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RAPID ESTIMATION OF PHOSPHOLIPED BY IMMOBILIZED PHOSPHOLIPASE D FROM PISTACHIA KHINJUK IN FIA SYSTEM

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ABSTRACT

Blue green gains of Pistachia khinjuk was collected from area of Harnai Balochistan and crude Phospholipase D was isolated by simple protean precipitation. This enzyme was covalently bounded on the surface of controlled porosity glass beads and packed in small glass columns. Immobilized PLD was used for quantitative determination of Phosphatidylcholine (Lecithin) and Phosphorylcholine in flow-injection analysis system. Immobilized columns of Phospholipase C from rice and Choline oxidase from chicken intestine were also prepared in same manner. The phosphatidylcholine was estimated by incorporation PLD column fallowed by choline oxidase column in flow stream. The resultant hydrogen peroxide produced as byproduct in flow injection system was detected amperometrically by electrochemical hydrogen per oxide detector. The Phosphorylcholine was estimated by insertion of PLC column in FIA system and final product was estimated using spectrophotometer at λ Max 640 nm. The isolated PLD showed rapid accuracy for estimation of lecithin and using immobilized PLC fallowed by Alkaline phosphatase found best for Phosphorylcholine estimation.

KEYWORDS: PLD, Pistachia Khinjuk, Harnai, Immobilization, Lecithin, Flow Injection Analysis.

INTRODUCTION

Animals and plants cells are mostly composed of phospholipids [1], "lecithin" which is important phospholipids have phosphatidylcholine, phosphatidylethanolamine and phosphotidylinositol in its composition [2]. In this mixture huge amount of Phosphatidylcholine is present, which on degradation produces choline [3]. Both lecithin and choline are believed to be the vital nutrients for human health as they together support memory function, reproductive system, and liver function, cardiovascular and physical health [4]. Lecithin is known to occur in various food items like egg yolk, milk, meat and legumes [5]. It can be degraded by carrying out its hydrolysis using either chemical or biological procedures. In chemical methods it requires treatment with acids or alkalis are not believed to be much suitable since they have the limitation of producing lecithin without controlled structures; enzyme hydrolysis is therefore considered as more appropriate [6]. Immobilized enzymes have been used as catalysts for the hydrolysis and esterification of lipids for many years. Previous investigators have found phospholipase A, C and D, to be efficient phospholipid degrading enzymes [7-8]. Out of these, phospholipase C (PLC) has the ability to breakdown lecithin into phosphorylcholine which can be converted into choline by Alkaline phosphatase as a catalyst [9]. Phospholipase D (PLD), on the other hand, has been found to be specific in the transformation of lecithin to phosphatidic acid and choline [10, 11] with the subsequent conversion of phosphatidic acid into diacylglycerol that works as a second messenger in human neuronal cell lines [10]. Another enzyme choline oxidase present in bacterium *Arthrobacter pascens*, is capable of catalyzing choline for its conversion into glycine betaine and H_2O_2 [12]**.** Thus PLD if present alongwith choline oxidase, transforms phosphatidylcholine into glycine betaine and H_2O_2 [13].

PLC and PLD widely distributed in vegetables like spinach leaves, carrot roots, celery, and cauliflower and are also found in rice grains, rice bran, wheat and barley [14, 15]. These enzymes can be isolated for their effective use as catalysts for the hydrolysis of phospholipids. Since enzyme degradation of phospholipids produces water soluble hydrolytic products therefore, their quantitative determination becomes quite easy [16]. Methods used for the assay of phospholipids are usually based on chromatographic and spectroscopic techniques like high

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performance liquid chromatography and fourier transform infrared spectroscopy; in addition, mass spectrometry in combination with electro spray ionization and liquid chromatography have also been reported [17-18]. Similarly, estimation procedures for choline are based on the spectrophotometric determination of hydrogen peroxide produced by using choline oxidase [19]. Besides all these time consuming and complex instrumental techniques, flow injection analysis (FIA) systems composed of covalently as well as non-covalently bonded immobilized enzyme reactors have also been used successfully by the previous scientists, for rapid determination of choline and phosphatidylcholine at nano-mole levels [20]. In the present study, a rapid and economical method has therefore been developed for the estimation of lecithin by hydrolyzing it using immobilized PLD from Pistachia khinjuk, PLC from rice, alkaline phosphatase isolated from chicken intestine and choline oxidase from *Alcaligenes* species, covalently bound to controlled porosity glass beads packed in small columns in a flow injection analysis system.

