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Rapid enzymatic method for the determination of phosphoryl choline using the fluorescence of the enzyme choline oxidase. Sequential determination of choline and phosphorylcholine in milk powder for children

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ABSTRACT

In this paper we present a rapid method for determining phosphorylcholine (ChoP). ChoP was first hydrolysed by the enzyme alkaline phosphatase (AP); the choline formed was then submitted to a reaction with Choline Oxidase (ChOx). Both reactions were carried out simultaneously, in the same test without previous steps of incubation. The analytical signals used were the intrinsic fluorescence of ChOx due to FAD and that corresponding to a fluorescein derivative bonded to ChOx (ChOx-FS); both can be related with the concentration of ChoP. Once the conditions were optimized, the response range for ChoP was 5.2×10^{-7}–1.0×10^{-5} M using the peak area as the analytical parameter with a precision of about 4% (RSD) at both ChOx and ChOx-FS wavelengths.
In the experimental conditions found, it was also possible to determine free choline (Ch) with figures of merit similar to those obtained for ChoP. These results have made possible the sequential determination of Ch and ChoP in milk powder, using only one aliquot of the mixture, with good results. The recoveries obtained for both analytes were close to 100 %.
The method is rapid because an incubation step is not necessary. Moreover, the enzymatic reaction is autoindicating and thus the additional detection step required by other published enzymatic methods is avoided.

1. Introduction

Phospholipids are the primary components of cell membranes, controlling the transfer of substances to the interior or exterior of the cell. They are in fact a mixture of different families, those containing choline (Ch) being the majority. Phosphatidylcholine (PC) is one of the most interesting of these components [1–3]. A by-product of PC is choline phosphate (ChoP), also called phosphorylcholine, formed by the action of the enzyme Phospholipase C on PC or from the breakdown of sphingolipids (sphingomyelin) by sphingomyelinase [4,5].

There has been a growing interest in ChoP in recent years. It is used as a coating for lenses [6,7] and, because of its biocompatibility, it is the basis of polymers used in the manufacture of coronary stents [8–11] and drug-eluting stents [12]. In addition, ChoP, like other phospholipids containing choline, is a component found in many foods [13] (acting as a Ch source for the body), especially milk. Because it is an essential nutrient for brain development [14], the American Academy of Pediatrics (AAP) [15] has recommended that infant formulas contain at least 7 mg of choline/100 Kcal (0.6 μmol/Kcal); consequently, Ch, ChoP and other phospholipids are usually determined in this kind of sample [16]. The choline content differs depending on the commercial source. In general, the total choline concentration values range from 31.1 to 227.0×10^{-5} M where free Ch is 4.3×10^{-5}–72.3×10^{-5} M and ChoP is up to 83.6×10^{-5} M.

Several analytical methodologies have been proposed for Ch and ChoP determination. Phillips [17] recently reviewed the analytical methods used for the determination of phospholipids containing Ch. Traditionally, the determination of phospholipids and consequently ChoP has been carried out using separation techniques [18] which require various steps in order to isolate both water-soluble compounds (Ch, ChoP) from the hydrophobic compounds (PC, SM) prior to their determination by GC-MS or LC-MS, among others [19–23]. In the last few years new alternatives have emerged. For example, Nzai et al. [24] have developed a simple and rapid method based on FTIR to determine the total phospholipid content in vegetable oil. One of the most interesting methods is the use of P-31 NMR spectroscopy [25]. This is based on the different chemical shift of the P atom in each phospholipid giving a NMR spectrum with the signal corresponding to the different phospholipids separated. The technique has low sensitivity but is non-destructive, so it has been applied for determining phospholipids in several samples, for example olive oil.

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For routine control, enzymatic methods are one of the most interesting options. Ch is usually determined by the enzymatic reaction with choline oxidase (ChOx). This enzyme catalyses the substrate oxidation by oxygen in a two-step process, according to:

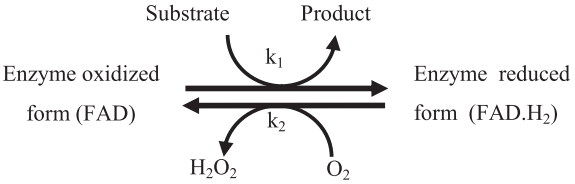
$$\begin{aligned} Ch + O_2 &\xrightarrow{ChOx} BA + H_2O_2 \\ BA + O_2 &\xrightarrow{ChOx} GB + H_2O_2 \end{aligned} \quad (1)$$

BA and GB being Glycine Betaine aldehyde and Glycine Betaine, respectively. The H_2O_2 is then submitted to a conventional second reaction involving a chromophore or a fluorophore. The enzymatic determination of ChoP [26,27] is based on the action of alkaline phosphatase (AP) for obtaining Ch.

$$ChoP + H_2O \xrightarrow{AP} Ch + Phosphate \quad (2)$$

Thus there are two alternatives depending on which product is determined: Ch by using (1) or phosphate with molybdate in the presence of a reducer, such as hydroquinone [28]. These methods have also been applied in flow analysis [29]: free Ch and Ch + ChoP are determined, ChoP being calculated as a difference. Similar methodologies have also been used for phospholipids.

