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Original article

Can we measure condensed tannins from tannin–protein complexes? – A case study with acid–butanol assay in boreal forest soil organic layer

Bartosz Adamczyk*, Oili Kiikkilä, Veikko Kitunen, Aino Smolander

Finnish Forest Research Institute, Vantaa Research Unit, P.O. Box 18, FI-01301 Vantaa, Finland

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ABSTRACT

Tannins may influence nitrogen cycle in forest soil. To understand the role of tannins in the decomposition process, we need to know their amount in the soil. Measurements of soil tannins meet great difficulties as tannins may undergo substantial retention. The main aim of this study was to assess the influence of complexation with proteins on condensed tannins (CT) retention. We measured the amount of extractable CT in the humus layer after additions of i) CT, ii) CT followed by addition of bovine serum albumin, BSA (uncomplexed before addition) or iii) CT–BSA complexes. Samples were taken from the humus layer of two sites; Norway spruce soil, which had high levels of organic matter and CT, and silver birch soil, which had lower levels of organic matter and CT. For tannin additions, we used CT extracted and purified from Norway spruce needles. To estimate extractable CT, we employed acid–butanol assay which is commonly used in condensed tannin chemistry. The complexation of CT with BSA remarkably decreased CT recovery from the humus layer in comparison to CT added alone or without prior complexation. The recovery of added CT also depended on the forest soil, and was higher in spruce soil. As complexation with proteins strongly decreases the recovery of CT it should be taken into account in studies concerning tannins in humus layer. According to our studies, only some of the CT from the CT–BSA complex in humus layer can be detected in the assay.

1. Introduction

Tannins are a widespread and highly diverse group of plant secondary metabolites, which are usually divided into two major classes, condensed tannins (CT) and hydrolysable tannins [1]. Different plant species have their own characteristic pattern of tannins [2]. Among the three common tree species in boreal forests, silver birch (*Betula pendula* Roth), contains both types of tannins while Norway spruce (*Picea abies* (L.) Karst) and Scots pine (*Pinus sylvestris* L.) contain only CT [1,3].

Tannins enter the soil with both above- and belowground litter. Several laboratory experiments indicate that tannins may slow down the degradation of organic nitrogen, making N unavailable in boreal forest soil (for review see Ref. [2]). It is commonly believed that tannins interfere with the decomposition of soil organic matter by three chemical activities: protein binding, metal complexation and antioxidant activity [4,5]. The response of forest soil processes to tannins may depend on the amount and the chemistry of tannins, and also on the biological, chemical and the physical characteristics of soil [2].

The knowledge about the role of tannins in soil is still suffering from numerous gaps. In contrast to a wide set of studies concerning tannins in plant material, tannins from soil have received less attention [6]. Some of the few results available indicate that amounts of tannins measured in soil vary widely from relatively high to undetectable levels, especially in mineral soils, even when purified tannins were added [4,7–9]. This may indicate the substantial sorption of tannins by soil constituents, rather than the rapid decomposition of tannins [4]. As far we know, recovery of CT added to the soil depends on the chemical structure of CT [8] and the solvent/solvents used [4,9]. We do not know whether tannins reach the soil as free tannins or as complexes with other plant ingredients (e.g. proteins). On the other hand, protein–tannin complexes may also be created in the soil. Northup et al. [10] suggested that most of the dissolved organic nitrogen may be associated with protein–tannin complexes. CT complexation may cause great

* Corresponding author.
E-mail address: Bartosz.Adamczyk@metla.fi (B. Adamczyk).

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underestimation of soil tannins, as we perhaps do not measure tannins from such complexes.

The aim of this study was to assess the influence of complexes on recovery of extractable CT from the humus layer. We compared this effect between soil organic layer from two forest humus layers, which have different fertility and native amount of extractable CT. We hypothesize that complexation may strongly decrease the recovery of CT and the soil organic layer may additionally increase retention. For additions we used CT that had been extracted and purified from Norway spruce needles. Since the concentration of hydrolysable tannins seems to be low in humus layer of boreal forest soils [11], we concentrated only on CT. Moreover, hydrolysable tannins in boreal forest soil may origin only from angiosperms, so in our study sites from birch and understory plants [1]. To estimate extractable CT, we employed acid–butanol assay which is commonly used in condensed tannin chemistry [12–14], and is still developed for quantifying CT in forages, foods, feeds, and foliage of herbaceous and woody plants (e.g. Ref. [15]). To our knowledge, this paper is showing for the first time the response of acid–butanol assay to protein–CT complexes in humus layer conditions.

2. Materials and methods

2.1. Study sites and sampling

Samples were taken from humus layer of two study sites, one located in Kivalo (66°20′N, 26°40′E, northern Finland) and the second located in Kerimäki (61°51′N, 29°22′E, south-eastern Finland). Characteristics of the soil can be found in Table 1. The Kivalo site includes an 81-year-old stand of Norway spruce (*P. abies* (L.) Karst.). The Kerimäki study site, originally a Norway spruce stand, was clear-cut in 1993 and re-planted with silver birch (*B. pendula* Roth.) in the following year. Samples for this study were taken from a previously N-fertilized plot (N fertilization totaling 860 kg N ha^{-1} during the 30 years before clear-cutting). A more detailed description of these two sites can be found in Refs. [16,17] for Kivalo and Kerimäki, respectively.

