Flow Injection Spectrophotometric Determination of choline using immobilized enzymes

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Summary: A flow injection spectrophotometric method is described for the determination of choline based on the production of hydrogen peroxide from choline using immobilized choline oxidase mini-column. The product is mixed downstream to oxidize iodide into triiodide at an elevated pH in the presence of molybdenum (VI), and the absorbance of the triiodide was monitored at 352 nm. The detection limit is lower than 0.01 mmol/L with relative standard deviation of 1.5% over the range 0.1-1.0 mmol/L. The sample throughput was 40/h.

Introduction

The combination of immobilized enzyme mini-columns with flow systems has emerged recently as a very powerful technique. Solutions carrying analyte can be made to pass through and react with enzyme in the column, the enzyme being retained for use over and over again, while the product is carried to the detector. The advantages of these mini-columns is the rapid termination of reaction and that they can be used with almost any detector system. Very sensitive and selective analytical methods have been reported for hydrolysis and synthesis of important substrates in aqueous and non-aqueous medium [1-13].

In the present work a flow injection spectrophotometer detector for the determination choline is described based on the oxidation of iodide into triiodide by hydrogen perioxide. This hydrogen peroxide is produced by the hydrolysis of choline using immobilized choline oxdase. Choline is enzymatically produced via cleavage of phosphatidylcholine by immobilized phospholipase-D. The sequence of enzymatic and chemical spectrophotometric reactions is as follows:

$$\text{Phosphatidylcholine} + H_2O \xrightarrow{\text{PL-D}} \text{Phosphatidic acid} + \text{choline}$$

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{ChOx}} \text{Betaine} + 2H_2O_2$$

$$H_2O_2 + 3I^- + 2H^+ \xrightarrow[\lambda max.\ 352\ nm.]{\text{Mo(VI)}} I_3^- + 2H_2O$$

Results and Discussion

Preliminary experiments were carried out to find the pH optimum for the activity of immobilized choline oxidase by using phosphate buffer (0.1 mol/L) of various pH values (7.0-9.0) as a carrier stream. The maximum activity was found at pH 8.0 as reported previously [14] and used for further investigation of conditions (Table-1).

Table-1: Effect of various parameters on the determination of choline.

Potassium iodide (mol/L)	0.01	0.05	0.1	0.15	0.2
Absorbance*	0.01	0.043	0.072	0.07	0.065
Amm.molybdate (mol/L)	0.001	0.0025	0.005	0.0075	0.01
Absorbance*	0.04	0.072	0.095	0.094	0.09
pH(PO_4 buff. 0.1 mol/L)	7.0	7.5	8.0	8.5	9.0
Absorbance*	0.06	0.074	0.085	0.077	0.054
Temperature (°C)	20	30	40	50	60
Absorbance*	0.05	0.07	0.08	0.085	0.05

*Mean of three readings.

The effect of potassium iodide (0.01-0.2 mol/L) and ammonium molybdate (0.001-0.01 mol/L) concentrations in phosphate buffer (0.1 mol/L, pH 8.0) on the oxidation process were studied by mixing the various concentration in the reservoir. As shown in Table-1, the highest signals of absorbance were obtained with 0.1 mol/L potassium iodide and 0.005 mol/L ammonium molybdate. The addition of ammonium molybdate to buffered iodide solution after the addition of hydrogen peroxide catalyzes their reaction and results in rapid conversion of iodide ion to triiodide.

The effect of flow rate of the buffer stream and reagent stream on the peak absorbance was also

characterized over the range 0.3-2.0 ml/min. At a flow rate of 0.3 ml/min for buffer stream, maximum response (peak height) was observed with a long residence time of the substrate within the immobilized column. Due to this drawback, a flow rate of 0.5 ml/min was selected. In the line of reagent stream a gradual increase in the peak absorbance was observed at a flow rate upto 1.0 ml/min and was therefore used throughout the experiment.

