EVIDENCE FOR CHEMICAL BINDING OF PROTEINACEOUS MATERIALS TO HUMIC ACIDS AS A MEANS FOR THEIR PRESERVATION IN THE ENVIRONMENT

DISSERTATION

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ABSTRACT

Many hypotheses have been proposed to explain the preservation of proteinaceous materials in sedimentary environments. These include both chemical binding and physical sorption. Quinone-like structures in humic substances have been suggested to form covalent linkages with molecules containing amide groups, however, there is no direct molecular evidence for chemical bonding of these substances to humic substances. In this study, model peptides, representing proteinaceous materials with ^{15}N-labeled amino acid residues, were mixed with various humic acids that vary in structural composition from predominantly aromatic to predominantly aliphatic. Several different peptides were selected; the shortest was a 4 amino-acid peptide (GGGR), two peptides having extremely different aromatic (SFFFYYS) and aliphatic (SLLLVIS) chemical properties were obtained, and a 9 amino-acid peptide (RGFFYTPKA) was selected for pepsin degradation experiments. In addition, a small protein, cytochrome c, was used to examine the interaction of a protein macromolecule with humic acids. The peptides with ^{15}N-labeled residues were examined by 2D NMR techniques to identify the presence of covalent binding and isotope ratio mass spectrometry was used for estimating the quantities of peptide hydrolyzed or preserved in enzyme degradation experiments. The small protein, cytochrome c, was subjected to enzymatic degradation in the presence of humic acids. Reaction products were monitored and evaluated by gel electrophoresis

coupled with different standard visualization methods to elucidate the interactions with humic acids. The results for all of the model experiments used in this study confirm the presence of both covalent and non-covalent bonds between peptides and proteins and humic acids. Not only do quinone-like structures in aromatic humic acids couple with peptide (shown by 2D ^{15}N-^{1}H HSQC NMR experiments), but aliphatic structures in humic acids are shown to exhibit a novel but uncharacterized interaction with peptides. The enzyme degradation experiments conclusively show that peptides and proteins linked to humic acids are not fully hydrolyzed and portions of the peptides remain preserved within the humic acid structure. Approximately 10 % of proteins may survive this enzyme hydrolysis, demonstrating that binding to humic acids is an important process that can lead to peptide preservation in environmental systems.

Dedicated to my parents

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Organic nitrogen forms in the environment

Nitrogen is an essential nutrient and the major element of many biochemical compounds, such as proteins, amino sugars, DNA/RNA and other key organic molecules. With carbon, hydrogen and oxygen, nitrogen is also the most common chemical element in the living organisms. The most significant source of nitrogen in the environment is nitrogen gas in the atmosphere. Although it is the most abundant element in the atmosphere (about 79 %), only a small fraction of the total global nitrogen is available for active cycling in biosphere (Bolin and Cook, 1983; Paul and Clark, 1996). A few microorganisms have the ability to use molecular nitrogen, however, most living organisms require organic nitrogen forms for life activities. This is because the nitrogen gas has to be chemically transformed (fixation) into inorganic forms such as ammonium or nitrate-N before plants can utilize it (Bolin and Cook, 1983). In many marine, freshwater and terrestrial ecosystems (Lerman et al., 1993; Vitousek et al., 1997), nitrogen is the most important element controlling the diversity of plants, the population dynamics of animals, and the vital ecological processes such as plant productivity and the carbon and soil mineral cycles (Bolin and Cook, 1983).

While a significant amount of research has been conducted for soil nitrogen, most of the work has been focused on the qualitative and quantitative determinations of proteinaceous materials and organic nitrogen forms in soils (Kelley and Stevenson, 1995; 1996; Schulten and Schnitzer, 1998), and the mineralization and importance of soil nitrogen to plants (Mengel, 1996). Organic nitrogen, the major form of nitrogen, accounts for more than 90 % of N in most of soils, with the remainder being inorganic nitrogen, mostly ammonium (Stevenson, 1994). The importance of organic nitrogen compounds in soil fertility has been recognized, however, not all of the organic nitrogen forms have been characterized. Only about one-half of the soil organic nitrogen chemical structures have been understood (Stevenson, 1994). Many studies on the known forms of organic nitrogen in soils are based on a strong acid extraction to release nitrogen compounds from clay and soil samples. The organic nitrogen compounds are then separated into several fractions including amino acid nitrogen, amino sugar nitrogen, acid insoluble nitrogen, and hydrolyzable unknown nitrogen. For all of these nitrogen forms, amino acid nitrogen is considered predominant (Stevenson, 1994; Anderson et al., 1989; Schnitzer, 1985).

Apparently, amino acid nitrogen in soil accumulates from the degradation of proteinaceous materials, mediated by microbial and abiotic processes. However, little is known of the chemical linkages that amino acid components form with the other soil materials.

1.2 Structures of organic nitrogen compounds in natural organic matter characterized by different techniques

To understand the molecular structures of organic nitrogen compounds in natural organic matter, traditional methods involve use of strong acid or base extraction of soils to release the organic nitrogen components. However, using these conventional wet chemical analysis techniques, only about half of the total organic nitrogen in soils and sediments is identified. Most of the identifiable organic nitrogen compounds are amino acids and amino sugars (Zhang and Amelung, 1996; Kelley and Stevenson, 1996; Stevenson, 1994; Vairavamurthy and Wang 2002). Since the detailed structures of the nitrogeneous materials are still not well understood, many attempts using the destructive techniques such as pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) (Schulten et al., 1997) and non-destructive spectroscopic techniques such as the nuclear magnetic resonance (NMR) (Knicker and Kogel-Knabner, 1998) or X-ray photoelectron spectroscopy (XPS) (Abe and Watanabe, 2004) have been applied to obtain the structural information.

The Py-GC/MS technique is a powerful technique to characterize organic materials in soil samples (Bracewell and Robertson, 1984). The organic nitrogen compounds are thermally degraded into small volatile fragments and analyzed by GC/MS. The Py-GC/MS technique is commonly used to identify the organic nitrogen in hydrolyzates and hydrolysis residues, that are released from soils by strong acid or base extraction (Schulten and Schnitzer, 1997; Schulten and Sorge-Lewin, 1995; Schulten et al., 1995; Schulten et al., 1997). The identified organic nitrogen compounds include pyrroles, imidazoles, pyrazoles, pyridines, pyrimidines, pyrazines, indoles, quinolines,

nitrogen-derivatives of benzene, alkylnitriles, and aliphatic amines (Schulten et al., 1995). Most of these nitrogen forms exist as heterocyclic nitrogen and are detected in most soils. In addition, by comparing the Py-GC/MS results for amino acids (Chiavari and Galletti, 1992; Sorge et al., 1993), peptides (Voorhees et al., 1994), proteins (Boon and de Leeuw, 1987; Munson and Fetterolf, 1987; Tsuge and Matsubara, 1985), amino sugars (Zhang and Amelung, 1996; Bahr and Schulten, 1983; Franich et al., 1984; van der Kaaden et al., 1984), and nucleic acids (Eudy et al., 1985; Jennings and Dimick, 1962; Posthumus et al., 1974), with those of soil organic matter, one can conclude that the Py-GC/MS data for soils is mostly derived from proteinaceous materials.

To further characterize the organic nitrogen forms in soils and sediments, an alternative technique, the tetramethylammonium hydroxide (TMAH) thermochemolysis, has been used as a complementary method to the Py-GC/MS technique. The TMAH method involves the depolymerization of the macromolecular materials to monomers by a thermally-assisted base hydrolysis and simultaneous methylation (Challinor, 1989). Moreover, the TMAH method solves some of the limitations of the traditional Py-GC/MS technique, that of limited volatility of polar products. In the TMAH technique, the polar products can be effectively converted to less polar derivatives via methylation which are compatible with the GC/MS analysis. Polar products are generally difficult to analyze by GC/MS due to the partial or complete adsorption or polymerization in the pyrolysis zone, injection system or capillary column (Challinor, 1989; de Leeuw and Baas, 1993; Hatcher and Clifford, 1994; Saiz-Jimenez, 1994). In addition, TMAH thermochemolysis prevents decarboxylation that can occur during the pyrolysis process. TMAH produces methyl esters and methyl ethers from carboxylic acids and certain

hydroxyl groups, respectively. Thus, the TMAH-GC/MS method can observe more structural information compared with the traditional Py-GC/MS technique.

Since both Py-GC/MS and TMAH-GC/MS techniques require the high temperature degradation process to break down molecules, thermal-induced secondary reactions can occur, leading to the formation of heterocyclic compounds and complicating the pyrolysis results. The thermal degradation of proteinaceous materials, for example, are suggested to result in the decomposition and formation of the rearranged nitrogen heterocyclic compounds, such as pyrrole, indole, alkylpyrrolediones and diketopiperazines (Bracewell and Robertson, 1984). The low structural resemblance between these pyrolysis products and the proteinaceous molecules complicates the interpretation of pyrolysis results. Thus, the dissociation and/or rearrangement reactions of nitrogen-containing molecules restrict the applications of Py-GC/MS and TMAH-GC/MS techniques in analyzing macromolecules.

In order to avoid the formation of products whose origin is uncertain during the pyrolysis process, non-destructive spectroscopic methods are used to examine soil organic samples. Solid-state ^{15}N NMR spectroscopy has been demonstrated as a very valuable technique to identify different types of functional groups and to determine their relative distribution in samples. It has been used to obtain structural information for organic nitrogen compounds in humified materials (Knicker et al., 1993; Knicker and Hatcher, 1997; Knicker and Kogel-Knabner, 1998; Mahieu et al., 2000). Amide nitrogen compounds are identified to be the major organic nitrogen form in the soils (Knicker et al., 1993; Knicker et al., 2000), sediments (Knicker and Hatcher, 1997; Knicker and Hatcher, 2001; Knicker et al., 1996), and deep oceanic seawater (McCarthy et al., 1997;

McCarthy et al., 1998) indicating the existence of proteinaceous materials. Further studies using the solid-state ^{15}N NMR analysis of the residues from acid hydrolysis of soil samples also indicates that amide nitrogen compounds are the primary structures of the extracted organic matter (Knicker and Hatcher, 1997). However, no significant signal is obtained by solid-state ^{15}N NMR for heterocyclic nitrogen compounds, such as pyridine, nitriles, phenazine and imine-like structures. The lack of these heterocyclic compounds in the solid-state ^{15}N NMR spectra was criticized as being unrepresentative of soil nitrogen due to the inadequate sensitivity of the solid-state ^{15}N NMR method. Thus, the sensitivity limitation restricts the use of ^{15}N NMR for identifying the chemical structures of organic nitrogen forms in natural organic matter.

X-ray photoelectron spectroscopy (XPS) is another non-destructive and semi-quantitative technique for determining the proportions of various nitrogen compounds. Based on the detection of the ejected photoelectrons, emitted from the surface of solid samples treated by x-ray irradiation, by an energy analyzer, XPS can assign and determine the relative abundance of different species of an element (Zubritsky, 2001). The characterization of functional groups in elements such as carbon, nitrogen, and oxygen in coals (Kelemen et al., 1994; Kelemen et al., 1999), soil (Yuan et al., 1998), and sediments (Patience et al., 1992) are determined by using XPS. The existence of heterocyclic nitrogen compounds and amide nitrogen are both confirmed in the soil and sediment samples. The small amount of sample required and the short experiment time for XPS are the major advantages over solid-state ^{15}N NMR. However, the insufficient spectral resolution obtained from XPS limits the assignments for the different chemical forms of organic nitrogen compounds and requires the deconvolution of signals for

revealing chemical structures. Lately, a more sensitive technique, the nitrogen K-edge X-ray absorption near edge structure (XANES) spectroscopy, has been performed to investigate the humic substances in order to provide better results than the XPS technique (Vairavamurthy and Wang, 2002). This method suggests that the main nitrogen form in humic substances and sediments is amide nitrogen.

Based on these destructive and non-destructive analysis techniques, some broad insights into the structure of organic nitrogen compounds are elucidated. Amide nitrogen and heterocyclic nitrogen have been confirmed to be present in natural organic matter. Consequently, the mechanism for formation and preservation of this nitrogen needs to be resolved.

1.3 Hypothesis for organic nitrogen preservation in natural organic matter

Molecular interactions occurring between proteinaceous materials and natural organic matter have been proposed to be primarily responsible for immobilization of nitrogen in soils. The proposed reactions include modification brought about chemical bonding and physical encapsulation within a complex three-dimensional structure of soil organic matter.

With regards to chemical modifications, two major hypotheses have been proposed. The first involves chemical reactions of the amide groups in amino acids with either quinone or phenolic structures in soils, or with other molecules like sugars or molecules possessing carbonyl functional groups (Anderson et al., 1989). Quinone or phenolic compounds, produced from the decomposition of lignin or from microbial synthesis, are known to form the covalent bonds with amino acids (Flaig et al., 1975;

Piper and Posner, 1972). Further reactions of the phenolic structures with amide nitrogen compounds can produce nitrogenous polymers, some of which have similar structural properties to humic acids (Stevenson, 1994). The mechanism involving formation of a nitrogenous polymer proposed that a portion of the nitrogen from proteinaceous molecules is incorporated into the polymer structure, and this portion is not released by acid hydrolysis.

The other mechanism for forming chemical linkages is that the amine groups in peptides or proteins can produce covalent bonds with aldoses (sugars with aldehyde groups) via a Schiff base intermediate, which subsequently undergoes Amadori rearrangements to form dark colored melanoidins (Maillard, 1912; Ikan et al., 1986; Ishiwatari et al., 1986). These covalently linked nitrogen adducts are also found to exhibit resistance to hydrolysis. The formation of the amine-sugar condensation reaction products are therefore postulated to be important in the preservation of the proteinaceous materials in the environment. However, recent studies (Zang et al., 2001) suggest that the Maillard reaction does not readily occur, at least in aqueous systems, although this reaction has been proposed in many studies to be important in humification (Schulten and Schnitzer, 1998). Thus, the chemical reactions for incorporating proteinaceous materials remain contentious and need new evidence to clarify the controversy.

