



## Short Paper

# Soil montmorillonite can exhibit peroxidase-like activity

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### Abstract

Montmorillonite (Mnt), belonging to the smectite group and representing a 2:1-type clay mineral with good cation-exchange and swelling capacities, is a common clay component of soils. It was observed that some Mnt preparations exhibit peroxidase-like (P-L) activity using N,N-diethyl-p-phenylenediamine sulfate salt as a substrate. Both native (non-swelling) and swelling Mnt exhibited similar P-L activity. Modification of Mnt with copper and iron influenced both P-L activity and phenol polymerization. Both free and textile-bound Cu-Mnt (Sigma-Aldrich) enabled phenol removal. Soil Mnt P-L activity is probably involved in lignin breakdown and decontamination of soils polluted with phenol-containing molecules.

**Keywords:** Clays; montmorillonite; peroxidase-like activity; phenol polymerization; soil nanozymology

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Soil enzymes have an extremely important role in organic matter formation and decomposition, stabilization of soil structure and nutrient cycling and transformation. Soil enzymes are also involved in the remediation of contaminated soils. Soil enzyme activities can serve as indicators of microbial growth, activity and soil ecosystem health. Many soil enzymes can be powerful biological indicators for assessing heavy metal toxicity. Large amounts of enzymes are present in soils, including amylases, aryl-sulfatases, glucosidases, cellulases, chitinases, dehydrogenases, phosphatases, proteases, ureases and peroxidases of plant, animal and microbial origin (Bakshi & Varma, 2011; Dotaniya *et al.*, 2019; Cui *et al.*, 2021; Fanin *et al.*, 2022).

Soil peroxidases represent important enzymes that use hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as an electron acceptor. This group of enzymes catalyses oxidation reactions *via* the reduction of H<sub>2</sub>O<sub>2</sub>; they are considered to be used by soil microorganisms as lignolytic enzymes because they can degrade molecules that lack a precisely repeating structure. Lignin breakdown results in significant contributions to soil N and C pools and makes available nutrients to soil microbes (Sinsabaugh, 2010; Plante *et al.*, 2015; Hassan *et al.*, 2022).

Recently, it was observed that soil magnetic iron oxide particles exhibiting peroxidase-like (P-L) activity can substantially influence peroxidase activity measured in soil suspensions (Safarik & Prochazkova, 2022). It has to be taken into account that this activity can be caused both by enzymes (peroxidases) of microbial, plant and animal origin and by inorganic peroxidase-mimetic minerals. A new research area called ‘soil nanozymology’, devoted to the study of soil-related nanozymes and other enzyme-mimetic materials, has been defined (Safarik & Prochazkova, 2022).

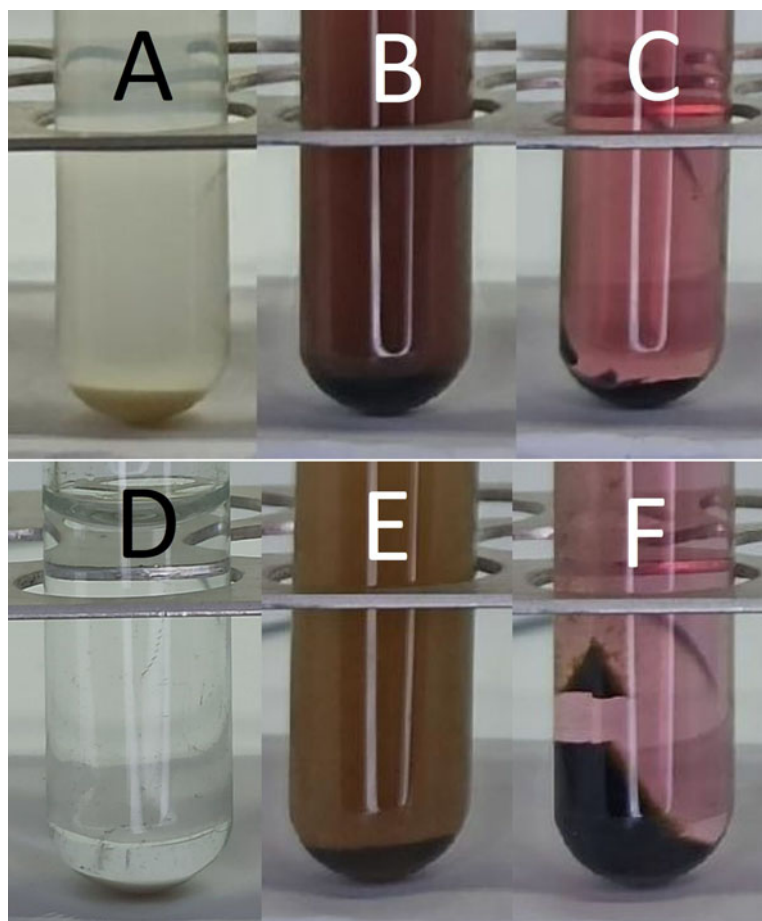
In further experiments, soil autoclaved twice at 121°C for 20 min and, after drying, heated in a hot air dryer (18 h, 200°C) to degrade natural peroxidases, with completely removed magnetic iron oxide particles, still exhibited P-L activity using N,N-diethyl-p-phenylenediamine sulfate salt (DPD) as the substrate. In fact, several soil constituents might be responsible for this behaviour, including antiferromagnetic hematite (Chaudhari *et al.*, 2012) or specific clays (Feng *et al.*, 2021). To test the behaviour of three commercially available soil-related clays, namely bentonite, halloysite and montmorillonite (Mnt; all from Sigma-Aldrich, USA), their P-L activity was tested with DPD as the substrate. It was observed that Mnt exhibited strong P-L activity (see Fig. 1), whereas bentonite and halloysite exhibited no measurable P-L activity, although bentonite consists mainly of Mnt.

