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Use of solid phase extraction for the sequential injection determination of alkaline phosphatase activity in dynamic water systems

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ABSTRACT

In this work, a solid phase extraction sequential injection methodology for the determination of alkaline phosphatase activity in dynamic water systems was developed. The determination of the enzymatic activity was based on the spectrophotometric detection of a coloured product, *p*-nitrophenol, at 405 nm. The *p*-nitrophenol is the product of the catalytic decomposition of *p*-nitrophenyl phosphate, a non-coloured substrate. Considering the low levels expected in natural waters and exploiting the fact of alkaline phosphatase being a metalloprotein, the enzyme was pre-concentrated in-line using a NTA Superflow resin charged with Zn^{2+} ions. The developed sequential injection method enabled a quantification range of 0.044–0.441 unit mL^{-1} of enzyme activity with a detection limit of 0.0082 unit mL^{-1} enzyme activity (1.9 μmol L^{-1} of *p*NP) and a determination rate of 17 h^{-1}. Recovery tests confirmed the accuracy of the developed sequential injection method and it was effectively applied to different natural waters and to plant root extracts.

1. Introduction

Phosphorus (P) is an important nutrient required by all organisms since it is present in the nucleic acids (DNA and RNA) and in phospholipids, located in the membranes. Furthermore phosphorous plays an essential role in energy metabolism as ATP, ADP, AMP, and PPi [1]. In dynamic water systems such as natural waters, phosphorous is present in two soluble sources: dissolved inorganic phosphate, orthophosphates ($H_2PO_4^-$ and HPO_4^{2-}) and organic phosphorus compounds. Orthophosphates are available for direct assimilation by organisms such as bacteria, algae (both micro and macro) and plants. However, organic phosphorus compounds need to be mineralized in order to be part of the soluble orthophosphate pool [2].

When a shortage of dissolved inorganic phosphate in waters compared to other nutrients (namely *N*) arises, phytoplankton and bacteria have the ability to obtain phosphorus from dissolved organic compounds, if available, as an alternative source for their metabolism. This feature results from the production of extracellular enzymes such as alkaline phosphatase (AP) that hydrolyze phosphate monoesters releasing inorganic phosphate and organic matter [3]. Thus, alkaline phosphatase may play an important role in the availability and recycling of phosphorous. Therefore it is regulated by inorganic phosphate concentrations and internal phosphorous levels, which makes this enzyme an excellent indicator of phosphorous status [4–6]. Routine assessment of alkaline phosphatase activity (APA) in waters is usually attained by incubating the enzyme with specific substrates, namely methyl-umbelliferyl phosphate (MUF-P), *p*-nitrophenyl phosphate (*p*NPP), or monofluorophosphate, and quantifying the resulted product with different detection methods, such as fluorescence, spectrophotometry and potentiometry, respectively. Therefore, APA determination is not only quite laborious but also time consuming due to the incubation step, which can go from 30 min [4] to 28 h [7]. The lower limits aimed, such as the expected in dynamic water systems, the higher incubation time needed.

The purpose of this work was the automation of the spectrophotometric APA determination based on catalysis of the *p*-nitrophenyl phosphate in inorganic phosphate and *p*-nitrophenol, a coloured product of the catalysis, in natural waters from dynamic water systems (estuarine and river waters with respective interstitial water and well water). Due to the expected low values of alkaline phosphatase in natural waters, an enzyme preconcentration step would be required to carry out the determination in an expeditious way. Solid phase extraction (SPE) can be an attractive approach to solve this problem, as it permits an efficient preconcentration with no consumption of organic solvents. The amount of solid material used can be minimized by packing a column for a reusable approach. Some problems

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associated with SPE, namely low reproducibility, solid-phase swelling and compaction, and washing can be minimised using sequential injection analysis (SIA). In SIA, the high level of automation [8] decreases reproducibility problems, furthermore solutions can flow through the column in opposite directions, thus minimizing possible compaction of the solid support. Pre-concentration and elution can also be easily achieved by selecting an appropriate sequence protocol, in which different solutions placed around the selection valve, namely conditioning and eluting buffers can be sent to or aspirated from the column and sent to the detector. The chosen solid phase material was Nitrilotriacetic Acid (NTA) Superflow resin, commercialized for protein purification when charged with nickel ions [9]. Nitrilotriacetic acid is an aminopolycarboxylic acid that can complex metal ions when fully deprotonated [10]. Thus, the NTA resin was charged with Zn^{2+} (instead of the commonly used nickel ions) in order to retain the enzyme since the enzyme requires that ion on its active site. In fact, alkaline phosphatase is a homodimeric metalloenzyme containing one Mg^{2+} and two Zn^{2+} ions in the active site. Magnesium is an important structural stabilizer of the enzyme, whereas the two zinc ions are directly involved in catalysis. One plays an important role in binding both the substrate and phosphate while the other stabilizes the amino acid responsible for the nucleophilic attack on the phosphate [11].

As far as we know, this is the first time that AP has been pre-concentrated with NTA resin charged with zinc ions. Moreover, we believe this was the first use of solid phase extraction coupled to pre-concentration for alkaline phosphatase activity determination. Although previous works have reported the determination of alkaline phosphatase activity using flow analysis techniques [12–23], they all relate to flow injection and only one was applied to water samples (seawater) [15]. Furthermore, within those reported works no extraction/pre-concentration was attained.

The developed SIA methodology was successfully applied to natural waters, namely estuarine, river, interstitial and well waters. Concomitantly, AP in root samples was also assessed in order to evaluate the potential relationship between plant root and soils [24,25].