In addition to formation of the covalent linkages, physical protection through non-bonding interactions between proteinaceous materials and molecules in soils and sediments have also been proposed to explain the long-term preservation of peptide. It is suggested that a portion of the proteinaceous materials in soils is entrapped in the void spaces of the three-dimensional humic acid structures (Schulten and Schnitzer, 1995;

Schulten and Schnitzer, 1997; Schulten and Schnitzer, 1998). In this model, physically trapped proteinaceous materials are associated with humic materials by formation of hydrogen bonds, electrostatic charge-charge interactions, or a set of bond dipole moments associated with polar bonds. Some have also suggested a mechanism to preserve organic matter in marine sediments involving adsorption (Hedges et al., 2001; Hedges and Keil, 1999; Keil, et al. 1994) to mineral surfaces or within mineral pores (Bock and Mayer, 2000; Mayer, 1994). According to this mechanism, labile materials, such as proteinaceous molecules, are adsorbed to mineral surfaces and stabilized by incorporation into mineral pores (mesopores). Proteinaceous material so trapped are too small to allow effective enzyme access and degradation (Mayer, 1994; Mayer 1995; Salmon et al., 1998). Experimental results provided by Knicker and Hatcher (1997) and Knicker et al. (1996) also suggest that proteinaceous materials survive in an organic-rich sedimentary system by trapping in the mesopores within organic matter. Proteinaceous materials appeared to be stabilized towards degradation when associated with a high molecular weight fraction of organic-rich sediments (Nguyen and Harvey, 1998; Nguyen and Harvey, 2001). Zang et al. (2000) demonstrated that ^{15}N-labeled proteins extracted from fresh algae can survive after 6 M HCl hydrolysis, likely due to physical encapsulation within humic acid structures.

Although both chemical bond formation and physical sorption protection are suggested as the mechanism to preserve the proteinaceous materials in the environment, direct evidence to demonstrate the presence of covalent bonds between amino components and humic substances or the existence of the proteinaceous molecules within mineral mesopores is still not available. In this research, an attempt to examine the

chemical bonding preservation mechanism is proposed by use of the non-destructive methods. The investigation of the chemical reaction between model peptides and humic substances is studied to clarify the details of nitrogen immobilization in the environment.

1.4 Analytical techniques available for testing the proposed preservation mechanism

For examining the interactions of organic nitrogen components with humic substances, many analytical techniques are available, including pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), X-ray photoelectron spectroscopy (XPS), and nuclear magnetic resonance (NMR) spectroscopy. Most of the applications for these methods involved only detection or evaluation of the organic nitrogen forms in humic substances. The intrinsic limitations of these analytical techniques, discussed above, restricts their ability to reveal how proteinaceous materials react with humic substances. For example, heterocyclic nitrogen compounds were discovered in the Py-GC/MS results and considered to be part of the organic nitrogen forms in humic substances. However, they can be artifacts of the pyrolysis. Although the XPS method can provide the structural information for nitrogen compounds in humics, the lack of spectral resolution limits obtaining details of molecular bonding mechanisms. Solid-state ^{15}N NMR is limited by sensitivity because of the low abundance of ^{15}N in natural organic matter; although it provides chemical structural information for organic nitrogen in humic acids. Therefore, our strategy for defining bonding interactions of peptides with humics is to develop a new approach.

1.4.1 The new approach: use of ^{15}N-labeled peptides

Use of ^{15}N-labeled peptides as model proteinaceous molecules in studies of the interactions with humic acids is the approach that best suits the goals of this study. Model molecules isotopically enhanced with ^{15}N provides for ^{15}N NMR spectroscopic signal enhancement and also provides the ability to track the distribution of model molecules in the system. Based on these advantages, several ^{15}N-labeled peptides in various sequences and lengths were synthesized and utilized in this study. The 4 amino acids peptide with the sequence GGGR, which has the three glycines (G) ^{15}N-labled, was utilized for investigating the interactions between short peptides and humic acid molecules. In addition, to evaluate the influence of amino acid functional groups on interactions with humic acids, two ^{15}N-labeled peptides with 7 amino acid chain lengths were used in this study. The peptide with the sequence of SFFFYYS, which has the three phenylalanines (F) ^{15}N-labled, represents the aromatic peptide since phenylalanine (F) and tyrosine (Y) are aromatic amino acids. The second peptide, the one with high a predominantly aliphatic structure, has the sequence SLLLVIS and the three leucines (L) are ^{15}N-labeled. Finally, a 9 amino acid peptide with the sequence of RGFFYTPKA, which has glycine (G) ^{15}N-labeled, was used in a simulated enzymatic degradation because the sequence was designed for optimum pepsin attack. Based on using these ^{15}N-labeled peptides with different sequences and lengths as model molecules, enriched nitrogen isotope content provides an intense signal to be measured in ^{15}N NMR studies of the interactions of proteinaceous molecules and humic substances.

1.4.2 Analytical techniques

Several analytical techniques are utilized in this study to examine the intermolecular interactions including nuclear magnetic resonance (NMR) spectroscopy, isotope ratio mass spectrometry (IR-MS), and gel electrophoresis coupled with silver staining and immunoblotting.

1.4.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) is a spectroscopic technique that reveals information about the environment of magnetically active nuclei. The active nuclei absorb electromagnetic radiation in the radio frequency region at frequencies governed by their chemical environment. The chemical environment around the examined molecule is influenced by chemical bonds, molecular conformations and dynamic processes. Thus, the structure of the molecule being inspected is deduced by measuring the frequencies at which resonances are observed for the active nuclei in the molecule. Therefore, NMR techniques are commonly used in chemistry to elucidate molecular structures and conformations. In this research, both the solid-state and liquid-state NMR experiments are performed to reveal the insights of molecular interactions by examining the chemical structural properties of humic substances and the model molecules.

The solid-state NMR has been proven as a very valuable technique since the whole sample can be investigated without the need to dissolve it or employ chemical pretreatments. Although the complexity of heterogeneous samples, such as humic acids, can make it difficult to obtain the detailed compositional information, it is possible to

identify various types of functional groups and to measure their average relative distribution by using the solid-state NMR technique.

Compared to the solid-state NMR, the liquid-state NMR techniques provide more resolved signals with sharp and intense peaks. Liquid-state NMR cannot only determine the functional groups in molecules but can also identify the detailed chemical structures of molecules. The liquid-state NMR technique applied in this research is primarily the multidimensional NMR, which usually provides the correlation signals for different nuclei to reveal the detailed structural characterization for macromolecules in solution. Many attempts to use the 2D NMR techniques to examine the chemical structures of macromolecules such as humic substances have been proposed (Simpson et al., 2002; Simpson, 2001). In this study, 2D HSQC (Heteronuclear Single Quantum Coherence) NMR spectroscopy is primarily applied to examine the interactions of ^{15}N-labeled proteinaceous molecules and humic acids.

The HSQC NMR technique is a two dimensional experiment to observe protons directly bonded to carbons or nitrogens in molecules (Bodenhausen and Ruben, 1980). The other basic 2D NMR technique that detects proton signals through the one-bond ^{13}C-^{1}H or ^{15}N-^{1}H shift correlation is the more widely used HMQC (Heteronuclear Multiple Quantum Coherence) experiment (Bax and Subramanian, 1986; Reynolds et al., 1997). In comparison with the 2D HMQC NMR technique, HSQC NMR provides much better resolution and signal/noise (Reynolds and Enriquez, 2002; Reynolds et al., 1997). The ^{15}N-^{1}H HSQC NMR experiments are commonly utilized to examine protein structures since each amino acid residue (except proline) gives one signal that corresponds to the amide group in proteins (Cavanagh et al., 1996; Reid, 1997). In 2D ^{15}N-^{1}H HSQC NMR

spectra, folded proteins usually display a broad distribution of the correlated nitrogen-proton signals since each amino acid in a protein molecule has a different chemical environment in the protein tertiary structure. On the other hand, the nitrogen-proton correlated signals in HSQC NMR spectra for unfolded or denatured proteins, which have no well-defined secondary or tertiary structures, share similar frequencies and this results in overlap of signals. The nitrogen-proton correlated signals can also be shifted when the amino acid residues experience changes in their chemical environment, that includes changes in pH or temperature, or the addition of binding ligands. Therefore, the ^{15}N-^{1}H HSQC NMR experiment is a very powerful technique for examining the protein conformational changes (e.g., folding/unfolding exchanges) or the structural difference (e.g., ligand binding reactions) based on the identification of nitrogen-proton correlated signals, which are shifted on affected amino acid residues.

In this study, the ^{15}N-^{1}H HSQC NMR experiments are performed to study the interactions between an ^{15}N-labeled peptide and humic acids with different chemical properties. The ^{15}N-labeled peptide with sequence GGGR, which has all three glycines ^{15}N-labeled, was utilized since it has short length without rigid conformation, good solubility and low cost for synthesis. The ^{15}N-labeled glycines in this synthesized peptide makes the NMR study of the chemical environments around the amide N-H group possible. When the covalent binding interaction occurs between ^{15}N-labeled peptide and humic acid molecules, the new peak(s) or shifted peak(s) will be observed in ^{15}N-^{1}H HSQC NMR spectra. On the other hand, when non-covalent binding or charge-charge interactions exists in the ^{15}N-labeled peptide and humic substance mixtures, not only will new signal(s) or signal(s) be detected but the signals will be possibly broadened

significantly, due to molecular relaxation changes. Therefore, the type of the interaction occurring between peptide and humic acid molecules can be investigated based on the ^{15}N-^{1}H HSQC NMR experiments. In a similar manner, ^{13}C-^{1}H HSQC experiments were also performed to study the interaction between peptide and humic substances.

1.4.2.2 Isotope ratio mass spectrometry (IR-MS)

In order to determine the amount of peptides with ^{15}N-labeled amino acid residues reacting with humic acids, isotope ratio mass spectrometry is applied. Samples containing the ^{15}N-labeled peptides and humic acids are analyzed by an elemental analyzer coupled with an isotope ratio mass spectrometer. The elemental analyzer measures the total amount of C, N, H, and O present in the samples by combustion. The isotope ratio of the product gases for each element may further be determined by coupling the elemental analyzer with an isotope ratio mass spectrometer.

A Carlo Erba NA 1500 Series II NC elemental analyzer is utilized in this research to combust solid and viscous liquid samples containing organic matter and ^{15}N-labeled peptides. The samples are placed in tin foil capsules and then crimped to a bead. The capsule bead including sample are flash-combusted in a stream of oxygen at 1800°C, and then carried by helium gas through an oxidizing furnace tube at 1025°C, a reducing furnace tube at 650°C, and a water trap at ambient temperature. The emerging anhydrous carbon dioxide and nitrogen pass through a 2 meter long packed chromatography column at 40 °C which separates the gases from each other due to their different affinities for the exchange medium in columns, with nitrogen eluting from the column before carbon

dioxide. The gases are then carried by helium to the mass spectrometer where they are analyzed in a continuous flow mode.

The elemental carbon and nitrogen compositions of the samples are measured by utilizing the thermal conductivity detector in the elemental analyzer and determined by comparing to the elemental composition of standards. The carbon and nitrogen isotopic composition of the sample is determined by comparison to the isotopic composition of reference standards. In this study, the determined isotope ratio of nitrogen can be applied to track the interactions of the addition of ^{15}N-labeled peptides to humic acid molecules. The isotopic enrichment of ^{15}N in samples provides information on the content of ^{15}N-labeled peptides in humic substances.

1.4.2.3 Gel electrophoresis coupled with silver staining and immunoblotting

Gel electrophoresis is the most popular method for protein separation and characterization (Weber and Osborn, 1969). Two different gel electrophoresis systems, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and tricine SDS-PAGE, are utilized in this study to examine the molecular interactions between small proteins and humic substances. While in the presence of the anionic detergent SDS, the protein migration is dependent on the molecular weight of the proteins and then the protein sizes can be estimated by comparing the migration with protein standards on the gels (Shapiro et al., 1967). In SDS-PAGE, proteins are denatured before the separation by heating in the presence of the excess SDS. SDS molecules bind strongly to the protein molecule to make the intrinsic charge of the protein insignificant. The SDS-protein complexes migrate in the gel according to their size since the conformational and charge

effects of proteins have been removed by SDS (Andrews, 1986). Therefore, an approximate molecular weight of proteins separated by gel electrophoresis can be determined by comparing with the protein standards.

The tricine SDS-PAGE gels are also used in our study since they are designed for the separation of peptides and small proteins which have molecular weights smaller than 10 kDa (Schagger and von Jagow, 1987). In Chapter 5, cytochrome c is the model protein to be investigated to incorporate with humic acids molecules. The molecular weight of cytochrome c is about 12.1 kDa, which is appropriate for analysis on the tricine SDS-PAGE. Like the conventional SDS-PAGE, the migrations of the peptide-SDS complexes on the tricine SDS-PAGE gel correspond to their molecular weights. Therefore, in order to examine the molecular interactions of cytochrome c to humic substances, the tricine SDS-PAGE gels provides better resolution than traditional SDS-PAGE gel for detecting the small molecular weight changes, which are due to the formation of the covalent bindings between molecules.

Two detection techniques for observing the proteins separated on gels are used, silver staining and immunoblotting. The silver staining method is the most sensitive method for permanently staining of proteins in gels. The gel with separated proteins is immersed in a solution containing soluble silver ions and developed by treatment with formaldehyde, which reduces silver ions to form an insoluble brown precipitate of metallic silver. The metallic silver will deposit on the protein molecules to form visible bands on the gel since protein molecules promote the reduction reaction (Heukeshoven and Dernick, 1985). The silver staining method is generally a universal, fast, convenient detection technique with high sensitivity and reproducibility.