Mnt is a common clay component of soils belonging to the smectite group and representing a 2:1-type clay mineral with good cation-exchange capacity (CEC) and swelling properties. Mnt is also an efficient, low-cost adsorbent, especially for various cationic contaminants (Zhu *et al.*, 2016). Typical Mnt-rich soils are Vertisols exhibiting deep black colour and seasonal severe cracking after drying and swelling upon being water-logged. Vertisols form both in tropical and subtropical regions with high precipitation and in subarid areas (McGarry, 1996; Pal *et al.*, 2012; Jordanova, 2017). Soils containing high Mnt contents have been tested as additives to improve the quality of sandy soils and to increase crop yields (Jia *et al.*, 2018; Yu *et al.*, 2022).

To characterize the P-L activity of Mnt in more detail, four different Mnt preparations were tested. Mnt K-10 powder was from Sigma-Aldrich (catalogue no. 69866; produced by the calcination of native Mnt). Dellite® HPS (Na-Mnt, CEC = 128 meq 100 g<sup>-1</sup>) and Dellite® LVF (Na-Mnt, CEC = 105 meq 100 g<sup>-1</sup>) were supplied by Laviosa Advanced Mineral Solutions (Italy), whereas Shrimp Nature Mnt powder (no detailed information available) was obtained from Shrimppworld.cz (Czech Republic).

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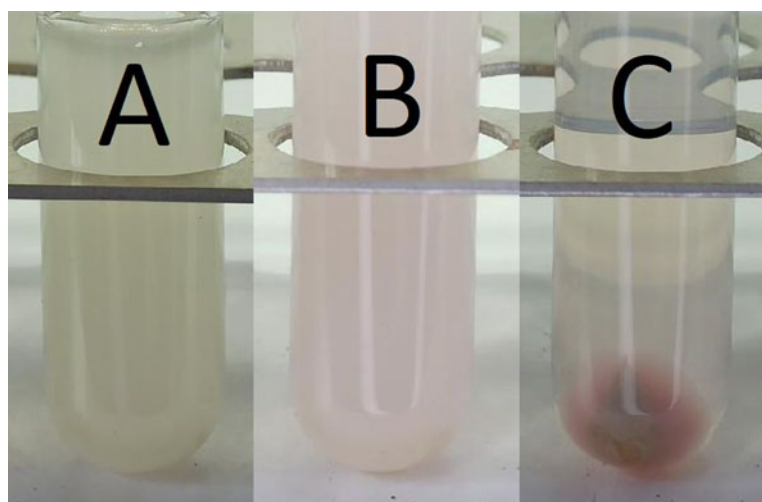


**Figure 1.** P-L activity of Mnt (Sigma-Aldrich). Upper figures – non-swollen Mnts: A = Mnt suspension without DPD; B = Mnt suspension with DPD + H<sub>2</sub>O<sub>2</sub> before centrifugation; C = Mnt with DPD + H<sub>2</sub>O<sub>2</sub> after centrifugation. Bottom figures – swollen Mnts: D = Mnt suspension without DPD; E = Mnt suspension with DPD + H<sub>2</sub>O<sub>2</sub> before centrifugation; F = Mnt with DPD + H<sub>2</sub>O<sub>2</sub> after centrifugation.

