Enzymatic Procedures for the Determination of Phospholipids

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Summary: Enzymatic and spectrophotometric procedures are described for the quantitation of phosphorylcholine and phosphatidycholine based on the release of phosphate by acid phosphatase and phospholipase-C respectively. The phosphate produced enzymatically is complexed with molybdate in the presence of hydroquinone, which is monitored at 720 nm. The detection limit for both species was less than 0.01 mM with relative standard deviation of 0.4 - 1.2% in the range investigated. The method is applied to the determination of phosphatidylcholine in rat brain and phospholipase-D activity isolated from *Pistachio khinjuk*.

Introduction

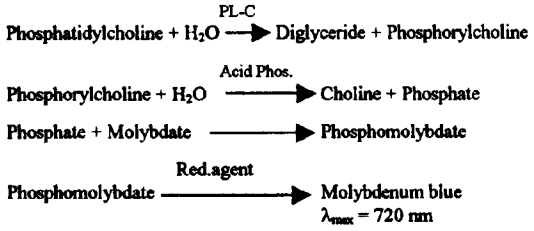
Phosphorylcholine is an important intermediate in the biosynthesis of phosphatidylcholine. Phosphorylcholine is formed in human body as a result of hydrolysis of lecithin and sphingomyelin by lecithinase (PL-C) and sphingomyelinase enzymes, respectively. Lecithin to sphingomyelin ratio in amniotic fluid is routinely determined in clinical laboratories to assess the fetus lung maturity [1]. Though the determination of phosphorylcholine levels for clinical diagnosis is a very rare test. However, this has attracted much attention as a result of increasing interest in phosphatidylcholine metabolism [2].

Phospholipase-D (PL-D, EC 3.1.4,4.) belongs to lypolitic enzymes, which function in a heterogeneous system, hydrolysing water-insolube substrates [3]. In several studies, the adsorption of the enzyme at the interface of lipid and water or oil and water was shown to be a crucial step for the performance of the catalytic reaction. PL-D activity has been identified in mammalians, in plants and in bacteria. A distinguishing feature of the PL-D reaction is the ability of the enzyme to perform "transphosphatidylation" in which a phosphatidyl-alcohol is produced in the presence of simple alcohol [4].

Various methods are available to measure the PL-D activity *via* choline determination by bromothymol blue [5], involving different enzymes such as choline oxidase/peroxidase/catalase [6, 7] and by synthetic substrates of PL-D such as phosphatidyl-*p*-nitrophenol [8]. It can also be determined by conductometric method, e.g., PL-D attacks the switterionic substrates phosphatidylcholine and phosphatidylethanolamine separating the two charges of the molecule to give two independently mobile ionic species [9]. A chemiluminescent flow sensing device for the determination of PL-D activity and / choline in biological samples using choline oxidase and peroxidase are being used in immobilized form and detecting 3.0 μM and 0.417 mIU, respectively, [10]. An electrogenerated luminescent method has also been used to measure choline-containing phospholipids in serum using an optical biosensor based on choline oxidase and oxygen transduction [11].

Phospholipase-C catalyzes the hydrolysis of phosphatidylcholine into diglyceride and phosphoryl-choline. This enzyme also catalyzes the hydrolysis of other phospholipids although less efficiently [12]. The combined action of PL-C and alkaline phosphatase has been utilized for quantitating phosphatidylcholine in assay procedures involving either choline kinase, pyruvate kinase and lactate dehydrogenase or choline oxidase and peroxidase [13, 14]. Chromatographic techniques also give siginificant response for the determination of total phosphatidylcholines in biological fluids. Quantitation of these compounds is performed by using spectrophotometer and by making complexes of phosphatidylcholines with erythrosin B [15].

The procedures reported here involve the reaction of PC with PL-C, releasing diglyceride and PHC. Acid phosphatase that catalyses the hydrolysis of PHC into choline and phosphate. The sequence of enzymatic and spectrophotometric reactions is as follow:



Results and Discussion

Optimization

The system was optimized by changing each variable in turn while keeping the other constant. This approach was adopted as it suits our assay system. The effect of ammonium molybdate (4 - 30 mM) and hydroquinone and (45 - 225 mM) concentrations on the absorbance was examined with in the range using phosphate standard (0.1 mM). The results obtained are shown in Table I. The ammonium molybdate concentration (16 mM) and hydroquinone concentration (135 mM) gave the most suitable response and therefore utilized for all further measurements.

The effect of sulphuric acid concentration over the range 50 - 800 mM on the reduction process was studied. Table I shows that the concentration of sulphuric acid (200 mM) gave highest absorbance which was used subsequently. The response of carbonate-sulfite mixture (13% sodium carbonate and 4% sodium sulfite) gave maximum absorbance and therefore, used in the experiment.

