57**, 142 ; 148 (1976).
3) O. HIRAYAMA, H. MATSUDA, H. TAKEDA, K. MAENAKA and H. TAKATSUKA, *Biochim. Biophys. Acta*, **384**, 127 (1975).
4) H. MATSUDA, T. MATSUMOTO and O. HIRAYAMA, *Nippon Nogeikagaku Kaishi*, **51**, 381 (1977).
5) P. S. SASTRY and M. KATES, *Biochemistry*, **3**, 1280 (1964).
6) M. M. ANDERSON, R. E. MCCARTY and E. ZIMMER, *Plant Physiol.*, **53**, 699 (1974).
7) H. MATSUDA and O. HIRAYAMA, *Agric. Biol. Chem.*, **43**, 563 (1979).
8) H. MATSUDA and O. HIRAYAMA, *Agric. Biol. Chem.*, **43**, 463 (1979).
9) H. MATSUDA and O. HIRAYAMA, *Agric. Biol. Chem.*, **43**, 697 (1979).
10) H. BEEVERS, "Recent Advances in the Chemistry and Biochemistry of Plant Lipids" ed. by T. GALLIARD and E. L. MERCER, Academic Press Inc., New York, 1975, p. 287.
11) D. A. WARDALE and T. GALLIARD, *Phytochemistry*, **14**, 2323 (1975).
12) O. HIRAYAMA, *J. Biochem.*, **57**, 681 (1965).
13) M. C. PANGBORN, *J. Biol. Chem.*, **188**, 471 (1951).
14) H. MATSUDA and O. HIRAYAMA, *Biochim. Biophys. Acta*, **573**, 155 (1979).
15) E. F. HARTREE, *Anal. Biochem.*, **48**, 422 (1972).
16) I. OHAD, I. Friedberg, Z. Ne'eman and M. Schramm, *Plant Physiol.*, **47**, 465 (1971).
17) O. HIRAYAMA and H. MATSUDA, *Agric. Biol. Chem.*, **36**, 2593 (1972).
18) T. GALLIARD, *Phytochemistry*, **9**, 1725 (1970).
19) T. GALLIARD and J. A. MATTHEW, *J. Sci. Fd Agric.*, **24**, 623 (1973).

摘　　要

糖脂質およびリン脂質を分解する脂質分解酵素の，いくつかの植物組織における分布，ならびに馬鈴薯塊茎における細胞内分布が調べられた．

酵素活性は，馬鈴薯塊茎，馬鈴薯，インゲン，大豆の各緑葉および米粒のホモジネート中に比較的強く存在したが，ホウレンソウ，キャベツ，さつまいもおよびニンジン等には，酵素活性は低いかまたは検出されなかった．

馬鈴薯塊茎の脂質は，ホモジネート中で内在の脂質分解酵素によって急激に分解され，ジイソプロピルフルオロホスヘートを添加すると脂質分解は強く抑えられた．細胞内分布は，阻害剤を無添加および顆粒の膜構造の保持のため添加の両条件で調べられた．その結果は，いずれの場合も酵素活性の約90%が可溶性画分（10.5万 g，上清）に分布していることを示した．しかしこれは，本酵素が空胞のようなこわれやすい器官の中に存在し，分画・操作中可溶性画分に混入してきたものと推察された．

可溶性画分とミトコンドリア画分中の酵素の分子量は約132,000と推定され，既報の精製酵素の分子量(70,000)とは異っていた．