Proteases activities of Andosols after the addition of clay and humic substances in the presence of cadmium

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Abstract
The addition of clay and humic substances to soils may control the negative effect of Cd through both ion adsorption and the stabilization of soil enzymes. In this regard we investigated the response of proteases activities in soils to Cd after the addition of allophanic clay or aromatic humic substances, separated from the soil samples used. The experiment was done at three levels of clay, humic substances and Cd (0, +5%, and +10% of original content of clay, 0, +5%, and +10% of original soil organic matter content as carbon, and 0, 10, and 50 mg Cd kg^{-1} soil). Two surface soil samples were obtained from Andosols under a forest and a cultivated field in an upland area of Japan's Kanto district. We determined proteases activities and Exchangeable Cd in both soil samples after 0, 2, 10 and 40 days of incubation. Cd addition decreased the proteases activities in both soil samples, while the addition of allophanic clay or aromatic humic substances significantly enhanced proteases activities even in the presence of Cd. Thus the addition of clay or humic substances did not have a significant positive effect on the inhibitory effect of Cd. These results indicate that both allophanic clay and aromatic humic substances separated from Andosols stabilize proteases of Andosols, but have little capacity to adsorb Cd^{2+}.

Key Words
Protease activity, cadmium, allophane, humic substances, soil improvement, Andosol.

Introduction
Heavy metals like Cd have long-term toxic effects on soil biological functions, including soil microbial communities and enzyme activities (Majer *et al.* 2002). Since Cd is not biodegradable, it is considered as one of the most serious groups of environmental contaminants (Bailey *et al.* 1999). Therefore the reclamation of contaminated soils with Cd is only possible by using the techniques that extract or stabilize the contaminant (Pérez-de-Mora *et al.* 2006). Our objectives of this study were to examine the response of the proteases activities of Andosols to the addition of clay or humic substances in the presence of Cd during a short-term incubation experiment, and also to find the factors affecting proteases activities. The hypothesis was that addition of clay or humic substances would reduce Cd solubility and increase soil proteases activities.

Methods
Study sites and soil samples
Two surface soil samples were collected from a forest and a cultivated field at the Agricultural and Forestry Research Center, University of Tsukuba, located in an upland area of Kanto district, Japan. The forest was a natural mixed wood stand of warm-temperate trees (mainly *Quercus serrata* and *Pinus densiflora*), and the field has been continuously cultivated with corn for more than 10 years. The two sites are 200 m apart. Both soils are classified as Umbric Andosols (FAO 2006). Soil samples were sieved through a 2-mm mesh and kept moist in plastic containers for one week at room temperature (20-25°C) before use. Clay fractions were separated from soil samples by the sedimentation method. Extraction and preparation of humic substances were carried out according to the combination of IHSS method (International Humic Substances Society, http://www.ihss.gatech.edu/, 1996) and Hiradate *et al.* (2007).

Characterization of clay and humic substances
Oriented clay fraction samples were subjected to X-ray diffraction (XRD), after the DCB treatment, using a X-ray diffractometer (RAD-X; Rigaku Intl. Co., Tokyo, Japan) with Cu-Kα radiation (λ=1.54 Å). XRD analyses of the samples were conducted after the separate treatments by air drying (Mg-saturated; K-saturated); ethylene glycol (Mg-saturated); and heating to 350°C and 550°C (both K-saturated). Infrared spectroscopic analysis of Mg-saturated clays was also carried out by a FT-IR spectrometer (FT-720; Horiba, Ltd., Tokyo, Japan) over the range 400-4000 cm^{-1}. Chemical analyses of Fe (Fe_d) and Al (Al_d) extracted by DCB and Si (Si_o), Fe (Fe_o) and Al (Al_o) extracted by acid ammonium oxalate (0.3 mol/L, pH 3) were done by

ICP-AES (ICAP-757, Nippon Jarrell-Ash, Tokyo, Japan). Weight loss (%) arising from the treatment by ammonium oxalate was also calculated. Elemental analysis (C, H and N) of humic substances was carried out in triplicate using Flash EA 1112 NCS Analyzer (Thermo Finnigan, Italy). Ratios of C/N and atomic ratio of H/C and O/C were calculated. Infrared spectroscopic analysis of humic substances was also carried out by a FT-IR spectrometer (FT-720; Horiba, Ltd., Tokyo, Japan) in the range of 4000-400 cm^{-1}. Solid-state CP/MAS ^{13}C NMR spectra of the humic substances were recorded with a FT-NMR system (Alpha 300, JEOL, Tokyo).

