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Micellar electrokinetic chromathography after pre capillary enzyme assay for the determination of phosphatase activity in semiarid soil.

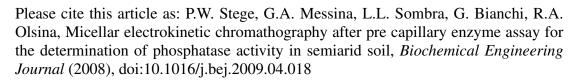Patricia W. Stege [*, 1], Germán A. Messina[1], Lorena L. Sombra[1], Guillermo Bianchi [2], Roberto A. Olsina [1]

[1]*INQUISAL, Department of Chemistry. National University of San Luis, CONICET. Chacabuco y Pedernera. D5700BWS. San Luis, Argentina.* [2]*Departament of Chemistry, Science Faculty. Los Andes University, Mérida, Venezuela.*

*Author to whom correspondence should be addressed: pwstege@unsl.edu.ar (e-mail). (Tel.) +54-2652- 425385; (Fax) +54-2652-43-0224. *INQUISAL, Department of Chemistry. National University of San Luis, CONICET. San Luis, Argentina.*

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Abstract

Inorganic phosphorous (P) is an essential plant nutrient that must be added to most soils to maintain plant growth and sustain crop yields. Phosphatases enzymes play a major role in the processes of mineralization of organic P substrates. In the present study we propose a precise and accurate method to determine the alkaline and acid phosphatase activity in soil using adenosine monophosphate (AMP) as a substrate. The adenosine released by the enzymatic reaction was quantified by micellar electrokinetic chromatography (MEKC) method. The background electrolyte (BGE) consisted in 20 mM phosphate buffer, 10 mM sodium dodecyl sulphate (SDS) and 10% acetonitrile (ACN). The LOD and LOQ for adenosine were $2.27 \ 10^{-4}$ and $6.4 \ 10^{-4}$ mg mL^{-1}, respectively. This sensitive method allowed the use a natural compound as enzymatic substrate, which is commonly found in soil. Moreover, we improve the detection method by diminishing the amount of waste and using a harmless substrate.

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1. Introduction

Sustainability of agricultural systems has become an important issue all over the world. Many of the issues of sustainability are related to the quality of soil and its changes through time [1]. Intensive cultivation leads a rapid decline in organic matter and in nutrient levels, besides affecting physical properties of soil. Conversely, management practices with organic materials influence agricultural sustainability by improving the physical, chemical and biological properties of soil. However, a better understanding of the nutrients cycle, and the factors that govern their decomposition in soil is imperative for the implementation of the sustainable practices. Nutrients cycle in soil involves chemical, biochemical and physico-chemical reactions. Furthermore, the biochemical reactions are catalyzed by soil enzymes principally associated with viable cells of microbial origin [2].

Enzymes activity are indicators of soil quality because: i) they are strongly connected with important soil properties such as organic matter, physical properties and microbial activity or biomass; ii) they respond earlier than other soil properties; and iii) they involve relatively simple testing methods compared to other soil quality bioindicators [3].

Phosphorus (P) is an essential nutrient element required for the growth and development of plant. However, P transformation in soil is regulated by biotic and abiotic factors. There are many studies about the use of P as mineral fertilizer [4, 5]. There is a small amount of knowledge about the mechanisms involved in the immobilization and mineralization of organic P and how these interact with the reactions of inorganic P in soil. The transformation of organic P is performed through enzymatic reactions [6] and the immobilization of P in the biomass play a fundamental role in P cycling. These reactions are likely to be affected by source of P [7]. Organic P

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in soil generally accounts for 30–50% of total soil P. Plants are unable to take up organic P directly from the soil. The enzymes presented in soil responsible of the mineralization may be of microbial origin [8, 9] or derived from root exudates [10]. The extent of the enzymes activity under different organic sources has not been shown clearly yet. The phosphatases (phosphoric monoester hydrolases) are required to mineralize organic P to release it as specie available for the plant [11]. The phosphatases are enzymes that remove a phosphate group from its substrates by hydrolysing phosphoric acid monoesters into a phosphate ion and also into a molecule with a free hydroxyl group. These enzymes are classified as acid phosphatase (AcP) and alkaline phosphatase (AlP) according to their optimum pH.