Enzymatic methods generally give good results, but they are time-consuming (incubation steps are necessary) and not fully reversible. These problems can be overcome by measuring the enzyme fluorescence changes which can be observed during reaction using oxidation reactions catalysed by flavoenzymes [30] and other enzymes [31,32]. The mechanism by which flavoenzymes catalyse the oxidation of substrates can be summarized as follows:



(3)

As noted, the flavin group of the enzyme oxidizes the substrate to 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 [33]: A) flavin fluorescence, since both FAD and FAD·H_2 have different fluorescence properties, the oxidized forms being more fluorescent than the reduced; B) tryptophan (Trp) fluorescence, due to an energy transfer mechanism with FAD, the fluorescence of the Trp also changes during the enzymatic reaction; C) probe fluorescence; some fluorophores linked to the enzyme, such as FS, also modify their fluorescence during the reaction. In this context, ChOx fluorescence has previously been used for determining Ch using alternatives B and C [34] at tryptophan and FS wavelengths, respectively.

In this paper we describe a method for ChoP determination linking reactions (1) and (2) in the same test and measuring the FAD or the FS fluorescence. The method has been applied to the sequential determination of Ch and ChoP in a milk sample. This methodology has three additional advantages over previously proposed methods: 1) it does not require the participation or the addition of reagents other than those involved in the reactions, 2) there are not incubation steps so is a rapid method and 3) the enzymes are regenerated during the process so they can be used several times. The process is therefore of great interest for implementation in optical biosensors.

2. Material and methods

2.1. Reagents and solutions

The buffer solutions were: carbonate solution 0.1 M, pH 9.0 and 10.0, prepared from solid $NaHCO_3$ and Na_2CO_3 (Sigma S5761 and 222321), and phosphate solution 0.1 M, pH 7.0 and 8.0, prepared from Na_2HPO_4 and NaH_2PO_4 solids (Panreac 123018.*1210* and Sigma S9763).

Enzymes: alkaline phosphatase (AP) from *Calf* intestine, 10000 IU mL^{-1} in 10 mM Tris–HCl (Sigma P4978) and finally diluted to 440 IU mL^{-1} in carbonate buffer at pH 9.0. Choline oxidase (ChOx) from *Alcaligenes* sp., 13 IU mg^{-1}, lyophilized solid (Sigma C5896), dissolved with carbonate buffer at pH 9.0, until obtaining a solution of 6.6 IU mL^{-1}. Solutions of the enzymes were separated in vials stored in the freezer and kept at a very low temperature (thus preventing denaturation). The fractions to be used were taken daily.

Substrates: choline phosphate (ChoP), (Sigma P0378); choline (Ch), 99%, (Sigma C1879). Stock solutions of ChoP and Ch were prepared daily in buffer solution.

The labelling agent solution (FS) was prepared by dissolving 1 mg of 6-[fluorescein-5(6)-carboxamido] hexanoic acid N-hydroxysuccinimide ester (Sigma 46940) in 0.1 mL of anhydrous dimethyl sulfoxide (Panreac 131954.*1611*).

2.2. Labelling procedure

1 mg of ChOx dissolved in 12.8 μL 0.1 M carbonate buffer at pH 9.0 was mixed with 8 μL of FS solution in an Eppendorf cap. The mixture was allowed to react in darkness at room temperature for 60 min. The excess of FS was then separated from the ChOx-FS using a home-made exclusion column.

The column consisted of a plastic syringe of 1 mL capacity, with glass wool placed at the bottom and filled with a solution of Sephadex G-50 (Sigma G-50-150), in water. To compact the enzyme, it remained for 1 min at 4800 rpm in the centrifuge.

The sample in the Eppendorf cap was collected with the help of a glass syringe, adding 20 μL of the mixture at the top of the column. The previously weighed Eppendorf body (which had been separated from the cap) was placed underneath the column. The Eppendorf serves to collect the enzyme fraction linked to the FS, so the excess of labelling is eliminated. The column was placed in the centrifuge, remaining for 5 min at 4800 rpm.

The column was later removed from the centrifuge. A yellow colour strip appeared at the top of the syringe, denoting that the excess of the label had not all been eluted. The FS molecular weight was 586.6 Da, much lower than ChOx 72000. By molecular exclusion, the ChOx enzyme bound to the FS fell by gravity into the Eppendorf. This solution was kept stable for about 4 days at 8 °C.

2.3. Instruments

Fluorescence measurements carried out in this work were developed using two kinds of instruments:

Plate reader: The Synergy HT Multi-Mode Microplate Reader, supplied by BioTek, is able to measure fluorescence, absorbance or luminescence. For fluorescence measurements, this instrument consists of a tungsten lamp (as the excitation source), a photomultiplier tube (as the detector) and two filter wheels for excitation and emission wavelength selection (380 nm; 420 nm; 440 nm; 485 nm; 508 nm; 530 nm; 560 nm; 620 nm; spectral bandwidths ranging from 20 to 50 nm). Other instrumental parameters which should be chosen are: sensitivity, illumination mode (top or bottom), and the optical fibre height. Measurements were performed on transparent 96-well plates. Data were collected using Gen5™ software.

Fluorometer: A Photon Technology International (PTI) Time Master Fluorescence Spectrometer (model TM-2/2003-PTI) was used for performing excitation and fluorescence spectra. In this case, a Xe arc lamp is used as a continuous excitation source. Two monochromators (excitation and emission) allow the measurement wavelength (λ) and the excitation and emission slit widths to be selected. The detector