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CHARACTERIZATION AND PURIFICATION OF A PHOSPHATIDASE FROM POTATO (*SOLANUM TUBEROSUM* L.) TUBERS

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Abstract

A phosphatidase from potato (*Solanum tuberosum* var. Ta-Yea) tubers has been demonstrated. The enzyme was purified to 48.31 folds by ammonium sulfate fractionation, DEAE cellulose column chromatography, and isoelectric focusing. The purified enzyme was an acidic protein with an pI 5.0. The enzyme was not affected by the presence of Mg^{2+}, Ca^{2+}, and surface active agents such as PEG, SLS, triton-x and linoleic acid. The enzyme released palmitic, linoleic acid and glycerophosphorylcholine from L-α-lecithin, and it was characterized as a phosphatidase B.

Introduction

Although our knowledge referring to lipases and phosphatidases (phospholipases) of microbial, animal and plant origins has increased rapidly, little information is available on the occurrence of phosphatide acyl-hydrolase enzymes such as phosphatidase A, B and lysophosphatidase in higher plant tissues. Contardi and Ercoli (1933) have firstly demonstrated the phosphatidase B in rice bran hulls. Galliard (1971) reported the presence of a lipid acyl-hydrolase activity in potato tubers and partially purified the enzyme. Hasson and Laties (1976) isolated and characterized three lipid acyl-hydrolases from potato tubers with different specificities and also suggested one of the enzymes was involved in the deterioration of mitochondria during their preparation and aging. This paper presents our findings of a B type of phosphatidase from potato tubers, and the purification, characterization as well as identification of this enzyme.

Materials and Methods

Stems, leaves and tubers of fresh potato (*Solanum tuberosum* var. Ta-Yea)

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plants were collected from Fung-Yen district, Taichung, Taiwan and stored at 6°C before use.

Substrate and chemicals were purchased as follows: refined soybean L-α-lecithin (phosphatidylcholine), linoleic acid and methyl esters of fatty acids (linoleic acid, oleic acid, palmitic acid and sterric acid) were obtained from Sigma Chemical Co. St. Louis, Mo., U. S. A.; methanolic BF (14%, w/v) from Applied Science Laboratories, State College, Pa.; ampholine carrier from LKB, Brevete S. G. D. G. Swedish; triton X-100 (Scintillation grade) from Koch-Light Laboratories Ltd.; polyethylene glycol 6,000 (PEG) and sodium lauryl sulfate (SLS) from Wako Pure Chemical Industries Ltd., and diethylaminoethyl cellulose (Whatmen, Colume Chromedia, DE 11, capacity 1.0 mequiv/g, medium fibrous powder) was obtained from W & R Balstom, Ltd. England. All solvents used for this experiment were analytical reagents.

Enzyme preparation

One hundred grams each of potato tissues were washed, dried and peeled, and then blended with 2 volumes (w/v) of distilled water by grinding for 3 min in a Virtis '45' homogenizer at high speed in cold (4°C). The brei was filtered through four layers of cheesecloth to remove debris, and the filtrate was centrifuged at 20,000 g for 20 min at 4°C. The supernatant was saved and lyophilized, and then stored in a freezer until use.

Enzyme assay

Refined soybean L-α-lecithin was used as the substrate in all assays. In preliminary study, both non-dialyzed and dialyzed enzyme preparations were qualitatively tested for the phosphatidase activity by the cup plate method as previously described (Tseng and Bateman, 1968). The quantitative assay for measuring the acyl-ester content remained in the reaction mixture was followed the method developed by Snyder and Stephens (1959). Reaction mixture contained 0.5 ml of enzyme preparation and 0.5 ml lecithin (5.5 μ moles) in 0.05 M citrate buffer at pH 4.5. The reaction was carried out at 30°C for an hour. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1.0 μ equivalent of acyl-ester bond in reaction mixture at 30°C for 1 hr. Protein was determined by the method of Lowry (1951).

Enzyme purification

1. Fractionation of enzyme preparation—Seven grams of lyophilized crude enzyme from potato tubers were dissolved in 100 ml of cold distilled water, and it was brought to 20% saturation with ammonium sulfate, stood at 28°C for 1 hr, centrifuged at 20,000 g for 20 min then the precipitate was saved.

The procedure was repeated for ammonium sulfate saturation of 40, 60 and 80%. The precipitate from each fraction was dissolved in 10 ml distilled water, dialyzed against one liter of distilled water, and assayed for phosphatidase activity.

