Biocatalysis and Agricultural Biotechnology 12 (2017) 142–147

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Phosphatidylcholine determination in dietary supplement by coupled enzymes immobilized in a single bioreactor

Anna Maria Girelli*, Azzurra Apriceno, Gloria Esposito

Department of Chemistry, Sapienza University of Rome, P.le A.Moro5, 00185 Rome, Italy

ARTICLE INFO

Keywords:
Dietary supplement
Enzymes immobilized
Phosphatidylcholine
Phospholipase C

ABSTRACT

A rapid and selective method for the determination of phosphatidylcholine (PC) is described. It is based on the use of a single packed bed reactor in which phospholipase C, alkaline phosphatase and choline oxidase are co-immobilized on long chain-alkylamino controlled pore glass. The detection of hydrogen peroxide, that is the final product of subsequent enzymatic reactions occurring within the bioreactor, is used for the determination of PC in a dietary supplement. The results of triplicate analysis show a coefficient of variation of 5%. The calibration curve is linear over a concentration range of 10–60 mg/L (R^2 = 0.942) and the detection limit is 4.25 mg/L. The bioreactor resulted to be stable for at least 1 month.

1. Introduction

Phosphatidylcholine (PC) represents the phosphorous portion of lecithin which is the most important natural phospholipid in animal tissues and plants. PC is rich of choline, a member of the B-vitamin complex, that can be employed by the body to produce the neurotransmitter acetylcholine which is useful for different diseases such as Alzheimer and memory loss (Kumar Singhal et al., 2012). Since PC is a surprisingly large array of dairy foods and also present in cosmetic and pharmaceutical products (Shurtleff and Aoyagi, 2007; Van Hoogevest and Wendel, 2014), the level that could be achieved in the human body through the additional intake of food supplements is certainly too high. It is well known that it is not toxic nor mutagenic but, if ingested in high level, it can cause side effects like gastrointestinal problems and heart-rhythm abnormality. In addition, it is unapproved by many countries when subcutaneously injected, as reported in the resolution on January 9, 2003, published by the Brazilian National Agency of Health Inspection (ANVISA) (Resolution 30, 2003). For all these reasons, the medical community is actually studying the action and effects of this phospholipid on the human health.

Chromatographic and spectrophotometric analysis are generally used for phosphatidylcholine determination. In the past, the analytical procedures were based on the separation of phospholipids by thin-layer chromatography (Moore, 1982; Touchstone et al., 1980) and their quantification was carried out by densitometry (Murray et al., 2007) or through the determination of phosphorus amount in each phospholipid (Tomassetti et al., 1984). Better results have been obtained by high performance liquid chromatography (Seri al., 2010) even if the analytical sensitivity depends largely on the detection method: e.g., when the ultraviolet-visible detector is employed, the measured values are affected by the different composition in fatty acids particularly if the unsaturated fatty acids are present. For all the above cited procedures, an extraction step with organic solvents is often required, thus affecting the precision of the analytical method. In addition, the separation of PC from other compounds is very tedious, and requires either a large amount of organic solvent and sometimes highly sophisticated instrumentation (Jangle et al., 2013). Spectrophotometric methods are instead simpler since the lipid extraction is not required. They are mainly based on the organic phosphate determination or on the formation of stable complexes between PC and different complexants (Yoshida et al., 1979). However, the interference of stable complexes as well as the formation of complexes with species different from PC could represent some drawbacks. ^{31}P-nuclear magnetic resonance is an alternative approach used since it is very selective: however, it is not very sensitive (Spyro and Dais, 2009) as well as it is very expensive and requires highly qualified operators.

Some enzymatic approaches have been reported in the literature for determination of phosphatidylcholine, sphingomyelin and phosphatidylglycerol in the biological fluid (Encinar et al., 1996; Hidaka et al., 2008) and in food samples (BoixMontaand Fibrega,1997) without any extraction step. The high costs, the impossibility of reuse and the instability of free forms, however, restrict the use of enzymes.