Microbial phosphatases are found to be more efficient in hydrolysis of organic P compounds than the phosphatases of plants [12]. Among the hydrolases, acid phosphomonoesterase activity is the most frequently used enzyme for estimating changes in soil quality. This enzyme is a good index of the quality and quantity of organic matter in the soil [13, 14].

In recent years, considerable interest has been raised in capillary electrophoresis (CE). This method is applicable in the analysis of a wide range of compounds including natural products and plant metabolites [15]. Compared to LC methods, capillary electrophoresis methods have some advantages. One of them is the use of a smaller volume of samples and the production of less amount of waste [16, 17]. Moreover, micellar electrokinetic chromatography (MEKC) has proved to be efficient to separate neutral compounds. MEKC development is performed by dissolving a surfactant in the CE background electrolyte at a concentration over the critical micelle concentration (CMC). The micelles present in MEKC form a pseudo-stationary phase with a charged surface and a non-polar core. In general, hydrophobic analytes spend more time inside

the micelle and as a consequence they are delayed compared to the hydrophilics. Finally, neutral analytes, which cannot be separated by capillary zone electrophoresis, are readily separated by MEKC [18, 19].

Several analytical methods were used to determinate enzymatic activities in different biological samples [20-22]. Nowadays, the use of capillary electrophoresis (CE) in enzyme assays has advantages over conventional assays in terms of time of analysis, and sample size required [23]. Also the ability to separate and quantify substrates and products, that are very similar in structure, could be easy to study using MEKC. Another advantage of CE is that organic buffers such as diethanolamine (DEA), which are compatible with bioanalytes, can be used. Recently, CE has become an attractive tool for bioanalysis due to its high efficiency for bioanalyte separation.

The aim of the present paper is to develop a selective, precise and accurate method to determine the acid and alkaline phosphatase activity in soil using MEKC. Moreover, we propose this method using a natural compound as a substrate (AMP) with the purpose of approaching our conditions in the laboratory to the natural ones.

2. Experimental

2.1 Instrumentation

A Beckman P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector and a data handling system comprising an IBM PC and P/ACE System MDQ Software (ESANCO) was used. The fused silica capillaries were obtained from MicroSolv Technology Corporation and had the following dimensions: 47cm total length, 40cm effective length, 75 µm ID, and 375 µm OD. The temperature of the capillary and the samples were maintained at 25ºC.

2.2 Reagents and Solutions

All reagents used, were of analytical reagent grade. The adenosine and AMP were purchased from Sigma Chemical Co. (St. Louis, MO) and acetonitrile (HPLC grade) were from Merck (Buenos Aires, Argentina). Sodium dodecylsulphate (SDS) were supplied by Tokyo Kasei Industries (Chuo-Ku, Tokyo, Japan). Disodium hydrogen phosphate dehydrate, sodium dihydrogen phosphate and sodium acetate were obtained from Merck (Darmstadt, Germany)

All the solutions were degassed by ultrasonication (Testlab, Argentina). The water used in all studies was ultrapure water (18 MΩ cm) obtained from a Barnstead Easy pure RF compact ultrapure water system. Running electrolytes and samples were filtered through a 0.45 µm Titan Syringe filters (Sri Inc., Eaton Town, NJ, USA).

2.3 Soil samples

Fourth soils samples were collected from the surface layer (0–15 cm) from the state of San Luis, Argentina. Soil 1 (33º19´ S, 66º20´ O), soil 2 (33º74´ S, 65º55´ O), soil 3 (32º32´ S, 65º14´ O) and soil 4 (34º06´ S, 66º44´ O)

The moist soil sample was sieved (≤ 2 mm) after removing the plant material and roots. Soil samples were kept at 4ºC in plastic bags for a few days to stabilize the microbiological activity disturbed during soil sampling, handling and then analyzed within two weeks. The important physical and chemical characteristics of the soil are given in Table 1.