The immunoblotting method, also called the Western blotting method, relies on the specificity of antibodies to identify single protein spots from protein gels. The detection process involves the separation of proteins by gel electrophoresis followed by the transfer of the separated proteins from the gel to a nitrocellulose membrane. The membrane, containing the separated protein bands in the same elution pattern as the gel, is then exposed to the antibodies which recognize and bind to a specific protein on the membrane. A secondary antibody, with an attached enzyme, that converts the added reagent to emit the detectable fluorescence signal, is incubated with the membrane and then binds to the primary antibody. Compared to the silver staining method, the immunoblotting technique has much higher sensitivity and specificity. Consequently, the immunoblotting method is an ideal technique to detect the protein of interest in samples with low or no interference from other molecules. In this study, both silver staining and immunoblotting are applied to investigate the interactions between cytochrome c and different humic acids molecules.

1.5 Objective

Although the processes of organic nitrogen preservation in the environments have been studied, the detailed mechanism has not been identified due to the lack of the direct evidence for intermolecular interactions. The hypotheses proposed to preserve the proteinaceous materials include covalent bond formation and non-covalent binding interactions. These involve the chemical modifications and physical sorption processes. The purpose of this dissertation is to identify these various interactions by using ^{15}N-labeled peptides reacted with different humic acids. Using different NMR techniques to

examine the molecular interactions between model peptides and humic acids, an overarching hypothesis that covalent bonding occurs with humic acids is being tested. Using isotope ratio mass spectrometry, the quantities of ^{15}N-labeled proteinaceous materials that can be protected from enzyme degradation are measured and related to the types of bonding interaction possible. Finally, gel electrophoresis with two different detection methods is used to investigate the interactions between protein molecules and humic acids, and the effect this has on enzyme digestion. The hypothesis is that a covalent and possibly non-covalent interaction with humic substances serves to protect proteins and peptides from enzymatic degradation, perhaps explaining their preservation in the environment.

1.6 Outline of the dissertation

A central issue relating to the overarching hypothesis above is that formation of covalent or non-covalent bonds between peptides and humic acids need to be demonstrated. Chapter Two describes the study to test our hypothesis that the proteinaceous materials form covalent and/or non-covalent bonds with humic acid molecules. Based on our observations made in testing this hypothesis, we also propose that these interactions occur between the amide groups in proteinaceous molecules and quinone structures in humic acids. We use the liquid-state two-dimensional HSQC NMR technique to obtain direct evidence that an ^{15}N-labeled tetrapeptide, GGGR, bonds with Everglades peat humic acids. The NMR results provide the crucial evidence that the chemical environment should change around the ^{15}N-labeled amide groups in peptides bounded to humic acids. In addition, by using 1,4-naphthaquinone to represent quinone

structures in humic acids and interacting it with ^{15}N-labeled peptide, GGGR, we can further support our hypothesis that quinone groups in humic acids are key structures facilitating the reaction.

Clearly, aromatic structures in humic acids are important because there is a high probability that they harbor quinones. We also hypothesize that the aromaticity of the peptides is important. Chapter Three presents the study to test our hypothesis that the functional groups in both humic acids and proteinaceous molecules influence the interactions occurring between them. We propose that the aromatic components in humic acids play an important role in reacting and preserving proteinaceous materials in the environment. We use the 2D NMR technique to examine the aromaticity on a set of humic acids and peptides having variable aromaticities. Mt. Rainier humic acids and Mangrove Lake humic acids are selected to represent the aromatic and aliphatic humic acids, respectively. Peptide SFFFYYS with three phenylalanines ^{15}N-labeled, exhibits mainly aromatic character, and peptide SLLLVIS, having three leucines ^{15}N-labeled, is chosen to represent low aromaticity aliphatic peptides.

Chapter Four evaluates the hypothesis that peptides that are covalently bonded with humic acids can be preserved from enzymatic degradation. We also hypothesize that the aromatic humic acids can provide better protection due to the stronger covalent interactions with proteinaceous materials. We design a simulation experiment to quantitatively test the protection efficiency of covalently bound peptides. The simulated preservation experiment includes use of an ^{15}N-labeled peptide reacting with humic acids and followed by enzyme degradation after adducts formed. Samples at each step of the preparation are analyzed by nitrogen isotope mass spectrometry to determine the residual

content of ^{15}N-labeled peptide. Thus, we test our hypotheses by measuring the amount of protected peptides in humic acids.

Chapter Five deals with the study of simulated preservation experiments of proteinaceous materials bound to humic substances using gel electrophoresis separation coupled with silver staining and immunoblotting. With regards to peptides, we hypothesize that the protein molecules, larger than the peptides used in earlier chapters, can survive enzymatic degradation in the environment by formation of covalent and/or non-covalent interactions with humic acids. To test this hypothesis, a model protein, cytochrome c, is used in this study. We conduct simulated preservation experiments that include interaction of model protein with humic acids followed by enzymatic degradation. This simulation experiment provides a model to examine the retardation of enzymatic degradation of proteinaceous materials in the presence of humic acids.

Chapter Six summarizes all the important findings and provides directions for future studies.

CHAPTER 2

NEW EVIDENCE FOR COVALENT COUPLING OF PEPTIDES TO HUMIC ACIDS BASED ON 2D NMR SPECTROSCOPY: A MEANS FOR PRESERVATION

Abstract

Nitrogen immobilization in soils and sediments involving the preservation of peptides is an important yet not well-understood process. Several hypotheses have been proposed for the preservation of peptides in these systems; however, to date, there is no direct molecular-level evidence. In this study, a synthesized peptide with ^{15}N-labeled amino acid residues was utilized to examine the mechanism by which humic substances from a peat might chemically interact with peptides to induce preservation. 2D ^{15}N-^{1}H HSQC NMR experiments were performed to evaluate the bonding and non-bonding interactions between ^{15}N-labeled peptide and an Everglades peat humic acid. The observed changes in cross peaks provide the first direct spectroscopic evidence for the covalent binding between these substances. Non-covalent interactions between the ^{15}N-labeled peptides and humic acid molecules are also observed in the spectra. Quinone structures in humic acids are suggested to be the important reactive groups, based on reaction of the labeled peptide with a model quinone which shows similar nitrogen-proton correlated peak patterns as is observed in the HSQC NMR spectra of humic acids reacted with the peptide. The Michael reaction with quinone structures is proposed, and

this type of reaction provides a mechanism that is consistent with previous observations for the chemistry of sedimentary nitrogen species.

2.1 Introduction

Nitrogen is a key nutrient element required for growth of plants and is the major elemental component of many biochemical molecules associated with plant biomass. When plants die and decompose in soils, sediments, and natural waters, most of this nitrogen is recycled as inorganic nitrogen during decomposition, mainly brought about by the microbes. A small fraction is incorporated into the organic biomass that escapes biodegradation and becomes soil and sedimentary organic matter (SOM), or dissolved organic matter (DOM). The organic nitrogen believed to be incorporated within SOM components are typically referred to as humic substances (Stevenson, 1994). The existence of nitrogen in humic substances has been cited as evidence that N-containing molecules in plants, mainly peptides, play an important role in humification (Anderson et al., 1989; Schnitzer, 1985).

Acid hydrolysis, a process used traditionally to cleave peptides into free amino acids, indicates that the major form of N in humic substances is peptidic N, representing about 40 to 50 percent of the elemental N in SOM (Stevenson, 1994; Schulten and Schnitzer, 1998). The remaining SOM nitrogen is largely unknown but thought to be primarily associated with heterocyclic organic compounds (Schulten and Schnitzer, 1998) and to a lesser degree known plant molecules such as amino sugars, chlorophyll, and microbial peptidoglycans (Kelley and Stevenson, 1996). Heterocyclic nitrogenous compounds do not normally exist in abundance in plants, so their presumed existence in

humic substances necessitates reactions that transform naturally existing nitrogen species to such heterocyclic molecules. The Maillard reaction has been proposed as one that can yield heterocyclic N (Maillard, 1912; Njoroge and Monnier, 1989). However, recent studies (Zang et al., 2001) suggest that the Maillard reaction does not readily occur, at least in aqueous systems. It has generally been recognized that that amino acids and peptides could react with microbial decomposition products of lignin compounds in SOM to produce humic substances that contain N (Stevenson, 1994). However, little is known of the structures and chemical linkages between amino compounds and other soil components. Moreover, we do not know whether peptides/amino acids are covalently linked as proposed above or are simply occluded within the structure of humic substances. Some recent studies indicate that occlusion (encapsulation) within the three dimensional structure of humic acids is possible (Zang et al., 2000). Certainly, encapsulation of peptides within macromolecular SOM can easily explain the persistence of peptides in some sedimentary environments (Knicker and Hatcher, 1997; Nguyen and Harvey, 2001).

While there have been numerous studies made of the readily extracted and hydrolyzable components of SOM (peptides, chlorophylls, and amino sugars), the search for identities of the "unknown N" has yielded two major contrasting views. Schulten and co-workers utilized pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) in a study of organic nitrogen in hydrolyzates and hydrolysis residues of SOM and suggested that these unknown N forms are primarily heterocyclic nitrogen compounds made up of pyridines and pyrroles (Schulten and Schnitzer, 1998; Schulten et al., 1995; Schulten et al., 1997). However, it is clear that the formation of heterocyclics may be due to the pyrolysis procedure itself (Jaegerstad et al., 1991; Sharma et al., 2003). To avoid artifacts

formed during the pyrolysis process, noninvasive methods have been applied to examine SOM samples to show that the major form of nitrogen in humic substances is peptidic nitrogen. X-ray photoelectron spectroscopy (XPS) of humic substances and SOM from soils and sediments shows that the main form of nitrogen in humic acids from a variety of different soils is peptide nitrogen (Abe and Watanabe, 2004) with some minor contribution from heterocyclic nitrogen. A more sensitive technique, the nitrogen K-edge X-ray absorption near edge structure (XANES) spectroscopy (Vairavamurthy and Wang, 2002) confirms that the amide nitrogen represents the dominant nitrogen type in the humic substances and SOM. Solid-state ^{15}N NMR spectroscopy, however, reveals amide nitrogen as the only form of organic nitrogen in soils (Knicker et al., 1993; Knicker et al., 2000), sediments (Knicker and Hatcher, 1997; Knicker and Hatcher, 2001), and deep oceanic seawater (McCarthy et al., 1997; McCarthy et al., 1998). The poor sensitivity of the solid-state ^{15}N NMR limits detection of less abundant nitrogenous species that may be heterocyclic in nature.

Considering the fact that "unknown N" constitutes such a large fraction of humic materials and that the spectroscopic methods indicate a clear predominance of peptidic nitrogen, we must surmise that a significant fraction of this non-hydrolyzable "unknown N" must be in the form of peptides. Previous studies demonstrated that non-hydrolyzable nitrogen in a marine sediment existed mainly as peptides (Knicker and Hatcher, 2001) and that the persistence of these peptides was related to a protection mechanism induced by the organic matrix. Furthermore, Zang et al. (2000) showed that peptides associated with humic acids are partially protected from strong acid hydrolysis. Nguyen and Harvey 1998; 2001 showed that hydrophobic and non-covalent associations were important for

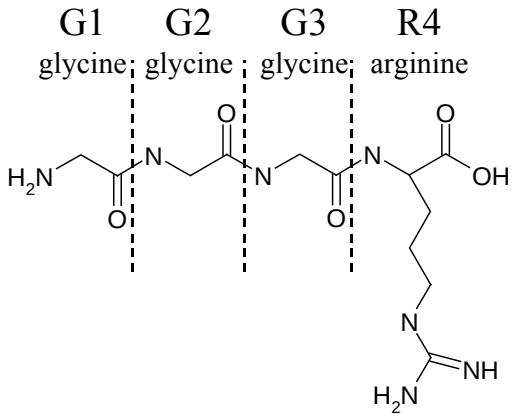
preservation. The above studies, conducted in predominantly organic sediments, suggested that a physical encapsulation phenomenon within a hydrophobic matrix or physical aggregation could be responsible for the protection.

There are numerous other ways in which protection of peptides has been suggested to occur in the environment. Some suggest that organo-mineral associations constitute an important mechanism for preservation (Keil et al., 1994; Hedges and Keil, 1995; Mayer, 1994). Others suggest that aggregation of organic matter within mineral assemblages offers protection from degradation (Guggenberger and Kaiser, 2003; Sollins et al., 1996). However, we propose that protection of peptides from hydrolysis, either by strong acid or via environmental enzymatic processes, can also be afforded by covalent linkage to the macromolecular SOM. This concept has been proposed by Kirchman (1989) who suggested that proteins underwent some sort of covalent binding to macromolecular organic matter during degradation of phytoplankton in oceanic systems.

To demonstrate covalent linking of peptides to macromolecular organic matter, 2D HSQC (Heteronuclear Single Quantum Coherence) NMR spectroscopy (Bodenhausen and Ruben, 1980; Reynolds et al., 1997; Reynolds and Enriquez, 2002) was utilized in conjunction with an isotopic enrichment experiment. The 2D ^{15}N-^{1}H HSQC NMR spectroscopy observes protons directly bonded to nitrogens in molecules and is sensitive to covalent bonds adjacent to the observed nitrogen-proton pair. ^{15}N-^{1}H HSQC NMR spectroscopy is commonly utilized to examine protein structures (Cavanagh et al., 1996; Reid, 1997), as changes in NMR spectra are observed when amino acid residues experience a change in their chemical environment. Therefore, the ^{15}N-^{1}H HSQC NMR experiment is a very powerful technique for examining protein

folding/unfolding (due to the pH or temperature change) and ligand binding based on identification of nitrogen-proton correlated signal changes on affected amino acid residues.

To delineate the covalent interaction between peptides and humic substances, an ^{15}N-labeled tetrapeptide with the sequence of amino acids glycine-glycine-glycine-arginine (GGGR, Structure 1) was added to humic acids from a sample of peat from The Everglades, Florida. We utilized ^{15}N-labeled GGGR having only the three glycines ^{15}N-labeled, primarily because this peptide has a short residue length, exists without a rigid conformation, demonstrates a high aqueous solubility consistent with that of humic substances, and is relatively inexpensive. The ^{15}N-labeling facilitates the NMR studies relating to the chemical environment surrounding the amide N-H group. We anticipate that covalent and non-covalent binding between ^{15}N-labeled peptides and humic acids will induce changes in the ^{15}N-^{1}H HSQC NMR spectra that will be recognizable. In addition, we compare the interaction with humic acids with the interaction between the labeled peptide and 1,4-naphthoquinone, a compound thought to be representative of oxygen-substituted aromatic structures in humic acids.