Addition of clay fractions or humic substances to the soil samples
In the present study two levels of clay fraction and or humic substances (separated from the soil samples used) were added to the soils, designated as low and high concentrations (LC and HC, respectively). Experiments were also performed with the soil samples containing the original concentration of clay and soil organic matter (OC: no addition of clay fractions or humic substances). These two amendments were added as their water suspensions in the LC and HC systems, giving 5% and 10% higher content than OC, respectively. In case of humic substances the content was calculated as carbon. Then doses of 0-, 1- and 5-fold of the critical Cd level, 10 mg Cd kg^{-1} soil (Chang and Broadbent 1981; Effron *et al.* 2004), were added to the soil samples using the solutions of $Cd(NO_3)_2$ of different concentrations, designated as Cd0, Cd10, and Cd50, respectively. Incubation periods were 0, 2, 10 and 40 days, designated as d0, d2, d10 and d40, respectively.

Proteases activities
Protease activity was measured after 0, 2, 10 and 40 days of incubation based on the method of Ladd and Butler (1972) with slight modifications by using colorimetric determination of leucine released by *N*-benzyloxycarbonyl L-phenylalanine L-leucine as substrate, followed by the incubation for 1 h at 40°C in dark. The absorbance at 570 nm was measured by a spectrophotometer (U-3210, Hitachi Co., Tokyo, Japan). Proteases activities were expressed as ng leucine g^{-1} dried soil sec^{-1}.

Exchangeable Cd
Incubated soil samples were subjected to the analysis of exchangeable Cd, based on the method of Sadamoto *et al.* (1994) in each incubation day. The extraction of Cd was done with $Ca(NO_3)_2$ (0.05 mol/L) with a soil/solution ratio of 1/10 after the end to end shaking (200 rpm) for 24 h at 30 °C. The supernatant, obtained after centrifuging at 1700 × g for 10 min, was filtered through a 0.2 μm filter (Millipore Co., Bedford, MA, USA), and Cd was measured by ICP-AES (Optima 5300 DV, Tokyo, Japan). Exchangeable Cd was expressed as mg Cd kg^{-1} soil.

Results
Characterization of clay and humic substances
X-ray diffraction patterns of oriented clay fractions from the forest and the cultivated field soil samples showed that the crystalline layer silicates are composed of kaolinite, illite and chlorite in addition to the primary minerals of quartz and feldspars. The atomic ratios of Al/Si extracted by acid ammonium oxalate were 2.02 and 1.95 for the forest and the cultivated field soil sample, respectively. Moreover considering the total weight loss (%) on acid oxalate treatment (56% and 61%, respectively), the two separated clay fractions are largely composed of allophane (probably including imogolite) and ferrihydrite in addition to the layer silicates mentioned above.

Humic substances from the forest soil sample had higher C, H and N contents than those from the cultivated field soil sample. The H/C ratio of humic substances from both samples was low, probably due to high O content in both samples. The higher O content and O/C ratio in the cultivated field soil than the forest soil may indicate higher amounts of more oxidized organic materials in the cultivated field sample.^{13}C NMR spectra showed that a small difference was observed in the alkyl region (0-60 ppm) between the forest and the cultivated field soil samples. In the cultivated field soil sample relative percentage of the alkyl group was less than the forest soil sample. Humic substances from the cultivated field soil sample included higher content of O-alkyl C (60-110 ppm). Percentage of aromatic C (110-160 ppm) in the forest soil sample was slightly higher than the cultivated field soil sample, while carbonyl-C (160-190 ppm) of the cultivated field soil was slightly higher than the forest soil. The ratio of C_{alip}/C_{arom} also indicated higher amount of aromatic C in the forest soil samples. Moreover hydrophobicity was less for the cultivated field soil sample. Thus the NMR spectra of both humic substances showed aromatic nature in general.