Therefore, the immobilization of enzymes onto an insoluble matrix is desirable in order to develop a powerful biocatalyst in a reusable form without loss of activity.

The combination of immobilized enzymes in column reactor (IMER)

* Corresponding author.
E-mail address: annamaria.girelli@uniroma1.it (A. Maria Girelli).

http://dx.doi.org/10.1016/j.bcab.2017.09.008
Received 21 February 2017; Received in revised form 11 August 2017; Accepted 10 September 2017
Available online 29 September 2017

with flow system is a very powerful technique. The analytes's solutions pass through the column and interact with the retained enzyme which can then be used several times, while the product can be easily separated. Further advantages are the high reaction rate and the possibility to couple different detection systems. In literature two different approaches are reported for phosphatidylcholine determination and they are based on the use of phospholipase D or phospholipase C. Phospholipase D, which catalyses the formation of choline and phosphatidic acid, is usually immobilized in a bioreactor coupled in series with immobilized choline oxidase (Masoom, 1988) or choline oxidase-based amperometric biosensor (Pati et al., 2005). When phospholipase C is employed, the product phosphoryl choline must be firstly hydrolysed from alkaline phosphatase and the choline thusformed must be subsequently oxidized by choline oxidase (Amini and McKelvie, 2005; Masoom, 1988; Masoom et al., 1990). In both systems, hydrogen peroxide is the final product, which can be determined by several methods and with different sensitivity (Amini and McKelvie, 2005; Masoom et al., 1990, 1992; Masoom and Yaqoob, 1995).

The aim of this paper is the development of a system in which phospholipase C (PLC), alkaline phosphatase (AP) and choline oxidase (ChO) are covalently co-immobilized on a single IMER through glutaraldehyde activation of long chain-alkylamino controlled pore glass support. In fact, the use of a multi-enzymatic system, in which more enzymes are fixed on the same carrier matrix, often results in an increase of activity allowed by intermediaries that can undergo subsequent enzymatic reactions more rapidly due to the reduced spatial proximity between enzymes.

The IMER, at the optimized experimental conditions, was tested for phosphatidylcholine determination in a dietary supplement through the chromogenic reaction of hydrogen peroxide, enzymatically formed, with 4-aminoantipyrine and 2-hydroxy-3,5-dichlorobenzene-sulfonate.

2. Experimental

2.1. Materials and methods

Phospholipase C (EC 3.1.4.3 from *Bacillus cereus*, 30.5 U/mg $_{solid}$), choline oxidase (EC 1.1.3.17 from *Alcaligenes Species*, 15 U/mg $_{solid}$), alkaline phosphatase (EC 3.1.3.1 from *Escherichia coli* 15.12 U/mg$_{solid}$), horse radish peroxidase (HRP), glutaraldehyde 25% (v/v) aqueous solution, were purchased from Sigma-Aldrich (Milan, Italy). Enzymes were co-immobilized onto long chain alkylamino-controlled pore glass (513 A°, 200–400 mesh particle size) obtained from CPG Inc. (Lincoln Park, NJ, USA). To prepare buffer solutions phosphoric acid (Carlo ErbaReagenti, Milan, Italy), potassium dihydrogenphosphate, dipotassium hydrogenphosphate heptahydrate, boric acid (Sigma) of analysis quality were used. All reagents used were of analytical grade and were as following: hydrogen peroxide (30%w/v), L-α-phosphatidylcholine (from frozen egg yolk, Sigma) p-nitrophenyl phosphate (p-NPP) (disodium salt, hexahydrate, Sigma), p-nitrophenol (NP), 4-aminoantipyrine (AAP), sodium 2-hydroxy-3,5-dichlorobenzene-sulfonate (Sigma).

Water, used to prepare both solutions and eluents, is from Milli-Q-Ultra pure system (Millipore, Bedford, Mass, USA) while 2-propanol was filtered on alumina.

