キャベツ葉およびStreptomyces chromofuscus起源Phospholipase Dのホスファチジル基転移能の比較

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Transphosphatidylation capacity of phospholipase D from cabbage (*Brassica oleracea* L. var. *capitata* L.) leaves and *Streptomyces chromofuscus*†

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The phospholipase D (PLD) was purified from leaves of cabbage (*Brassica oleracea* L. var. *capitata* L.). The molecular weight of the enzyme was estimated as 87 and 73.5-kDa on SDS-PAGE and gel filtration using Superdex 200 HR 10/30 column. The transphosphatidylation capacity of cabbage PLD was shown to be higher than that of *Streptomyces chromofuscus* PLD. Although cabbage PLD showed a single band, *Streptomyces chromofuscus* PLD gave several bands on nondenaturing and denaturing PAGE. These results indicated that the cabbage PLD was a monomer protein, while the *Streptomyces chromofuscus* PLD was hetero oligomer, having several types with different molecular weights. Furthermore, the cabbage PLD included the duplicated HxKxxxxD catalytic motifs in the molecule, but the *Streptomyces chromofuscus* PLD did not. Previously, YAMANE *et al.* reported that the transphosphatidylation capacity of *Streptomyces* sp. PLD was higher than that of *Streptomyces chromofuscus*. These results indicated that the high degree of the transphosphatidylation capacity of cabbage PLD was due to the existence of HxKxxxxD catalytic motifs.

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In recent years, the development of novel phospholipids was attempted in the oil and fat industries. Especially, phosphatidylcholine (PC) obtained as a by-product during purification of oil and fat or from yolk lecithin is widely used.

Phospholipase D (PLD; EC 3.1.4.4) hydrolyzes phospholipids at the phosphorus diester bond, leading to the formation of phosphatidic acid (PA). Further, the enzyme catalyzes not only degradation of phospholipids, but also transphosphatidylation transferring a phosphatidyl group to primary hydroxyl groups in the presence of primary alcohol. This transphosphatidylation activity makes it possible to synthesize phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylglycerol (PG) from phosphatidylcholine (PC). Since these phospholipids have superior solubility and dispersibility to PC, they are useful for oil and fat manufacturing and pharmaceutical industry.[1)~3)]

YANG *et al.*[4)] and DAWSON[5)] reported that PLD catalyzes transphosphatidylation. They demonstrated that the PLD purified from leaves of cabbage catalyzed the transphosphatidylation of PC. Thereafter, PLD from microorganisms was shown

† Transphosphatidylation capacity of phospholipase D II

to catalyze transphosphatidylation. PLD from genus *Streptomyces* was reported to have superior transphosphatidylation capacity.[6)~11)]

Previously, YAMANE *et al.*[12)] reported that the transphosphatidylation capacity of *Streptomyces chromofuscus* PLD was lower than that of *Streptomyces* sp. PONTING *et al.*[13)] reported that the HxKxxxxD motifs in the amino acid sequences of PLD were involved in phosphatidylalchol synthase activity. We inferred that the low degree of the transphosphatidylation capacity of *Streptomyces chromofuscus* was due to a lacking of duplicated HxKxxxxD catalytic motifs. In this paper, we will discuss the transphosphatidylation capacity of cabbage PLD having HxKxxxxD motifs.

Materials and Methods

1. Materials

Fresh cabbage (*Brassica oleracea* L. var. *capitata* L. cv. Kinsyun) produced in Hokkaido was obtained from a vegetable market. *Streptomyces* (*Str.*) chromofusucus and *Str.* sp. phospholipase D were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lecithin (PC-98S, containing 99.1% PC and 0.4% lysophosphatidylcholine) was obtained from Q.P. Co. (Tokyo, Japan). Mini ProBlott™ membranes were purchased from Applied Biosystems (Foster City, CA, U.S.A.). Coomassie Brilliant Blue (CBB) R-250 was from Fluka Chemie AG (Buchs, Switzerland). All other reagents used were of the highest grade commercially available.

2. Purification of PLD from cabbage leaves

Purification of PLD from cabbage leaves was carried out as previously reported[14)]. Briefly, crude lyophilized acetone powder was prepared from cabbage leaves by heat coagulation and acetone precipitation using the procedure described by DAVIDSON and LONG.[15)] PLD was purified from the precipitate by the methods reported by LAMBRECHT and ULBRICH-HOFMANN[16)] and ABOUSALHAM *et al.*[17)].