2. Ion-exchange chromatography—DEAE cellulose was equilibrated with 0.05 M citrate buffer (pH 4.5). The slurry was poured into a column with inner dimension of 1.6 cm × 21 cm. Elution was carried out at 4°C by a citrate buffer (pH 4.5) gradient. Fractions of 5 ml were collected.

3. Isoelectric focusing—Electrofocusing was carried out in a LKB 8101 Ampholine electrofocusing apparatus equipped with a 110-ml column (LKB Produkter AB, Bronna, Sweden) containing pH 3.5–10 ampholine carriers, and it was carried out at 4°C for 48 hrs. After electrophoresis, the content of the column was cut into 5 ml fraction, followed by measuring pH values. Each fraction was dialyzed against distilled water at 4°C for 24 hrs and then assayed for protein and enzyme activity.

Determination of fatty acid by gas-liquid chromatograph

Fatty acids which released from L-α-lecithin, after incubation with purified phosphatidase from potato tubers, were estimated by gas-liquid chromatograph.

Reaction mixtures contained 0.5 ml of lecithin (2.75 μ moles) in 0.05 M citrate buffer pH 4.5 plus 0.5 ml of purified enzyme (0.03 mg), and it was incubated at 30°C for 1 hr. After incubation, reaction mixtures were extracted for free fatty acids by the addition 9.0 ml 95% ethanol followed by the addition of 10 ml of CCl_4. The mixture was shaken vigorously and the CCl_4 layer retained. Fifteen ml of 0.2 N KCl was added to the CCl_4 fraction and shaken vigorously. The aqueous layer was saved. The CCl_4 was washed with 2 more 10-ml portions of 0.2 N KCl and the 3 washes combined. The CCl_4 fraction was then washed with 10 ml 0.2 N KCl containing 0.5% NH_4OH and 4% ethanol, and was repeated. The latter two washes were combined with the former three. The combined aqueous washes were acidified by adding 1.5 ml of 1 N HCl. The acidified solution was extracted with 20 ml cold ether, and ether layer was saved. The ether was evaporated after transfer to screwcap vials. Two ml methanolic BF were added to each sample, and the vials were caped and heated in a sand bath at 90°C for 10 min. After the samples were cooled, 2.0 ml of distilled water and 5.0 ml of hexane were added to each sample, and the mixture was shaken vigorously. The fatty acid methyl esters were removed from the hexane layer which was evoporated to about 3 μl. These samples were assayed for fatty acid methyl esters by gas-liquid chromatograph.

Varian Aerograph gas chromatograph (Series 1200) equipped with 1/8 inch O. D. 9-ft column packed with 15% diethylene glycol succinate (DEGS) on

60/80 mesh Chromasorb W and a flame detector was served for analyzing fatty acid methyl esters. During analysis, injector, column, and detector temperatures were maintained at 200°C. Helium was used as carrier gas at the rate of 60 ml/min.

Results

In the preliminary study, both dialyzed and non-dialyzed crude enzyme preparations from potato stems, leaves and tubers were examined for their phosphatidase activities. The cup plate assay was conducted at pH 4.5 and 7.8. The positive phosphatidase activity was shown by the appearance of a white zone around the wells containing 0.15 ml of enzyme preparation. The result was shown in Table 1. There was no enzyme activity at pH 7.8. However, at pH 4.5, enzyme preparations from potato tubers, both young and mature, showed distinctive white reaction zone with similiar activity. The enzyme activity seems not to be affected by dialysis process. In contrast, the enzyme preparations from potato stems and leaves exhibit slight activity or none, either before or after dialysis. The result of acyl-ester assay for quantitatively determination of phosphatidase activity was illustrated in Table 2. The enzyme preparations from potato tubers showed the positive ability to deacylate the lecithin by decreasing the acyl-ester content in the reaction mixture at pH 4.5. In general, the result of acyl-ester assay is compatible with that of cup plate assay.

Since both enzyme preparations from potato stems and leaves only exhibit slight or non phosphatidase activity, thus the crude dialyzed and partially purified enzymes from potato tubers were used for the following experiments, and the phosphatidase activity was only estimated by acyl-ester assay.