Proteases activities and exchangeable Cd after the addition of clay in the presence of Cd
As shown in Figure 1 the addition of separated clay to both the forest and the cultivated field soil samples significantly increased proteases activities ($p < 0.01$) compared with the control (OC) on each incubation time. Proteases activities were almost in the order of HC > LC > OC. Addition of Cd significantly inhibited proteases activities during incubation periods ($p < 0.01$) in most of the two soil samples. The effect of time on proteases activities also was significant ($p < 0.01$), fluctuated over the incubation periods. This may relate to the changes in the microbial community during the incubation time (Pérez-de-Mora *et al.* 2006). Exchangeable Cd increased in proportion to the total added Cd in both soil samples. However there were little changes in the exchangeable Cd in term of the amount of added clay or incubation time. Exchangeable Cd in the forest soil samples was higher than the cultivated field soil samples. This can be attributed to the difference in pH between these soils, since soil pH has the greatest impact on the availability and mobility of metal cations. Exchangeable Cd showed no clear change in LC and HC systems in the forest soil samples, and seemed to fluctuate among the different incubation times. In the cultivated field soil samples the addition of clay led to a significant decrease of exchangeable Cd at all doses of Cd, but again it was fluctuated during incubation times.

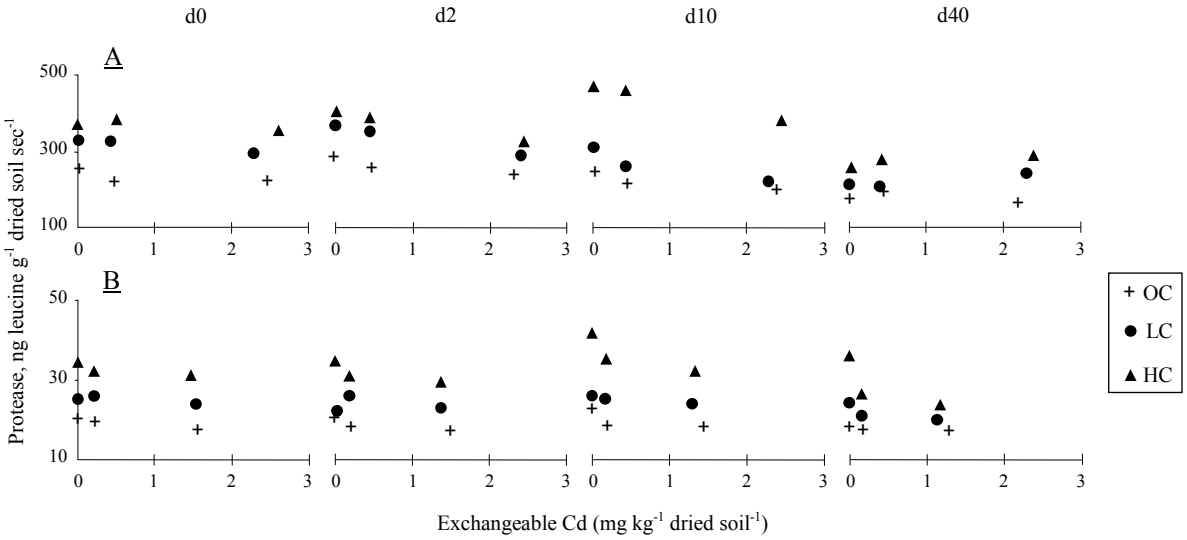

Figure 1. Correlation between proteases activities and exchangeable Cd in (A) forest and (B) cultivated field soil samples after the addition of clay fractions and Cd.