MATERIALS AND METHOD

Material

PL-D [EC 3.1.4.4 13.25 U/mg] from Pistachia khinjuk, PLC [EC 3.1.4.3 28U/mg] from rice were indigenous isolated enzymes. Alkaline phosphates [EC 3.13.17, 6U/mg] from chicken intestine, choline oxidase [EC 1.1.3.17, 10U/mg] from *Alcaligenes* species, phosphatidylcholine from Sigma, (St Lous, MO, USA), glutaraldehyde, controlled porosity glass (pore size (10-1400 A°, 200-400 #) and 3-aminopropyltricthoxysilane were purchased from Sigma Aldrich chemicals. All other chemicals were of analytical grade.

Instrumentation

The flow system consists of a simple peristaltic pump, a Rheodyne injection valve equipped with a 20ul sample loop and an amperomtric detector. Standard size (0.5 mm id) Teflon tubing's were used to assemble the components.

Immobilization procedure

The enzymes used in this study were immobilized on glass according to established procedures [21]. Controlled porosity glass beads were boiled in 5% nitric acid for 30 min, washed with water and dried. The dried beads were derivatized by treatment with 20 ml of a 10% aqueous solution of 3-aninopropyltriethoxysilane at pH 3.45 for 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) for 1 hour at 20°C. The beads were washed thoroughly with water. PLD (100U) isolated from pistachia khinjuk, PLC (100U) isolated from rice, and choline oxidase (50U) was separately immobilized on 0.4 g aliquots of the derivatized glass beads. The immobilization process was carried out by incubating the derivatized glass beads overnight at 4°C with the enzyme dissolved in 0.5 ml of 0.1 M phosphate buffer (pH 6.0). The combined solutions were lyophilized. The protein content of residue was measured according to Lowery et al, to evaluate the yield of the immobilization procedure. The derivatized beads were packed in glass columns (2.5 x 30 mm) plugged with glass wool at both ends. The column was washed with a stream of 0.1 M Tris- HCl buffer (pH 7.5).

Construction of electrochemical detector

The electrochemical detector, which is used for the detection of hydrogen peroxide, released as end product of enzymatic reaction. It consists of flow through glass cell (4.9 mm x 30mm) containing two rectangular platinum electrodes (6x3 mm) Fig.1. The potential of 0.6 V is applied across electrodes. When hydrogen peroxide is reduced at one of platinum electrodes, the current is fed to recorder and the peak height is measured.

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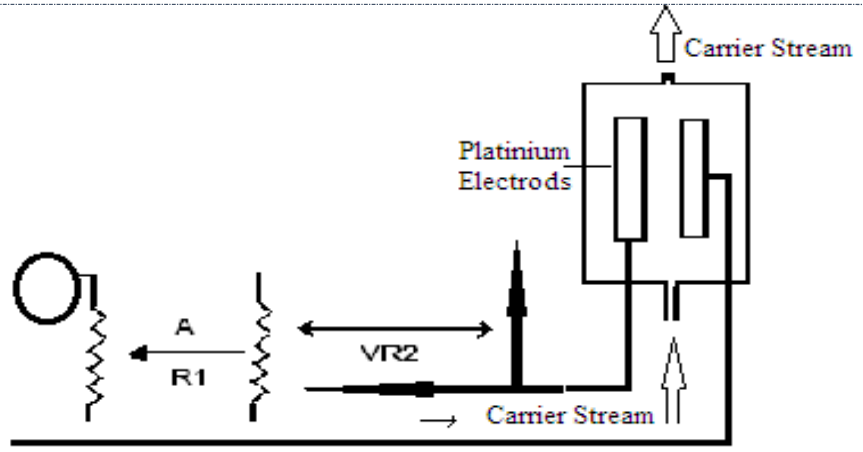

Fig. 1: Electronic circuit of electrochemical cell and flow of carrier stream from cell body

Construction of FIA system for estimation of choline by immobilized choline oxidase

The determination of choline was carried out by using a manifold in which choline oxidase column was inserted between injection valve and detector Fig. 2 having phosphate buffer (0.1 M pH 8) in career stream. The temperature of choline oxidase column was maintained by flowing water across the column in a water jacket from the thermostat.