3. PLD assay

PLD activity was assayed spectrophotometrically using a modified method by Imamura and HORIUTI[18)]. The amount of free choline generated was estimated colorimetrically, after oxidation to betaine by choline oxidase with the simultaneous production of H_2O_2, which was oxidatively coupled with 4-aminoantipyrine and phenol by peroxidase to give a chromophore with a maximal absorbance at 500nm. Egg yolk lecithin emulsion (1% (w/v) concentration, 100mg of PC-98S in 10mℓ of 40 mM sodium deoxycholate solution) was prepared with a sonicator (model; Sonifier 450, Branson Co., Danbury, CT, U.S.A.) in ice cold conditions. The mixture (0.9mℓ) composed of 0.1mℓ of 20mM Tris-HCl (pH 8.0) containing 40mM $CaCl_2$, 0.7mℓ of distilled water and 0.1mℓ of enzyme solution was preincubated at 37℃. After 5min, the mixture was started the reaction by adding 0.1mℓ of 1% (w/v) lecithin emulsion as a substrate, and then the reaction mixture (1.0mℓ) was incubated at 37 ℃. After 10 min, the reaction mixture was stopped by adding 0.2mℓ of 0.5M Tris-HCl (pH 8.0) containing 50mM EDTA. To this 1.2mℓ of mixture, 0.4 mℓ of distilled water containing 1.5 unit of choline oxidase, 0.2 unit of peroxidase, 1.5 μmol of 4-aminoantipyrine and 2.1 μmol of phenol, was added. The mixture was incubated at 37℃ for 30 min. The mixture was stopped by adding 2mℓ of 1% (w/v) Triton X-100, and the optical density was read at 500nm. As a blank test, 0.1mℓ of distilled water was used instead of the enzyme solution, and the reaction mixture was treated in the same way as described above. The amount of choline liberated was calculated from the absorbance obtained by subtracting the absorbance of the blank from that of the sample, using the standard curve obtained with a known amount of choline chloride. One unit of PLD was defined as the amount of the enzyme that produced 1 μmol of choline/min under the conditions described above.

4. Gel filtration

Gel filtration chromatography was carried out on an AKTA system (model; Purifier 900, Pharmacia, Uppsala, Sweden) equipped with a Superdex 200 HR 10/30 (Pharmacia) column calibrated with thyroglobulin (669-kDa), ferritin

(440-kDa), aldolase (158-kDa), bovine serum albumin (67-kDa), ovalbumin (43-kDa) and ribonuclease A (13.7-kDa) as molecular weight standards (Pharmacia). Proteins were eluted with 30mM PIPES buffer (pH 6.7) containing 50mM $CaCl_2$ and 0.15M NaCl at a constant flow rate of 0.25ml/min.

5. Polyacrylamide gel electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed by the methods described by ORNSTEIN [19] and DAVIS [20]. The slab gel consisted of 7.5% (w/v) acrylamide (pH 8.9) in the resolving phase and 3.5% (w/v) acrylamide (pH 6.8) in the stacking phase and, performed using a discontinuous system. The gel was run at a constant voltage of 80 V for 30 min and then increased to 120V at 4℃. After electrophoresis, the gel was soaked in 2 mM Tris-HCl (pH 8.0) containing 4mM $CaCl_2$. Each lane was cut into serial 1.6mm slices perpendicular to the direction of electrophoretic migration, and then each slice was crushed and extracted with 0.7mℓ of 2mM Tris-HCl (pH 8.0) containing 4 mM $CaCl_2$, left overnight and removed by centrifugation at 4℃. The supernatant were assayed for PLD activity. On the other hand, the gel was briefly stained with 0.25% (w/v) CBB R-250 and destained. Each stained protein band was excised the gel, soaked in 0.125M Tris-HCl (pH 6.8) containing 0.1% (w/v) SDS for 30min in equilibrium, and then the band was crushed and extracted with 0.125M Tris-HCl (pH 6.8) containing 0.1% (w/v) SDS, overnight. The eluted protein was concentrated, and then subjected to reducing SDS-PAGE. SDS-PAGE was performed with a 15% (w/v) acrylamide gel as described by LAEMMLI [21]. Protein samples containing 10mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 20% (v/v) glycerol were boiled for 5 min. After electrophoresis the gels were stained with 0.25% (w/v) CBB R-250 and then destained.

6. Transphosphatidylation reaction and extraction of phospholipids

The mixture (0.9mℓ) composed of 0.1mℓ of 20mM Tris-HCl (*Str. chromofuscus*; pH 8.0, cabbage; pH 7.0) containing 40 mM $CaCl_2$, 0.1 mℓ of enzyme solution and glycerol to the desired concentrations was preincubated at 37℃. After 5min, the mixture the reaction was started by adding 0.1mℓ of 1% (w/v) lecithin emulsion as a substrate, and then the reaction mixture (1.0mℓ) was incubated at 37℃. The reaction was stopped by adding 0.2mℓ of 1N HCl. To this 1.2 mℓ of mixture, 5.0 mℓ of Folch solution (chloroform-methanol, 2:1) and 0.9mℓ of distilled water was added. After mixing well, the solution was centrifuged for 5min at 2500r.p.m. and the lower layer was collected and the extractions were evaporated with a vacuum rotary evaporator. The evaporated samples were dissolved in a proper quantity of chloroform.

