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RAPESEED PHOSPHATIDYLCHOLINE HYDROLYSIS TO PHOSPHATIDIC ACID USING PLANT EXTRACTS WITH PHOPSPHOLIPASE D

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Abstract: Phosphatidic acid (PA) has a crucial role in cell membrane structure and function. For that reason it has a possible application in the treatment of some health disorders in humans, can be used as a natural and non toxic emulsifier and the component of drug carriers in pharmaceuticals and cosmetics as well as a component for synthesis of some new phospholipids. PA is short-lived in the cell and is difficult to extract directly from the biological material. PA may be easily prepared by hydrolysis of phospholipids, especially phosphatidylcholine (PC), using cabbage phospholipase D (PLD). Hydrolytic activity of purified by us PLD extracts from cabbage towards rapeseed phosphatidylcholine (RPC) was investigated. Hydrolysis was carried out in the biphasic system (water/diethyl ether) at pH 6,5 and temp 30˚C. Influence of enzymatic extracts from three cabbage varieties, reaction time, Ca^{2+} concentration and enzyme extracts/PC ratio, on activity towards RPC resulting in rapeseed phosphatidic acid (RPA) formation were examined. Our study shows that the PLD extracts from savoy cabbage (PLD_{sc}), white cabbage (PLD_{wc}) and brussels sprouts (PLD_{bs}) used in experiments exhibit hydrolytic activity towards RPC resulting in rapeseed RPA with different yield. The highest activity towards RPC shows PLD extract from PLD_{sc} with the RPC conversion degree to RPA (90%) was observed at 120 mM Ca^{2+} concentration, reaction time 60 min and ratio of PLD extract to RPC 6 : 1 (w/w). Our study shows that purified by us PLD_{sc} extracts exhibit hydrolytic activity towards RPC giving new RPA with satisfying conversion degree for use in pharmacy, cosmetics and as a standard in analytical chemistry.

Keywords: rapeseed phosphatidylcholine , phospholipase D from cabbage, rapeseed phosphatidic acid, phospholipid hydrolysis

Using phospholipases as biocatalysts is an interesting concept in the modification of oxygen and heat sensitive plant phospholipids. Contrary to the chemical modification, enzymatic methods are more safe and distinguished, especially for the products used in pharmaceuticals, cosmetics and food. Enzymes are selective and stereospecific catalysts, so the hydrolysis by them can be carried out under mild conditions, eliminating the use of toxic and harmful solvents, which often are present in a form of residue, when the chemical modifications were used (1). Phosphatidic acid (PA) was first reported as a component of cabbage leaf cytoplasm (2) and is an acid, negative charged phospholipid, the structural component of many cell membranes. PA is generated in animal and plant cells mainly *via* hydrolysis of phospholipids, in particular phosphatidylcholine (PC) by phospholipase D (3-5). In cells PA acts as biosynthesis precursor of many other phospholipids and intracellular lipid second messenger. This presents new opportunities for development of therapeutic approaches relevant to reproduction, metabolism and neurodegenerative disease (6). Rutenberg reported that combination of phosphatidylserine and PA has been shown to have anti-depressant, stress suppressor and mood improver component (7). PA has also been examined as a dietary supplement which enhanced strength, muscle thickness and lean tissue during training program. The ability to augment muscle strength is important for various population groups (elderly people, athletes) (8). Orally administrated PA presents opportunity to prevent nonsteroidal anti-inflammatory drug induced gastric ulcer, and can be considered as a potential antiulcer phospholipid (9, 10). It was also found that formulation composed of PA and α-lactoglobulin is

