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Chemical characteristics and influences of two fractions of Chinese lignite humic acids on urease

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ABSTRACT

Soil urease is crucial for the nitrogen cycle and its association with humic acids (HAs) is a fundamental requirement for its stability. In this work, the chemical characteristic of two HA fractions (HA1, ≥50 kDa; HA2, 10–50 kDa) extracted from lignite was evaluated, and their effects on the activity and stability of Jack Bean urease were also studied. HA1 and HA2 exhibited different structural properties in the micro-FT-IR and ^{13}C NMR spectra and influences on urease stability during 12 days of incubation: HA1 stabilized the urease activity. After 12 days, the residual activity of urease, at pH 6.0, 7.0 and 8.0, was 2.1, 2.6 and 3.9 times higher in the treatment of HA1-urease than in the free urease, respectively. With pH values increasing, the stability of free urease decreased and that of HA1-urease increased, which indicated that HA1 improved the stability of urease in the solution, especially at the alkaline condition.

1. Introduction

The use of nitrogenous fertilizers in agriculture has serious consequences both for water quality and climate change. The disruption to ecosystems posed by fixed-N pollution has been described as one of the major threats to the environment [35]. Several reports have linked increasing levels of anthropogenic N oxides in the atmosphere to increasing fertilizer usage [16,19,32]. Urea fertilizers are the most abundant form of nitrogenous fertilizer applied to agricultural soils [13]. They are decomposed in soil by urease into ammonia, making it available to plants. However, large amounts of ammonia not absorbed by plants immediately, are lost during volatilization, or fixed by clay minerals; soil organic matter can undergo nitrification to NO_2^- and NO_3^- under aerobic conditions. NO_2^- and NO_3^- in turn may undergo denitrification to NO and N_2O under anaerobic conditions [6], which cause loss of nitrogenous fertilizer and atmosphere pollution.

As urease makes urea N available to plants, it plays an important role in the utilization of nitrogenous fertilizer and is crucial to the nitrogen cycle. The worldwide use of urea as a N fertilizer is made possible by the ubiquitous presence of urease in soils, where it is produced by a vast number of both eukaryotic and prokaryotic organisms [27]. Like many other soil enzymes, it can be either intracellular or extracellular, and the extracellular urease can be either free or immobilized on mineral matter or soil organic matter [5,30,31].

Soil organic matter consists mainly of natural macromolecular structures such as humic acids (HAs) which are amorphous, colloidal and polydisperse substances yellow to brown-black in color. In various ways HAs participate in biogeochemical processes in the carbon and nitrogen cycles

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and differently modify the behavior of anthropogenic pollutants in soil [15,28]. In addition, they can stabilize the extacellular enzymatic activity in soil [30]. These enzymes, especially hydrolases (e.g. urease, sulfatase and phosphatase), play a very important role in both soil microbial ecology and the availability of nutrients for soil biomass and green plants [4]. Humic–urease associations have been studied and different approaches have been reported for preparing humic–urease complexes: (1) direct extraction of naturally occurring soil humic-urease; (2) complexes obtained in vitro between HAs extracted from soil or peat and pure urease. The association of the extracellular enzymes with the HAs is a fundamental requirement for their stability in soil [30].

HAs from low-grade coal have had a long history of use as a compound fertilizer with urea to enhance the efficiency of nitrogen fertilizers. However, little analytical information about the mechanism is available. There are abundant lignite resources in China (~130 million tons) [38]. Considering that lignite contains a high content (>30%) of HAs and little information about the chemical characteristics of lignite HA fractions and their effects on urease stability is available, the aims of this work were to investigate the chemical character of two lignite HA fractions (HA1, ≥50 kDa; HA2, 10–50 kDa) and their effect on urease stability. The data may offer basic information relating to the mechanism for enhancement of the efficiency of urea and the application of lignite HAs to different kinds of soil.