7. Phospholipids analysis

Phospholipids analysis was performed by the methods described by JUNEJA *et al.*[22]. Thin layer chromatography with a frame ionization detector (TLC/FID) (model; Iatroscan Analyzer new MK-5, Iatron Laboratories Co., Tokyo, Japan) connected with an integrator (model; Chromatopac C-R6A, Shimadzu Co., Kyoto, Japan) was used to analyze the phospholipids quantitatively in the reaction mixture. The evaporated samples dissolved in a proper quantity of chloroform were spotted on Chromarod S III quartz rods coated with silica gel (Iatron Laboratories, Tokyo, Japan). Just before use, the blank rods were activated by passing them through the flame of the TLC/FID attached to the integrator. Disposable micropipettes were used for spotting. An aliquot (1-3μl) of each sample together with standards of phospholipids were spotted on the chromarods. The spotted rods enclosed in a frame were put into a glass tank having first developing solvent (acetone-acetic acid, 70 : 0.25, v/v). After development, the rods were burned again leaving 20mm from the bottom and developed again with second developing solvent (chloroform-acetone-methanol-acetic acid-distilled water, 6.5:2:1:1:0.3, v/v). After development, the solvent was removed by heating the rods with a hot dryer. The rods were then transferred to the instrument and scanned. The TLC/FID was conducted at a

hydrogen gas flow rate of 160mℓ/min, an air flow rate of 200mℓ/min and a scanning speed of 40 sec/scan. The concentration of PC, PG and PA were estimated from their peak areas with the help of the integrator.

Results

1. Purification of cabbage PLD on chromatography

PLD from cabbage leaves was fractionated by acetone precipitation from crude extract. The precipitate was dissolved in 30mM PIPES buffer (pH 6.2) containing 50mM $CaCl_2$, and subjected to an Octyl Sepharose CL -4 B (Pharmacia) hydrophobic chromatography column equilibrated with the same buffer. The elution was performed by stepwise gradient of $CaCl_2$ concentration. The fraction containing PLD activity was dialyzed against 20mM Tris-HCl buffer (pH 7.5), and applied to Mono Q HR 5/5 (Pharmacia) anion exchange chromatography column equilibrated with same buffer. The absorbed PLD was eluted by a linear gradient of NaCl concentration (0~0.5 M). Elution profiles obtained by chromatography are shown in Fig. 1.

2. Determination of molecular weight on gel filtration

Purified PLD was concentrated to minimum

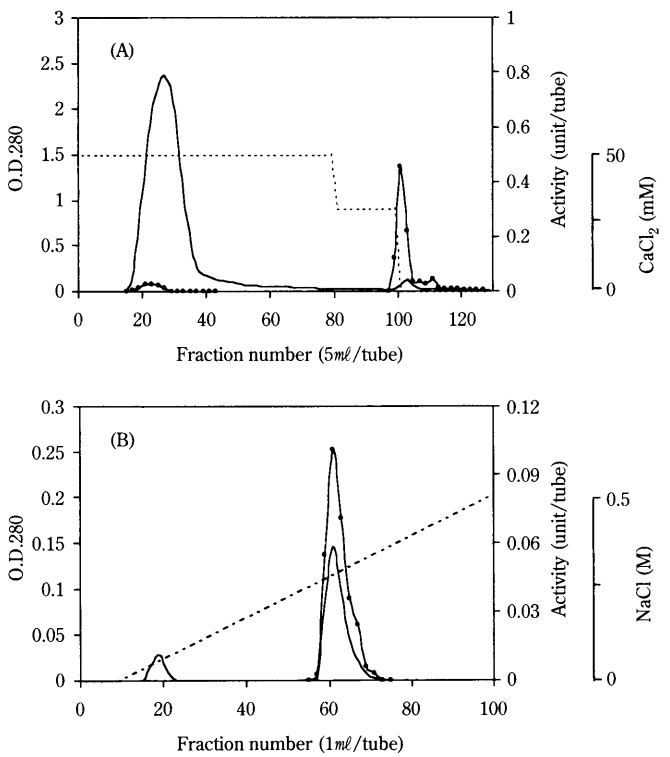

Fig. 1 Elution profiles of PLD from cabbage leaves on Octyl Sepharose CL-4B column and Mono Q HR 5/5 column

(A) The sample was applied to Octyl Sepharose CL-4B column (2.2 × 30cm) equilibrated with 30mM PIPES buffer (pH6.2) containing 50mM $CaCl_2$. Elution was performed with stepwise gradient of $CaCl_2$ concentration.