Table 1. *Phosphatidase activity of enzyme preparations from potato tissues by cup plate method* (Tseng and Bateman, 1968)

Enzyme source	Activity (mm)/mg protein(1)			
	pH 4.5		pH 7.8	
	Non-dialyzed	Dialyzed	Non-dialyzed	Dialyzed
Stem	58.33	0	0	0
Leaf	45.59	26.71	0	0
Young tuber	519.54	493.00	0	0
Mature tuber	616.25	648.68	0	0

(1) The area of reaction is calculated according to the formula: (radius of reaction zone)$^2\pi$ − (radius of well)$^2\pi$. The radius of wells is 10 mm.

Table 2. *Deacylation of lecithin (phosphatidylcholine) by phosphatidase extracted from potato tissues*(1)

Enzyme source	Specific activity (units/mg protein)	
	Non-dialyzed	Dialyzed
Stem	0	0.52
Leaf	0	0
Young tuber	1.11	0.91
Mature tuber	1.22	1.55

(1) Deacylation of lecithin by phosphatidase was measured by acyl-ester method. Reaction mixtures contained 0.5 ml of crude enzyme preparation and 0.5 ml of lecithin (5.5 μ moles) in 0.05 M citrate buffer at pH 4.5. The reaction was carried out at 30°C for 1 hr.

Effect of pH on phosphatidase activity

The pH response of the enzyme activity was examined between pH 3.0 and 8.0. Two buffer systems were used, 0.05 M citrate buffer for pH 3.0–6.0 and 0.05 M phosphate buffer for pH 6.5–8.0. The optimal pH range was from 4.5 to 5.0, and no enzyme activity was detected above pH 6.0 (Fig. 1).

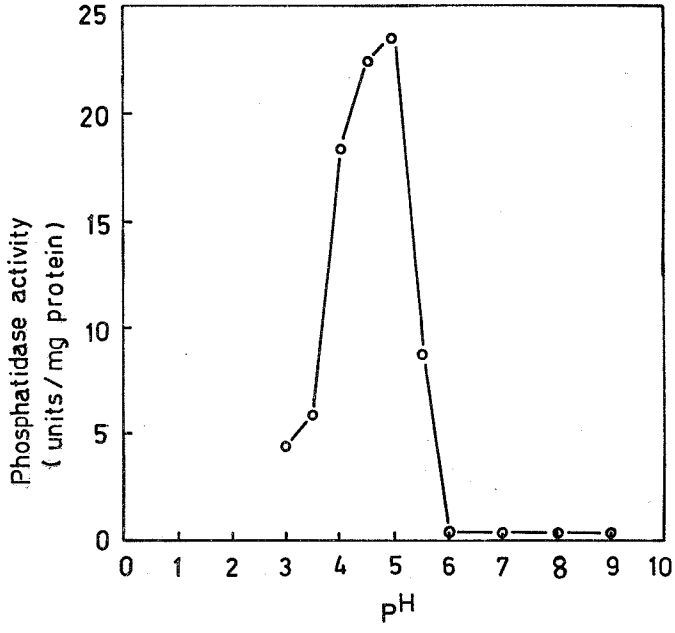

Fig. 1. Effect of pH on potato phosphatidase activity. The enzyme activity was measured by acyl-ester method. Reaction mixtures contained 0.5 ml of partially purified enzyme (0.225 mg protein) and 0.5 ml lecithin (5.5 μ moles) in 0.05 M of various buffers. Citrate buffer for pH 3.0-6.0; phosphate for pH 6.5-8.0. The reaction mixture was carried out at 30°C for 1 hr.

Stability of potato phosphatidase

In this studies, 0.5 ml of partially purified enzyme was pretreated at different temperatures and incubated for 30 min. After treatment, the enzyme were bronght back to ice, and the substrate (lecithin) was added. There is insignificant effect of temperatures between 10–50°C, however, the enzyme activity was completed inactivated at 60°C (Fig. 2).