(Structure 1)

2.2 Materials and Methods

2.2.1 Preparation of humic acids and ^{15}N-labeled peptide

A peat sample obtained from The Everglades National Park, Florida was collected from a core obtained near Alligator Alley, west of Hialeah, Florida. The freeze-dried sample was treated to obtain humic acids by the standard isolation procedure involving sequential extraction with 0.1 M HCl followed by 0.5 M NaOH extraction of the residue from acid treatment. The dark alkali extract was treated with cation exchange resin to remove sodium and humic acids were precipitated by adjusting the solution to pH 2 (Hatcher et al., 1986). The humic acids were washed several times with dilute HCl and then freeze-dried. Weighed amounts of the dried humic acids were redissolved in water whose pH was adjusted to 10 by dropwise addition of 0.5 M NaOH to assist in dissolution. The final solution of humic acids was adjusted to pH 7.6 by dropwise addition of 0.1 M HCl.

The ^{15}N-labeled peptide with the sequence GGGR and with the three glycines ^{15}N-labeled was purchased from Genemed Synthesis, Inc. (South San Francisco, CA). The custom synthesis assured 90 % purity. No further purification procedure was applied on the ^{15}N-labeled peptide before use. A stock aqueous solution of 10 mg/mL was prepared by dissolving the powdered peptide in deionized water.

A solution containing 0.1 mg ^{15}N-labeled peptide (10 μL of peptide stock solution) was mixed with 2.5 mg Everglades peat humic acids (5 mg dissolved in 500 μL H_2O) and made to a volume of 600 μL by addition of H_2O and 60 μL D_2O to 10 % D_2O/90 % H_2O (v/v) solution which was adjusted to pH 7.6 by dropwise addition of 0.1 M NaOH. The

mixture was then vortexed (VWR MV-1 mini vortexer, VWR, PA) at room temperature for at least 48 hr before running NMR experiments.

2.2.2 Reaction of 1,4-naphthoquinone with labeled GGGR

Approximately 1.58 mg of 1,4-naphthoquinone (Sigma-Aldrich, St. Louis, MO) was dissolved in 500 μL H_2O, mixed with a solution containing 0.1 mg ^{15}N-labeled peptide (10 μL of peptide stock solution), and made to a volume of 600 μL by addition of H_2O and 60 μL D_2O to 10 % D_2O/90 % H_2O (v/v) solution which was adjusted to pH 7.6 by dropwise addition of 0.1 M NaOH. The mixture was then vortexed at room temperature for at least 48 hr before running NMR experiments. In another experiment, the pH of the 1,4-naphthoquinone/GGGR mixture was adjusted to pH 10 by addition of appropriate amounts of 0.5 M NaOH.

2.2.3 Solid-state ^{13}C NMR

A solid-state ^{13}C NMR spectrum of Everglades peat humic acids was obtained on a Bruker DMX-300 MHz spectrometer (Bruker Biospin Corporation, Billerica, MA) using the cross polarization-magic angle spinning (CP-MAS) technique. Approximately 100 mg dry weight of humic acids is placed in a 4 mm (outside diameter) NMR rotor with a Kel-F cap (3M, Minneapolis, MN). The sample is spun at a frequency of 13 kHz. Experiments were conducted at 300 K and 8192 scans were accumulated using a contact time of 2 ms and a 1 s recycle delay time. The spectral width was 22 kHz with 2048 data points collected on the free induction decay (FID). The FID was zero-filled to 4096 data points and subjected to exponential multiplication equivalent to 50 Hz line broadening.

The chemical shifts are plotted using the carboxyl signal of glycine as the external standard (176.03 ppm) and referenced to tetramethylsilane at 0 ppm.

2.2.4 Liquid-state HSQC NMR

The 2D ^{15}N-^{1}H HSQC NMR spectra were obtained on a Bruker DMX-600 spectrometer with a 5 mm TXI (triple-resonance inverse) probe (^{1}H, ^{13}C, ^{15}N) equipped with a z-gradient coil. The HSQC spectra (64 scans, with the FID time domain F2 having a digital acquisition of 2048 data points and the time domain F1 having 256 data points) were acquired using a relaxation delay of 1.5 sec with phase-sensitive States-TPPI (Time Proportional Phase Incrementation). All liquid-state NMR experiments were performed at 293 K except for one experiment performed at 303 K to evaluate T_2 effects. The acquired NMR data are then processed by XWIN-NMR, version 3.1 (Bruker Biospin Corporation, Billerica, MA). The data were processed with a 90^{o} shifted squared sine multiplication (QSINE) window function in the F1 dimensions (^{15}N) and a Gaussian function in the F2 dimensions (^{1}H), and a 1 Hz line broadening in both dimensions. The 2D ^{13}C-^{1}H HSQC NMR spectra were acquired from a Bruker DMX-400 spectrometer with a 5 mm BBI (broad band inverse) probe equipped with a z gradient coil. The same 2D parameters as above were used. The ^{15}N chemical shift scale used was the δ-scale, a common nitrogen scale for protein NMR researchers, which sets liquid NH_3 as 0 ppm and nitromethane as 380.23 ppm.

2.3 Results and Discussion

2.3.1 Solid-state ^{13}C NMR of Everglades peat humic acids

Figure 2.1 shows the solid-state ^{13}C NMR spectrum of Everglades peat humic acids. The spectrum shows broad bands characteristic of the many environments for structures present in these humic acids and is similar to spectra of humic acids from many peats (Hatcher et al., 1980; Simpson et al., 2003). Signals between 110 and 150 ppm represent the aromatic components in Everglades peat humic acids, with peaks at about 150 ppm representing O-substituted aromatic groups characteristic of lignin-derived materials and other phenolic substances in peat. The methoxyl carbons associated with lignin are reflected by signals around 56 ppm in the spectrum. The most intense signal at 33 ppm is the characteristic peak of paraffinic carbons associated with polymethylenic structures. The peak at 72 ppm is mainly attributed to the O-substituted alkyl group of lignin-derived structures because other possible contributors such as polysaccharides are unlikely, due to the absence of the peak for anomeric carbons at 105 ppm. Finally an intense peak at 175 ppm is likely that of carboxyl or amide groups, and the broad signal at 200 ppm is attributed to ketone/aldehyde carbons. It is clear that the ^{13}C NMR spectrum shows the presence of both aromatic and aliphatic components and that these are similar to what is typically observed from most peat humic acids (Orem and Hatcher, 1987).

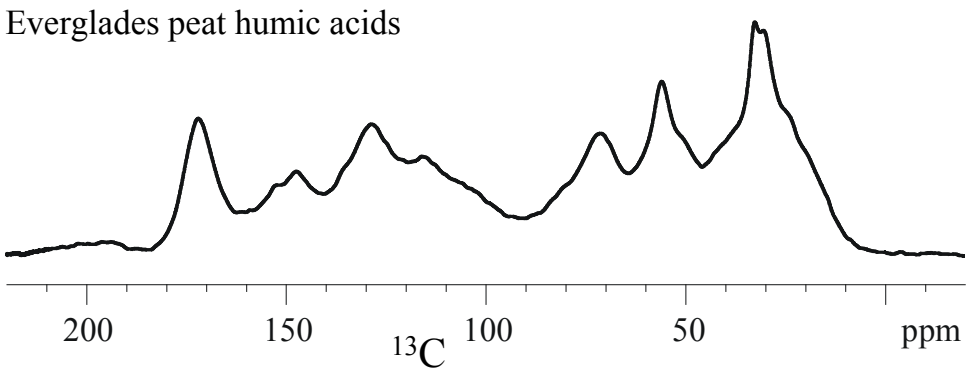


Figure 2.1: Solid-state ^{13}C NMR spectrum of Everglades peat humic acids.

2.3.2 Liquid-state ^{15}N-^{1}H HSQC NMR

Shown in Figure 2.2(a) is the ^{15}N-^{1}H HSQC NMR spectrum of the peptide GGGR. Three signals represent the three ^{15}N-labeled glycines. The N's of arginine are not observed as major signals because the N in arginine is not enriched in ^{15}N. The NH group in the N-terminal glycine, represented as G1, is observed at 8.15 ppm at the ^{1}H chemical shift and 116 ppm at the ^{15}N chemical shift. The NH groups of the second and the third ^{15}N-labeled glycines, G2 and G3, are observed at 8.45 ppm and 8.54 ppm on the ^{1}H axis and 109 ppm and 110 ppm on the ^{15}N axis, respectively. We currently cannot discriminate as to which specific nitrogen-proton pairs these two signals belong. It is well known that the chemical shifts of glycine are very unpredictable and greatly influenced by their local environment. We have been unable to calibrate ourselves to any published data on such molecules.

Figure 2.2(b) shows the ^{15}N-^{1}H HSQC NMR spectrum of peptide GGGR interacted with the Everglades peat humic acids. Comparing Figure 2.2(a) and 2.2(b), one

observes that the signal for G1 is unchanged by the presence of humic acids, suggesting that either the N-terminal amine group is remote from any reaction site or that the chemical shifts are insensitive to reactions near the site. We can definitely state that this terminal amine group is not involved in any covalent reaction with the humic acids. This is likely due to the fact that this amine group probably exists as a protonated species at the pH of the reaction (7.6).

However, for the G2 and G3 cross peaks, the signals are significantly broadened. Peak broadening effects usually indicate a non-bonding interaction at the site (G2 and G3). These non-bonding interactions may be from the charge-charge interactions or Coulombic attractions between peptide GGGR and Everglades peat humic acids. The broadening may arise from T_2 effects, because humic acids are known to possess broad lines and can be expected to have very short T_2's. Non-covalent interactions with these humic acids can, thus, lead to T_2 broadening. An additional ^{15}N-^{1}H HSQC NMR experiment was performed at a higher temperature in an attempt to diminish such an effect (303 K) and the same broadening effect was observed. This suggests that the broadening is due to inhomogeneous broadening arising from a large distribution of non-covalent interactions with the humic acids.

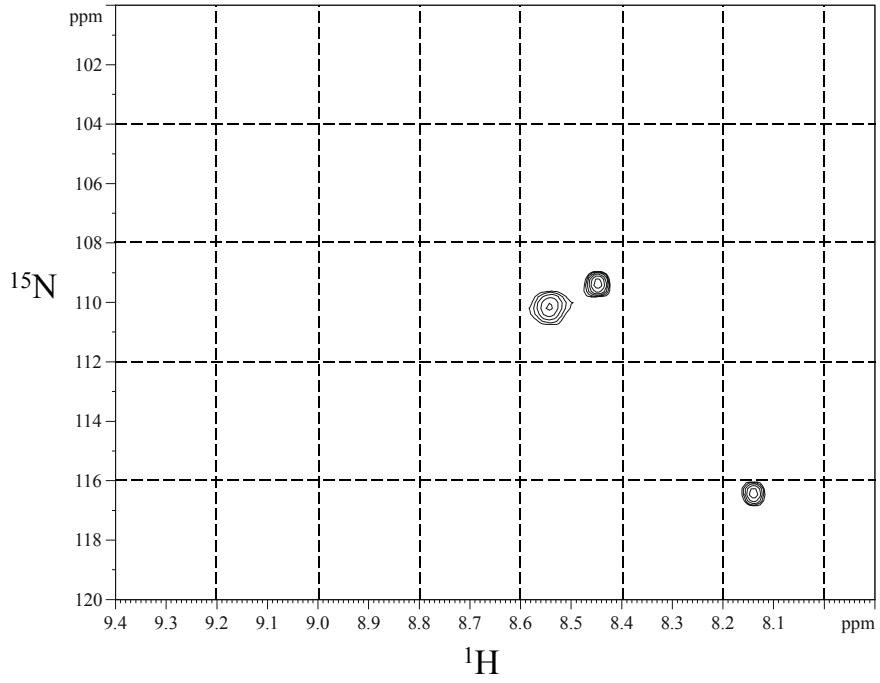
In addition to the broadening effects, there are also two new peaks observed in Figure 2.2(b), at 8.72 and 8.85 ppm on the ^{1}H frequency and 109 and 111 ppm on the ^{15}N frequency. These unambiguously indicate the presence of covalent bindings that have formed between the peptide and humic acid molecules. Based on the ^{15}N chemical shift values, these two new signals probably represent adducts formed at G2 and G3. It is unlikely that an adduct with G1 causes these changes in chemical shifts because, as

discussed above, no significant change in the peak G1 was observed. The G1 nitrogen is most likely protonated and unreactive, and the amide groups of G2 and G3 in peptide GGGR are the only possible reaction sites for nucleophilic addition. It is possible that the amide N associated with arginine is a reaction site and this influences the chemical shift of the labeled glycines. We do not expect guanidine groups of arginines to be reactions centers because they are protonated at pH 7.6. Even if they were reaction centers, their remoteness to the labeled glycines may not have a large effect. We cannot rule out their reactivity because they are not labeled and, consequently, signals are not observed.

2.3.3 Liquid-state ^{13}C-^{1}H HSQC NMR

Figure 2.3 shows the ^{13}C-^{1}H HSQC NMR spectrum of peptide GGGR. The $(CH)_{\alpha}$ groups for the four amino acids of GGGR are identified as the following cross peaks (given as the ^{1}H frequency and ^{13}C frequency in parentheses): arginine- 4.10 ppm (55 ppm), G1- 3.75 ppm (41 ppm), and G2 or G3 (unable to discriminate)- 3.92 ppm (43 ppm), 3.85 ppm (43 ppm). For the arginine side chain, the $(CH)_{\delta}$ group gives a cross peak at 3.05 ppm (41 ppm), the $(CH)_{\beta}$ group gives two cross peaks, one at 1.72 ppm (29 ppm) and the other at 1.59 ppm (29 ppm), and the $(CH)_{\gamma}$ group gives a cross peak at 1.46 ppm (24 ppm). All proton-carbon correlations are observed in Figure 2.3(a) since none of the carbon atoms are selectively ^{13}C enriched and, as a result, they have similar sensitivities.