(B) The sample was applied to Mono Q HR 5/5 column equilibrated with 20mM Tris-HCl buffer(pH7.2). Elution was performed with linear gradient of NaCl concentration.

—,O.D.280; ,Activity; ···,$CaCl_2$ or NaCl

volume by using VIVAPORE 10/20 (Viva Science Limited, Binbrook, U.K.), and applied to Superdex 200 HR 10/30 (Pharmacia) column equilibrated with 30mM PIPES buffer (pH 6.7) containing 50mM $CaCl_2$ and 0.15M NaCl. The elution was performed with a flow rate of 0.25mℓ/min. As a result, the molecular weight of cabbage leaves PLD was estimated as approximately 73.5-kDa by retention volumes relative to protein standards as shown in Fig. 2.

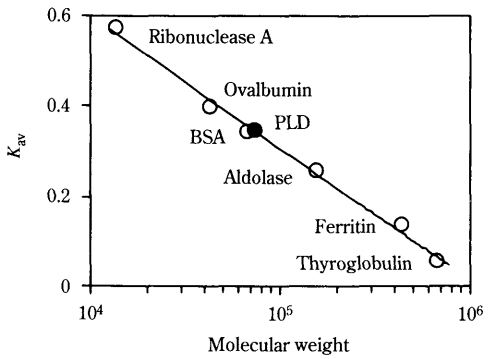

Fig. 2 Calibration of molecular weight of purified PLD from cabbage leaves
Proteins were applied to Superdex 200 HR 10/30 (bed volume, 24 mℓ, V_t) equilibrated with 30 mM PIPESbuffer (pH6.7) containing 50 mM $CaCl_2$ and 0.15 M NaCl. The protein standards gave a linear plot of log molecular weight versus K_{av} $((V_e-V_o)/(V_t-V_o)$, V_o was determined by using blue dextran 2000). Other conditions are described in Materials and Methods.

3. Electrophoretic analysis

Cabbage, *Str.* sp. and *Str. chromofuscus* PLD preparations were subjected to nondenaturing PAGE by the method of ORNSTEIN[19] and DAVIS[20] using 7.5% (w/v) acrylamide gels. As shown in Fig. 1, on nondenaturing PAGE, cabbage and *Str.* sp. PLD demonstrated only one band, While *Str. chromofuscus* PLD gave five protein bands (Fig. 3). On SDS-PAGE, cabbage and *Str.* sp. PLD gave one band with a molecular mass of approximately 87 and 67- kDa, on the other hand, *Str. chromofuscus* PLD yielded five peptide bands with molecular masses of approximately 57, 40, 18, 17 and 14-kDa. The nondenaturing PAGE gel of *Str. chromofuscus* PLD was sliced at intervals of 1.6mm parallel to the protein bands. Protein in the gel slices was extracted into 2mM Tris-HCl buffer (pH 8.0) containing 4mM $CaCl_2$. The PLD activity of each extract was measured. As shown in Fig.

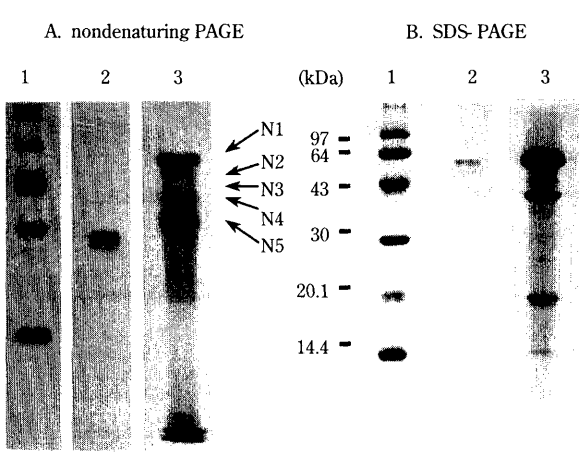

Fig. 3 Comparison of separation patters of PLD from different strain, resolved on nondenaturing PAGE and SDS-PAGE
1; cabbage leaves, 2; *Str.* sp., 3; *Str. chromofuscus*. (A) Each protein was loaded onto 7.5% polyacrylamide gel. (B) Each protein was boiled for 5 min in the presence of 2-mercaptoethanol, and loaded onto 15% SDS-polyacrylamide gel, M; molecular standards (97kDa; phosphorylase b, 64 kDa; bovine serum albumin, 43 kDa; ovalbumin, 30 kDa; carbonic anhydrase, 20.1kDa; trypsin inhibitor, 14.4kDa; α - lactalbumin)