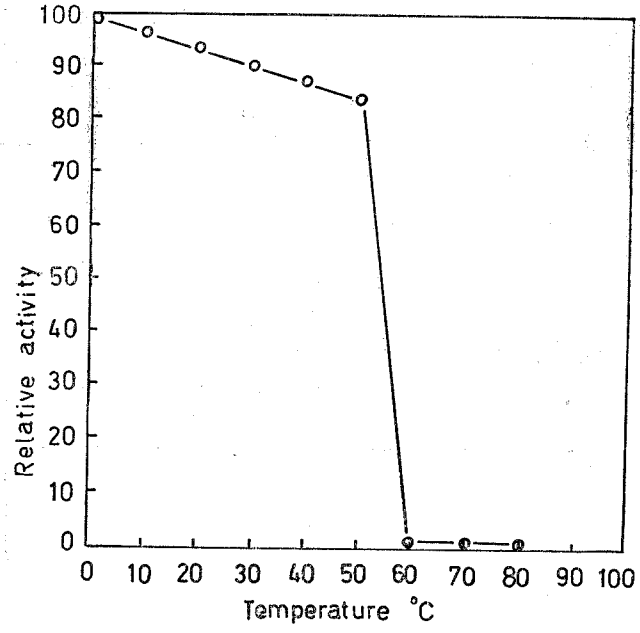

Fig. 2. The stability of potato phosphatidase. The enzyme activity was measured by acyl-ester assay and the conditions were the same as described in Fig. 1.

Effect of salts and EDTA on potato phosphatidase activity

The effect of chloride salts of Ca^{2+} and Mg^{2+} as well as of EDTA at various concentrations of reaction mixtures was tested. Result in Table 3 indicated that the enzyme activity at pH 4.5 was not appreciably affected by any of the addities in reaction mixtures.

Effect of surface active agents and fatty acid on potato phosphatidase

The effect of surface active agents, PEG, SLS and triton x–100, on the phosphatidase activity was measured by acyl-estsr assay. Reaction mixtures contained 0.5 ml lecithin (5.5 μ moles) in 0.05 M citrate buffer (pH 4.5), 0.5 ml of partially purified enzyme (0.35 mg protein) or 0.5 ml H_2O and various concentrations of surface active agents and a fatty acid. Results in Table 4

Table 3. *Effect of addities (EDTA and salts) on potato phosphatidase activity*[1]

Treatment	Phosphatidase activity (pH 4.5) (units/mg protein)
1. Control[2]	23.71
2. EDTA (20 mM)	23.46
3. Ca^{2+} (1 mM)	23.82
Ca^{2+} (2 mM)	22.91
Ca^{2+} (1 mM) + EDTA (20 mM)	23.15
4. Mg^{2+} (1 mM)	24.00
Mg^{2+} (10 mM)	23.71
Mg^{2+} (20 mM)	23.71
Mg^{2+} (1 mM) + EDTA (20 mM)	24.00
Mg^{2+} (10 mM) + EDTA (20 mM)	21.71

(1) The enzyme activity was measured by acyl-ester assay as described in Fig. 1, except in reaction mixtures containing 0.5 ml of partially purified enzyme (0.35 mg protein) and 0.5 ml of lecithin (5.5 μ moles) in 0.05 M citrate buffer (pH 4.5) with various concentrations of addities.

(2) Reaction mixture without adding any addities.

illustrated that PEG has no effect on the enzyme activity at the concentrations of 1 to 100 mg in reaction mixtures. However, SLS, triton x-100 and linoleic acid reacted with the substrate and split the acyl-ester bond in the absence of enzyme. It was suggested the results of assay in the presence of SLS, triton x-100 and linoleic acid were not dependable.

Purification of potato phosphatidase

When crude dialyzed enzyme was subjected to ammonium sulfate fractionation, very little protein was obtained from 20% and 80% saturations, however, in 40% and 60% fractions contained the bulk of protein but with very low phosphatidase activity when it was measured by acyl-ester method. On the contrary, both fractions showed strong enzyme activity in cup plate assay. It seems that there may have inhibitor present in these fractions. Attempt was made to remove the inhibitor by dialyzing the fractions against distilled water but failed. However, the unknow inhibitor was able to remove simply by dilution method (Table 5) and through DEAE cellulose column chromatography.

Since 40% $(NH_4)_2SO_4$ saturation contained the bulk of the phosphatidase activity (assayeed by cup plate method), and also has high protein content, thus this fraction was then subjected to DEAE cellulose column (1.6 cm × 21 cm). The phosphatidase activity was recovered as a single peak in the elution volumes between 90 and 135 ml (Fig. 3).