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inhibitory on bitterness and may be used for practical purposes to inhibit the bitterness of food and drugs (11, 12). PA was also used to stabilize liposomes encapsulating an emulsion nanodroplets used for ultrasonically activated drug delivery (13). A number of studies have revealed a decreasing toxicity of amphotericin B administered in the vesicles, liposomes, micelles and emulsions based on negative charged phospholipids, like PA and phosphatidylglycerol (14, 15). PA monosodium salt was also examined as an additive in cationic arginine glyceride conjugates which were used as cationic surfactants (16). PA can be easily prepared by phospholipids hydrolysis using plant extracts with phospholipase D (PLD). PLD was first isolated from carrot (17), is widespread in plants, animals and microorganisms and is well known for hydrolytic and transphosphatidylation activity towards natural phospholipids and their synthetic analogs. PLD catalyzes hydrolysis of the terminal phosphodiester bond on phospholipid giving PA and free aminoalcohol group. In the presence of an another alcoholic nucleophile, PLD catalyzes exchanging the polar head group, giving a new phosphoester bond (18-20). Many previous studies, using crude and purified, free or immobilized PLD from plant and microbial, showed that this enzyme is capable of hydrolyzing a broad range of phospholipids. However, significant differences of the substrate for PLD may occur, PC is assumed as the best preferable substrate for this enzyme. It is well known that PLD activity depends on source and purity of enzyme, structure and form of phospholipid, concentration of Ca^{2+} and pH level. The most important difference in phospholipid structure (from different sources) is their fatty acids profile, especially length and degree of unsaturation of carbon chain. It is known from previous studies on egg, soybean and synthetic phospholipids that the degree of fatty acids saturation in phospholipids have influence on the PLD activity. PLD from cabbage and Streptomyces sp. are exploited for a number of phospholipid-transforming reactions in the laboratory as well as on industrial scale (21-23). Phospholipids from soya and egg yolk are used as a source of natural PA. Although hydrolytic activity of PLD from different sources in hydrolysis of egg, soybean and synthetic phospholipids is well described in many studies, activity of cabbage PLD extracts in hydrolysis of rapeseed phospholipids has not been reported. Lower sensitivity to oxidation of rapeseed phospholipids compared to soya ones give a chance to use them as a more stable phospholipids derivatives. Furthermore, there is no standards for rapeseed PA, as yet. For that reasons, in our study the hydrolysis of rapeseed phosphatidylcholine (RPC) to rapeseed PA (RPA) in a biphasic system was examined. Purified by us PLD extracts, from three varieties of cabbage were used and the results were compared with standard cabbage phospholipase (PLD_{st}).

MATERIALS AND METHODS

Enzymes and chemicals

PLD_{st} from cabbage (type IV, activity 335 units/mg solid, liberated 1 μmol/h choline from egg at pH 6.5 and temp. 30°C) and egg PA sodium salt (purity 99%) were from Sigma-Aldrich (St. Louis, MO, USA). Solvents of HPLC grade and TLC silica gel G 60 plates were purchased from Merck (Darmstadt, Germany). All other solvents and reagents of analytical grade were from POCH (Gliwice, Poland).

Preparation of rapeseed phosphatidylcholine

Crude commercial rapeseed lecithin, free of erucic acid and glucosinolates (00-type rapeseed), was obtained from the Company Kruszwica SA (Brzeg, Poland). The raw material was deoiled with acetone and fractionated with 95% ethanol, to obtain rapeseed lecithin ethanol soluble fraction, by the method described elsewhere (24). Further purification of lecithin ethanol soluble fraction was performed by column chromatography on aluminum oxide as previously described (25). As a result the RPC with 99% purity (HPLC) was obtained.

Preparation of phospholipase D cabbage extracts

Fresh cabbage varieties: savoy cabbage (*Brassica oleracea* var. *sabauda*), white cabbage (*Brassica oleracea* var. *capitata f. alba*) and brussels sprouts (*Brassica oleracea* var. *gemmifera*), were obtained from the local market. Crude PLD extracts were prepared by the method of Davidson and Long (26). Hundred grams of cabbage leaves or sprouts were homogenized with 200 mL of water at 4°C for 5 min at 4000 rpm (MPV 120 Homogenizer, MPV MED Instruments, Poland). Homogenate was stored at 4°C for 60 min and filtered. After that, the homogenate was centrifuged twice at 4000 rpm for 15 min at 4°C (MPV 325 centrifuge MPV MED Instruments, Poland). The supernatant was heated at 50°C for 5 min, immediately cooled, and centrifuged as described. Finally PLD extracts were freeze-dried at -50°C, 0.03 mBar (Freezone VI, Labconco, USA) to yield a stable pale beige powder.