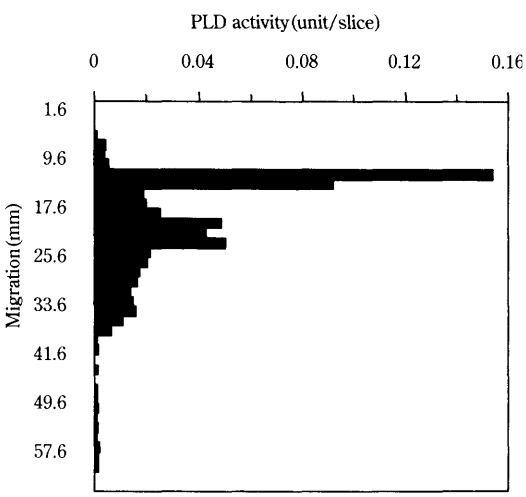

Fig. 4 Nondenaturing PAGE separation of *Str. chromofuscus* PLD
Protein (100 μg/lane) was loaded onto 7.5% (w/v) acrylamide gel. PAGE conditions and method of protein elution were described in Materials and Methods. The gel was sliced (1.6mm) and assayed for PLD activuty.

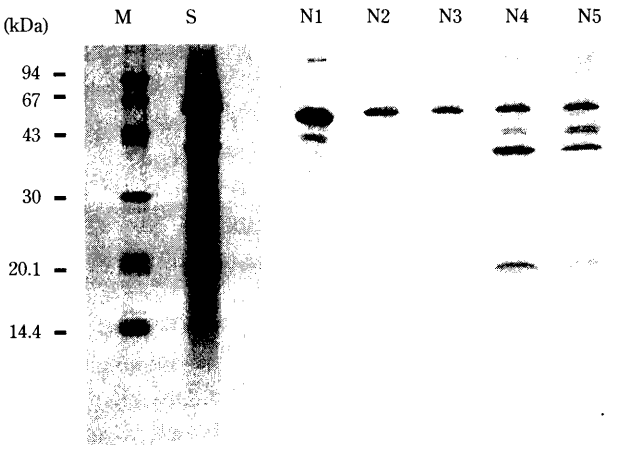

Fig. 5 SDS-PAGE separation of *Str. chromofuscus* PLD and each protein band resolved on nondenaturing PAGE
M; molecular standards (94kDa; phosphorylase b, 67kDa; bovine serum albumin, 43kDa; ovalbumin, 30kDa; carbonic anhydrase, 20.1 kDa; trypsin inhibitor, 14.4kDa; α- lactalbumin), S; *Str. chromofuscus* PLD, N1, N2, N3, N4 and N5 ; proteins, respectively, eluted from gel slices that corresponded to the position of bands on nondenaturing PAGE gel (7.5% acrylamide). Each protein was boiled for 5 min in the presence of 2- mercaptoethanol, and loaded onto 15% (w/v) acrylamide gel.

4, these proteins separated on nondenaturing PAGE demonstrated PLD activity. N1 showed the highest activity. The five native *Str. chromofuscus* PLDs with different molecular sizes, obtained by nondenaturing PAGE, were subjected to SDS-PAGE using 15% (w/v) acrylamide gels. As shown in Fig. 5, these proteins were separated into several polypeptides with molecular masses of 57, 40, 18, 17 and 14-kDa, although the molecular ratios of these polypeptide were different from each other. This result indicated that native *Str. chromofuscus* PLD was formed by association of several polypeptides with different molecular masses.

4. Transphosphatidylation capacity

The transphosphatidylation capacities of cabbage and *Str. chromofuscus* PLD preparations were measured using egg yolk PC as the substrate in the presence of 0, 5, 10, 15, 20 and 30% (v/v) glycerol. PC as a substrate and the PG and PA as products were isolated by TLC. The areas of spots on TLC were measured using TLC/FID, and the transphosphatidylation capacity was calculated from these values. As shown in Fig. 6, the maximum transphosphatidylation capacities of cabbage was more than 90% in 5% (w/v) glycerol, whereas, that of *Str. chromofuscus* PLD was 46.89 % in 20% (w/v) glycerol. These results indicated that cabbage PLD had a high affinity to glycerol and low transphosphatidylation capacity compared with *Str. chromofuscus*.