The effect of temperature on the activity of immobilized enzyme column was studied over the range 20-60°C. There was an increase in peak absorbance with increase in temperature upto 50°C (Table-1). The column was maintained at 30°C to protect the enzyme from denaturation and to increase the life time of the enzyme column. The effect of mixing coil length was also calibrated to obtain the best overall response of the system in terms of sensitivity. Optimum peak absorbance was obtained when using a mixing coil length of 30 cm and selected for further studies.

Calibration data

From the stock solution of choline, a series of standard solutions were treated under the optimum conditions. The calibration graph shown in Fig. 1, is linear covering the range 0.2-1.0 mmol/L. The correlation coefficient for choline is 0.9991 (n=6) with regression equation y=0.013x + 0.044[y=Abs; x=Conc. (mmol/L)]. The limit of detection is lower than 0.01 mmol/L, with relative standard deviation generally 1.5% over the range investigated and the sample throughout is 40/h. For the determination of hydrogen peroxide, standards were prepared in phosphate bufer (0.1 mmol/L, pH 8.0) and treated according to the method described for choline without utilizing immobilized column. Fig. 2 shows a linear calibration graph over the range 0.02-0.1 mmol/L, having a correlation coefficient of 0.9992 (n=5) with regression equation y=0.0006x + 2.021. The calibration data of hydrogen peroxide, choline and PC are given in Table-2.

Analysis of PC Via PL-D and choline oxidase

Phospholipase-D catalyzes the phosphatidyl-choline to phosphatidic acid and choline. As choline is liberated, the PL-D is linked with the system used for choline to determine the phosphatidylcholine. For the purpose, phospholipase-D (PL-D; EC 2.1.4.4, ex. streptomyces species) was immobilized (30 units/0.5 g of CPG), packed in a column (2.5 x 30 mm) and incorporated into the manifold used for choline determination prior to the choline oxidase column (Fig. 1). The immobilized PL-D column was utilized for about 300 h without any change in their activity and the enzyme activity was completely preserved for three months storage at 4°C. The buffer stream comprised sodium diethylbarbitone buffer (20

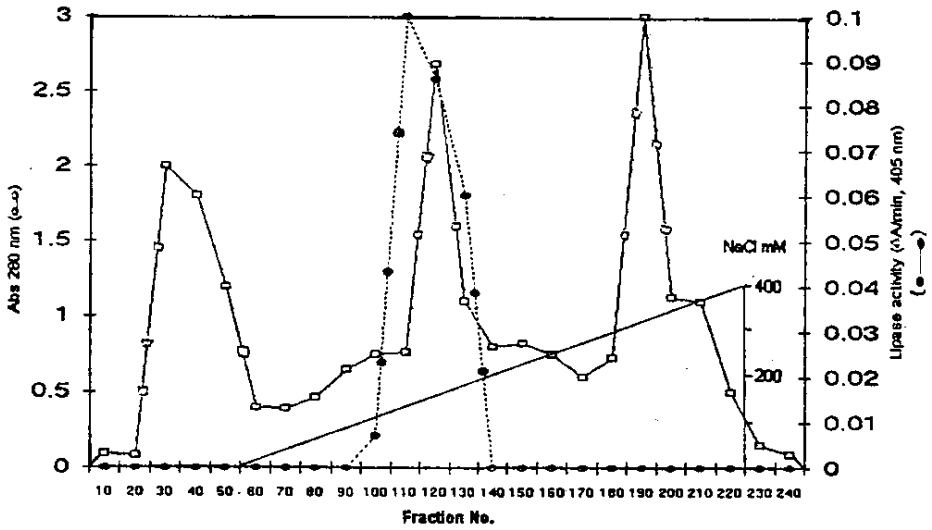

Fig. 1: Calibration graphs for choline and phosphatidylcholine determination.

