

# Structural characterization of natural nanomaterials: potential use to increase the phosphorus mineralization.

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## Abstract

Nanomaterials like nanoclays occur widely in nature. The use of nanomaterials as immobilization support improves enzymatic stability and catalytic activity against other materials. The aims of this study were: i) to characterize natural allophanic clay and nanoclay fraction with and without organic matter from an Andisol and ii) to evaluate allophanic and montmorillonite nanoclays as support to immobilize acid phosphatase. The used Andisol belongs to Piedras Negras Series from Southern Chile. Clays and nanoclays extracted were characterized by TEM, SEM, EDX, ED and AFM. The enzymatic activities were measured with  $\alpha$ -nitrophenylphosphate ( $\alpha$ -NPP) as substrate. The kinetics parameters ( $V_{\max}$  and  $K_m$ ) were calculated according to Michaelis–Menten equation. The microscopy analysis showed that the methodology of the nanoclays extraction allowed obtaining aggregates with high proportions of mesoporous, which are suitable to enzymatic immobilization. The nanoclay fractions showed that organic matter governs the feature behavior and is very recalcitrant, suggesting that this nano-fraction plays an important role in carbon sequestration. Finally, we demonstrated that the immobilized phosphatase has a significant increase both in catalytic efficiency ( $V_{\max}/K_m$ ) and in substrate affinity ( $1/K_m$ ) when allophanic and montmorillonite nanoclays were used as material support.

## Key Words

Natural nanoclays; allophane; aluminosilicate, enzyme immobilization, acid phosphatase.

## Introduction

Nanoparticles occur widely in the natural environment (Theng and Yuan 2008). Particles within nanoscale range have been studied in soil sciences a few decades ago to understand the behavior of soil environment (Wada 1987; Parffit *et al.* 1983). However, these particles have not been studied from the nanoscience point of view. In nanoscience, material design and surface feature of nanomaterials play an increasing role in many fields of technological application such as: electronics, medicine, biocatalysis, material science and other. Although, synthetic nanomaterials are relevant for a wide possibility of applications in nanoscience (Garrido *et al.* 2010), the production of nanoparticle with small diameters and a narrow size distribution is expensive and difficult (Hofmann *et al.* 2008). However, in the environment we can find many types of morphologies that actually are synthesized, thereby an interesting alternative is to obtain and use natural nanomaterials. Nanoclays is a real alternative to get a material with nanometric size. In soils derived from volcanic ashes as Andisols (Calabi *et al.* 2009), different structures of nanoparticles like aluminosilicate with nano-ball (allophane) and nano-tube (imogolite) morphology (Wada 1987; Abidin *et al.* 2007) within clay fraction are possible to find. The most important constituent of Andisols is allophane which is a non-crystalline ('short-range order') aluminosilicate with Al/Si ratio varying between 1 and 2. Independent of composition the unit particle of allophane is a hollow spherule with an outer diameter of 3.5–5.0 nm. Imogolite is more crystalline than allophane. The unit particle of imogolite is a hollow tubule with an outer and inner diameter of about 2 and 1 nm, respectively (Wada 1987; Abidin *et al.* 2007). Allophane clays have a high specific surface area. These nanoparticles form stable microaggregates with pores within the nanoscale range with similar physical characteristics to silica nanomaterials which are very important in biocatalysis (Kim *et al.* 2006), because these improve the catalytic efficiency of the enzyme. Previous results from our investigation group showed that acid phosphatase immobilized on allophanic clays from Andisols of Southern Chile increase its catalytic efficiency (Rosas *et al.* 2009). The aims of this study were: i) to characterize natural allophanic clay and nanoclay with and without organic matter from an Andisol and ii) to evaluate allophanic and montmorillonite nanoclays as support material to immobilize acid phosphatase.