Cabbage extracts hydrolytic activity

Hydrolytic activities of cabbage PLD extracts towards RPC were compared to activity of standard PLD. Hundred milligrams of RPC was dissolved in 10 mL of diethyl ether and added to the mixture of 0.4 mg PLD_{st} or 400 mg cabbage PLD extract, 40 mM $CaCl_2$, in 10 mL of acetate buffer (pH 5.6), and then incubated at 30°C under stirring for 30 min. Product of hydrolysis was extracted three times with 40 mL of diethyl ether. Extracts were combined, dried with anhydrous sodium sulfate and evaporated to dryness at 40°C under vacuum. The phosphatidylcholine and PA content was analyzed by TLC and HPLC as a 1% w/v solutions in chloroform.

Hydrolysis of rapeseed phosphatidylcholine

Hundred milligrams of RPC was dissolved in 10 mL of diethyl ether and added to the mixture of 200-1000 mg PLD extract from savoy cabbage, 20-160 mM $CaCl_2$ in 10 mL of acetate buffer of pH 5.6. The obtained mixture was then incubated at 30°C under stirring for different times in the range of 15-120 min. Next, the product of hydrolysis was extracted three times by 40 mL of diethyl ether. Extracts were combined, dried with anhydrous sodium sulfate and evaporated to dryness at 40°C under vacuum. The PC and PA content was analyzed by TLC and HPLC as a 1% w/v solutions in chloroform.

TLC analysis

TLC was performed on 20 × 20 cm silica gel plates. Samples of 20 μL of chloroform phospholipids solutions were applied and the plates were developed with chloroform-methanol-water (45 : 25 : 0.2, v/v/v) in saturated glass chamber to 15 cm high. The plates were sprayed with 5% phosphoromolybdate ethanol solution and dried at 105°C.

HPLC analysis

Further calculation of the products was performed by HPLC analysis using a Waters 600 unit, fitted with a UV photodiode array detector Waters 996 PDA and a data processor (Millenium 32, Waters). The chromatograms were evaluated at 205 nm. A 250 × 5 mm column packed with Lichrospher 100-10 (Knauer) was used. Column flow rate was 1 mL/min and the column temperature was maintained at 20°C. Phospholipids were separated by isocratic elution with hexane/2-propanol/water at ratio of 1 : 4 : 1, v/v/v. Samples of 10 μL (1% w/v) were injected in the same solvent used for elution. The peaks were identified on the basis of their retention times using standard phospholipids. PA concentration was calculated as a peak area from the standard curve.

RESULTS AND DISSCUSION

We have characterized the hydrolytic activity of cabbage PLD extracts towards RPC. The partially purified enzyme extracts have been used, and the routine purification has not been continued beyond heat precipitation step. The purified by us cabbage PLD acts readily upon PC (22, 26, 27). Cabbage PLD of the highest purity proved rather unstable and the purification was accompanied by significant reduction of enzyme activity and yield (26-28).

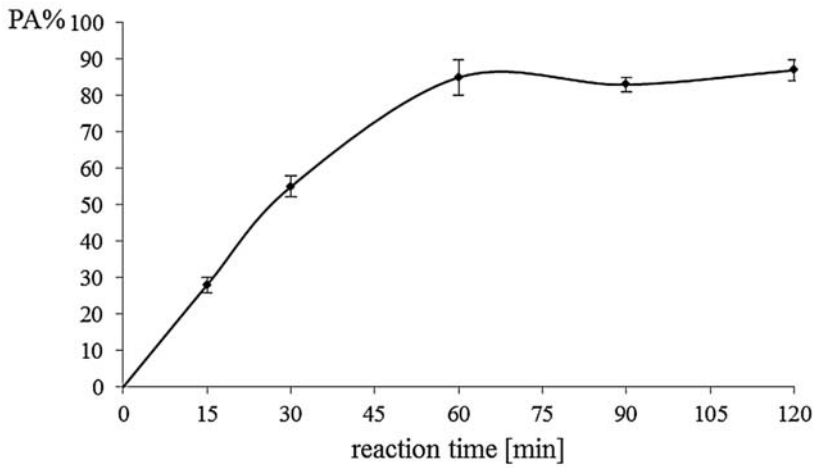

Figure 1. Effect of reaction time on phosphatidic acids concentration (PA%) in hydrolyzed RPC (RPC 100 mg, PLD_{sc} 400 mg, 30°C). The calculations are given as the mean values of triplicate measurements