Proteases activities and exchangeable Cd after the addition of humic substances in the presence of Cd
The response of proteases activities after the addition of aromatic humic substances was analogous with that of allophanic clay addition. As shown in Figure 2 the addition of different concentrations of humic substances significantly increased proteases activities ($p < 0.01$) compared with control (OC) in both soil samples on each incubation days, except 40 days of incubation (d40) of the forest soil samples. The increase in the activity was observed in three levels of Cd addition. However these results have shown that the activity enhancement did not occur in proportion to the increase in the levels of humic substances. In the present study the enhancement of enzyme activity is more evident for forest soil samples than the cultivated field sample. This may be attributed to the difference in the contents of functional groups of humic substances, as described in the characterization of humic substances used in the present study.

Levels of exchangeable Cd became higher in proportion to the total Cd content of the soils after the addition of Cd in both the forest and the cultivated field soil samples. But the response of exchangeable Cd in the forest and the cultivated field soil was different after the addition of humic substances. In the forest soil samples it didn't show clear change after the addition of different contents of humic substances and the exchangeable Cd decreased significantly in LC, but it was fluctuated in HC during different incubation days. While, in the cultivated field soil samples, it significantly increased in both LC and HC ($p < 0.01$) at all levels of Cd doses.

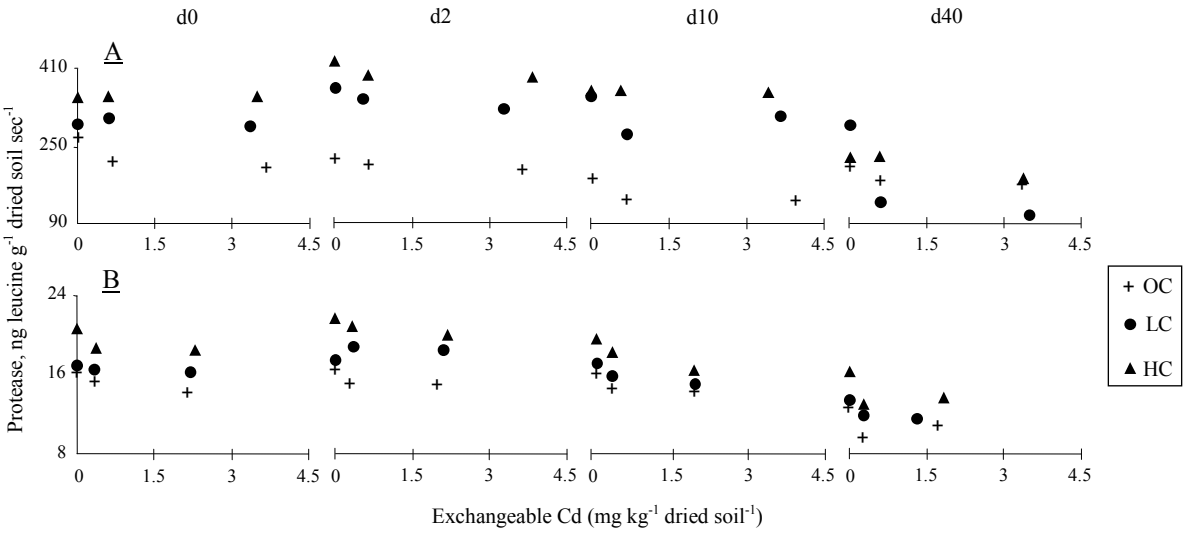

Figure 2. Correlation between proteases activities and exchangeable Cd in (A) forest and (B) cultivated field soil samples after the addition of humic substances and Cd.

Conclusion

Soluble and exchangeable fractions of heavy metals are the most important pools regarding the toxicity and bioavailability of heavy metals. One of the main reasons for the application of both clay minerals and humic substances was to change bioavailability of Cd in soils. Although the addition of humic substances seems to be an important factor affecting proteases activities in the presence of Cd, the addition of both amendments showed the stabilization of proteases, but had little contribution on the inhibitory effect of Cd.

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