Table 4. *Effect of surface active agents and fatty acid on potato phosphatidase*[1]

Treatment	Acyl-ester content (μ. equiv.)	Phosphatidase activity (units/mg protein)
Lecithin + H_2O	9.90	
Lecithin + H_2O + PEG (1 mg)	9.90	
Lecithin + H_2O + PEG (10 mg)	9.90	
Lecithin + H_2O + PEG (100 mg)	9.90	
Lecithin + enzyme	5.60	24.57
Lecithin + enzyme + PEG (1 mg)	5.60	24.57
Lecithin + enzyme + PEG (10 mg)	5.60	24.57
Lecithin + enzyme + PEG (100 mg)	5.85	23.14
Lecithin + H_2O + triton (5 mg)	5.40	
Lecithin + H_2O + triton (10 mg)	5.50	
Lecithin + enzyme + triton (5 mg)	3.35	37.42
Lecithin + enzyme + triton (10 mg)	4.80	29.14
Lecithin + H_2O + SLS (1 mg)	4.20	
Lecithin + H_2O + SLS (2 mg)	2.65	
Lecithin + enzyme	5.75	23.71
Lecithin + enzyme + SLS (1 mg)	5.00	28.00
Lecithin + enzyme + SLS (2 mg)	3.60	36.00
Lecithin + H_2O + Linoleic acid (2 mM)	4.80	
Lecithin + enzyme + Linoleic acid (2 mM)	5.00	28.00

(1) Reaction mixtures contained 0.5 ml lecithin (5.5 μ moles) in 0.05 M citrate buffer pH 4.5, 0.5 ml H_2O or partially purified enzyme (0.35 mg protein) and various concentrations of surface active agents and a fatty acid. The reaction mixture was incubated at 30°C for 1 hr.

Table 5. *Ammonium sulfate fractionation of potato phosphatidase*[1]

Saturation (%)	Protein content (mg/ml)	Unit[2]	Specific activity (units/mg protein)
20	0.65	6.30	9.69
40	6.05	4.50	0.74
60	6.30	4.07	0.65
80	0.95	4.50	4.74
1:5 dilution of 40% fraction	1.21	2.30	1.90
1:5 dilution of 60% fraction	1.23	2.30	1.80

(1) The enzyme activity was measured by standard acyl-ester method as described in text.

(2) One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1.0 μ equivalent of acyl-ester bond in reaction mixture at 30°C for 1 hr.

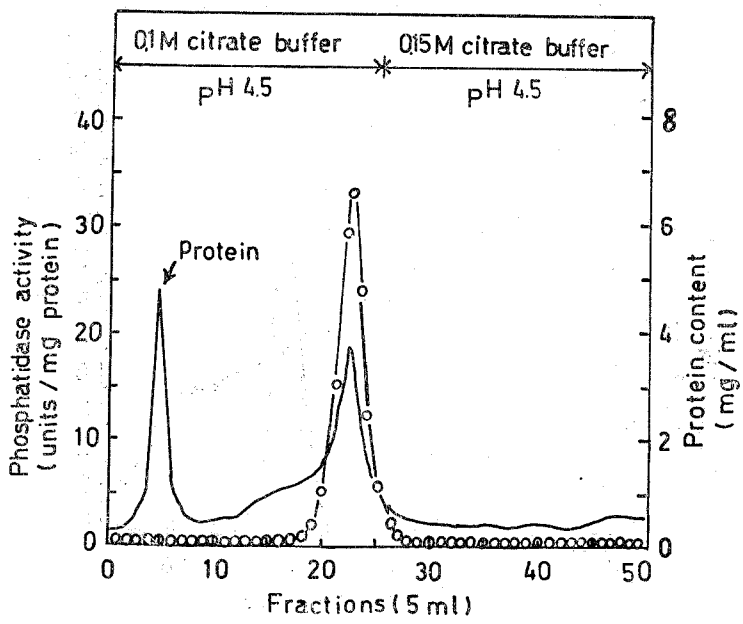

Fig. 3. Purification of potato phosphatidase by DEAE collulose column chromatography. Ten ml of dialyzed enzyme from 40% ammonium sulfate fraction were applied to a column (1.6 cm × 21 cm) at 4°C. Five ml fraction was collected and the enzyme was assayed by standard acyl-ester method.

The peak fractions were pooled and assayed for phosphatidase activity. This partially purified enzyme was dialyzed, and then subjected to isoelectric focusing. Result in Fig. 4 illustrates that only one peak has phosphatidase activity with isoelectric point at 5.0. Summary of the purification of potato phosphatidase followed by ammonium sulfate fractionation, DEAE cellulose column chromatography and isoelectric focusing is showed in Table 6.