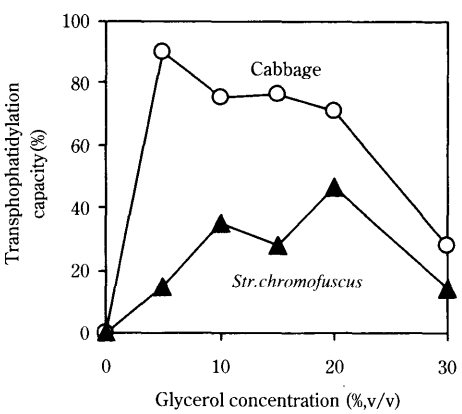

Fig. 6 The transphosphatidylation capacity of PLD from cabbage and *Streptomyces chromofuscus* in the presence of glycerol at various concentrations

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cabbage PLD1     1 MAQHLLHGTLHATIYEVDDLHTGGLRSG-FFGKILANVEETIGVGKGETQLYATIDLQRA  59
cabbage PLD2     1 MAQHLLHGTLHATIYEVDALHTGGLRSAGFLGKIISNVEETIGFGKGETQLYATIDLQKA  60
S. sp.           1 ------------------------------------------------------MPLSQAVA   8
S. chromofuscus  1 ------------------------------------------------------VLAGPLA   7

cabbage PLD1    60 RVGRTRKIKDEAKNPKWYESFHIYCAHLASDIIFTVKDDNPIGATLIGRAYVPVDQVIHG 119
cabbage PLD2    61 RVGRTRKITDEPKNPKWYESFHIYCAHMASDIIFTVKDDNPIGATLIGRAYVPVDEVING 120
S. sp.           9 RS-RRRPVR----T---VLSLALVLAG-ASASATPALAAPPSGASAATAA-VPAAASVAA  58
S. chromofuscus  8 AA---LPAR--------AA--D--QAP-AFLHGVASGDPLPDGVLLWTRV-TPVPEAIPG  50

cabbage PLD1   120 EEVDQWVEILDNDRNPIHGGSKIHVKLQYFGVEADRNWNQGIKSAKFPGVPYTFFSQRQG 179
cabbage PLD2   121 EEVEKWVEILDDDRNPIHGESKIHVKLQYFAVEADRNWNMGVKSAKFPGVPYTFFSQRQG 180
S. sp.          59 P---TGSPAGSPGGSPG-GSPTPHLD----AVEQ-------VLRQVSPGLEGTVWQRTEG 103
S. chromofuscus 51 S----GVGPDTEVGWVV-ARDKAFTD----VVAK--------GSTTARAGSDHTVKADIRG  94

cabbage PLD1   180 CKVSLYQDAHIPDNFVPRIPLAGGKNYEPQRCWEDIFDAISNAQHMIYITGWSVYTEIAL 239
cabbage PLD2   181 CKVSLYQGAHVPDNFVPKIPLAGGKNYEPHRCWEDIFDAITNAKHLIYITGWSVYTEITL 240
S. sp.          104 NAL----DAPAGD---P-----GGWLLQTPGCWGDPSCATRPGSQALLAKMTANIAAATR 151
S. chromofuscus  95 -------LAPATD----------YWFRFTAGGTDSPVARTRTAP-----AADAAVSSLRF 132

cabbage PLD1   240 VRD--SRRPKPGGDVTVGELLKKKASEGVRVLLLVWDDRTSVDVLKKDGLMATHDEETEN 297
cabbage PLD2   241 VRD--SRRPKPGGDMTLGELLKKKATEGVRVLLLVWDDRTSVDVLKKDGLMATHDEDTEN 298
S. sp.          152 TVDISSLAPLPNG---A---FEDAIVAGLKSAVASG-HRLQVRIL--VGAAPLYNITTL-201
S. chromofuscus 133 GVV--SCANWEAG------YFAAYRHLAARNDLDAWLHLGDYIYEYKSGEYAARGTVVR-183

cabbage PLD1   298 FFRGSDVHCILCPRNPDDGGSIVQNLQ--VSAM--FT-HHQKIVVVDSEMPSRGG-SQMR 351
cabbage PLD2   299 YFNGSEVHCVLCPRNPDDGGSIVQNLQ--VSAM--FT-HHQKIVVVDSEVPSQGGGSEMR 353
S. sp.          202 ---PSSYRDELVGKLGDAAGSVTLNVASMTTAKTSFSWNHAKLLVVD-------G----Q 247
S. chromofuscus 184 ---PHAPANEILT-LADYR-TRHAKYK--TDPD--LQGLHLLKAPVVA----------IW 223

