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Enzymatic methods for choline-containing water soluble phospholipids based on fluorescence of choline oxidase: Application to lyso-PAF

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ABSTRACT

In this paper we present methods to determine water soluble phospholipids containing choline (wCh-PL). The analytes were hydrolyzed by the enzyme phospholipase D and the choline formed was oxidized by the enzyme Choline Oxidase (ChOx); the fluorescence changes of the ChOx are followed during the enzymatic reaction, avoiding the necessity of an indicating step. Both reactions (hydrolysis and oxidation) can be combined in two different ways: 1) a two-step process (TSP) in which the hydrolysis reaction takes place during an incubation time and then the oxidation reaction is carried out, the analytical signal being provided by the intrinsic fluorescence of ChOx due to tryptophan; 2) a one-step process (OSP) in which both enzymatic reactions are carried out simultaneously in the same test; in this case the analytical signal is provided by the ChOx extrinsic fluorescence due to a fluorescent probe (Ru (II) chelate) linked to the enzyme (ChOx-RuC).

The analytical capabilities of these methods were studied using 1,2-dioctanoyl-sn-glycero-3-phosphocholine (C_8PC), a water soluble short alkyl chain Ch-PL as a substrate, and 1-O-hexadecyl-*sn*-glyceryl-3-phosphorylcholine (lyso-PAF).

The analytical features of merit for both analytes using both methods were obtained. The TSP gave a 10-fold sensitivity and lower quantification limit ($1.0^{*}10^{-5}$ M for lyso-PAF), but OSP reduced the determination time and permitted to use the same enzyme aliquot for several measurements. Both methods gave similar precision (RSD 7%, n = 5). The TSP was applied to the determination of C_8PC and lyso-PAF in spiked synthetic serum matrix using the standard addition method.

The application of this methodology to PLD activity determination is also discussed.

1. Introduction

Glycerophospholipids (PLs) constitute a class of lipids with very important biological roles in human health and nutrition. One of the most interesting PLs families is that containing choline (Ch-PLs). In fact, the most common biological PLs are phosphatidylcholines (PCs) [1], produced by PLs esterification with a Ch molecule. PC is the major constituents of the cell membrane. It has many common applications in the pharmaceutical industry (as a drug, in drug delivery systems [2] or simply as an excipient [1]), in the food industry [3,4] (as part of food preparations and as emulsifiers) and many others.

Since PC has two fatty chains (C16 or C18), it is a water insoluble compound, which limits some of its applications. However, there are other Ch-PLs which are fairly soluble (wCh-PLs), especially those having only one large chain (-lyso compounds) or those having short chains.

The Platelet Activating Factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a wCh-PL having only one chain. It is a potent bioactive mediator related with platelet aggregation, inflammatory response [5] and other processes [6]. Lyso-PAF (1-O-alkyl-sn-glycero-3-phosphocholine) is a precursor [7] and a metabolite [8] of PAF. Since PAF is quickly deacetylized by the enzyme acetylhydrolase yielding Lyso-PAF, the concentration level of the former in blood or biological samples [9] is about three

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orders of magnitude lower than that of the latter (blood [10,11] and organ [12] levels are in the part-per-million range). It was believed that the lyso-PAF lacks bioactivity, but in recent years it has been attributed a biological role balancing some of the bioactivities of PAF [13] and it has acquired relevance as a biological PAF marker.

Short chain wCh-PLs refer to those PLs having one or two C_6 to C_9 chains. These are increasingly being used as biological surfactants, because they are less aggressive against protein denaturation [14], and also in new chemical drug delivery systems (virosomes) [15].

From the analytical point of view, there is no well-established methodology for short-chain wCh-PLs determination. wCh-PLs have been used as substrates for determining the activity of phospholipase D (PLD) [16]. For lyso-PAF determination, methods based on the capability of PAF as a platelet aggregation (lyso-PAF is previously acetylated) have been used [9], but for the selective determination of the analyte, HPLC [12,17] (reverse phase and detection by fluorescence) or GC (with MS detector) [10] are more common.

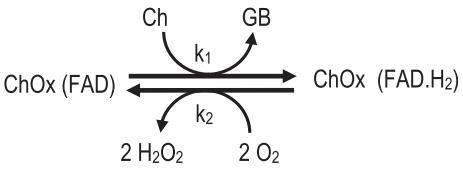
Enzymatic methods [18–20] are an interesting alternative for the analysis of Ch-PLs. They involve a hydrolysis step catalyzed by PLD

$$\text{Ch-PL} + H_2O \leftarrow \text{PLD} \rightarrow \text{Ch} + \text{phosphatidic acid} \quad (1)$$

and an oxidation step of the Ch to Glycine Betaine (GB) using Choline Oxidase (ChOx)

$$\text{Ch} + 2O_2 \leftarrow \text{ChOx} \rightarrow \text{GB} + 2H_2O_2 \quad (2)$$