2. Methods

2.1. Extraction and fractionation of lignite HA

HA was extracted from lignite collected from the Huolingele Minerals Administration Coalmine (Inner Mongolian Autonomous Region, Northwest China)., Air-dried lignite was pulverized and sieved with a 70-mesh sieve. An aliquot of the powder (2 g) was suspended in 100 ml 0.1 M NaOH according to the procedure described by Dong et al. [12] and N_2 bubbled through the mixture for 5 min to displace the air. The vessel was sealed and the mixture stirred at 20 °C for 24 h and then centrifuged at 6000 × *g* for 15 min. The supernatant was filtered through Whatman No.1 paper and the pH was adjusted to 2.0 with 1.0 M HCl. The solution was allowed to settle for at least 12 h and was centrifuged at 8000 × *g* for 5 min to precipitate the humic acids which was washed with distilled water three times and dried at 60 °C.

The dried HAs was then dissolved in alkaline solution (the pH was then adjusted to 7.0 with 1.0 M HCl). The solution was placed in a 500 ml Amicon ultrafiltration cell under N_2 pressure and desalted through a 500 molecular mass cutoff membrane. Ultrafiltration membranes with molecular mass cutoffs (10 kDa and 50 kDa) were then used to obtain humic fractions with a known range of nominal molecular size. The fractions were dried at 60 °C and stored at 4 °C until use.

2.2. Chemical analysis of HA fractions

Elemental analysis (C, H, N and S) of HA fractions was carried out using a Vario-EL Elemental Analyzer (Germany). Analysis was performed in triplicate. Light absorbance of the fractions dissolved in 0.05 M $NaHCO_3$ was measured with a Perkin Elmer Lambda 35 UV/VIS Spectrometer (USA) over the range 200 to 800 nm. The absorbances at 465 nm and 665 nm were used to calculate E_4/E_6 ratios [7].

2.3. Micro FT-IR analysis

As the samples were too hard to pulverize sufficiently, we used localized micro FT-IR spectroscopy with homogeneous and representative sliced samples (instead of KBr discs). Micro FT-IR spectra of local areas of the slices were obtained using a Nicolet Magna-IR 750 spectrophotometer (USA), which was connected to a Nicolet NicPlan IR microscope and a MCT detector. The resolution was 4 cm^{-1} and the spectral range 4000–650 cm^{-1}.

2.4. Solid state CP/MAS ^{13}C NMR spectra

Solid state CP/MAS ^{13}C NMR spectra were obtained with a Bruker av-300 spectrometer (Switzerland) at a frequency of 75.47 MHz with magic angle spinning at 4 kHz and a contact time of 3 ms. The pulse delay for the spectra of the samples was 5 s. Approximately 2290 scans were acquired for each spectrum. The ^{13}C chemical shifts were calibrated to the tetramethylsilane scale (0 ppm) as external standard. For quantification, the spectra were divided into different chemical shift regions assigned to specific carbon groups; the assignments are shown in Table 2.

2.5. Urease preparation and assay of activity

An aliquot (2 mg) of commercial jack bean urease powder (Worthington Biochemical Corporation) was dissolved in 10 ml doubly distilled water and mixed with 40 ml Gomori buffer (pH 7.0, 2 mM) [25]. The solution was passed through an ultrafiltration cell, using a membrane with a cut-off of 200 kDa (the molecular mass of urease is 480 kDa), until a volume of 10 ml was obtained; 40 ml of Gomori buffer was again added to the ultrafiltration cell and the procedure was repeated 5 times. The final concentration of the enzyme stock solution was 75 μg ml^{-1} using a protein assay kit (Bio-Rad protein assay). The range of Gomori buffer (pH 5.0–8.5) was enough to cover the range of the experimental pH values.

The urease activity was measured in triplicate using a pH-stat method (PHS-3C pH meter, LeiCi, Shanghai; [14,26,34]. The activity was tested by recording the volume of a 0.1 M HCl solution necessary to maintain a constant pH in the assay solution (30 ml) containing urease or HAs-urease (ratio 2:1) and 36 mM urea as substrate. One unit (U) of urease activity is defined as the amount of urease needed to hydrolyze 1 μmol urea min^{-1} at 25 °C.

The pH activity profiles for free urease and HAs-urease were acquired by changing the pH of the buffer from 6.0 to 8.0 and using the pH-stat method at the corresponding pH value.

2.6. Stability of free urease and HAs-urease with time

The stability of free urease and HAs-urease with time was monitored at pH 6.0, 7.0 and 8.0, by assaying the activity at the 0, 1st, 2nd, 4th, 7th, 9th and 12th days, respectively.