(a):



(b):

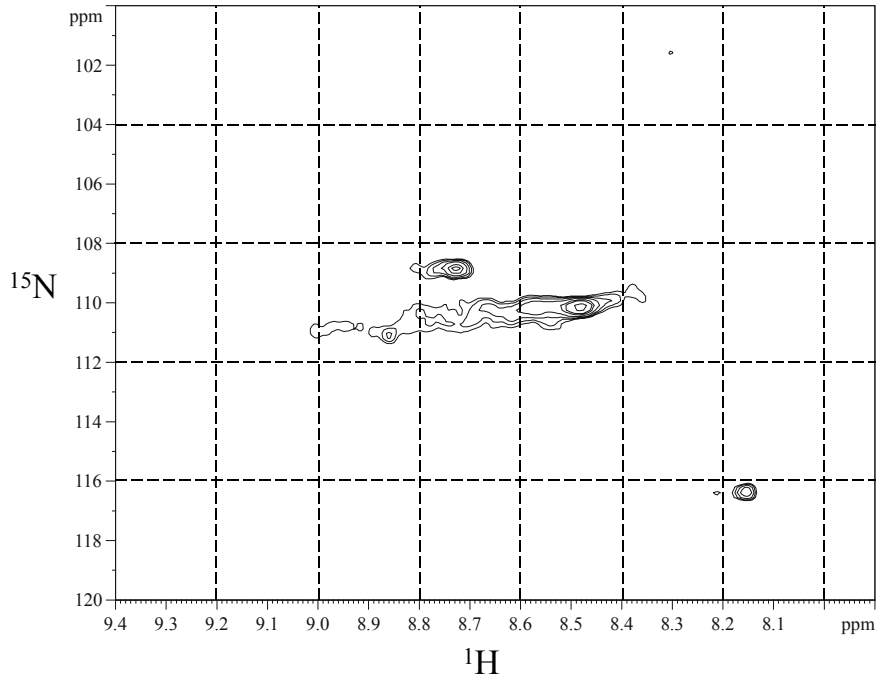


Figure 2.2: ^{15}N-^{1}H HSQC NMR spectrum of (a) ^{15}N-labeled peptide GGGR showing labeled glycines G1, G2 and G3; (b) ^{15}N-labeled peptide GGGR mixed with Everglades peat humic acids.

Figure 2.4(a) shows a selected region of the ^{13}C-^{1}H HSQC NMR spectrum of humic acids from the Everglades peat that have been mixed with the peptide GGGR. While a large number of cross peaks can be identified, some representing the peaks for GGGR and others representing the complex distribution of structures present in humic acids, only the aliphatic region of the 2D spectrum is shown because this region represents that in which peptide cross peaks are observed. The spectrum is difficult to completely interpret but some cross peaks are obviously assignable to specific structures known to exist in humic acids. Assignment of cross peaks to humic acid structures was made by obtaining a ^{13}C-^{1}H HSQC NMR spectrum for Everglades peat humic acids alone (not shown). An intense peak is observed for methoxyl carbons in peat humic acids at 56 ppm at the carbon frequency. One can observe that this peak correlates with at least 4 different types of protons (3.7, 3.8, 3.85, and 4.0 ppm). The other proton-carbon correlated peaks in the ^{13}C-^{1}H HSQC NMR spectrum that are assigned to Everglades peat humic acids are denoted in Figure 2.4. An additional cross peak at 3.2 ppm (49 ppm) is assigned to methanol used in cleaning of the NMR tube. Apparently removal of this methanol from the cleaned tube was incomplete.

Figure 2.4(b) is the enlarged area of the ^{13}C-^{1}H HSQC NMR spectrum representing the GGGR peptide that was mixed with the Everglades peat humic acids. Shown in the figure are the cross peaks assigned to humic acids and those assigned to the peptide GGGR. Note that two additional peaks are observed at positions that were devoid of cross peaks in spectra of the GGGR and the humic acids alone. In addition, one cross peak observed in Figure 2.3 for G1 has totally disappeared. We interpret these results in the following manner. The disappearance of the cross peak for G1 at 3.75 ppm (41 ppm)

and the appearance of two new cross peaks at 4.02 ppm (62 ppm) and 3.38 ppm (42 ppm) are consistent with formation of new bonds between humic acids and either G2 or G3. A bond formed at G2 would cause a disappearance of its assigned cross peak as well as a shift in the position of G1 and G3. One of the new cross peaks could be that of these two glycines. Likewise, a bond formed between humic acids and G3 would cause a disappearance of the cross peak for G3 and a shifting of the cross peak for G2 and the $(CH)_\alpha$ of arginine would also be affected. It is likely that one of the new cross peaks formed is from G2 that has shifted due to bond formation at G3. The $(CH)_\alpha$ of arginine appears to be altered somewhat from its symmetrical shape even though the peak position is unchanged. We rule out bond formation at G1 because we expect the N-terminus of this glycine to be in its protonated form at the pH of the reaction (7.6).

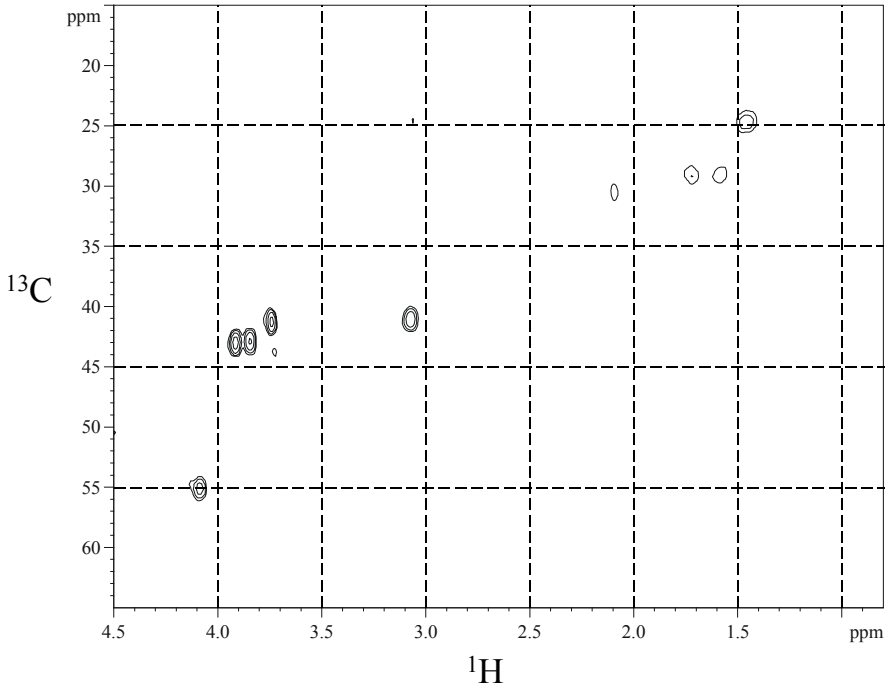
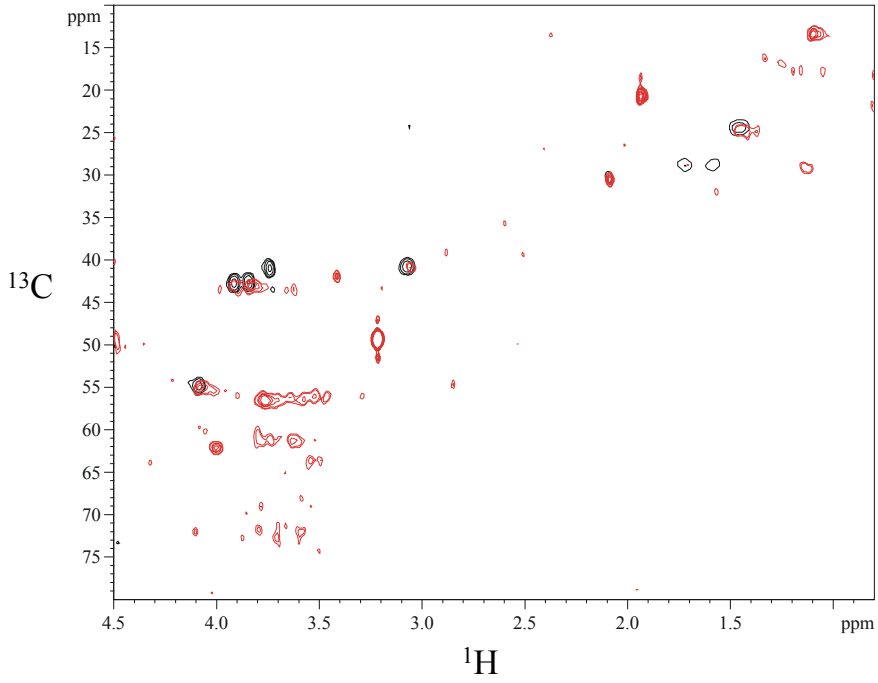


Figure 2.3: ^{13}C-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide GGGR.

(a):



(b):

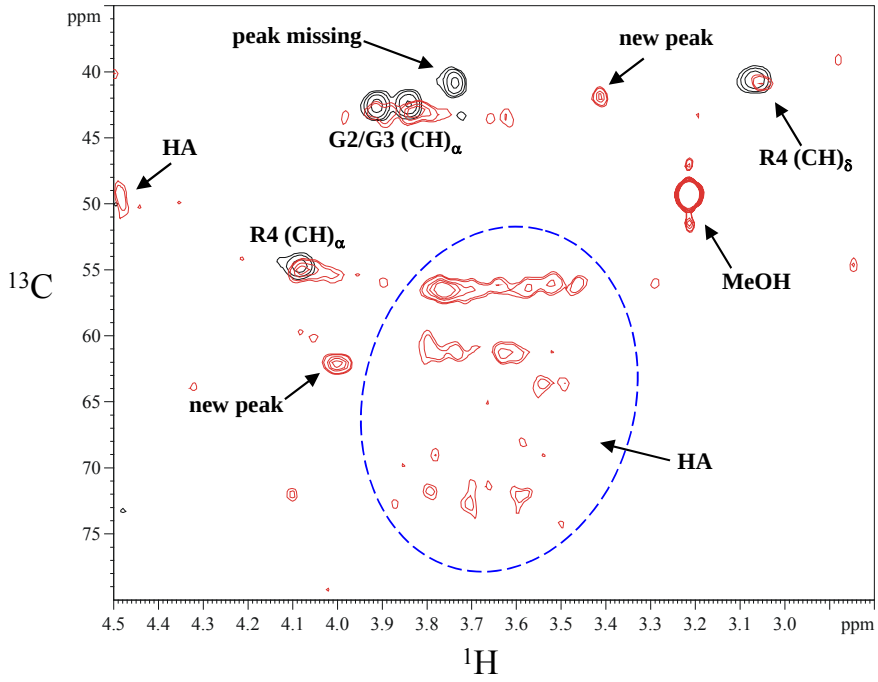


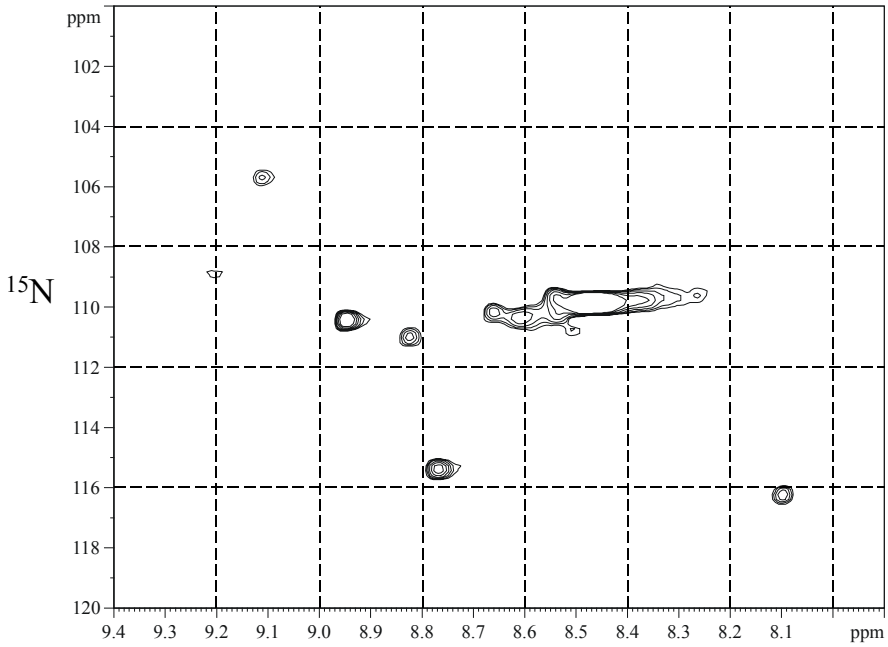
Figure 2.4: The ^{13}C-^{1}H HSQC NMR overlay spectrum of ^{15}N-labeled peptide GGGR (black) and GGGR/Everglades peat humic acids mixture (red), (a) aliphatic region; (b) enlarged area of peptide $(CH)_\alpha$ cross peaks region.

2.3.4 Reactions of peptide with 1,4-naphthaquinone, liquid-state ^{15}N-^{1}H HSQC NMR

The existence of quinone structures in humic substances is well known (Stevenson, 1994; Schnitzer, 1985); thus, 1,4-naphthaquinone was utilized as a model compound to interact with the ^{15}N-labeled peptide GGGR in an effort to evaluate the binding/reaction mechanism. Figure 2.5(a) is the ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide GGGR mixed with 1,4-naphthoquinone at room temperature for 48 h at pH 7.6. Comparing this with Figure 2.2(a), one observes that there are three new signals in Figure 2.5(a) at 8.95 ppm (110.4 ppm), 8.82 ppm (110.9 ppm), and 8.77 ppm (115.3 ppm). These three new cross peaks in the ^{15}N-^{1}H HSQC spectrum very likely represent covalent bonding between 1,4-naphthoquinone and amide groups in the ^{15}N-labeled peptide GGGR. The cross peak at 8.85 ppm (110.9 ppm) was also observed when the labeled GGGR was reacted with Everglades peat humic acids (Figure 2.2(b)) which suggests that quinone structures are the most likely functional groups in Everglades peat humic acids reacting with the ^{15}N-labeled peptide GGGR.