The effect of temperature on the activity of phospholipase-C and acid phosphatase was investigated for PC determination by incubating the reaction mixture in a thermostated water bath for 10 min at various temperatures. The results are shown in Table I. There is an increase in absorbance with an increase in temperature up to 50 °C; however, for all subsequent studies reaction mixtures were maintained at 37 °C.

Calibration Data

Standard solutions of PHC, PC and phosphate were treated according to the procedures described. The calibration graphs for PHC and PC over the range of 0.2 - 1.0 mM and for phosphate over the range of 0.02 - 0.1 mM are shown in Fig. 1 and 2. The correlation coefficient for PHC and PC are 0.9993 and 0.9994 (n=5 for each) with regression equations y=0.097 x - 0.004 and y=0.067 x - 0.0036 [y = absorbance, x = concentration (mM)], respectively. Similarly the correlation coefficient for phosphate is 0.9996 (n=5) and regression equation y=0.92 x + 0.012. The limit of detection for PHC and PC was less than 0.01 mM and for phosphate 0.001 mM with relative standard deviations 0.4 -1.2% over the range investigated.

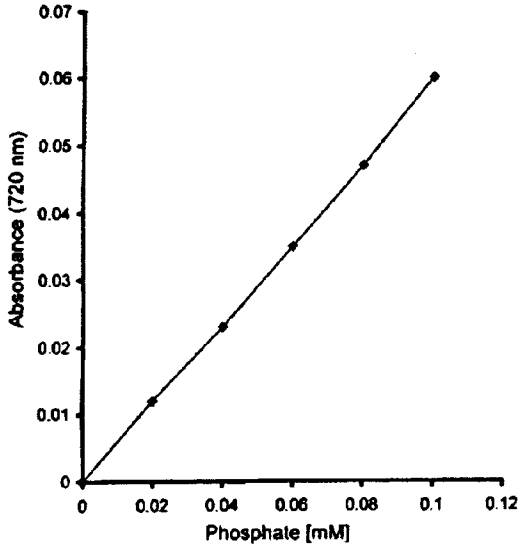


Fig. 1: Calibration graphs for phosphate.

Table I:Effect of variables on the reaction conditions.

Ammonium molybdate (mM)	4.0	8.0	16.0	24.0	30.0
Absorbance*	0.07	0.09	0.13	0.10	0.08
Hydroquinone (mM)	45.0	90.0	135.0	180.0	225.0
Absorbance*	0.09	0.11	0.12	0.10	0.09
Sulphuric acid (mM)	50.0	100.0	200.0	400.0	800.0
Absorbance*	0.04	0.10	0.12	0.09	0.05
Temperature (°C)	20	30	40	50	60
Absorbance*	0.03	0.05	0.06	0.07	0.05

*Mean of three readings.

Table II. Calibration data of PHC using phospholipase-D (from *Pistachia khinjuk*).

Phosphorylcholine (mM)	0.2	0.4	0.6	0.8	1.0
Absorbance*	0.030	0.056	0.083	0.105	0.120
RSD(%)	0.4	0.9	0.7	1.2	1.0

*Mean of three readings.

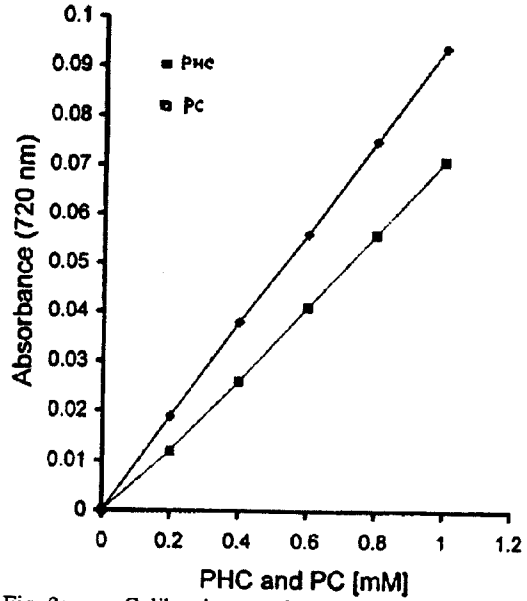


Fig. 2: Calibration graph for PHC and PC.

Application of the procedure

The method is applied for the analysis of PC to real samples (rat brain). The results obtained using this method was 4.30±0.20 μM of PC per brain (CV = 3.40, n = 10). The procedure is also applied to assay PL-D during the isolation from a seed source *(Pistachia khinjuk)*. The results obtained in Table II shows that PL-D isolation is simple and economic to be used for PHC analysis.

Experimental

Reagents and solutions

Phospholipase-C (PL-C; EC 3.1.4.3, ex C. perfringens (C. welchi), Acid phosphatase (AcP; EC 3.1.3.2, ex potato), phosphorylcholine and dimyristoyl phosphatidylcholine (from ox brain or synthetic) were obtained from Sigma (St. Louis, MO). All other chemicals used were analytical grade (E. Merck, Darmstadt, Germany) and distilled/ deionized water was used throughout.

