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An enzymatic flow analysis method for the determination of phosphatidylcholine in sediment pore waters and extracts

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Abstract

A sensitive and selective flow injection method for the determination of phosphatidylcholine (PC) in sediment pore waters and extracts is described. It involves the use of phospholipase C, alkaline phosphatase and choline oxidase co-immobilized on controlled pore glass in a packed column reactor. The final product of the enzymatic reaction of phosphatidylcholine is hydrogen peroxide, and this is detected by measuring the chemiluminescence emission resulting from cobalt(II) catalysed reaction with luminol. The flow injection method is rapid (30 injections/h), reproducible (1.4% R.S.D. at 3 μM PC, $n = 10$) with a detection limit of 0.14 μM (estimated from $3\sigma_{n-1}$ of the measured blank). A linear calibration response was obtained over a concentration range of 0.5–9 μM ($r = 0.999$). The method has been applied to the determination of phosphatidylcholine in sediment extracts and sediment pore waters.

Keywords: Phospholipase C; Alkaline phosphatase; Choline oxidase; Immobilized enzyme(s); Luminol

1. Introduction

Eutrophication of lakes and waterways is an increasing problem. Nitrogen and phosphorus are thought to be the most important of the nutrients responsible for eutrophication, as they are the limiting factor controlling productivity of aquatic plants and algae [1,2]. Phosphorus is most commonly the limiting nutrient for algal growth in freshwater environments [3].

Inorganic phosphorus in the form of orthophosphate has long been regarded as the most bioavailable form of phosphorus, and the focus of most environmental studies has been on this form. However, organic phosphorus species may also constitute a sizeable proportion of the total P; this organic phosphorus fraction includes sugar phosphates, nucleic acids, inositol phosphates and phospholipids which are derived from the decomposition of plants and animals [4]. While the majority of studies of the cycling and bioavailability of phosphorus in aquatic systems have focused on inorganic phosphorus, the potential algal bioavailability of organic phosphorus has been largely ignored, and in part, this is perhaps due to a lack of simple quantification methods.

One group of organic phosphorus species, the phospholipids, occurs mostly in cellular membranes [5,6] and in higher plants and animals phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant, whereas in soils phosphatidylcholine followed by phosphatidylserine (PS) and phosphatidylethanolamine [5] are the dominant species [7]. In waters, the phospholipid concentrations are generally low, e.g. eutrophic lake waters were found to contain 0.7–6.4 $\mu g\,l^{-1}$ as P [8], and this may reflect the rapid degradation of phospholipids that occurs within hours following cell death [9,10].

However, substantial phospholipid concentrations have been reported in marine coastal sediments (1000–5000 $\mu g\,g^{-1}$ of dry weight [11], and this raises the possibility that sediment pore waters may contain

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higher concentrations of phospholipids. If this is the case, diffusion of pore water into overlying waters may thus constitute an important and relatively labile source of dissolved organic phosphorus. The ability to detect and quantify phospholipids in pore waters, sediment extracts and overlying waters has been the driver for the work described here.

A number of methods have been published for phospholipid analysis by high performance liquid chromatography (HPLC), usually involving ultraviolet (UV) detection. The poor UV absorbance of saturated phospholipids, limitations in the choice of eluents with suitable spectral characteristics that would not interfere with phospholipid absorbance, combined with the low sensitivity and non-linear response obtained using light scattering detection [12] makes these methods unsuitable for phospholipid detection in environmental samples. Detection of phospholipids on thin layer chromatography has also been reported [13] but the procedure is laborious and time consuming. Therefore, there is a need for a more rapid and selective method for the quantification of phosphatidylcholine in environmental samples, and particularly in sediment pore waters and extracts.

Enzymatic methods have been extensively reported for the determination of PC. In these methods, enzymes have been used in their soluble [14,15] or immobilized forms [16,17]. The use of enzymes in the their soluble form results in a method that is slower and more expensive compared to that when immobilized enzymes are employed. In a method developed by Kotsira and Clonis, two enzymes phospholipase D (PLD) and choline oxidase (ChO) and an indicator dye analogue, bromothymol blue–glutathione (BTB–GSH) conjugate, were co-immobilized on a gel [16]. Changes in the pH of the microenvironment of the immobilized enzymes results in a shift of the absorbance wavelength of the co-immobilized indicator dye conjugate (BTB–GSH). The change in the absorbance at λ_{max} is proportional to the PC concentration in the sample. However, this method is relatively slow, taking about 20 min before the change of absorbance can be measured. Furthermore, the immobilized system (PLD, ChO, and BTB–GSH conjugate) only has a lifetime of 2 weeks. Masoom et al. reported two methods for the determination of PC, both of which employed the use of immobilized enzyme reactors (IMERs) in flow injection systems with amperometric detection. The detection limits of these methods were 0.1 and 1.0 mM, respectively [17], which may have been due to the low conversion of PC by the enzymes, although this is unspecified.