The peaks for G2 and G3 of the ^{15}N-labeled peptide GGGR are observed to be broadened in Figure 2.5(a) in a very similar manner to that observed in Figure 2.2(b), where the GGGR is reacted with humic acids. The broadening of these peaks represents a non-covalent binding mechanism that likely exists in both the peptide/humic acid system and the peptide/1,4-naphthaquinone system.

(a):



(b):

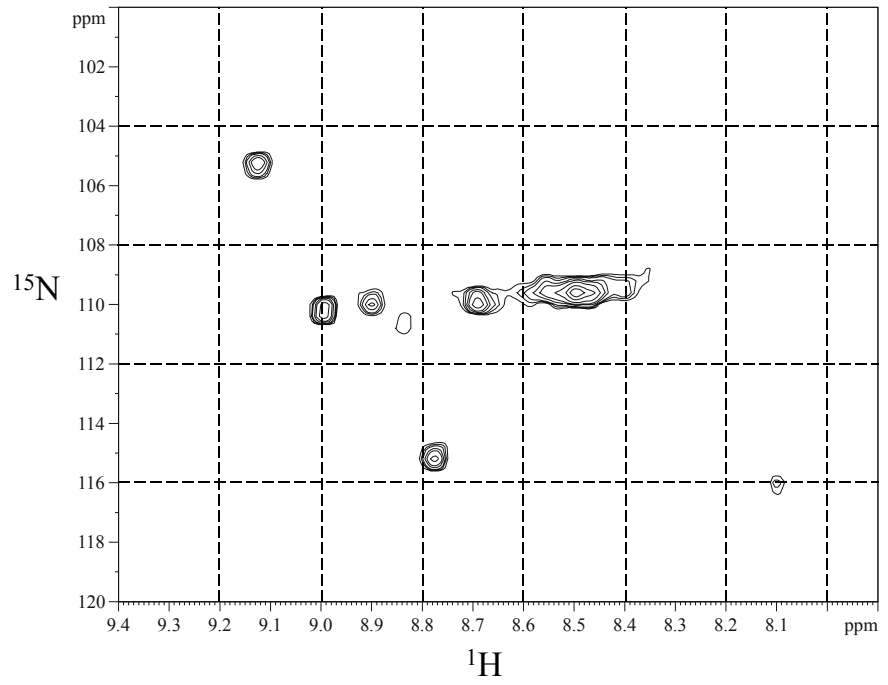


Figure 2.5: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide GGGR mixed with 1,4-naphthoquinone, (a) at pH 7.6; (b) at pH 10.

Because the net charge on the GGGR peptide is positive at pH 7.6 and the N-terminus at G1 is protonated, we sought to examine the reaction under conditions where G1 was present in the amine form. The ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide GGGR mixed with 1,4-naphthoquinone at pH 10 is shown in Figure 2.5(b). The cross peak at 8.10 ppm (116) is significantly decreased, compared to its intensity in Figure 2.2(a), indicating that a bonding interaction is observed between peptide GGGR and 1,4-naphthaquinone was through the N-terminal glycine (G1). In addition, a new cross peak in the region of 8.87 ppm (110 ppm), not observed in Figure 2.5(a), denotes the effect of bonding at the G1 site. Broadening of the peaks for G2 and G3 is also detected in Figure 2.5(b). These changes in the ^{15}N-^{1}H HSQC NMR spectra indicate that all amide/amine groups in the GGGR peptide, or proteins in general, can react with quinone-like structures that might exist in humic acids.

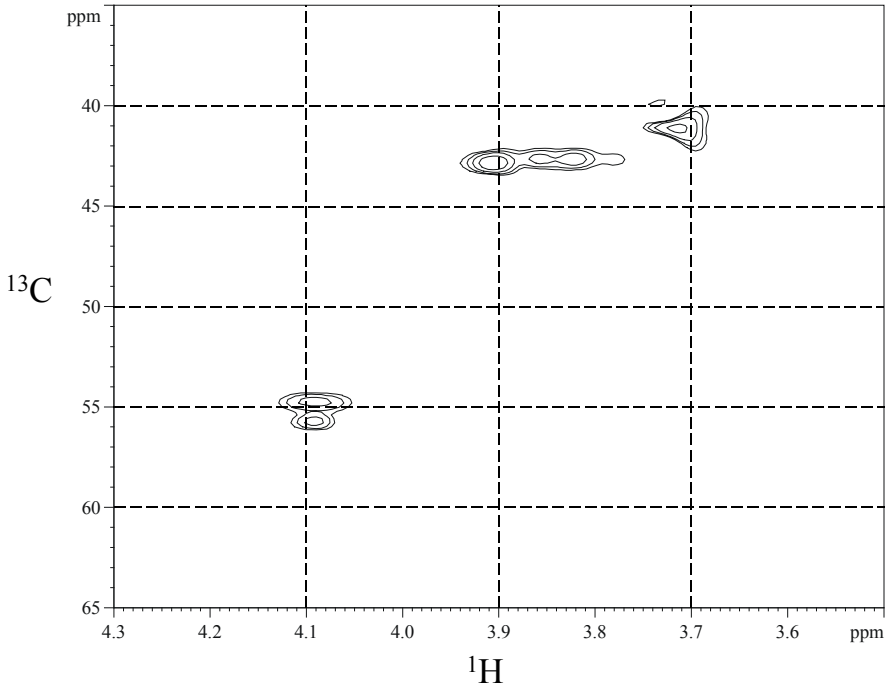
2.3.5 Reactions of peptide with 1,4-naphthaquinone, liquid-state ^{13}C-^{1}H HSQC NMR

The ^{13}C-^{1}H HSQC NMR spectra of ^{15}N-labeled peptide GGGR and 1,4-naphthoquinone mixtures at different pHs are shown in Figure 2.6. No carbon-proton correlated signals are observed in the aliphatic region of the ^{13}C-^{1}H HSQC NMR spectrum for 1,4-naphthaquinone since it contains only aromatic carbons (data not shown). Therefore, all signals in the aliphatic region in the ^{13}C-^{1}H HSQC NMR of the reaction mixture results from the ^{15}N-peptide GGGR. Figures 2.6(a) and 2.6(b) display only the $(CH)_{\alpha}$ group carbon-proton correlations because signals in the ^{1}H aliphatic1 region (0-2.5 ppm) are from arginine side chains which show no changes after mixing with 1,4-naphthaquione. In Figure 2.6(a) representing a reaction at pH 7.6, a pair of split

cross peaks around 4.09 ppm (55 ppm), which are from the arginine $(CH)_\alpha$ group, indicating at least two different chemical environments. Moreover, the carbon-proton correlated signal around 42 ppm on ^{13}C axis and 3.83 ppm on ^{1}H axis, from the $(CH)_\alpha$ group of G2 or G3, also shows a splitting into two peaks indicating that more than one product is formed from the reaction of peptide GGGR and 1,4-nathphoquionone. A new peak of low intensity at 3.78 ppm (42 ppm) also emerges from this reaction. The newly formed carbon-proton correlated peaks from the reaction of GGGR with 1,4-naphthoquinone all support the fact that covalent bonding occurs between peptide GGGR and 1,4-naphthoquinone. However, comparing the carbon-proton cross signal for N terminal glycine G1 in Figures 2.3(a) and 2.6(a), no peak shift is observed. This indicates that no chemical bonding occurs at the N-terminus of peptide GGGR. This is likely due to the fact that the amide group in G1 is protonated at pH 7.6.

In Figure 2.6(b) representing the reaction of GGGR with 1,4-naphthoquinone at pH 10, a new cross peak at 3.58 ppm (41.8 ppm) is observed. This new peak is believed to that of the N-terminal group (G1) of peptide GGGR, which disappeared from its previous position in the NMR spectrum (Figure 2.6(a)). The N-terminal group of peptide GGGR, a primary amine group, readily undergoes nucleophilic addition to 1,4-naphthoquinone because it is not protonated at pH 10. The reaction rate of primary amine groups is faster than amide groups (G2, G3, and R4) in nucleophilic addition reactions. Therefore, the dominant adduct formed is between the N-terminal amide group and 1,4-naphthoquinone as shown in Figure 2.7. The reaction changes the chemical environment around the labeled nitrogen in G1 to induce the observed peak shift in Figure 2.6(b).

(a):



(b):

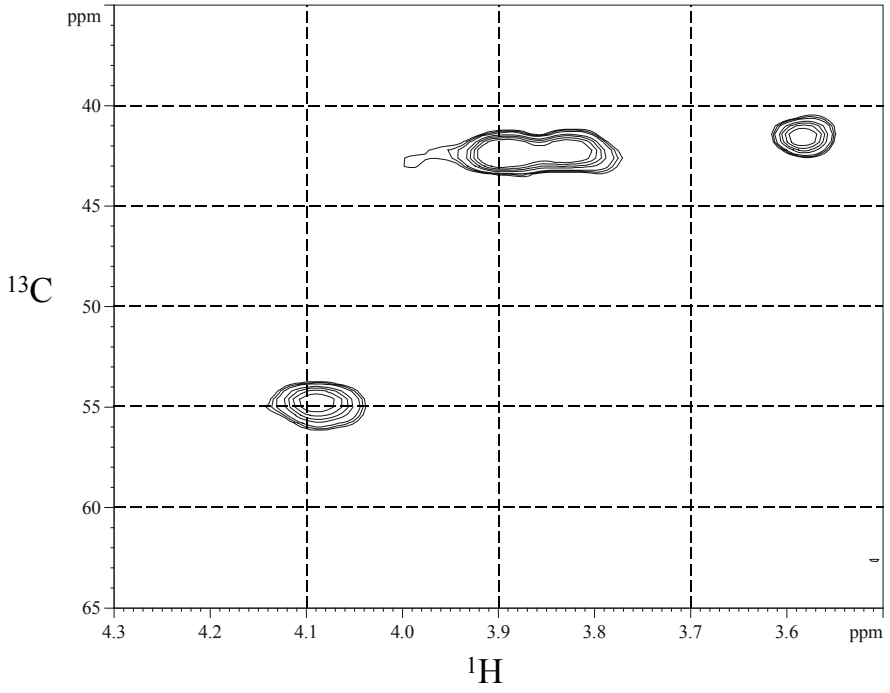


Figure 2.6: ^{13}C-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide GGGR and 1,4-naphthoquinone mixture, (a) at pH 6; (b) at pH 10.

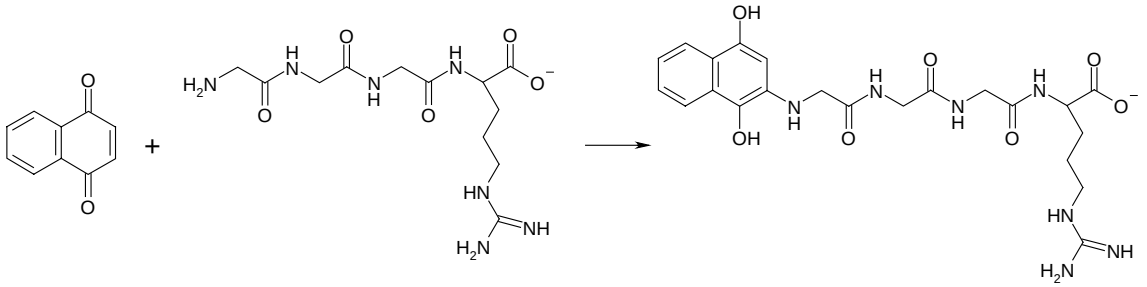


Figure 2.7: The proposed reaction of peptide GGGR and 1,4-naphthoquinone at pH 10.

The results shown in Figures 2.6(a) and 2.6(b) demonstrate clearly that chemical bonding of the model GGGR peptide occurs readily with quinoid structures. The likely reaction is known as the Michael Reaction whereby primary amines and secondary amides in peptides react with at least one or more possible available sites on the quinone molecules. Similar reactions are expected with humic acids that likely contain quinonoid structures. In addition, it is also clear that pH influences the availability of N-terminal sites on peptides towards addition reactions to quinones. Therefore, environmental pHs can be expected to exert a similar influence on reactivity of humic acids with peptides.

2.4 Conclusions

HSQC NMR techniques, combined with isotope enrichment experiments, provide direct spectroscopic evidence for the reaction of nitrogen-containing molecules with macromolecular organic matter in the form of humic acids. To our knowledge this is the first molecular-level evidence that peptides form both covalent and non-covalent bonds

with humic acids. The HSQC NMR spectra clearly reveal shifts in nitrogen-proton or carbon-proton correlated signals that can only be interpreted as the formation of covalent and non-covalent bonds between peptides and humic acid molecules. When comparing ^{15}N-^{1}H HSQC NMR spectra, one finds that the formation of new peaks and loss of peaks indicates that the covalent bonds involve the amide groups of the peptide. Broadened peaks suggest possible non-covalent bonds or charge-charge interactions between the ^{15}N-labeled peptide and humic acid molecules. In ^{13}C-^{1}H HSQC NMR experiments, comparable peak shifts are also observed providing clear evidence that the covalent bonding occurring between peptides and humic substances is through a nucleophilic attack of the nitrogen in amide groups of the peptide to appropriate functional groups in humic substances.