2. Materials and methods

2.1. Reagents and solutions

Solutions were prepared with analytical grade chemicals and deionised water (specific conductance less than 0.1 $\mu s\,cm^{-1}$), previously boiled.

Alkaline phosphatase (AP) from bovine intestinal mucosa was purchased from Sigma Aldrich (EC 3. 1. 3. 1). Enzyme long term stock solution of 179.3 unit mL^{-1} was prepared according to the instructions of the product supplier [26], and kept at 2–8 °C in a storing buffer composed by: 10 mmol L^{-1} Tris–HCl (pH=8), 2.5 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 0.15 mmol L^{-1} $ZnCl_2$, and 50% glycerol.

The diethanolamine buffer was daily prepared by dissolving 2.6 mg of magnesium chloride hexahydrate in water, adding 2.4 mL of diethanolamine (d=1.09, Merck) and diluting to 25 mL of water to final concentrations of 1.0 mol L^{-1} diethanolamine and 0.5 mol L^{-1} of magnesium chloride. The pH was adjusted to 9.8 using a 4 mol L^{-1} HCl solution, obtained by proper dilution of the concentrated acid (d=1.19; 37%). A 5 fold dilution of the diethanolamine buffer was used in the sequential injection manifold (DB_{SI}).

Enzyme stock solution 0.441 unit mL^{-1} was prepared by proper dilution of the long term stock solution (179.3 unit mL^{-1}) in diethanolamine buffer. The working solution of 0.2 unit mL^{-1} of AP, and the AP standards in the range 0.022–0.441 unit mL^{-1} were daily prepared by appropriate dilution in diethanolamine buffer.

The substrate solution, *p*-nitrophenyl phosphate (Calbiochem) 30 mmol L^{-1}, was also daily prepared by dissolving 28 mg in 2 mL of water.

The elution buffer NPI-250 (NTA—QIAGEN handbook [9]) used in preliminary studies, EB_{PS}, was obtained by dissolving 0.136 g of KH_2PO_4, 0.351 g of NaCl and 0.340 g of imidazole in 20 mL of water to final concentrations of 50 mmol L^{-1} KH_2PO_4, 300 mmol L^{-1} of NaCl and 250 mmol L^{-1} of imidazole. The pH was adjusted to 8.0 using NaOH.

The elution buffer (buffer E in the NTA—QIAGEN handbook [9]) used in the sequential injection method for eluting the enzyme in denaturing conditions, EB_{SI}, was prepared dissolving 24 g of urea, 0.69 g of KH_2PO_4 and 0.79 g of Tris–HCl, in 50 mL of water. The pH was adjusted to 4.5 (with HCl) and the final concentrations were: 8 mol L^{-1} urea, 100 mmol L^{-1} of KH_2PO_4 and 100 mmol L^{-1} of Tris–HCl.

The buffer solution used for the assays with plant roots, B_{MOPS}, was based in the work of George et al. [25] where the MES (2-(*N*-morpholino) ethanesulfonic acid) reagent was replaced by MOPS (3-(*N*-morpholino) propanesulfonic acid), a structural analog expected to have better compatibility with the NTA resin [8]. Stock solutions of 25 mmol L^{-1} of cysteine and 255 mmol L^{-1} of MOPS were obtained by dissolution of the solids: 0.2 g cysteine to a final volume of 50 mL and 2.7 g of MOPS to a final volume of 50 mL. The buffer for plant roots assays, B_{MOPS}, 5 mmol L^{-1} of cysteine and 15 mmol L^{-1} of MOPS, was prepared daily by proper dilution of the stock solutions cysteine and MOPS, respectively.

A stock solution of the coloured product, *p*-nitrophenol (*p*NP) 560 μmol L^{-1}, was prepared by dissolving 15.6 mg of the solid in 200 mL of water. The working standards in the range 19–280 μmol L^{-1} were obtained by proper dilution of the stock solution.

Nitrilotriacetic Acid Superflow resin, highly cross-linked 6% agarose, 60–160 mm of bead diameter, 50% suspension in 30% ethanol (30510, Qiagen, UK) was used for solid phase extraction/preconcentration of the enzyme. For metal charging the column a 2% Zn^{2+} solution was obtained from the solid $ZnCl_2$.

2.2. Preparation of the NTA beads column

An acrylic column with 3 mm i.d., 20 mm length and 140 μL inner volume was used to pack the NTA resin (60–160 μm). Filters (10 μm pore size, MoBiTec M2210) were placed at both ends to prevent resin leaking. Three milligrams of NTA resin were introduced in the acrylic column with a Gilson micropipette, and connected to the SI system (Fig. 1).

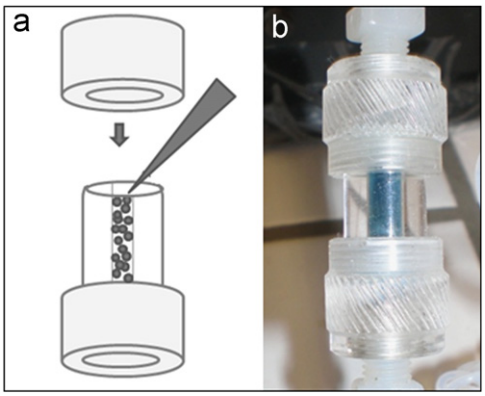

Fig. 1. NTA resin micro column: (a), schematic representation of the packaging process; (b), photography of the column connected to the selection valve.