2.4 Enzymatic assay

The developement of the phosphatase assay 0.1 g of soil was added in a 1.5 ml Eppendorf tube with 1 mL of a solution the of DEA buffer 0.1 M (pH=9) contain different concentrations of AMP for the alkaline phosphatase. On the other hand, acetate buffer 20 mM (pH=6) was used for the acid phosphatase assay, contain different concentration of AMP. The flask was placed in a shaker at 37ºC. After 1 hr, 500 µl of

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0.5 M sodium hydroxide was added. Immediately after the flask containing the mixture was swirled for a few seconds, and centrifuged at 3000 rpm for 5 min. A 500 μl aliquot of the supernatant phase was filtered through 0.45 µm Titan Syringe filters (Sri inc., Eaton Town, NJ. USA) and was transferred into the CE sample vial.

2.5 MEKC development

The MEKC operation parameters were as follows: the detection was performed at 264 nm; the capillary temperature was maintained at 25°C, the voltage was set at 25 kV. Samples were pressure-injected at the cathodic side at 0.5 psi for 10 seconds. In order to achieve high reproducibility of migration times and to avoid solute adsorption, the capillary was washed between analyses with pure ACN for 1 min., sodium hydroxide for 1 min., followed by water for 1 min., and then equilibrated with the running buffer (20mM phosphate buffer containing SDS 10 mM and 10% of ACN, pH 8) for 1 min.

3. Results

3.1 Determination of de enzymatic product by MEKC

3.1.1. Development of the separation conditions

The first step in the development of a MEKC-based enzyme assay for monitoring phosphatase activity was the development of a suitable CE separation method. The choice of buffer for the separation of analytes by MEKC is very important. Different concentrations of sodium phosphate and SDS were studied and we observed optimal results with concentrations of a 20 mM of sodium phosphate and 10 mM SDS. Increases in migration times as well as current were observed when the concentration of buffer increased. A high resolution was obtained with increased buffer concentrations. However, we didn't observe appreciable improvements over the separation with

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concentrations above 20 mM. With higher SDS concentrations, the system current was too high (over 100 μA).

Organic modifiers are very important to improve separation in many systems because they can change the partition coefficient and polarity of the sample [24]. Thus, ACN was used as an organic modifier in order to enhance the resolution. Different amounts of ACN (5-30% v/v) were added into the BGE. The compounds were baseline separated when 10% v/v of ACN was added. Consequently, a 20mM phosphate buffer containing SDS 10 mM and 10% of ACN, pH 8 was chosen as the BGE for the best detection of adenosine. The sample injections were made in the hydrodynamic mode (0.5 psi) for a period of 10 seconds.

The effect of the pH was investigated within the range of 7.5-10.0 at a fixed buffer concentration, adjusted by 0.1 M NaOH and 0.1 M HCl. It was found that the resolution decreased as the pH increased, while the time analysis decreased. The separation was achieved at pH 8.

Figure 1 shows the electropherograms of adenosine and AMP standards using the optimized experimental conditions. The migration time of adenosine and AMP were found at 3.6 and 11.1 min, respectively. The throughput was 3.75 samples per hour.

Identification of the adenosine and AMP were performed by comparison of the migration times obtained in actual samples with those of the standard solutions. Furthermore, spiking experiments (standard addition method) were performed to confirm the peak identity. The repeatability of the separation system was evaluated by replicated analysis of solution (0.5 mM) of adenosine; the relative standard deviations of migration time were 1.02% (n = 5, intra-day) and 3.08% (n = 15, inter-day).

3.1.2. Study of the enzymatic process

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Reactions catalyzed by enzymes have long been used for analytical purposes in the determination of different analytes such as substrates, inhibitors, and also the activity of the enzymes [25]. A schematic representation of the catalytic mechanism of phosphatase enzyme process is shown in figure 2.

Temperature, pH, and substrate concentration influence the enzymatic reactions rates. In order to study the activity of AP, the substrate must be present in excess amount. Thus, the reaction rate must be independent of the substrate concentration. For this reason, the first step in this study was the determination of the concentration at which the enzyme activity was not modified due to the substrate concentration. Thus, increasing concentration of AMP was studied in the range of 0.5-24 mM of AMP. Figure 3 shows the curve of the enzymatic activity in response to the increasing substrate concentrations. As a result, we decided to perform the following studies with 18mM of substrate concentration.