cabbage PLD1   352 RIVSFVGGIDLCDGRYDTPFHSLFRTLDTVHHDDFHQPNFTG-AAITKGGPREP-WHDIH 409
cabbage PLD2   354 RIMSFVGGIDLCDGRYDTPFHSLFRTLDTVHHDDFHQPNFTG-ASITKGGPREP-WQDIH 411
S. sp.          248 SVIT--GGINDWKADYLETSH---PVTD----ADL---ALTGPAAATAGRYLDTLWSWT-294
S. chromofuscus 224 DDHEF--ADNAWSG--GAVNH----TEG----AEG---TWSARQAAAKQAYFE--WMPVR 266

cabbage PLD1   410 SRLEGPIAWDVLYNFEQRWSKQGGKDILVKLRELSDIIITPSPVMFQEDHDVWNVQL-FR 468
cabbage PLD2   412 SRLEGPIAWDVLYNFEQRWSKQGGKDILVKLRELSDIIITPSPVMFQEDHDVWNVQL-FR 470
S. sp.          295 CRNSGP--------FSAAWFASSNGAGCLATLEQDSNPASPAATGSLPVIAVGGLGVGIQ 346
S. chromofuscus 267 PAIAGT-------TYRRLRFGKLADLSLLDLRSFRSQQAS-TASGSVDDPDRTLTGR--A 316

cabbage PLD1   469 SIDGGAAAGFPESPEAAAEAGLVSGKDNIIDRSIQDAYIHAIRRAKDFIYIENQYFLGSS 528
cabbage PLD2   471 SIDGGAAAGFPDSPEVAAEAGLVSGKDNVIDRSIQDAYIHAIRRAKDFIYIENQYFLGSS 530
S. sp.          347 SVDP--ASTFQPTP--VNPAGTPATSCGPIK--VPD---HTN-ADRDYAT---------- 386
S. chromofuscus 317 QLDW-----------LKAGLKASDTRWRL--VGN----------SVM---------- 340

cabbage PLD1   529 FAWAADGITPEDINALHLIPKELSLKIVSKIEKGEKFRVYVVVPMWPEGLPESASVQAIL 588
cabbage PLD2   531 FAWAADGITPEDINALHLIPKELSLKIVDKIEKGEKFRVYVVVPMWPEGIPESASVQAIL 590
S. sp.          387 -------VNPEES-AL----RALVASATSHIEISQ----QDLNGTCPP-LPR-------Y 422
S. chromofuscus 341 -------ISPFAVGSLS---ADLLKPLAKLLGLPQEG--IAVNTTQWDGYTD-------- 380

cabbage PLD1   589 DWQRRTMQMMYKDIVQALRAQGLEEDPRNYLTFFCLGNREVKKEGEYEPAERPDADSSYM 648
cabbage PLD2   591 DWQRRTLEMMYKDVTQALRAQGLEEDPRNYLTFFCLGNREVKKEGEYEPAERPDPDTDYM 650
S. sp.          423 D--ARLYDTLAAKLAAGVKVRIVVSDP---------ANRGAVGSGGYSQMK--------- 462
S. chromofuscus 381 D--RRELLAHLRSNAIGNTVFLTGDIHMAW------ANDVPVDAGTYPLSA--------- 423

cabbage PLD1   649 KAQEARRFMIYVHTKMMIVDDEYIIIGSANINQRSMDGARDSEIAMGGYQPHHLSHRQPA 708
cabbage PLD2   651 RAQEARRFMIYVHSKMMIVDDEYIIVGSANINQRSMDGARDSEIAMGGYQPHHLSHRQPA 710
S. sp.          463 ----------SLS---EISDVLLDRIGAATGQDR--AGAKATMCQN--LQ---LAAFRAA 502
S. chromofuscus 424 ----------------SAATEFVVTSVTSDNLD--DIVKVPEGTVSAVA---SP-VIKA 460

cabbage PLD1   709 RGQIHGFRMSLWYEHLGMLDETFLDPSSVECIEKVNRISDKYWDLYSSESLEHDLPGHLL 768
cabbage PLD2   711 RGQVHGFRMSLWYEHLGMLDETFLDPSSLECIEKVNRIADKYWDFYSSESLEHDLPGHLL 770
S. sp.          503 PG-------DTWAD--GH-------P--YALHHKLVSVDGA--AFYLGS--KNLYPAWLQ 540
S. chromofuscus 461 AN-----RHVHWVD----------T----DRHGYGVLDIT---ADRAQ--MDYY----- 490