In the indicating step, in the presence of peroxidase enzyme (HRP), the hydrogen peroxide reacts with a dye precursor to generate a fluorophore or chromophore. Several commercial kits have been developed. These methods give good results, but they are time-consuming and not fully reversible. The optical properties of proteins have been widely used in bioanalysis [21] and these problems can be partially overcome by exploiting the spectroscopic properties of the enzymes, especially the fluorescence of flavoenzymes (but not only this [22]) or the absorption of metalloenzymes [23]. This methodology is better understood if the mechanism by which flavoenzymes catalyse the oxidation of substrates (i.e. ChOx in (2)) is considered:



(3)

As noted, the flavin group of the enzyme oxidizes the substrate giving the product and it becomes reduced to FAD.H_2; then, the oxygen regenerates the FAD and H_2O_2 is simultaneously formed. This reaction can be followed using three alternatives [24–26]:

A) tryptophan (Trp) fluorescence. Due to an energy transfer mechanism with FAD, the fluorescence of the Trp also changes during the enzymatic reaction; depending on the Ch concentration, the fluorescence intensity changes during the reaction as indicated in Fig. 1.
B) flavin fluorescence; since both FAD and FAD.H_2 have different fluorescence properties (the oxidized forms being more fluorescent than the reduced) the fluorescence intensity changes during the reaction as a mirror image to that shown in Fig. 1.
C) probe fluorescence; some fluorophores linked to the enzyme, such as fluorescein (FS), also modify their fluorescence during the reaction; signals similar to those given in Fig. 1 are obtained. Option A requires a lower enzyme concentration than B. Option C is recommended when a high background is observed.

This paper proposes a methodology based on the combination of PLD and ChOx for wCh-PLs determination, which can also be extended to the determination of the PLD activity. Two configurations have been tested. In the first one, reactions (1) and (2) are carried out sequentially, and the tryptophan fluorescence is used (method A). In the second one, both enzymatic processes are carried out simultaneously and the indicating method C (using a Ru(II) chelate as probe) is optimized and used. Both methods have been evaluated with 1,2-dioctanoyl-sn-glycero-3-phosphocholine (C_8PC), a water soluble short alkyl chain Ch-PC, and successfully applied to the determination of this compound and lyso-PAF. Since a preconcentration step is not applied, the proposed method does not improve the sensitivity of some previously reported HPLC and GC methods, but it is faster and easier to apply. The application of this method to PLD activity determination is also discussed.

2. Material and methods

2.1. Reagents

Acetate buffer solution (0.1 M, pH 5.5) prepared from CH_3COOH (Poch, 568760114) and solid NaOH (Scharlau, SO0469). Carbonate solution (0.1 M and 0.01 M, pH 8.0, 9.0 and 10.0) prepared from solid $NaHCO_3$ and Na_2CO_3 (Sigma S5761 and 222321). Phosphate buffer solutions (0.1 M and 0.01M, pH 6.0, 7.0 and 8.0) prepared from Na_2HPO_4 and NaH_2PO_4 solids (Sigma S9638 and S9763). TRIS buffer solution (0.1 M, pH 9.0) prepared from TRIS (Biorad 161-0719) and HCl (Poch 575283115).

For the choline (Ch) stock solution, 0.14 mg of choline chloride (Sigma C-1879) were dissolved in10 mL of buffer. The C_8PC stock solution was prepared from 50 mg of 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (C_8PC), (Avanti Polar Lipids 850315P) dissolved in 5 mL of doubly distilled water. The lyso-PAF C-16 solution was obtained by dissolving 0.51 mg of 1-O-hexadecyl-*sn*-glyceryl-3-phosphorylcholine (Cayman 60906) in 0.50 mL of acetic buffer or water milliQ.

The Ruthenium labelling agent solution (RuC) was prepared by dissolving 1 mg of bis(2,2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium N-succinimidyl ester-bis(hexafluorophosphate) (Sigma 96631) in 0.50 mL of dimethyl sulfoxide (Panreac 131954.1611).

The Choline Oxidase (ChOx) from *Alcaligenes* sp solution was prepared by dissolving 100 U of the solid (Sigma C-5896, EC 1.1.3.17, 13 U mg^{-1}) in 1 mL of doubly distilled water and divided into fractions which were immediately frozen. The lipase enzyme solution was prepared by dissolving 13 mg of Phospholipase D from *Arachis hypogaea* ($_{AH}$PLD), (Sigma P0515, 36.5 U mg^{-1}) in 0.50 mL buffer solution.

Further dilution was carried out with the corresponding buffer solution.

2.2. Labelling procedure

200 μL of ChOx (100 U mL^{-1}), 100 μL of RuC (2 mg mL^{-1}), and 200 μL of pH 9.0 carbonate solutions were mixed. The mixture was allowed to react in darkness at room temperature with continuous stirring for 120 min. The excess of RuC was then separated from the ChOx-RuC using the chromatographic system later described. The