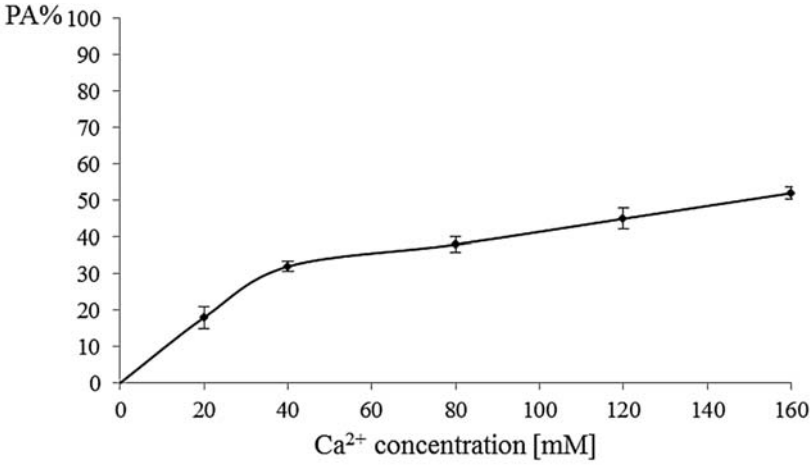

Figure 2. Effect of Ca^{2+} concentration (mM) in reaction mixture on phosphatidic acids concentration (PA%) in hydrolyzed RPC (RPC 100 mg, PLD_{sc} 400 mg, 30°C, 60 min). The calculations are given as the mean values of triplicate measurements

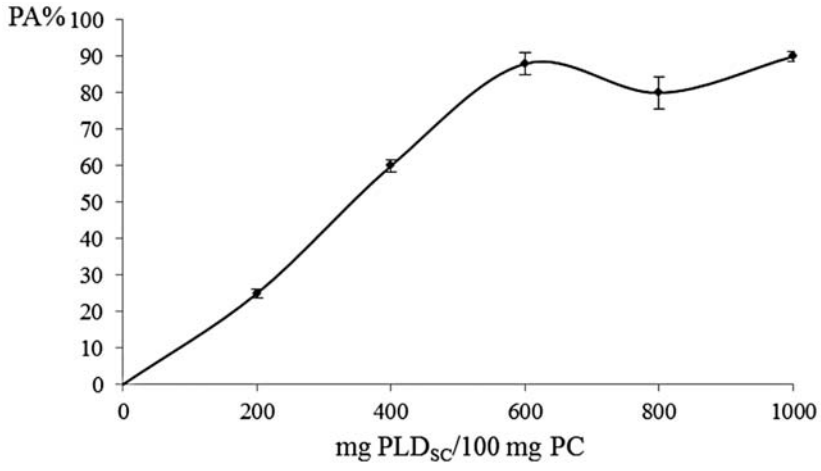

Figure 3. Effect of cabbage phospholipid D extract amount (mg PLD_{sc}/100 mg RPC) on phosphatidic acids concentration (PA%) in hydrolyzed RPC (RPC 100 mg, Ca^{2+} concentration 120 mM, 30°C, 60 min). The calculations are given as the mean values of triplicate measurements

Hydrolytic activity of crude cabbage phospholipase D

Biphasic system with the diethyl ether as a phospholipids solvent was used. Substrate and product differ in their R_f values, for RPC and RPA spots of 0.25 and 0.61, respectively. As shown in Table 1, all of cabbage extracts have PLD hydrolytic activity towards RPC. The highest calculated activity exhibit PLD extract from savoy cabbage (4.17×10^{-3} [mg PA/min × mg PLD extract]). This enzyme extract was used in all the following experiments. The results are in accordance with hydrolytic activity of cabbage PLD towards egg, soya and synthetic PC. Some reports have demonstrated hydrolytic activity of savoy cabbage extracts towards many natural and synthetic phospholipids, especially PC (21, 22, 26, 28) but not of RPC.

Effect of reaction time on rapeseed phoshatidylcholine conversion to rapeseed phosphatidic acid

It is known that the amount of PC hydrolyzed to PA is strongly dependent on physicochemical properties of reaction mixture. Generally, PC is regarded as the best, readily hydrolyzed substrate for cabbage PLD. The reaction time of the RPA formation in our study was examined in the range of 15-120 min. As shown in Figure 1, TPA level is rapidly increased up to 60 min of reaction time, and

Table 1. Hydrolytic activity of phospholipase D cabbage extracts from white cabbage (PLD_{wc}), savoy cabbage (PLD_{sc}), brussels sprouts (PLD_{bs}) compared to standard phospholipase D (PLD_{st}) towards rapeseed phosphatidylcholine (RPC 100 mg). (PA – phosphatidic acid, $CaCl_2$ concentration 40 mM, temperature 30°C, reaction time 30 min).