The P-L activities of all four Mnt materials were studied using DPD as the substrate, as described previously (Safarik & Prochazkova, 2022). The reaction mixture contained 3.4 mL of deionized water, 400  $\mu$ L of DPD solution (12.53 mmol L<sup>-1</sup>) and 200  $\mu$ L of 2% H<sub>2</sub>O<sub>2</sub>. A total of 5–30 mg of Mnt materials was used for the assays performed at 20°C; the incubation time was 3 or 8 min, followed by centrifugation for 2 min. Corresponding blank samples containing all of the reagents except the Mnt materials were also prepared. Absorbance at a wavelength of 551 nm

was measured. In addition to the P-L assay with natural (non-swelling) Mnt samples, Mnt samples swollen in 3.4 mL of deionized water for 1 week at 20°C before the assay were also analysed.

Figure 1 shows the peroxidase-mimetic activity of the most efficient Mnt sample (commercial preparation from Sigma-Aldrich); a red-coloured oxidized DPD product was formed during the assay. Due to the fact that Mnt is a well-known adsorbent, the red-coloured reaction product was bound to Mnt powder,



**Figure 2.** P-L activity of non-swollen Mnts (Dellite® LVF). A = Mnt suspension without DPD; B = Mnt suspension with DPD + H<sub>2</sub>O<sub>2</sub> before centrifugation; C = Mnt with DPD + H<sub>2</sub>O<sub>2</sub> after centrifugation.

**Table 1.** Specific P-L activities of native and modified Mnt samples (nkat mg<sup>-1</sup>) and absorbances at 600 nm after Mnt sample reaction with phenol in the presence of H<sub>2</sub>O<sub>2</sub> for 24 or 48 h (phenol polymerization).

Mnt type	P-L activity (nkat mg <sup>-1</sup> )	Phenol polymerization (absorbance at 600 nm)	
		24 h reaction	48 h reaction
Mnt (Sigma-Aldrich)	8.81	0.06	0.11
Fe-Mnt (Sigma-Aldrich)	7.42	0.01	1.44
Cu-Mnt (Sigma-Aldrich)	2.14	0.88	1.55
Mnt (Dellite® HPS)	– <sup>a</sup>	0.39	0.65
Fe-Mnt (Dellite® HPS)	7.49	1.16	0.80
Cu-Mnt (Dellite® HPS)	3.30	0.44	0.70
Mnt (Dellite® LVF)	– <sup>a</sup>	0.21	0.35
Fe-Mnt (Dellite® LVF)	9.60	1.32	1.10
Cu-Mnt (Dellite® LVF)	2.17	0.56	0.71
Mnt (Shrimp Nature)	0.66	0.04	0.09
Fe-Mnt (Shrimp Nature)	9.75	1.47	1.04
Cu-Mnt (Shrimp Nature)	2.63	0.44	0.48

<sup>a</sup>Red-coloured oxidized DPD product was bound to Mnt particles.

causing its dark red colouration. A similar situation was observed using another peroxidase substrate, namely ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), forming a blue reaction product. The observed P-L activity of this Mnt material is in agreement with a previously analysed Chinese Mnt material that was assayed for its P-L activity with 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate (Feng *et al.*, 2021). Swelling of Mnt in water did not substantially influence its ability to act as a P-L catalyst (Fig. 1d–f).

The Dellite® LVF Mnt material (Fig. 2) exhibited substantially lower P-L activity, which resulted in a weak red colouration of sedimented Mnt after the assay; no free reaction product remained in the supernatant. In addition, the two remaining Mnt materials exhibited only weak P-L activity. This suggests that materials with identical chemical compositions might not all exhibit enzyme-mimetic activity; other typical examples are cerium dioxide particles of various particle sizes (Hamidat *et al.*, 2016).

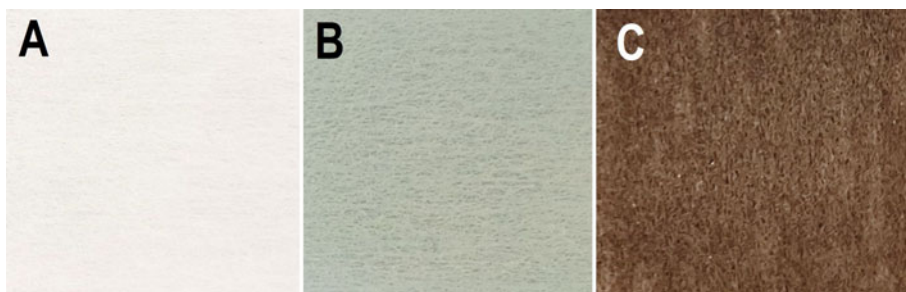
To evaluate the P-L activities of the tested Mnt materials, we measured the absorbance values of the coloured DPD reaction products in the supernatant after centrifugation; however, these were also strongly adsorbed on the Mnt surface in the sediment (Figs 1 & 2). This does not allow for measuring catalytic activity accurately. This is why 5 mg of the Mnt samples was used in the assays to limit the effects of adsorption. Therefore, arbitrary values of the specific P-L activities of the Mnt samples are given as the values expressing P-L activities not considering the adsorption of the reaction products (Table 1). The Mnt from Sigma-Aldrich exhibited the highest specific P-L activity despite the strong adsorption of the reaction product. Other Mnt preparations exhibited substantially lower specific P-L activities, and in

some cases the reaction product was completely bound to the Mnt surface.