In this paper, we describe the development of a selective enzymatic flow analysis method for the detection of phosphatidylcholine. This is based on the use of co-immobilized phospholipase C (PLC), alkaline phosphatase (AP) and choline oxidase (ChO) with detection of the chemiluminescence produced by the reaction between hydrogen peroxide and luminol, as shown in the enzymatic scheme in Fig. 1. The method is sufficiently sensitive to detect phosphatidylcholine in waters and sediment pore waters without preconcentration, and rapid when compared with liquid chromatographic techniques.

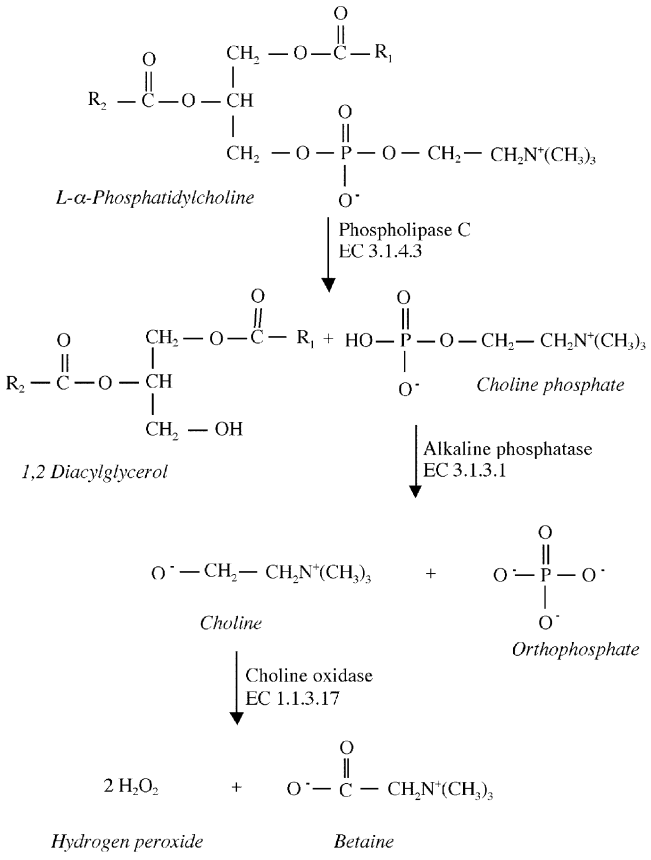

Fig. 1. Scheme for the enzymatic determination of PC using immobilized PLC, AP and ChO. Hydrogen peroxide produced from choline by ChO in the last step is detected using the chemiluminescence of luminol.

2. Experimental

2.1. Materials

The enzymes used were: AP (EC 3.1.3.1, from *Escherichia coli*, Sigma), ChO (EC 1.1.3.17, from *Alcaligenes* species, Sigma) and PLC (EC 3.1.4.3, from *Bacillus cereus*, Sigma). Enzymes were immobilized or co-immobilized onto aminopropyl-controlled pore glass (1273 Å, 125–177 μm particle size) obtained from CPG Inc. (NJ, USA).

All reagents used were of analytical grade and were as following: adenosine-5′-monophosphate hexahydrate (Sigma), anhydrous sodium carbonate (BDH); cobalt(II) chloride (BDH); disodium adenosine-5′-diphosphate (Sigma); disodium adenosine-5′-triphosphate (Sigma); disodium D-glucose-6-phosphate hydrate (Sigma); β-nicotinamide adenine dinucleotide (reduced form; Sigma); disodium phenyl phosphate (Sigma); glutaraldehyde (Ajax Chemicals; 25% solution); hydrochloric acid (BDH); hydrogen peroxide (BDH; 30% w/v); magnesium–potassium phytic acid