PLD extract		PA content in the hydrolysis product [mg]	PLD extract activity [mg PA/min × mg PLD extract]
Kind of cabbage	Amount [mg]		
PLD_{wc}	400	45	3.75×10^{-3}
PLD_{sc}	400	50	4.17×10^{-3}
PLD_{bs}	400	28	2.33×10^{-3}
PLD_{st}	0.4	90	7.50

The calculations are given as the mean values of triplicate measurement.

then is almost constant, probably because of PLD inhibition by formed PA. Fast hydrolysis of soya PC by cabbage PLD in biphasic system was also observed in previous studies (29). For the next experiments, 60 min time of hydrolysis was chosen.

Effect of calcium ions concentration on rapeseed phosphatidylcholine hydrolysis to rapeseed phosphatidic acid

PLD is a metal dependent enzyme. Especially Ca^{2+} concentration play a crucial role in plant enzyme activation (30). In present study, Ca^{2+} concentrations were determined in the range from 20 to 160 mM. As shown in Figure 2, the optimum activity of PLD extract from savoy cabbage towards RPC was observed at 120 mM Ca^{2+}. This result is somewhat higher than it was observed for another plant PLD used in other study (30), where used Ca^{2+} concentration was up to 100 mM. It may be due to fatty acid profile and to substrate (RPC) concentration in the reaction mixture. The effective Ca^{2+} concentration as a dependence on substrate amount was previously observed (26). Similar Ca^{2+} concentration (120 mM) was reported in the enzymatic hydrolysis of egg yolk PC by rapeseed phospholipase D extract (31).

Effect of phospholipase D/rapeseed phosphatidylcholine ratio on rapeseed phosphatidylcholine conversion to rapeseed phosphatidic acid

RPA concentration in hydrolyzed RPC in experiments with various enzyme/substrate ratios from 200 to 1000 mg PLD_{sc}/100 mg RPC are shown in Figure 3. From the previous study where cabbage and garlic phospholipase D extracts were used (23) it is known that substrate concentration does not directly affect the hydrolysis rate. But there was observed a strong interdependence of substrate form (monomeric or aggregated forms), their critical concentration in reaction mixture and activation by Ca^{2+} (23, 27). In our study, it is shown (Fig. 3) particularly significant conversion of RPC to RPA observed at enzyme/substrate ratio 600 mg PLD_{sc}/100 mg RPC. Enzyme/RPC ratios over 600 mg PLD_{sc}/100 mg RPC was not significant for RPA content in the hydrolysis product of RPC. Pantazi et al. have observed a significant decrease in the PA amount formed in the hydrolysis of synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine applying in the study a constant amount of enzyme extract and increasing quantities of phospholipids substrate (22).

Lambrecht and Urlich-Hoffman (27) have observed, in the study of short chain egg PC hydrolysis with cabbage phospholipase D, occurrence of the inflection point in the effect of substrate amount on phospholipase D activity. Above this point, phospholipase D activity significantly increased. In our study, where was used RPC, such phenomenon was not observed. This may be caused by differences in the fatty acids profile of egg and RPC. RPC may form in the reaction mixture other forms of phospholipid aggregates, compared to egg phosphatidylcholine (32).

CONCLUSIONS

The hydrolytic activity of cabbage phospholipase D towards RPC has been reported. Our results show PLD_{sc} activity towards rapeseed phosphatidylcholine similar to phosphatidylcholine from egg, soya or synthetic. It is difficult to make a fair comparison, because a lack of information concerning the variety of cabbage used for extracts with phospholipase D preparation and the differences in fatty acids profiles of rapeseed phosphatidylcholine compared to the profiles of substrates phosphatidylcholine used in other studies.

In conclusion, presented study shows that the hydrolysis of RPC to RPA using purified by us PLD_{sc} gives a product, with the satisfactory conversion degree up to 90% and it is not necessary to use expensive, high purity phospholipase D cabbage extracts.

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