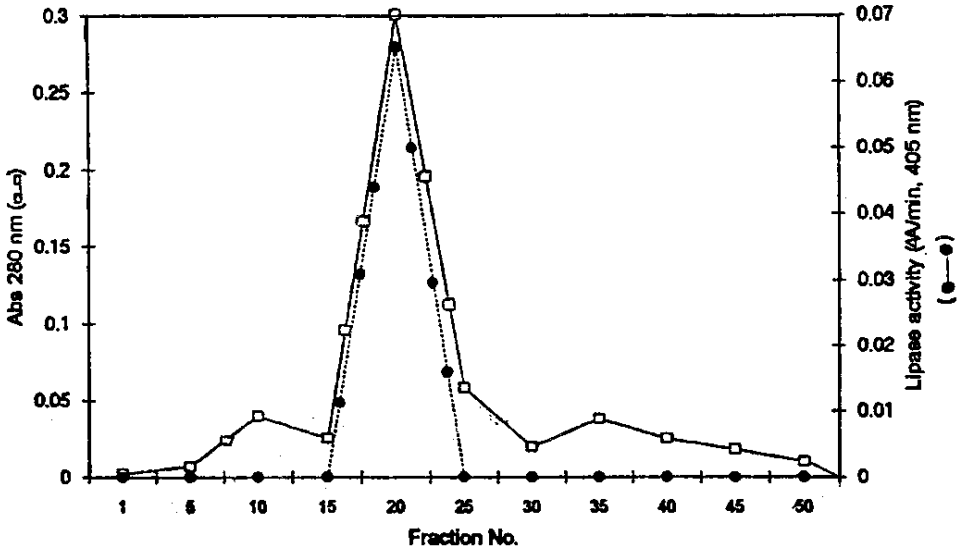

Fig. 2: Calibration graph for hydrogen peroxide determination.

mmol/L, pH 6.5), 0.3% Triton X-100 and 30 mmol/L in calcium chloride dihydrated, previously optimized [15]. PC stock solution (10 mmol/L) was prepared by dissolving the required amount of PC in 1 ml of chloroform and evaporated the solvent on a rotary evaporator and residue was dissolved in barbitone buffer and fresh standards solutions (0.2-1.0 mmol/L) prepared in the carrier buffer were injected. The calibration graph shown in Fig. 2, was linear in the range investigated. The method was applied to the determination of egg yolk PC obtained by acetone precipitation [16] and the results were compared with the analysis carried out by the enzymatic-amperometric detection [2]. The value PC in egg yolk (% by weight) obtained was 30.2 ± 1.2 (n=6) with this method as compared to 32.4 ± 1.0 (n=6) when analyzed by the enzymatic-amperometric system.

Experimental

Choline oxidase (ChOx, EC 1.1.3.17, ex. alcaligenes species), choline hydroxide, dimyristoyl phosphatidylcholine (PC from ox brain), Triton X-100, controlled pore glass (CPG, 120-200, pore diameter 116°A), 3-aminopropytriethoxysilane were obtained from Sigma chemical Co. (St. Louis, MO, USA). All other chemical were of analytical reagent grade (Merck, BDH) and deionized/distilled was used throughout.

Table-2: Calibration data for spectrophotometric determination of hydrogen peroxide, choline and PC.

Hydrogen Peroxide					
Concentration (mmol/L)	0.02	0.04	0.06	0.08	0.10
Absorbance*	0.018	0.035	0.056	0.076	0.096
RSD (%)	0.85	0.63	0.94	1.00	1.22
Choline					
Concentration (mmol/L)	0.2	0.4	0.6	0.8	1.0
Absorbance*	0.011	0.021	0.034	0.042	0.055
RSD (%)	1.20	0.98	1.42	1.56	1.58
PC					
Concentration (mmol/L)	0.2	0.4	0.6	0.8	1.0
Absorbance*	0.007	0.016	0.025	0.033	0.038
RSD (%)	1.50	1.48	2.00	1.96	2.20

*Mean of three readings.

Choline stock solution (1.0 mol/L) was prepared from a 45% choline hydroxide in methanol by taking an appropriate volume of choline hydroxide in a tube and evaporated on a rotary evaporator at 40°C. The residue was dissolved in phosphate buffer (0.1 mol/L, pH 8.0). Fresh standards of choline were prepared by appropriate dilution with the same buffer.