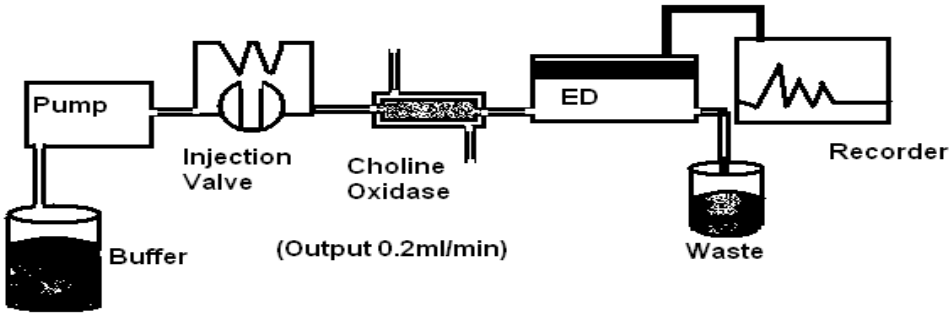

Fig. 2: FIA manifold immobilized choline equipped with oxidase column

A flow injection system was constructed for determination of PLD activity by connecting peristaltic pump, injection valve, and electrochemical detector simply by Teflon tubing having 0.5 mm internal diameter. The PLD and choline oxidase columns were sequentially inserted as shown in Fig. 3, between injection valve and detector for determination of lecithin (phosphatidylcholine).

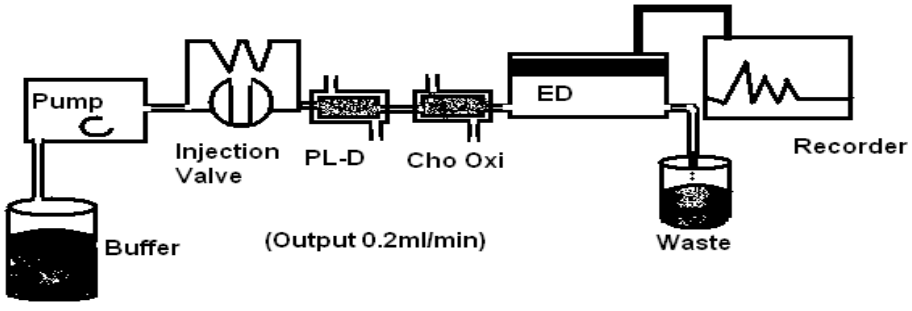

Fig. 3: FIA manifold for estimation of lecithin

Construction of System to estimate the Phosphatidylcholine using immobilized PLC.

Lecithin + H_2O + PLC ⟶ 2 diacylglycerol + phosphorylcholine

Phosphorylcholine ⟶ Alkaline phosphatase Choline + phosphate

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Phosphate + Molybdate ⟶ Phosphomolybdate (λ Max 640 nm)

A FIA manifold was constructed for estimation of lecithin by insertion of immobilized PLC column after injection valve Fig. 4**.** As lecithin passes from PLC column it produces phosphorylcholine. This Phosphorylcholine was treated with liquid Alkaline phosphatase manually which results to liberate inorganic phosphate. The phosphates were analyzed as Phosphomolybdate at λ Max 640 nm.

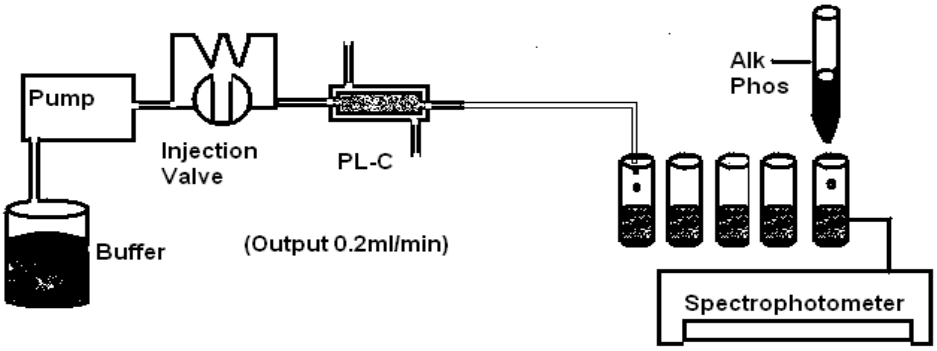

Fig. 4: FIA manifold equipped with immobilized PLC to estimate Phosphatidylcholine & Phosphorylcholine

RESULTS AND DISCUSSION

Yield of cross-linked immobilized enzyme

The controlled porosity glass beads were found a suitable support for the cross linkage of enzymes. After the incubation process as earlier described in the method and material section, it was estimated that about more than 85% of total enzymes were covalently bonded with glutaraldehyde treated glass beads and only 10-12% of the proteins were detected in the supernatant after immobilization process. As once these enzymes linked with the support via glutaraldehyde, and packed in the column having stream of related buffers, this may be used continuously for the product development. If these columns incubated at low temperature with sealed conditions, it may be reused after months or years. The economic feasibility of these immobilized enzymes is ensured by its reusing.