We suspected that quinone structures were the most likely candidates for formation of adducts with peptides, considering the fact that such structures are known to exist in humic acids and their affinity for nucleophiles like peptides is well known (Mason and Liebler, 2000; Briggs et al., 2003). Corroboration of their reactivity was substantiated when we chose 1,4-naphthaquinone as a model compound to react with the ^{15}N-labeled peptide. In the ^{15}N-^{1}H HSQC NMR spectra, new peaks and peak broadening effects were observed when ^{15}N-labeled peptide was mixed with 1,4-naphthaquinone. Similar changes in NMR spectra were observed in the peptide/humic acids mixture, indicating that the quinone structure in humic substances is the most likely functional group to form a covalent bond with amino acids or peptides. Furthermore, the ^{13}C-^{1}H HSQC NMR experiments provided complementary evidence supporting the formation of covalent bonds.

pH was found to be an important factor influencing the reaction between peptides and quinone. When the solution pH is lower than that of the peptide pI, the nucleophilic addition reaction is suppressed for the peptide N-terminal site since the amine group is protonated. The most available nucleophilic centers in the peptide under such conditions are the amide groups. The 2D HSQC NMR results show that peptide amide groups are capable of forming covalent bonds with the quinone. At higher solution pH, greater than that of the peptide pI, the N-terminal amine group of the peptide becomes the preferred nucleophile. This change in reactivity imparted by pH has important implications for reaction of peptides in environmental systems whose pH may fluctuate or vary. Thus, the propensity for humic acids to covalently interact with peptides may be entirely dictated by pH of the medium.

We recognize that the system examined in this study is a very simplistic representation of the complicated sets of interactions occurring in nature. However, our results indicate that covalent bonding can readily occur between model compounds that represent some of the components of real systems. It is likely that covalent bonds other than the types demonstrated here can occur. One can expect that covalent bonding to a generally recalcitrant type of organic matter, humic substances, might be a prerequisite for the eventual preservation of peptides in natural systems. We also recognize non-covalent interactions in our study and, thus, realize that both covalent and non-covalent bonding can play a role in preservation of peptides.

It is important to evaluate previous findings on the nature of organic nitrogen in environmental systems with regard to our current discovery. Numerous studies find that peptides partially survive biodegradation to be incorporated into recalcitrant organic

matter in sediments and soils (Zang et al., 2000; Knicker and Hatcher, 1997). Chemical bonding to the recalcitrant organic matter can explain some of this, as can non-covalent bonding and encapsulation processes that might protect peptides from enzymatic hydrolysis. However, there has always been the belief that chemical bonding would produce compounds that could assume a heterocyclic nature, in part because a mechanism was needed to explain the fact that they became incorporated into a non-hydrolyzable fraction of the sediment or soil. While the work of Zang et al. (2001) showed that encapsulation could protect peptides from acid hydrolysis, and by inference enzymatic hydrolysis, we now recognize that bonding to humic substances could also protect them from hydrolysis. Moreover, the need to reorganize into a heterocyclic nature (e.g., formation of melanoidins, Maillard, 1912; Njoroge and Monnier, 1989) is removed with such a concept. However, the bonding we demonstrate here involves formation of a Michael adduct in which the form of the N as a peptide is maintained. Thus, N remains in a peptide bond, thus explaining why ^{15}N NMR spectra of hydrolyzed residues of humic acids (Zang et al., 2001) and sedimentary humic substances (Knicker and Hatcher, 1997) retain their peptidic signature.

CHAPTER 3

COVALENT COUPLING OF PEPTIDES TO HUMIC ACIDS: STRUCTURAL EFFECTS INVESTIGATED BY 2D NMR SPECTROSCOPY

Abstract

Preservation of proteinaceous materials in soils and sediments has been suggested as an important mechanism for immobilization nitrogen in numerous types of environments. Several preservation mechanisms have been proposed, including chemical bonding to macromolecular organic matter and physical sorption or encapsulation within sites, both mineral and organic, that protect the proteinaceous materials from access of enzymes that would degrade them. In a previous study (Chapter 2), we have provided molecular evidence for covalent bonding between ^{15}N-labeled peptides and humic acid molecules using the 2D HSQC NMR techniques. Non-covalent interactions are also observed in the NMR spectra, suggesting that both covalent and non-covalent interactions contribute to the preservation of proteinaceous materials. In this report, we examine the influence of aromaticity and aliphaticity of both the peptides and the humic materials on covalent and non-covalent interactions. ^{15}N-labeled peptides with different aromatic and aliphatic properties are mixed with three humic acids, that vary in degree of aromaticity, and we use the 2D HSQC NMR technique to evaluate bonding interactions. The peptide containing primarily aromatic amino acid residues is observed to form

covalent and non-covalent bonds with aromatic-rich humic acids. Like the results of previous studies, bonding with quinones is the likely mechanism. The peptide that is composed of aliphatic amino acid residues, on the other hand, shows only bonding interactions with aliphatic-rich humic acids. These observations provide the first direct molecular level evidence that aliphatic functional groups are involved in the bonding with proteinaceous materials. This process may play an enormous role in sequestration of proteinaceous materials in sedimentary systems such as marine systems where the humic materials are mainly aliphatic in nature.

3.1 Introduction

Nitrogen constitutes a major elemental component of humic materials in the environment (Schnitzer and Khan, 1978), constituting approximately 2-7 % of the total organic matter in these substances. Much of this nitrogen originates from proteinaceous material (proteins, polypeptides, and amino acids) that is released from living organisms as they die and become humified. When humic susbtances are treated with strong acid to hydrolyze the N-containing substances to their monomer forms, numerous compounds are identified including amino acids, amino sugars, and hydrolyzable unknown nitrogen-containing species. Approximately 50 % of the nitrogen in humic substances can be released by hydrolysis (Stevenson, 1994), and of this, most is identified as amino acid nitrogen. The non-hydrolyzable nitrogen that remains as a residue of hydrolysis is thought to be mostly heterocyclic nitrogen (Schulten and Schnitzer, 1998), but this view is rapidly changing as many studies now show that the non-hydrolyzable nitrogen is likely to exist as non-hydrolyzable peptides (Knicker et al., 1996; Knicker and Hatcher,

1997; Knicker and Hatcher, 2001). The recent information that has been used by Knicker et al. (1993) to suggest that all, if not most, of the nitrogen in humic acids is proteinaceous comes from solid-state ^{15}N NMR that show nearly all humic acids have identical spectra showing exclusively peaks for amide and amino nitrogen. Using synchrotron X-ray absorption spectroscopy, Vairavamurthy and Wang (2002) generally substantiate these findings but also identify minor amounts of heterocyclic nitrogen not seen in the NMR data. Nonetheless, little is known about the specific chemical interaction between proteinaceous materials and humic substances during humification due to the chemically complex and physically heterogeneous nature of humic substances.

Several possible interaction mechanisms of proteinaceous materials with humic substances have been proposed, and they include the physical adsorption and/or entrapment (encapsulation) and chemical bonding. It has long been known that humic substances, while partially soluble in aqueous solvents, are colloidal polyelectrolytes that demonstrate surfactant behavior (Stevenson, 1994) and can exhibit both hydrophobic and hydrophilic domains (Wershaw et al., 1986; Schwarzenbach et al., 2003). It is also well recognized that small molecules can become entrapped within this colloidal network (Stevenson, 1994). Knicker and Hatcher (1997) and Knicker et al. (1996) have recently suggested that proteinaceous materials that become entrapped or encapsulated within this macromolecular network in humic substances can be protected from degradation and survive for extended periods of geologic time. Riboulleau et al. (2002) suggested that such a mechanism could explain the preservation of proteinaceous materials in sediments as old as the Late Jurassic. Zang et al. (2000) found that proteinaceous extracts of living algae, when mixed with humic acids, are physically encapsulated within the humic acid

structures and are resistant to the chemical hydrolysis. Because the humic acid structures exist as highly branched networks of aliphatic components with varying degrees of aromaticity (Almendros et al., 1996; Hatcher et al., 1981; Hatcher et al., 1980), they suggested that physical encapsulation is caused by association of encapsulated proteinaceous materials with aliphatic moieties in humic acids. In addition to encapsulation, small (or large) molecules can become associated with humic substances by sorption onto exterior surfaces of colloids. Schulten and Schnitzer (1998) and Schulten et al. (1997) suggested that, while a portion of the proteinaceous material in soil is trapped in the three-dimensional humic acid structure, a fraction of these are either physically or chemically preserved on the surface of humic acids.

Another plausible mechanism for the existence and preservation of proteinaceous material in humic substances is formation of chemical bonds such that the bound peptides retain their peptidic nature, are somewhat protected from degradation, and show preservation of amide nitrogen functional groups that are identified in ^{15}N NMR spectra. Saxena and Bartha (1983a) proposed that covalent bonding between aromatic amines and quinone structures in humic substances can occur through nucleophilic addition of the amine group with carbonyl functional groups in quinones. Subsequently, oxidation of this newly formed adduct produces amino nitrogen that can be incorporated into a heterocyclic ring system to form organic heterocyclic molecules (Saxena and Bartha, 1983b). Obviously, the suggested pathway is built upon the concept that formation of heterocyclic nitrogen is needed to retain resistance to further degradation. While primary aromatic amines used in these studies are not functional groups found in proteinaceous substances, primary amines do exist and the hypothesis has some merit. Parris (1980)

substantiated the importance of the quinone functional groups of humic substances in some kinetic studies involving aromatic amines reacting with humic acids.

The reaction of proteinaceous materials with plant degradation products has been suggested as a primary mechanism for incorporation of nitrogen into humic substances. Maillard (1912) first proposed that amino acids, degradation products of peptides, react with reducing sugars, degradation products of carbohydrates, to form a Schiff base followed by rearrangements to eventually yield heterocyclic nitrogen (Stevenson, 1994). While this reaction has been proposed in many subsequent studies to be important in humification (Schulten and Schnitzer, 1998), some recent studies suggest that it is an unlikely reaction in algal-dominated systems (Zang et al., 2001). Other studies, by Flaig and co-workers (1975) show that aromatic components typically found in humic substances, namely quinones, react readily with amino acids, forming covalent linkages that escape hydrolysis (see review by Stevenson, 1994). It is clear that humic substances are thought to possess the ability to covalently incorporate proteinaceous materials; however, direct molecular evidence for this has been lacking, until recently.

In a previous study (Chapter 2), 2-dimensional HSQC (Heteronuclear Single Quantum Coherence) NMR techniques were used to investigate the interaction of an ^{15}N-labeled model peptide to Everglades peat humic acids. The 2D NMR results provide the first direct molecular evidence for covalent bond formation between amine/amide groups and humic substances and 1,4-naphthoquinone, supporting the idea that one of the possible preservation mechanisms for proteinaceous materials in the environment is through chemical linkage with quinone structures in the humic acid under investigation. The tetrapeptide used for these studies was composed of three glycines and arginine. We

do not know if the reaction was promoted by the structure of the peptide and the humic acid chosen or was universal for peptides in general or for all humic acids. To evaluate the reaction more fully, we embarked on a series of studies designed to test several different types of peptides and humic substances for their propensity to form covalent bonds.

Two peptides containing several ^{15}N-labeled amino acids in a specific sequence are reacted with three different humic acids. Our strategy was to examine the influence of the structural nature of the peptides on the formation of covalent bonds. The ^{15}N-labeled peptide, with the sequence serine-phenylalanine-phenylalanine-phenylalanine-tyrosine-tyrosine-serine (SFFFYYS) and which has the three phenylalanines ^{15}N-labeled, is considered a peptide with high degree of aromaticity. On the other hand, the second ^{15}N-labeled peptide with the sequence serine-leucine-leucine-leucine-valine-isoleucine-serine (SLLLVIS), which has the three leucines ^{15}N-labeled, represents proteinaceous materials with entirely aliphatic functional groups. The 2D HSQC NMR technique (Bodenhausen and Ruben, 1980) is applied to investigate the interaction of these two ^{15}N-labeled peptides to various humic substances. The 2D HSQC NMR experiment only observes protons directly bonded to carbons or nitrogens in molecules; thus, the chemical interaction of the peptides with three different humic acids can be readily discerned by changes in the HSQC spectra as shown previously (Chapter 2). We chose three different humic acids for this investigation, one whose structure is principally aliphatic, one whose structure is mainly aromatic as it is derived from degraded wood and has a structure similar to lignin, and another whose structure is both aromatic and aliphatic.

3.2 Materials and Methods

3.2.1 Preparation of humic acids and ^{15}N-labeled peptides

The three humic acids with different chemical properties are 1) Everglades peat humic acids, 2) Mt. Rainier humic acids, and 3) Mangrove Lake humic acids. The Everglades peat humic acid was from a peat collected in The Everglades National Park, near Alligator Alley, west of Hialeah, Florida (Hatcher et al., 1986). Mt. Rainier humic acids were extracted from a wood sample that had undergone degradation by brown rot fungi, collected on the slopes of Mt. Rainier, Washington (Hatcher, 1987). Mangrove Lake humic acids were extracted from a lacustrine sediment sample, which was collected with a 12.7 cm diameter piston core at a depth of 370 to 390 cm, in Mangrove Lake, Bermuda (Hatcher et al., 1983). All humic acid samples were prepared by the standard procedure involving extraction with 0.5 M NaOH, removal of sodium by cation exchange resin, and precipitation after adjusting the solution to pH 2. The humic acids were then washed several times with dilute HCl and then freeze-dried. Weighed amounts of the dried humic acids were redissolved in water whose pH was adjusted to 10 by dropwise addition of 0.5 M NaOH to assist in dissolution. The final solution concentrations of humic acids were 5 mg/mL and the solution pH's were adjusted to pH 8 by dropwise addition of 0.1 M HCl.

Two ^{15}N-labeled peptides with the sequence SFFFYYS, which has the three phenylalanines ^{15}N-labeled, and SLLLVIS, which has the three leucines ^{15}N-labeled, were purchased from Genemed Synthesis, Inc. (South San Francisco, CA) who certified 90% purity. No further purification was applied on these two ^{15}N-labeled peptides before

use. The concentration of the stock solution for peptide SFFFYYS was 1 mg/mL in H_2O and for peptide SLLLVIS was 1 mg/mL in 50 % deuterated acetonitrile (CD_3CN)/50 % H_2O.