Iodide solution (0.1 mol/L) was prepared by dissolving oven dried potassium iodide (1.66 g) in phosphate buffer (0.1 mol/L, pH 8.0) containing 0.62 g of $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.005 mol/L).

Hydrogen peroxide stock solution (0.1 mol/L) was prepared from commercially available hydrogen peroxide (30%, w/v, 9.8 mol) by taking 1.0 ml and diluting it to 100 ml with water. Standards were prepared in phosphate buffer when required.

Immobilization procedure

Choline oxidase (28 units) was immobilized on 0.5 g of controlled pore glass (CPG) by cross-linking with glutaraldehyde, following the procedure described previously [14]. The immobilization was carried out by incubating the glass beads overnight at 4°C with the enzyme dissolved in 0.5 ml of phosphate buffer (0.1 mol/L, pH 6.0). After immobilization, the aqueous phase was measured for protein contents according to the reported method [17] to evaluate the yield of the immobilization procedure. The immobilized choline oxidase was packed in a glass column (2.5 x 30 mm) plugged with glasswool at both ends, washed with a stream of phosphate buffer (0.1 mol/L, pH 8.0) and used as needed. The immobilized enzyme column was utilized for about 280 h without any changes in their activity at 30°C by flowing water through a water jacket around the column and the enzyme activity was completely preserved for three months storage in phosphate buffer (0.1 mol/L, pH 8.0) at 4°C.

Apparatus and procedure

The flow injection manifold shown in Fig. 3 was used. The activity of the immobilized choline oxidase was characterized by incorporating the packed column in the flow manifold. A stream of phosphate buffer (0.1 mol/L, pH 8.0) was made to flow through the system at a flow rate of 0.5 ml/min using a peristaltic pump (Ismatec Reglo 100). Teflon tubing (0.5 mm, i.d.) was used throughout the remainder of the system. Aliquots of choline (1 mmol/L) were injected to pass through a packed column using a rotary injection valve (Rheodyne 5020, 30 µl sample loop). The hydrogen peroxide produced enzymatically merged with another stream of phosphate buffer (0.1 mol/L, pH 8.0) at a flow rate of 1.0 ml/min containing potassium iodide and ammonium molybdate at concentration levels 0.1 and 0.005 mol/L respectively. The absorbance of triiodide was monitored at 352 nm using a spectrophotometer (Hitachi U-1100) with a flow through cell 30 µl, connected to a chart recorder (Kipp & Zonen BD 40).

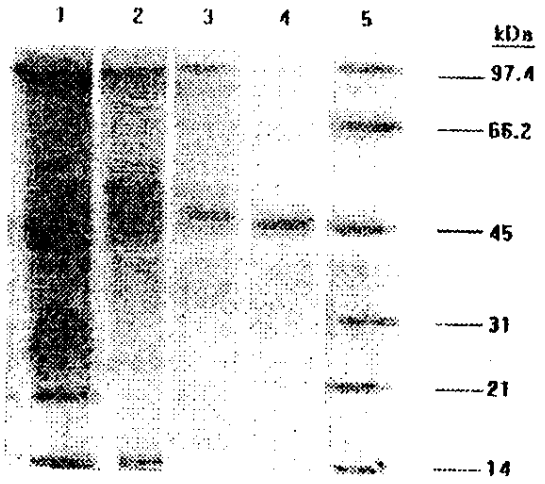

Fig. 3: FI-Sepctrophotometric manifold for the determination of choline.

Conclusion

The use of spectrophotometer in conjunction with a flow injection system using immobilized enzymes provides a sensitive and selective procedure for the determination of choline and phosphatidylcholine as compared with the previously reported results [15]. The limit of detection for choline is improved at 100 fold compared to the enzymatic amperometric procedure. The immobilized enzyme columns used online further added to the stability and reliability of FI-spectrophotometer system.

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