Estimation of hydrogen peroxide by constructed flow injection system

The working of constructed electrochemical cell equipped in FIA System was checked by varying concentrations of H_2O_2. .The standards were prepared in acetate buffer (0.2M, pH 5.6) in the range of 1-10 mM. The system gave a linear response for standard hydrogen per oxide calibration graph and typical recorded output is shown in Fig. 5.

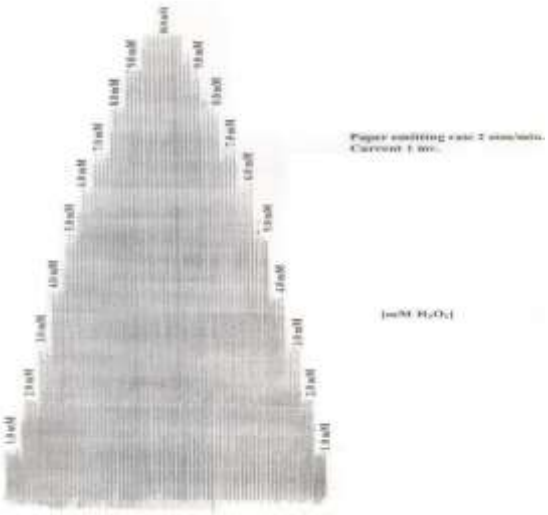

Fig. 5: Recorder output for estimation of Hydrogen peroxide

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Estimation of choline on FIA system by using immobilized Choline Oxidase column

1 -10 mM stander solutions were used to determine the activity of immobilized enzyme and constructed FIA system. As the choline passed through immobilized choline oxidase column, choline is converted into betaine and H_2O_2. The librated hydrogen peroxide was rapidly detected by electrochemical cell, equipped in the system after column as described in the method and material section. It is also revealed that 1.0 mM of choline produce 2 mM of hydrogen peroxide. This system was found more accurate and rapid for the estimation of varying concentration of choline. The recorder out put is shown in the Fig. 6.

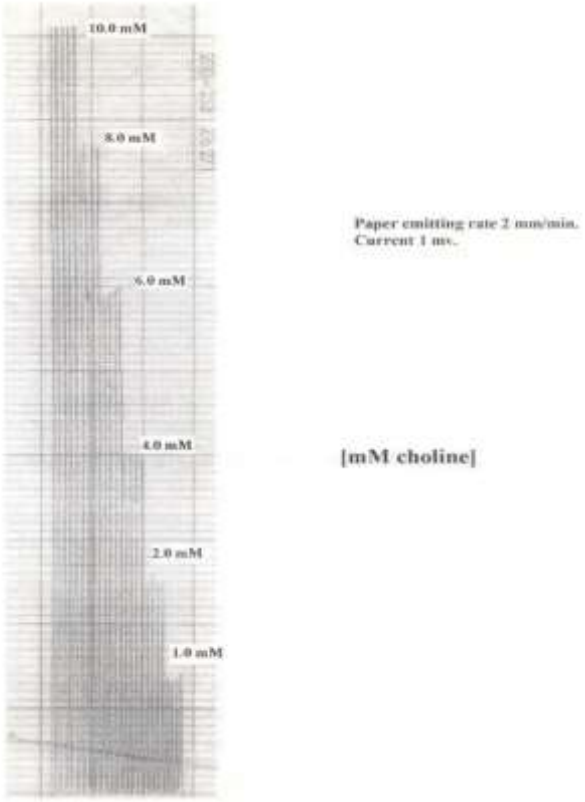

Fig. 6: Calibration graph for choline on FIA system.