## Methods

### *Extraction of nanoclay and Structural and chemical characterization*

An Andisol, from Piedras Negras Series, taken from Southern Chile was sampled within 0–20 cm of depth, sieved to 2 mm mesh and air-dried. One part of the soil was treated with 30% hydrogen peroxide to remove the soil organic matter. The separation of particle-size < 2  $\mu\text{m}$  fractions was performed by sedimentation

procedures based on Stoke's law. The nanoclays were extracted from allophanic clays and montmorillonite clay (AppliChem A6918, LOT 7W007719), these were extracted using clays pre-treated in the similar way as described by Li and Hu (2003). 5 g of clay were suspended in 100 ml of 1 M NaCl. This solution was dispersed applying 214 J by ultrasonic dispersion. The clay suspension was centrifuged at 3000 rpm for 40 min. The pellet was collected, and nine-fold washed with 50 ml of distilled water (these supernatant is the nanoclays suspension). Previous to washing nanoclays, these were moderately stirred for 40 min. The nanoclay suspension was dialyzed (1000 kDa membrane) in pure water until conductivity in ranging 0.8-0.5  $\mu$ S, and later it was vacuum lyophilized. Allophanic clays and nanoclays were characterized throughout energy dispersive X ray (EDX), transmission electron (TEM) and scanning electron (SEM) microscopy, electronic diffraction (ED) and atomic force microscopy (AFM).

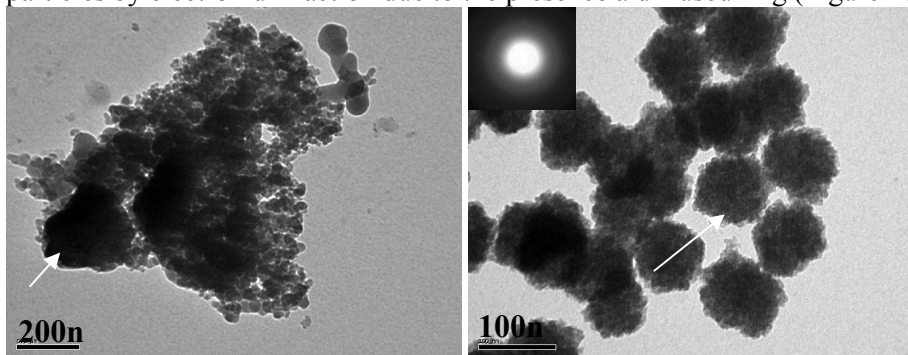
#### *Phosphatase assay*

The complexes were formed by interaction between acid phosphatase and allophanic and montmorillonite nanoclays, and used as model systems to simulate enzymatic reactions in heterogeneous environment. The enzymatic activities of free and immobilised phosphatase were assayed with 6 mM *p*-nitrophenylphosphate (*p*-NPP) and the concentration of *p*-nitrophenol was determined by measuring the absorbance at 405 nm with a spectrophotometer (Rao *et al.* 2000, Rosas *et al.* 2009). To avoid interference by turbidity, the samples were centrifuged at 14,000 g for 4 min prior to measurement. The kinetics parameters ( $V_{max}$  and  $K_m$  values) were calculated according to Michaelis–Menten equation varying *p*-NPP concentrations ranging from 0 to 6 mM. The enzymatic determinations were made in triplicate and analyzed using the SPSS software, version 14.0. The Tukey test was applied to data using a 0.05 significance level of probability.

## **Results**

### *Structural and chemical characterization*

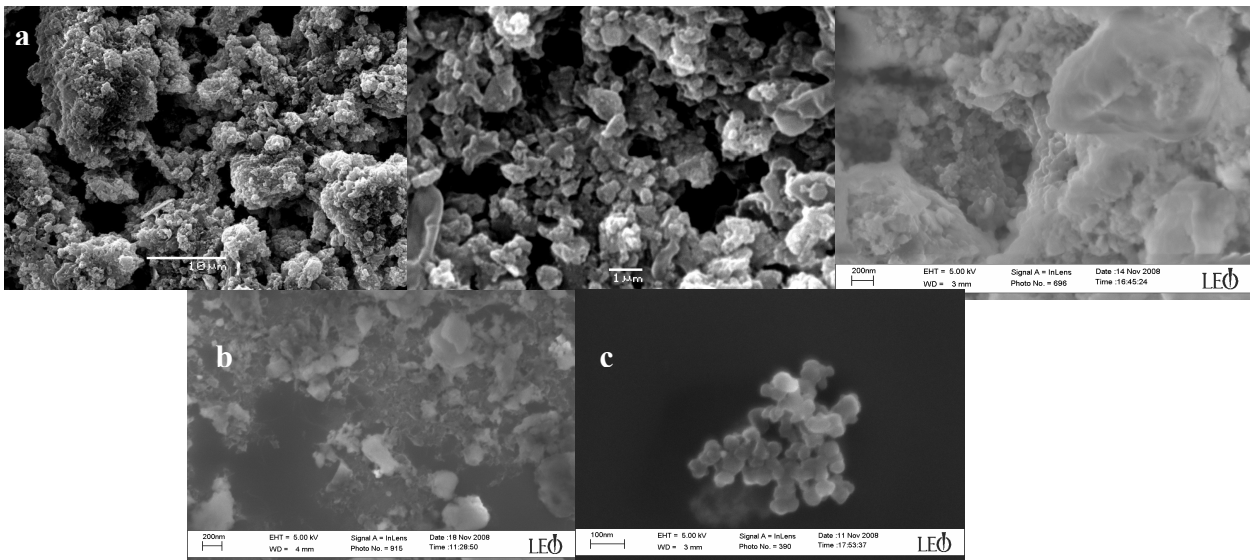
The TEM analysis showed the allophanic nanoclays in the samples, with predominant size < 50 nm with organic matter (OM). We could appreciate the high occurrence of allophane nanoparticles (outer diameter around 5 – 3 nm) with two kinds of morphology: as gel (arrow short) or condensed aggregates (arrow long), which are commonly reported for allophane materials. The Figure 1a showed the presence of high nanoclay content in the clay samples and some crystalline material. Also, we observed the occurrence of amorphous particles by electron diffraction due to the presence a diffused ring (Figure 1b).