Afterwards, the reaction time was set up. With this purpose six reaction mixture were incubated at different times: 30-480 min. Figure 4 shows a linearity relationship between time of incubation and the amount of adenosine released. Thus, we could demonstrate that this method is not affected by microbial growth or assimilation of enzymatic reaction products by soil microorganisms.

With the aim of quantifying the adenosine freed, a calibration curve was obtained by plotting the corrected peak area (Y), versus the corresponding concentrations of the adenosine (C; mM). By linear regression analysis the following equation was obtained: Peak Area $= 2819 + 18530$ x $C_{Adenosine}$; $R^2 = 0,999$. The sensitivity data calculated as LOD and LOQ were $2.27 \ 10^{-4}$ and $6.4 \ 10^{-4}$ mg mL^{-1} (CV = 3.5%; n = 54), respectively (figure 5) at the detection wavelength of 264 nm.

The enzymatic activity values for both enzymes were obtained using the equation:

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$$EA = \frac{2(A - a)}{b \ T \ W}$$

EA: enzymatic activity (µmol of free adenosine)/(soil g x digestion time(h)), A: corrected peak area, a: interception adenosine calibration curve, b: slope adenosine calibration curve, T: incubation time (h), W: dry soil mass (g).

In order to improve the quantification of phosphatases activity assays, the amount of soil (0.1g) used in this study was minimized compared with the amount of soil usually used in other spectrophotometric systems (1g) [6, 10] (Table 3). The assay was performed with the same four soils samples (n= 5) used in this study. The results obtained were reasonably close, indicating a good correspondence between the values in both amounts of tried soils (Table 3). The possibility to work with less amount of soil is a very important advantage due to the fact that the reduction of waste generation, which is a basic requisite in green analytical chemistry [26].

As a certified value for alkaline and acid phosphatase activity, applied to determine the quality of soil in which adenosine is the natural substrate, does not exist and the most used method for determine the activity of those enzyme use other substrate, the method of standard addition could be considered with the aim of evaluate the accuracy of the proposed method. The development of this assay consisted in warm up a portion of the soil at 200º C during 24h. Thus, a soil without enzyme activity or microorganism able to synthesize the enzyme was obtained. Afterwards, increasing amount of non-heated soil with normal enzyme activity was added (0-100%). The optimized enzymatic assay-micellar electrokinetic chromatography was applied to the different mixtures of soil. The figure 6 shows the results obtained for this assay.

The values of the activities of ALP and ACP in semiarid soil are showed in Table 2. The high precision of the proposed method is probably due to the fact that the

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used technique, for the determination of the freed adenosine, was quantitative and the entire assay procedure was simple and readily standardized.

4. Discussion

Numerous efforts are till made to develop bioanalytical methods to determine phosphatase activity in soil. Firstly, the phosphatase activity was measured by estimating the inorganic phosphate released by the incubation of soil with organic phosphates. These methods are deficient because inorganic phosphate is fixed for soil constituents [27]. Kroll et al. [28] estimated soil phosphatase activity by determining phenol released. This substrate is used in several investigations of soil phosphatase activity [29-31]. Skujiņš et al. [32] assayed soil phosphatase activity through a procedure in which the amount of glycerophosphate hydrolyzed is estimated by analyses thought extractable total and inorganic phosphorus after incubation. This method is tedious, time consuming, and low precise [27]. Other methodology in order to measure phosphatase activity involves a fluorimetric assay of the β-naphthol. This method is complicated by sorption of β-naphthol by soil constituents [33]. Actually, the most used methodology to determine the phophatase activity in soil was proposed by Tabatabai et al. [34]. This method has some disadvantages: i) the suggested concentration of the p-nitrophenilphosphate for the reaction is extremely high, ii) this solution has an intense yellow coloration because the substrate solution already has some p-nitrophenol concentration, iii) the substrate stability is very poor, iv) p-nitrophenol is absorbed by soil components. Consequently it is difficult to obtain a suitable reproducibility in the measurement using this method.