2.2. Immobilization procedure

Phospholipase C (PLC), choline oxidase (ChO)and alkaline phosphatase(AP) were co-immobilized on long chain-alkylamino controlled pore glass (LCA-CPG) with the "in situ" technique (Girelli et al., 2009) as follows: a stainless steel column (3 mm * 40 mm) dry packed with ~ 70 mg LCA- CPG beads was connected to an HPLC pump and the glutaraldehyde solution (2.5%) in 0.05 M phosphate buffer solution pH 7 was recycled through the column at a flow rate of 0.2 mL/min for 2.5 h. The column was then washed with deionized water for 2 h at 0.2 mL/min. Afterwards the column was thermostated at 15 °C in a tailor made glass water jacket connected to a cryo-thermostat Regoterm3000 (ISCO, Mila, Italy) and the solution containing PLC, AP and ChO in phosphate buffer 0.1 M pH 6, maintained at about 4 °C in an icebox, was recycled in the column for 4 h at 0.2 mL/min flow rate. The immobilization procedure was concluded washing the bioreactor with phosphate buffer 0.1 M pH 7 for 1.5 h at a flow rate of 0.25 mL/min. Then a solution of 1% sodium cyanoborohydride in phosphate buffer 0.1 M pH 6.5 was fluxed in the bioreactor for 1 h at 0.25 mL/min to reduce Schiff's base. At the end, deionized water was used to wash the column for 1 h.

2.3. Determination of immobilized protein

The % of immobilized protein was spectrophotometrically determined at 280 nm by the difference of the absorbance values obtained prior (A_i)and after (A_f) recycle as following:

$$\% \text{ immobilized protein} = ((A_i - A_f)/A_i)*100$$

2.4. Removed activity ratio

Removed activity ratio is the activity removed during immobilization process. It was mathematically expressed by:

$$\text{Removed activity ratio } (\%) = ((U_i - U_f)/U_i)*100$$

Where U_i and U_f were the initial and final enzymatic activity of the immobilization process, respectively.

2.5. Enzyme activity determination

2.5.1. Determination alkaline phosphatase

Alkaline phosphatase was determined spectrophotometrically by measuring the rate of p-nitrophenol formation at 410 nm.

Analysis of free AP: 10 μL of the enzyme solution was added to 5 mL of TRIS buffer (1.0 M, pH 8.0) containing the substrate p- nitrophenylphosphate ($5*10^{-4}$ M); the mixture was mixed and the change in absorbance at 410 nm was monitored in the first 2 min compared to a blank solution which contained H_2O instead of enzyme. The assay was conducted at 25 °C and in path length cell of 1 cm. To obtain the alkaline phosphatase activity, a calibration curve was first built by relating AP activities with the absorbances corresponding to the p-nitrophenol formed after 2 min (equation $y = 6.5562x + 0.0353$, $R^2 = 0.984$). A linear trend was obtained up to a value of 0.02 U/mL of AP activity.

Analysis of immobilized AP: The bioreactor was placed between the HPLC pump and the detector was first equilibrated with TRIS buffer pH8 for 15 min. Then, 20 μL of a solution containing 0.1 mM p-NPP in TRIS buffer pH 8, were injected into the bioreactor at 0.4 mL/min flow rate and 25 °C. The amount of p-nitrophenol formed was determined at 410 nm by interpolation of the peak area, obtained by integration, on the experimentally determined calibration curve. The curve, which is linear up to 0.3 mM of p-nitrophenol, showed the equation $y = 6\ 10^8 \times -3150$ ($R^2 = 0.991$). The immobilized alkaline phosphatase activity was determined as follows:

$$\text{Immobilized AP activity } (U/L) = ((\text{area} - k)/(\text{m}))/t_r *10^6$$

Where t_r is retention time of p-NP, k and m are parameters of the calibration curve.

2.5.2. Determination of choline oxidase

Choline oxidase activity was determined by measuring the enzymatically peroxide hydrogen formed after its conversion to an optically measurable substance by reaction with a suitable chromogenic indicator. The reaction mixture was let stand for 15 min at pH 8.0 in order to complete the color formation; then was measured at 505 nm, against a blank prepared without H_2O_2.