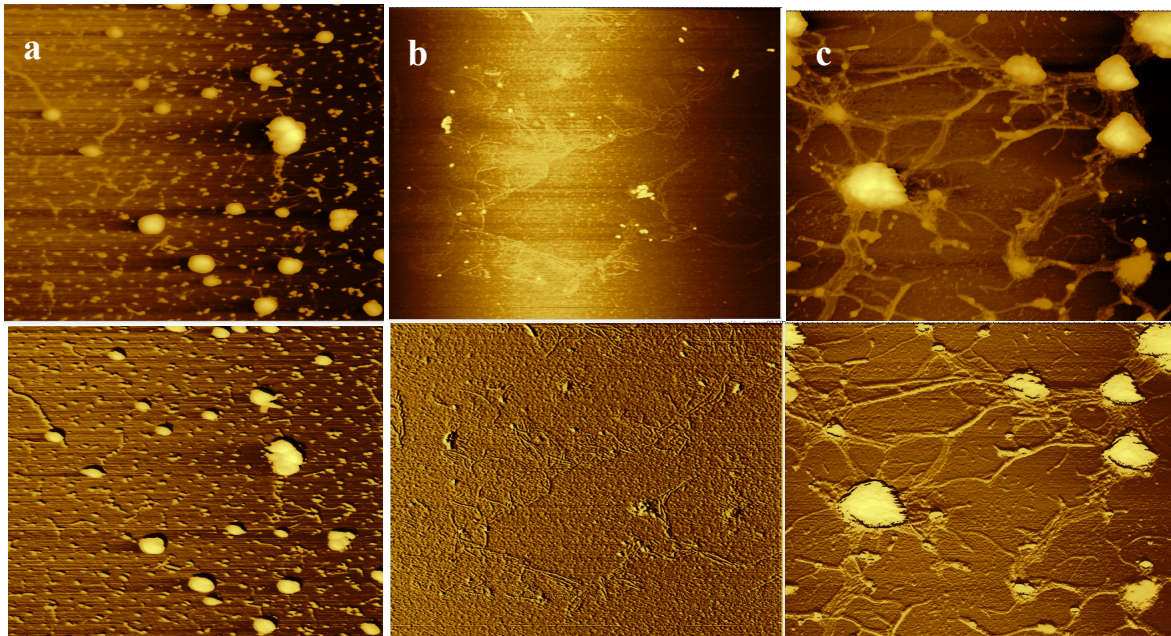
**Figure 1. TEM and ED of nanoclays and clays in the presence of organic matter.**

The SEM analysis showed difference between clays and nanoclays from allophanic soil with OM and after treatment to remove OM in size (Figure 2). The SEM images showed that nanoclay aggregates with OM (Figure 2a) have high amount of mesoporous with different sizes, this can absorb water from surrounding environment (Okada *et al.* 2008). Thus, providing an aqueous microenvironment which could be useful for biotechnological applications (Li and Hu 2003), allowing an adequate mass transport for both substrates and products. The OM removal exhibited nanoclay aggregates (Figure 2c) of size less than 50 nm and showed cavities in the nanoscale.