In this study we propose an easy, fast, and powerful CE method to determine the alkaline and acid phosphatase activity using a natural substrate (AMP) by quantitative

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UV detection. The possibility to apply a separation method allowed us to check not only the adenosine concentration but also the AMP concentration during the enzyme activity assay. The CE method has demonstrated to be reliable to evaluate enzymatic activity, representing a suitable and convenient alternative to the spectrophotometric method.

5. Conclusions

The results of the present study clearly demonstrate the potentiality and versatility of MEKC method, which could be applied to the routine monitoring of soil enzymes activity. The most important advantage of this method is that allows the use of natural substrate in the enzymatic assay.

The use of nano-procedures has contributed to achieving greener analytical methods not only by automation and miniaturization, but also by replacing toxic reagents by non-contaminating reagents. In this sense, MEKC has the advantages of minimizing both reagent consumption and waste generation.

6. Acknowledgements

7. References

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Figure 1

Electropherogram of the AMP and Adenosine for a) standard solution of Adenosine (10 mM) and AMP (18 mM); b) Alkaline Phosphatase activity; c) Acid Phosphatase activity. Conditions: 20 mM phosphate buffer (pH 8) 10 mM of SDS and 10% ACN; capillary, 47 cm full length, 40 cm effective length, 75 µm ID, 375 µm OD; hydrodynamic injection at 0.5 psi, 5 sec; 25 kV constant voltage; detection by UV absorbance at 264 nm.

Figure 2

Schematic representation of the catalytic mechanism by which the phosphatases enzymes take out the phosphate group from the AMP.

Figure 3

Enzymatic activities in relation to the increase of the substrate concentration (AMP).

Figure 4

Effect of time of incubation concerning with the release of Adenosine in assay of soil phosphatase activities by described method.

Figure 5

Calibration curve; corrected area of the peak of adenosine vs. the different concentrations of the adenosine (C; mM).

Figure 6

Method of the standard addition for both enzymes.

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Table 1. Physical and chemical characteristics of soils

Characteristic	Soil 1	Soil 2	Soil 3	Soil 4
pH	8.1	8.0	8.3	6.9
Clay (%)	5.3	6.5	8.2	9.0
Sand (%)	80	75	63	64
Organic matter (%)	0.63	0.84	0.97	0.59
Total N (mg kg^{-1})	842	1070	1208	798

Table 2. The values for the activities of ALP and ACP in semiarid soil.

Soil nº	Enzyme activity			
	Alkaline phosphatase		Acid phosphatase	
	Mean[a] $\pm$ SD	CV%	Mean[a] $\pm$ SD	CV%
1	44.89 $\pm$ 1.05	2.35	21.95 $\pm$ 0.37	1.67
2	36.75 $\pm$ 0.80	2.18	17.48 $\pm$ 0.57	3.28
3	48.90 $\pm$ 0.92	1.86	15.96 $\pm$ 0.42	2.60
4	21.26 $\pm$ 0.76	3.50	15.38 $\pm$ 0.45	2.94

[a] μmol of free adenosine g^{-1} soil h^{-1}

Table 3. Results obtained from the comparison between different amounts of soil using the proposed method. Conditions: 18 mM of AMP, incubation of 1 h. at 37º C.

Soil nº	Alkaline phosphatase		Acid phosphatase	
	0.1* $\pm$SD	1*$\pm$SD	0.1*$\pm$SD	1*$\pm$SD
1	44.89[a]$\pm$1.05	51.03 [a]$\pm$1.63	21.95 [a]$\pm$0.37	21.17 [a]$\pm$0.45
2	36.75 [a]$\pm$0.80	36.21 [a]$\pm$1.2	17.48 [a]$\pm$0.57	17.94 [a]$\pm$0.24
3	48.90 [a]$\pm$0.92	47.74 [a]$\pm$1.04	15.96 [a]$\pm$0.42	16.14 [a]$\pm$0.52
4	21.26 [a]$\pm$0.76	22.10 [a]$\pm$0.54	15.38 [a]$\pm$0.45	15.54 [a]$\pm$0.29

* Amount of soil in gram.
[a] μmol of free adenosine g^{-1} soil h^{-1}

a

b

c

Time (min)

0   4   8   12

AMP + H₂O → Adenosine + HPO₄²⁻ (via Phophatase)