Identification of the phosphatidase from potato tuber

It revealed that palmitic acid and linoleic acids were released only in the presence of active enzyme (Fig. 5). These fatty acids represent major fatty acid constituents in lecithin molecule. The fatty acid were identified by their retention times comparing with the chromatograms of known fatty acids methyl esters. Since palmitic and linoleic acids were released, it indicated that the potato phosphatidase was capable of releasing the fatty acids from both the α and β positions of lecithin. In addition, the release of fatty acids from L-α-lecithin was confirmed by determining the decrease in acyl-ester content of reaction mixtures when incubated with the potato phosphatidase. The nonhydrolyzed lecithin in 1.0 ml reaction mixtures consisting of 0.5 ml

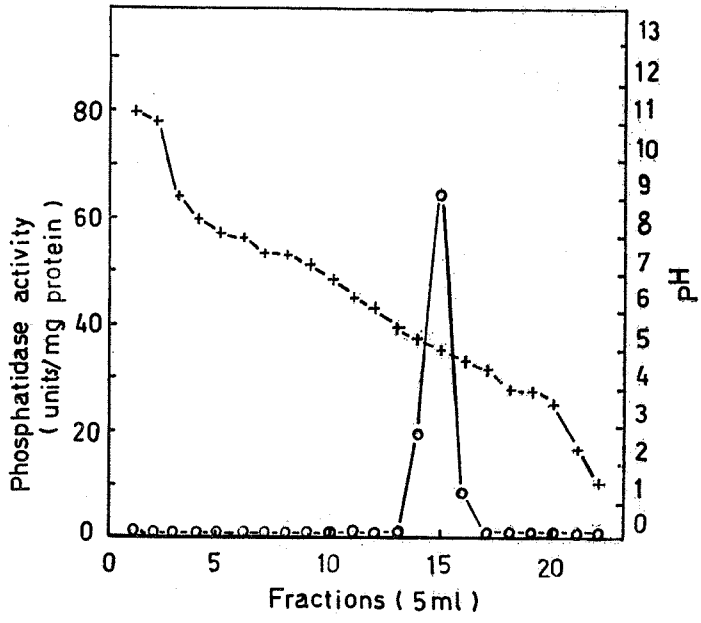

Fig. 4. Isoelectric focusing of potato phosphatidase positive fractions from DEAE cellulose column chromatography (Fig. 3) using ampholine carrier (pH 3.5-10) at 4°C for 48 hrs. Five ml fractions were collected and immediately measured for pH values. After dialyzation at 4°C for 24 hrs, the enzyme activity was measured by standard acyl-ester method.

Table 6. *Purification of phosphatidase from potato tuber*(1)

Stage	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)
Crude extract	425.0	1.19	1.00
40% $(NH_4)_2SO_4$ fraction	60.5	0.74	0.62
DEAE cellulose	14.0	23.25	19.53
Isoelectric focusing	0.5	57.50	48.31

(1) Seven grams of crude lyophilized enzyme (4.25 mg/ml protein) were dissolved in 100 ml distilled water and dialyzed at 4°C for 24 hrs, and then it was brought to 40% saturation with ammonium sulfate. The precipitate from 40% fraction was dissolved in 10 ml distilled water (6.05 mg/ml protein), dialyzed and subjected to DEAE cellulose column. The positive phosphatidase fractions (0.31 mg/ml protein) from the column were pooled, dialyzed and further purified by isoelectric focusing.

enzyme (0.03 mg) and 0.5 ml lecithin (2.75 μ moles) in 0.05 M citrate buffer at pH 4.5 was precipitated by the addition of 0.15 ml 5% bovine serum albumin and 0.5 ml 2% TCA. The precipitate was recovered by centrifugation, and its acyl-ester content was determined by the method of Snyder and Stephens (1959).

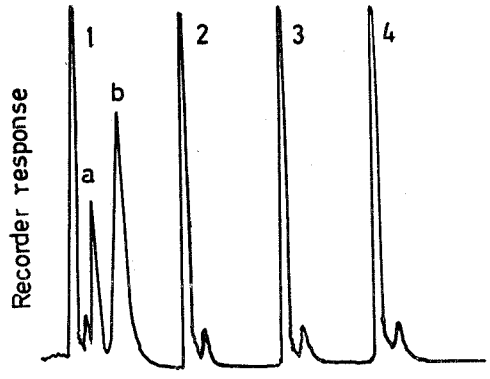

Fig. 5. Profile of the results of gas-liquid chromatographic assay for the release of fatty acids by potato phosphatidase. Reaction mixtures contained 0.5 ml of purified enzyme (0.03 mg/ml protein) and 0.5 ml lecithin (2.75 μ moles) in 0.05 M buffer at 30°C for 1 hr.
(1) Active enzyme at pH 4.5 (3) Active enzyme at pH 8.0
(2) Boiled enzyme at pH 4.5 (4) Boiled enzyme at pH 8.0
a = palmitic acid b = linoleic acid