Representative samples (20 cores, diameter 5.8 cm) were taken from the humus layer (Ofh – organic soil layer without litter, consisting of Of = weakly decomposed layer, i.e. fermented layer and Oh = highly decomposed, i.e. humified layer). Sampling took place in September 2012 from both birch (Kerimäki) and spruce (Kivalo) sites. Samples were then combined to give one composite sample per site. Samples were sieved using 4 mm mesh after removing the plant material and roots. Samples were then frozen until the laboratory experiments began and preincubated at +1–2 °C for 3 days prior to use. The soil water holding capacity (WHC), dry matter, and soil organic matter (SOM) contents and pH were measured as described by Ref. [18].

2.2. Formation of CT–BSA complexes

Studies were conducted with bovine serum albumin (BSA) as this protein is commonly used in studies concerning precipitation of proteins by tannins, and with CT extracted from Norway spruce needles. Tannin extraction, purification and characterization are described precisely in a previous paper [19]. Briefly, dried needles were extracted and fractionated as described by Ref. [20], modified by Ref. [3]; ground needles were soaked in hexane, remaining plant material was extracted with acetone–water (70:30), filtered, and concentrated by roto-evaporation and extracted with 100% ethyl acetate. Ethyl acetate and water were separated; ethyl fraction was labelled as fraction 1. Acetone–water fraction was loaded on Sephadex LH-20, column was eluted with methanol–water (50:50) and by acetone–water (70:30) until the eluate was colourless. Acetone–water fraction was loaded into clean Sephadex LH-20 and eluted with 100% ethanol; eluate was labelled as fraction 2. The extract in column was eluted with 100% methanol; eluate was labelled as fraction 3. The extract was finally eluted with acetone–water (70:30); eluate was labelled as fraction 4. Fractions 1, 2, 3 contain CT but also monomers of CT and some other compounds, fraction 4 used in this study does not have monomers and is of high purity (see Ref. [3]). We characterized fraction 4 as described in Ref. [21], using reversed-phase and normal-phase high-performance liquid chromatography coupled with electrospray ionization mass spectrometric detection in the negative ion mode. On the basis of mass spectral data, tannins were found to be built up from procyanidin (dominating) and prodelphinidin units; the compounds detected were mainly tetramers, pentamers, hexamers and heptamers as well as higher polymers (Mw 1154–2130 Da, and also higher).

Condensed tannins were dissolved in water (in concentration of 1 mg CT per 1 ml of water), and undissolved material was removed by centrifugation (5000 *g*, 15 min). Undissolved material was dried and weighed; 11% of initial CT weight was not dissolved. Protein (BSA) was dissolved in 0.2 M acetic buffer with a pH of 4.9. Complexes were prepared as described previously [22] by mixing 0.165% BSA with 0.1% CT solution for 30 min in a planar shaker at room temperature. The samples were then centrifuged (5000*g*, 15 min), amount of CT and BSA in complexes were calculated from a difference between added amounts and residual amounts from the supernatant, these were studied as described below. Pellets (containing complexes) were washed with 0.05 M acetic buffer (pH 4.9) and centrifuged (5000*g*, 15 min) two times; in the supernatant after the second washing the amounts of compounds (BSA, CT) were undetectable, which means that no uncomplexed CT or BSA remained. After preparation, the complexes were stored at +1–2 °C overnight and added to humus layer on the following day.

Measurements of residual amounts of CT and BSA were done as described in Ref. [22]; since CT may cause problems with protein measurements, at first we precipitated BSA with trichloroacetic acid. After 1 h incubation +1–2 °C and centrifugation the supernatant was precisely removed and the precipitate was dissolved in 1 M NaOH, Bradford reagent was added and absorbance was measured at 595 nm. To confirm amount of CT in precipitate, we used one more method [23,24], in which the precipitate, after washing with buffer, is dissolved in sodium dodecyl sulfate/triethanolamine solution. Later $FeCl_3$ is added and the absorbance is read at 510 nm. To confirm protein concentration in precipitates washed and dissolved complexes (as above for CT) were exposed to [25] method modified by Ref. [26], which is capable of estimating

Table 1
Characteristics of the studied soil.

Site	Soil type	Humus type	Soil texture	N (g/kg o.m.)	C (g/kg o.m.)	C-to-N ratio	Organic matter, %	pH	CT, mg/g SOM
Kivalo	Podzol[†]	Mor[†]	Loamy sand[†]	20[‡]	600[‡]	30[‡]	85	3.8	5.7
Kerimäki	Podzol[¶]	Mor[¶]	Fine sand till[a¶]	27[§]	532[§]	19.5[§]	31.5	4.0	0.4

Abbreviations: o.m. – organic matter. Data marked with [†] are taken from Ref. [38], those marked with [‡] are taken from Ref. [16], those marked with [§] are taken from Ref. [39], and those marked with [¶] are taken from Ref. [40].

[a] Glacial till was the parent material and the dominant fraction in till was fine sand.