The AFM analysis confirmed the presence of allophane and imogolite (Figure 3), and the abundance of aggregates with similar size such as those observed by TEM. AFM observations from allophanic clay samples showed clearly a high content of OM forming networks between particles, and also inside the aggregates as coating (Figure 3c) but, we can not observed clearly the OM content in nanoclays (Figure 3a). However, the elemental analysis showed that the nanoclays (28%) have a 20% higher OM content than clays (19%). This could be explained due to the strong interaction between OM and nanoclays, as we evaluated when we tried to remove it repeatedly with hydrogen peroxide. The analysis of AFM images of allophanic nanoclays after treatment to remove OM (Figure 3b) allowed identify smaller and cleaner allophane and imogolite nanoclays.



**Figure 2. SEM images from allophanic clay and nanoclays. (a) nanoclays with OM at different amplification, (b) allophanic clays with OM and (c) allophanic nanoclays without OM.**



**Figure 3. AFM from allophanic nanoclays and clays. (a) nanoclays with OM, (b) allophanic nanoclays without OM and (c) allophanic clays with OM.**

By means of microtopography AFM we confirmed nanoparticles presence with basal length and thickness of single particle aggregates in the nanoscale. The OM removal in nanoclays produced a decrease of maximal-height aggregates from 103.3 to 12 nm for nanoclays (Figure 3a,b). However, we observe no differences in the average values of smaller height. In fact, this is another evidence of the strong interaction between OM and nanoclay. Therefore, the recalcitrant OM content is able to affect the structural and physicochemical features of this material (Mora and Canales 1995). Thus, allophanic nanoclays are a very important fraction for carbon sequestration in Andisols.

#### *Phosphatase assay*

Results showed that acid phosphatase immobilization on allophanic and montmorillonite nanoclays reduced the enzymatic activity in 23 and 30 %, respectively. Huang *et al.* (2005) used different support materials from soil colloids to enzyme immobilization and reported an important specific activity reduction between 28 and 61 %. In addition, Rao *et al.* (2000) obtained high deactivation values (37 at 77 %) after acid phosphatase immobilization on montmorillonite clay, tannic acid and organo-mineral complex. Therefore, allophanic and montmorillonite nanoclays are suitable as material support. The kinetic parameters obtained from Michaelis-Menten equation showed that the enzymatic immobilization on allophanic and montmorillonite nanoclays increased both the catalytic efficiency in 55 and 120 %, and the substrate affinity

( $1/K_m$ ) in 50 and 60 % (Table 1). Therefore, nanoclays are suitable support materials to acid phosphatase immobilization.

**Table 1. Kinetic parameters of acid phosphatase free (P) and immobilized in allophanic nanoclays (AN-P complexes) and montmorillonite nanoclays (MN-P complexes).**

	$V_{max}$	$K_m$	$V_{max}/K_m^*$	R
	( $\bar{\square}$ NP $\square$ mol/ml/min)	(mM)		
P	0,269	0,260	1,04	0,993
AN-P complexes	0,217	0,135	1,61	0,989
MN-P complexes	0,195	0,082	2,37	0,985

\* $V_{max}/K_m$ : catalytic efficiency.

## Conclusions

The methodology of Li and Hu (2003) modified by our Group, reduced the time of nanoclay extraction by changing the shaking by ultrasonic system and allowed more dispersed particle aggregates. Furthermore, we obtained more homogeneous nanoclay materials. A recalcitrant fraction of OM in the nanoclays was observed. This fraction affected the structural and physicochemical material characteristics.

We observed a significant increase both of the catalytic efficiency in 55 and 120 %, and the substrate affinity in 50 and 60 %, when allophanic and montmorillonite nanoclays were used as support materials for phosphatase immobilization.

In general, the nanomaterials are more promising candidates for enzyme immobilization than conventional supports because they can carry a high enzyme load, while substrate diffusion is relatively unhindered. Then, enzymes immobilized on nanoclay can be show a high mobility and activity, suggesting that the molecules are not rigidly attached to the support materials, also the stereochemistry of the immobilized enzymes is more able to attach to the substrate. Moreover, the good match between the pore dimension of nanomaterials and enzyme molecular size has a stabilizing effect on the immobilized enzyme.

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