The supernatants from the above reaction mixtures were assayed for TCA-soluble products released from lecithin by the potato phosphatidase. No evidence was obatined from the release of free choline or phosphorylcholine. Supernatants, which obtained from reaction mixtures containing active enzyme, constituted glycerolphosphorylcholine. Furthermore, the hydrolysis of 5.24 μ eq. of acyl-ester bonds (2.62 μ moles lecithin) in the L-α-lecithin by the potato phosphatidase results in the appearance of the choline in the sample as glycerophosphorylcholine. These results indicate that the potato phosphatidase from 40% $(NH_4)_2SO_4$ fraction was a phosphatidase B.

Discussion

Recently, many attempts were made to isolate and characterize lipid acyl-hydrolases which occur in potato tuber. Our present findings indicate that the phosphatidase activity (assayed by cup plate and acyl-ester methods) found in potato stems and leaves is very low as compared to that from tubers. Since the enzyme prepared from potato tubers exhibits high phosphatidase activity, the enzyme was characterized and purified by ammonium sulfate fractionation, ion exchange column chromatography and isoelectric focusing. It is found the purified enzyme is an acidic protein of pI 5.0 similiar to that reported by Hirayama *et al.* (1975). The enzyme is not affected by the presence of Mg^{2+} or Ca^{2+}, and also not by surface active agents such as PEG, SLS, triton-x and linoleic acid (Table 3 and 4). On the contrary, Galliard (1971) reported that triton-x and various fatty acids can stimulate the acyl

hydrolase activity. The enzyme prepared by Galliard has poor activity in the absence of triton-x or fatty acids, but the enzyme prepared in his laboratory has activity up to 25 (μeq./mg/hr) in the absence of either surface active agents or fatty acid. The difference might be interpreted as being due to the enzymes prepared from different variety of potato tubers.

When crude enzyme preparations from potato tubers were brought to various ammonium sulfate fractions, very little protein precipitated in 20 and 80% fractions was found; however, enzyme activity of both fractions was very high. On the other hand, in 40 and 60% $(NH_4)_2SO_4$ fractions a large amount of protein was precipitated, but very low phosphatidase activity, even lower than crude preparation (Table 5) was detected. These results suggest that there is certain inhibitor like substance present in 40 and 60% $(NH_4)_2SO_4$ fractions. Such inhibitor can not be removed by dialysis, but the inhibition effect can be reduced by simply diluting the enzyme preparation. The inhibitor like substance also can be removed by passing through DEAE cellulose column. The specific enzyme activity increases about 20 times after DEAE cellulose column chromatography. The yield of this partially purified enzyme is about 75% of the protein applied to the column.

Protein assay for the column fractions shows two main peaks (Fig. 3). The first peak emerges from the 4th to 6th fractions has no phosphatidase activity. The second peak, from 20 to 25 fractions, shows positive phosphatidase activity. The protein pooled from fractions 4–6 is called unknown protein temporarily. Further study should be made to understand the characteristics of this protein, which is a new enzyme or inhibitor.

Multiple lipid acyl-hydrolases have been recently demonstrated by Hasson and Laties (1976). Whether the enzyme extracts from potato (*Solanum tuberosum* Var. Ta-Yea) tubers which served for this study also contain multiple types of phosphatidases are now under way to eluciate. In this report, however, we find out the enzyme being isolated and purified from potato tuber is a phosphatidase B.

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馬鈴薯塊莖磷脂分解酵素之純化與鑑定

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自馬鈴薯塊莖分離到一種磷脂分解酵素，該酵素經硫酸銨分劃法，DEAE 纖維色層分析法和等電點分劃法，已將之純化爲 48.31倍，並確定其等電點 pI 爲 5.0。

馬鈴薯磷脂分解酵素之活性不受鉀，鈉，PEG，SLS，triton-x 和 linoleic acid 等化合物之影響，這種純化酵素已被確定爲B型磷脂分解酵素。