Estimation of phosphatidylcholine by immobilized PLD and choline oxidase column

Another FIA system was prepared as described in methods and material section, in which immobilized PLD and Immobilized PLC were used fallowed by the same electrochemical detector. In carrier stream acetate buffer (0.2 mM, pH 6.5) was used. The temperatures of both columns were maintained at 37°C. The standards of lecithin 2-20 mM were injected in carrier stream through injection valve. PLD have the ability to cleave lecithin into choline and phosphatidic acid. When free choline was passed through the column of choline oxidase, H_2O_2 was liberated and detected by electrochemical detector and response was detected electrochemically. The recorder response is shown in Fig. 7.

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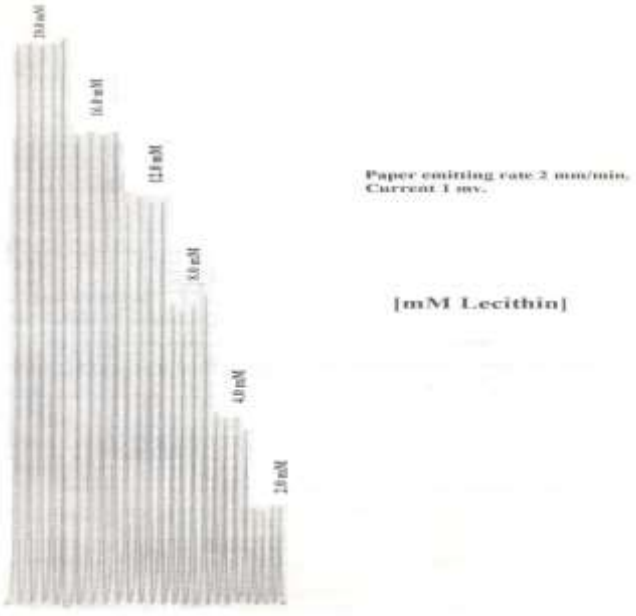

Fig. 7: ***Recorder output for varying concentration of lecithin, catalyzed by immobilized PLD and Choline Oxidase.***

Estimation of Phosphorylcholine by using immobilized phospholipase C

The lecithin was also estimated by using immobilized PLC. In carrier stream Tris- HCl buffer (0.1M, pH 7.3) was used. Substrate solutions were injected into the carrier stream, passed through the immobilized PLC column and collected into the tube. 1.0 ml of carbonate buffer (pH 10.4) and 100 ul (0.5units) of alkaline phosphatase were added and incubated at 37°C for 15 min. the phosphate released was detected by the Phosphomolybdate method the results are shown in Fig. 8.

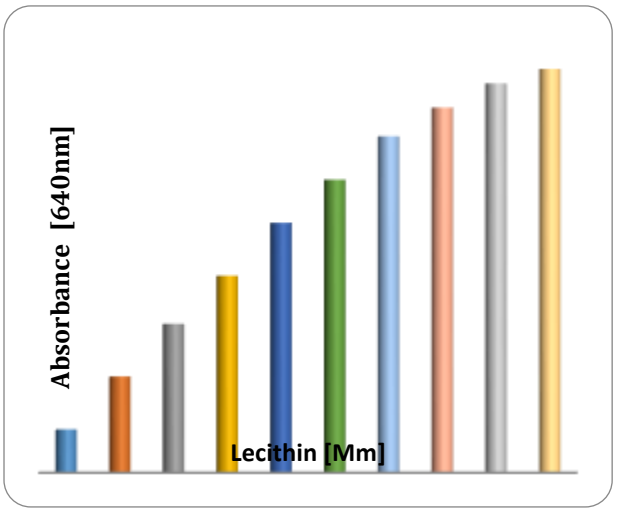

Fig. 8: ***Estimation of Phosphorylcholine and Lecithin with PLC and Alkaline phosphatase***

CONCLUSIONS

In this investigation a novel source of PLD has been identified and partially purified enzyme was immobilized using controlled porosity glass beads as sporting material. The covalently attached PLD, Choline oxidase and PLC were used successfully for the estimation of Phospholipases in flow injection analysis system. The enzymes showed a good response for rapid, accurate and economical estimation of lecithin. Isolated enzyme behaved similarly as previously reported from Soybean, citrus fruits, cabbage and bacterial source [6, 11, and 16]. The manifold prepared for analysis of phosphatidylcholine, choline and Phosphorylcholine have good flow, quick

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response and reproducible. Clinical and industrial applications of these immobilized enzymes are possible for rapid estimation of phospholipids its reuse and rapid estimation. The activity of enzyme may be enhanced by further chromatographic purification steps. The enzyme may also be used for transphosphatidylation of phospholipids.

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