cabbage PLD1   769 RYPVDVDGEGDVTEFPGFEFFPDTKARILGTKSDYLPPILTT                     810
cabbage PLD2   771 RYPISVDNEGNITELPGFEFFPDSKARILGNKVDYLPPILTT                     812
S. sp.          541 DFGYVTEDQTAAAQLDAQLLAPEWQYSQAAATVDYTRGLCSA                    582
S. chromofuscus 491 ----VLSDRTDANATSAWVRSYRTRSGTQRVERTYDP-V---                    524
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Fig. 7 Alignment of deduced amino acid sequences of PLD from cabbage (two isozymes of PLD1 and PLD2, respectively, designated by PANNENBERG *et al.*)[23], *Streptomyces* sp.[24] and *Streptomyces chromofuscus*[25]. Dashes indicate breaks inserted to maximize algnments using the multiple alignments program Clustal W[26]. The conserved histidine, lysine and aspartic acid residues in the duplicated HxKxxxxD catalytic motifs involving phosphatidylserine synthase and cardiolipin synthase from *Escherichia coli*[13] are indicated by boxes.

Discussion

The PLD was purified from extract of cabbage leaves by acetone precipitate, hydrophobic chromatography and anion-exchange chromatography. The molecular weight of purified enzyme was estimated as 87 and 73.5-kDa on SDS-PAGE and gel filtration, indicating the enzyme was a monomer.

The *Str. chromofuscus* PLD preparation was separated into five protein bands on nondenaturing PAGE. These proteins had PLD activities. SDS-PAGE analysis of these proteins clarified that these were hetero-oligomers formed by association of several polypeptides with noncovalent bonds. These results indicated that the native *Str. chromofuscus* PLD had several types of oligomers comprised of several polypeptide with different molecular weights. The transphosphatidylation capacities of *Str. chromofuscus* and cabbage PLD were compared. The maximum transphosphatidylation capacity and affinity to glycerol of *Str. chromofuscus* PLD was lower than those of cabbage PLD. Previously, YAMANE *et al.*[12] reported that the transphosphatidylation capacity of *Str. chromofuscus* PLD was lower than that of *Str.* sp. PONTING *et al.*[13] reported that the HxKxxxxD motifs in the amino acid sequence of PLD were involved in phosphatidylalchol synthase activity. As shown in Fig. 7, although duplicated HxKxxxxD motifs were observed in sequences of PLD from cabbage[23] and *Str.* sp.[24], they were not in the sequence of *Str. chromofuscus* PLD[25]. These results indicated that the differences in amino acid sequences, especially lack of HxKxxxxD motifs, might be involved in the differences in transphosphatidylation capacity of PLD. It was considered that the cabbage PLD was useful for development of phospholipid due to the high degree of transphosphatidylation capacity.

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キャベツ葉および*Streptomyces chromofuscus*起源*Phospholipase* Dのホスファチジル基転移能の比較

(PhospholipaseDのホスファチジル基転移能に関する研究　第2報)

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本論文では起源の異なるPhospholipase D (PLD)のホスファジル基転移能を比較し、さらにその電気泳動法による解析、そして先に報告された全アミノ酸配列を比較・検討した。

キャベツの葉から精製されたPLDのホスファジル基転移能は*Streptomyces chromofuscus* PLDよりも高かった。

キャベツの葉から精製されたPLDはnondenaturing PAGEおよびSDS-PAGE上で単一であった。しかし、*Streptomyces chromofuscus*のPLDは、nondenaturing PAGEおよびSDS-PAGEにおいていくつかのバンドを与えた。この結果は*Streptomyces chromofuscus* PLD標品にはいくつかのポリペプチドの会合によって形成されたいくつかのヘテロ多量体が存在することを示唆した。

また、先に報告されているキャベツ起源PLDと*Streptomyces chromofuscus* PLDのアミノ酸配列を比較した。キャベツ起源PLDにはN末端より330残基近傍と660残基近傍にPONTINGらによってホスファチジルアルコール合成酵素に関与することが示唆されたHxKxxxxDモチーフの存在が確認された。一方、*Streptomyces chromofuscus* 起源のそれにはHxKxxxxDモチーフに類似した配列が存在していたものの、本モチーフの存在は確認できなかった。

先にYAMANEらは、*Streptomyces*属由来PLDは優れたホスファチジル基転移能を有するものの*Streptomyces chromofuscus*由来PLDを用いた場合、転移反応はほとんど進行しないことを見出した。また、彼らは転移能の高いPLDのアミノ酸配列はそのタンパク質全体において相同性が高いことを報告している。

これらのことから、キャベツ起源PLDのホスファチジル基転移能が*Streptomyces chromofuscus* PLDそのそれより高いことはアミノ酸配列の差異、特にHxKxxxxD モチーフの有無に起因することが考えられた。これらの事実はタンパク工学的手法からの追究により確実に証明されるだろう。さらに、キャベツ起源PLDは、その優れたホスファジル基転移能より、リン脂質の改良に適した試料であると考えられた。

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