Phospholipase-C (20 units/mg) solution was prepared by dissolving 2.0 mg in 2.0 ml of Tris-HCl buffer (0.001 mM, pH 7.4), stored at 4 °C and used whenever needed.

Acid phosphatase (10 units/mg) solution was prepared by dissolving 4.0 mg in 2.0 ml of acetate buffer (100 mM, pH 5.0), stored at 4 °C and used whenever needed.

Phospatidylcholine solution (5.0 mM) was prepared by dissolving 34 mg in 1.0 ml of chloroform and evaporated at 40 °C. The residue was dissolved in 10 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 0.2% Triton X-100 and 0.4 mM $ZnCl_2$ to give a final concentration of 5.0 mM dimyristoyl phosphatidylcholine (DMPC) and standards were prepared by further dilution with the same buffer.

Phosphorylcholine solution (10 mM) was prepared by dissolving 23 mg of compound in 10 ml of acetate buffer (100 mM, pH 5.0) and standards were prepared by further dilution with the same buffer.

Phosphate solution (10 mM) was prepared by dissolving 0.14 g of potassium dihydrogen phosphate in water and diluted to 100 ml, stored in a polyethylene bottle. Standard solutions were prepared by further dilution.

Ammonium molybdate solution (16 mM) was prepared by dissolving 2.0 g of $(NH_4)_6Mo_7O_{24}.4H_2O$ in 100 ml of water.

Hydroquinone solution (135 mM) was prepared by the addition of 1.5 g of compound in 100 ml of water.

Carbonate-sulfite mixture solution (13:4) was prepared by dissolving 13.0 g of sodium carbonate (anhydrous) and 4.0 g of sodium sulfite in 100 ml of water.

Sulphuric acids solution (200 mM) was prepared by diluting 11.0 ml of concentrated H_2SO_4 in 1 litre of water.

Apparatus

All spectral measurements were carried out on a Hitachi U-1100, uv/visible spectrophotometer, using 10 mm silica cuvettes. A thermostated water bath was used to maintain the temperature during the enzymatic reactions.

Procedure for phosphorylcholine

Standard solution of PHC (0.5 ml) in acetate buffer (100 mM, pH 5.0) was mixed with 0.4 ml of the same buffer and 0.1 ml of acid phosphatase enzyme solution (40 units/2.0 ml) in a tube. The reaction mixture was incubated for 10 min in the thermostated water bath at 37 °C. The phosphate liberated was determined as follows: A 0.5 ml aliquot of the incubated mixture was taken in a tube and 1.25 ml of sulphuric acid followed by 0.5 ml of ammonium molybdate solution and 0.25 ml of hydroquinone solution were added. After 10 min at room temperature, 0.25 ml portion of carbonate-sulfite mixture solution was added and the volume was made upto 3.0 ml with water. The solution was allowed to stand for further 10 min for full colour development and the absorbance was monitored at 720 nm against reagent blank prepared in the same manner without phosphate.

Procedure for phosphatidylcholine

Standard solution of PC (0.5 ml) in tris-HCl buffer (100 mM, pH 7.4 containing 0.4 mM $ZnCl_2$) was mixed with 0.3 ml of the same buffer, 0.1 ml of phospholipase-C (40 units/2.0 ml) and 0.1 ml of acid phosphatase (40 units/2.0 ml) in a tube. The reaction mixture was incubated for 10 min at 37°C in a thermostated water. The phosphate released was determined as described for PHC.

PL-D assay in Pistachia Khinjuk

PL-D catalyzes the hydrolysis ester bond between the phosphate group and choline in the phosphatidylcholine molecule. In this assay procedures phosphorylcholine is used as the PL-D substarte and the results indicate that PL-D can also catalyze the ester bond between phosphate and choline in phosphorylcholine molecule. In this assay procedure, after ammonium sulphate precipitation step, the activity of isolated PL-D was investigated by taking 0.5 ml of phosphorylcholine from each standard (0.2 - 1.0 mM) prepared in acetate buffer (100 mM, pH 5.5 containing 30 mM $CaCl_2.2H_2O$) and it was mixed with 0.4 ml of acetate buffer and 0.1 ml of enzyme extract. The reaction mixture was incubated at 37 °C for 10 min, free phosphate was determined as described for PHC.

Conclusions

Simple spectrophotometric procedures are described for the determination of these important metabolites and for the assay of enzyme. Isolation of PL-D from *Pistachia Khinjuk* and its assay is facile and economic. The detection system based on spectrophotometry can easily be adopted in almost every laboratory. The detection limits are quite sufficient for the quantitation of these metabolites in biological samples, which shows the applicability, and advantages of the methodology developed.

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