The Mnt easily interacts with metal ions (Bhattacharyya & Sen Gupta, 2008). It was observed previously that copper and iron ions bound to solid surfaces can exhibit high P-L activity (Castro *et al.*, 2013; Wu *et al.*, 2014; Pospiskova & Safarik, 2022). Thus, Mnt with bound metal ions may exhibit even higher P-L activity than native Mnt. To test this possibility, Mnt samples were immersed in excess of 5% CuSO<sub>4</sub>·5H<sub>2</sub>O or FeSO<sub>4</sub>·7H<sub>2</sub>O solutions for 24 h. After thorough washing to remove free (unbound) metal ions, the modified clays were dried and used for P-L activity measurements. The results are presented in Table 1. As expected, metal modification usually increased specific P-L activities; iron modification led to higher values.

Materials with P-L activity have been successfully used in various biotechnology and environmental technology applications instead of natural peroxidases – for example, for the decolourization of organic dyes (Safarik *et al.*, 2021) or for the removal of phenol, aniline and related compounds (Zhang *et al.*, 2009; Jiang *et al.*, 2018). Phenol polymerization by plant peroxidases and the formation of dark reaction products have been described previously (Reihmann & Ritter, 2006). To test the ability of Mnt and its metal derivatives to polymerize phenol, 5 mg of Mnt derivatives was added to 4.9 mL of 5% phenol solution, and then 100 µL of 30% H<sub>2</sub>O<sub>2</sub> was added. After 24 and 48 h, the absorbances of the reaction supernatants at 600 nm were measured (higher absorbance corresponds to higher phenol polymerization), and the results are presented in Table 1. Native Mnt exhibited rather low phenol-polymerizing activity; however, this activity increased substantially after Mnt interaction with iron and copper ions. Interestingly, Cu-Mnt from Sigma-Aldrich demonstrated substantially higher phenol-polymerizing activity than the iron derivative after 24 h of incubation, but after 48 h the results were comparable. For other Mnt types, the iron modification was more effective for phenol polymerization than the copper modification. In any case, an increase in phenol-polymerization activity in vineyard soils containing higher copper concentrations due to the regular application of copper-based fungicides (Droz *et al.*, 2021) is to be expected.

In addition, the abovementioned activity was displayed not only by free particles of Mnt and its derivatives, but also by immobilized particles. Figure 3 shows the example of Cu-Mnt (Sigma-Aldrich) particles bound to a low-cost colour catcher sheet (Color & Dirt Catcher Sheets, Brauns-Heitmann GmbH & Co. KG, Germany) before and after interaction with phenol in the presence of H<sub>2</sub>O<sub>2</sub> (see above). The dark-coloured reaction product (polymerized phenol) was efficiently bound to the sheet, thus representing the combination of both enzyme-like and adsorbent properties. This result has interesting potential implications for environmental technology applications.



**Figure 3.** Pieces of colour catcher sheet (Color & Dirt Catcher Sheets, Brauns-Heitmann GmbH & Co. KG, Germany): A = native sheet; B = sheet after binding Cu-Mnt (Sigma-Aldrich); C = modified sheet after 24 h of interaction with phenol in the presence of H<sub>2</sub>O<sub>2</sub>.

Commercially available Mnt preparations have been tested for their P-L activity. It is expected that the potential P-L activity of soil Mnt and its complexes with metal cations will be utilized for activities usually performed by peroxidases, namely lignin breakdown and the decontamination of soils polluted with phenol-containing molecules (Hassan *et al.*, 2022). Even at a low reaction rate, these long-term catalytic processes may lead to substantial decontamination of polluted soils.

In conclusion, specific mineral nanoparticles present in soils and exhibiting intrinsic enzyme-like activities could play important roles in the environmental biogeochemical cycles of elements, nutrients and pollutants (Chi & Yu, 2021). Our study could encourage further research in the area of soil nanozymology to detect other interesting soil-derived enzyme-mimetic materials.

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**Author contributions.** Ivo Safarik: Supervision, Conceptualization, Writing – original draft, review & editing. Jitka Prochazkova: Methodology, Investigation, Writing – review & editing.

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**Conflicts of interest.** The authors declare none.

**Data availability.** Data will be made available on request.

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