A solution containing 0.1 mg ^{15}N-labeled peptide (100 μL of peptide stock solution) was mixed with 2 mg humic acids (400 μL of humic acids stock solution) and made to a volume of 600 μL by addition of H_2O and 60 μL D_2O to 10 % D_2O/90 % H_2O (v/v) solution which was adjusted to pH 8 by dropwise addition of 0.1 M NaOH. The mixture was then vortexed (VWR MV-1 mini vortexer, VWR, PA) at room temperature for at least 48 h before running NMR experiments.

3.2.2 Solid-state ^{13}C NMR

Solid-state ^{13}C NMR experiments of humic acids were performed on a Bruker DMX-300 MHz spectrometer (Bruker Biospin Corporation, Billerica, MA) with cross polarization-magic angle spinning (CP-MAS). Approximately 100 mg dry weight per sample was placed in a 4 mm (outside diameter) NMR rotor with a Kel-F cap (3M, Minneapolis, MN). Each sample was spun at a frequency of 13 kHz. Experiments were conducted at 300 K and 8192 scans were accumulated using a contact time of 2 ms and a 1 s recycle delay time, and 2048 data points collected on the free induction decay (FID). The FID was zero-filled to 4096 data points and subjected to exponential multiplication equivalent to 50 Hz line broadening. The chemical shifts were plotted using the carboxyl signal of glycine as the external standard (176.03 ppm) and referenced to tetramethylsilane at 0 ppm.

3.2.3 Liquid-state ^{15}N-^{1}H HSQC NMR

The 2D ^{15}N-^{1}H HSQC NMR spectra were obtained on a Bruker DMX-600 spectrometer with a 5 mm TXI (triple-resonance inverse) probe (^{1}H, ^{13}C, ^{15}N) equipped with z gradient. Heteronuclear Single-Quantum Coherence (HSQC) spectra (64 scans, with 2048 data points for the time domain F2 and 256 data points for time domain F1) were acquired using a relaxation delay 1.5 sec with phase-sensitive States-TPPI (Time Proportional Phase Incrementation). All liquid-state NMR experiments were performed at 293 K. The acquired NMR data are then processed by XWIN-NMR (version 3.1, Bruker Biospin Corporation, Bruker, Billerica, MA). The data was processed with 90^{o} shifted sine squared (QSINE) function in F1 dimensions (^{15}N) and the Gaussian window function in the F2 dimensions (^{1}H), and 1 Hz line broadening in both dimensions. The ^{15}N chemical shift scale used in this research was the δ-scale, a common nitrogen scale for protein NMR researchers, which sets liquid NH_3 as 0 ppm and nitromethane as 380.23 ppm.

3.3 Results and Discussion

3.3.1 Solid-state ^{13}C NMR

Figure 3.1 shows the stacked solid-state ^{13}C NMR spectra of Everglades peat humic acids, Mt. Rainier humic acids and Mangrove Lake humic acids. Signals between 110 and 150 ppm represent the aromatic components which are observed in Everglades peat humic acids and Mt. Rainier humic acids but relatively suppressed in Mangrove Lake humic acids. The intense peak at about 150 ppm in the Mt. Rainier humic acids

represents O-substituted aromatic groups that are characteristic for lignin-derived materials and other phenolic substances. The other intense peak at 56 ppm in Mt. Rainier humic acids is assigned as methoxyl carbons associated with lignin and this peak is also observed in Everglades peat humic acids. Lignin and its degradation products are obviously important component structures in these two humic acids. The predominant

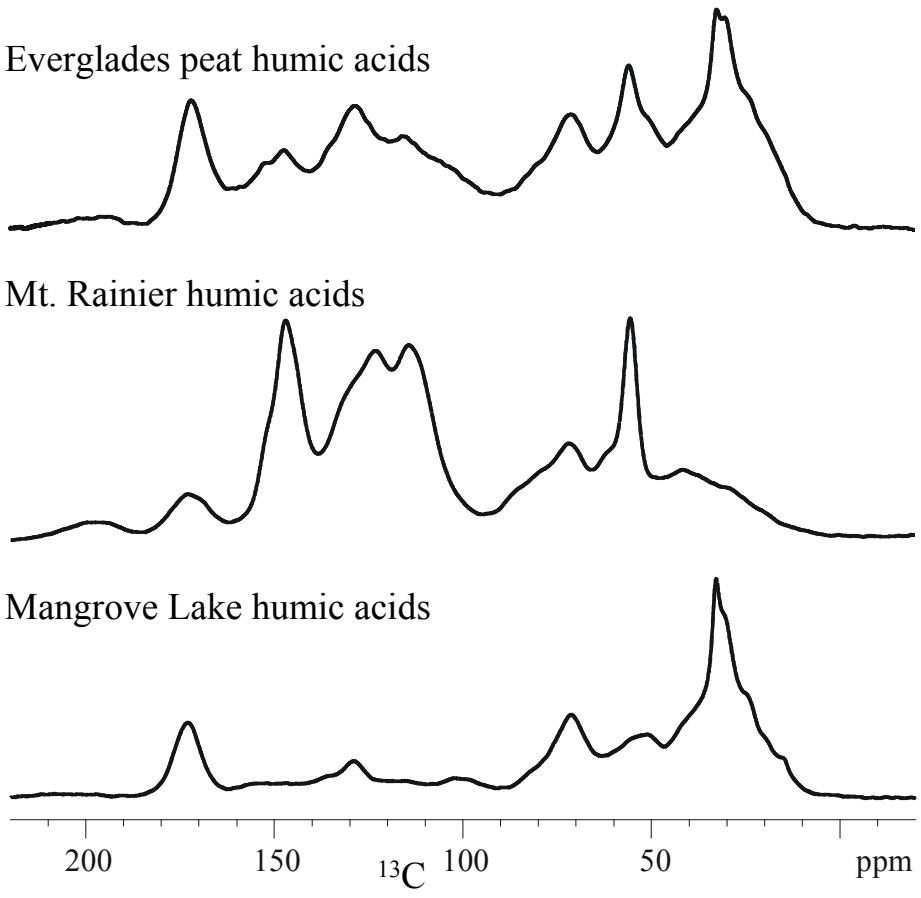


Figure 3.1: Stacked solid-state ^{13}C NMR spectra of Everglades peat humic acids (shown previously in Zang et al., 2000), Mt. Rainier humic acids (shown previously in Hatcher, 1987), and Mangrove Lake humic acids.

signal in Mangrove Lake humic acids at 33 ppm represents the presence of paraffinic carbons; it, too, is observed in Everglades peat humic acids. The signal at 72 ppm observed in all three humic acids is the O-substituted alkyl group found usually in either carbohydrates or lignin. An intense peak at 175 ppm is likely that of carboxyl or amide groups, and the broad signal observed at 200 ppm is attributed to ketone/aldehyde carbons. Based on these solid-state ^{13}C NMR results, we conclude that the Mt. Rainier humic acids represent humic substances with high aromaticity and Mangrove Lake humic acids represents humic substances with more aliphatic properties. The Everglades peat humic acids are a mixture of the two end-members, containing both aromatic and aliphatic properties. This humic acid is most representative of typical humic acids. Therefore, Mt. Rainier and Mangrove Lake humic acids exhibit vastly different chemical structural properties whose influence on the interaction with proteinaceous materials can be discerned by the studies planned herein.

3.3.2 Liquid-state ^{15}N-^{1}H HSQC NMR of peptide SFFFYYS

In Figure 3.2, showing the ^{15}N-^{1}H HSQC NMR spectrum of the unreacted peptide SFFFYYS, there are only two nitrogen-proton correlated signals observed even though all three phenylalanine amino acids are ^{15}N-labeled. The nitrogen-proton correlated signals are at 8.16 ppm and 8.10 ppm on the ^{1}H axis and 119.9 and 120.3 ppm on the ^{15}N axis, respectively. The NMR data can be explained by the fact that two of the three phenylalanines have similar chemical environments such that both chemical shifts are identical. If this is the case, then one would expect one of the peaks to show greater intensity than the other, a situation not observed as both peaks are of equal intensity.

Analysis by electrospray ionization-mass spectrometry (ion trap) performed at the Campus Chemical Instrument Center at Ohio State University confirmed that all three phenylalanines are ^{15}N-labeled (data not shown). If two of the phenylalanine nitrogens show overlapping signals, then the intensities are not additive in proportion to the one intensity not composed of overlapping resonances perhaps due to the fact that the HSQC experiment is not quantitative.

In order to clarify that only two of the three nitrogen-proton correlated signals are observed in the ^{15}N-^{1}H HSQC NMR spectrum in Figure 3.2, we subjected the structure to molecular modeling. Possible 3D structures of peptide SFFFYYS are calculated by Chem3D, and the one with the lowest energy minimum is shown in Figure 3.3. The aromatic rings of the third phenylalanine (F3) and fourth phenylalanine (F4) stack

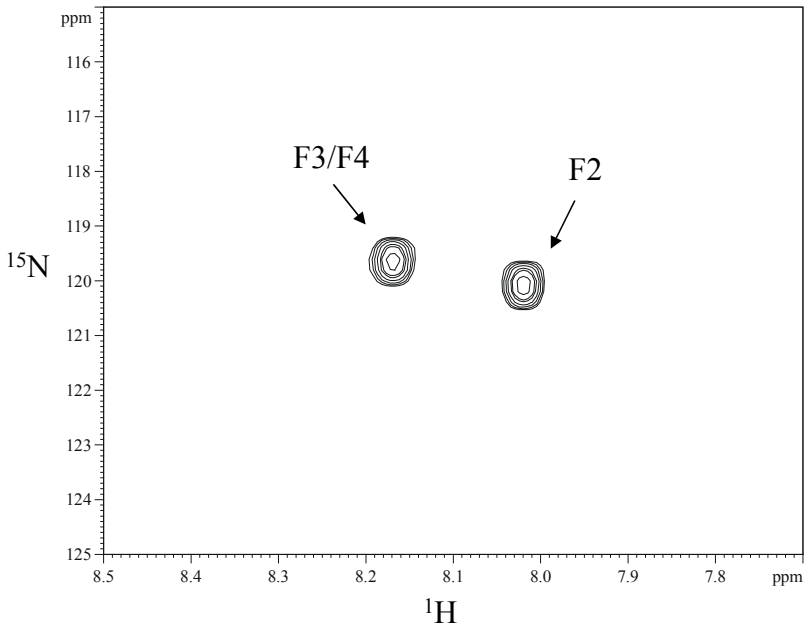


Figure 3.2: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide SFFFYYS.

vertically, as do the aromatic rings of Y5 and Y6. The hydrogen atoms in the amide group of three phenylalanines are all pointed to the opposite side of the aromatic side-chains and are less hindered sterically. However, based on a closer examination at the phenylalanine amide groups, the amide group in F2 is affected by the proximal serine side-chain (S1 in Figure 3.3). Amide groups in F3 and F4, in contrast, are not influenced by other amino acid side-chains. Therefore, the amide groups of F3 and F4 probably have

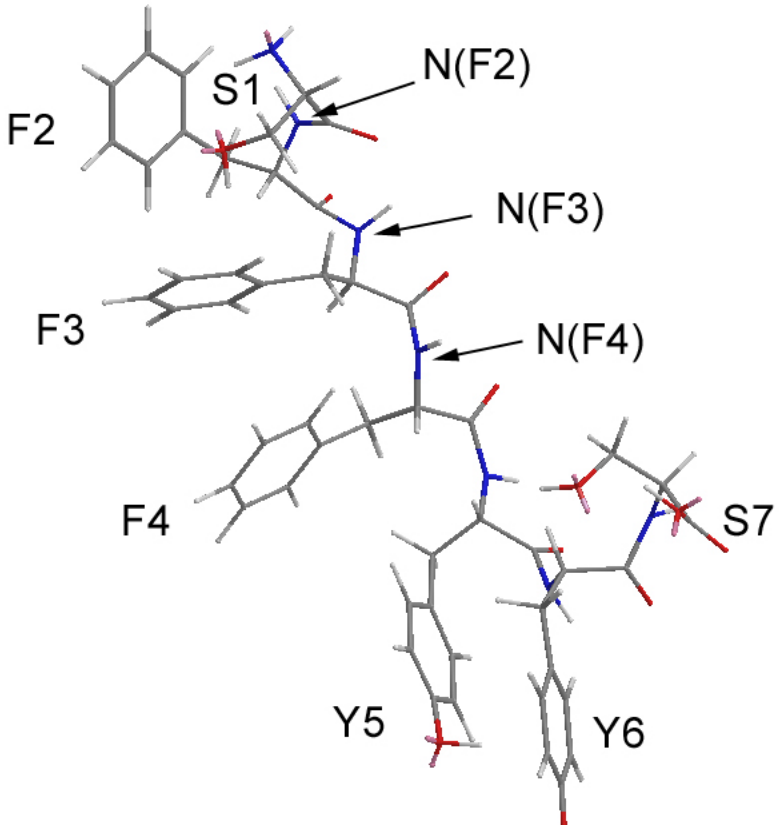


Figure 3.3: A model structure of peptide SFFFYYS with minimized energy (Chem3D). F2, F3, and F4 represent the three ^{15}N-labeled phenylalanine amino acid residues and the N(F2), N(F3), and N(F4) indicate the positions of the three ^{15}N atoms. S1, Y5, and Y6 represent the other amino acids in the structure (serine and tyrosine).

similar chemical environments, resulting in identical chemical shifts. The amide group of F2 has a different chemical shift due to a slightly different chemical environment induced by interaction with the serine side-chain. Also, we expect that the nitrogen-proton correlated signals for amide groups in F3 and F4 to have downfield shifts compared with the signal for the amide group in F2 since the adjacent amino acid residues (tyrosine) have more aromatic properties.

3.3.3 Liquid-state ^{15}N-^{1}H HSQC NMR for peptide SFFFYYS mixed with humic acids

Figure 3.4 represents the ^{15}N-^{1}H HSQC NMR spectrum of the peptide SFFFYYS mixed with Everglades peat humic acids (red) overlain by the spectrum of peptide SFFFYYS (black, Figure 3.2). Comparing the original nitrogen-proton correlated signals of the free peptide, which are at 8.16 and 8.10 ppm on the ^{1}H frequency and 119.9 and 120.3 ppm on the ^{15}N frequency, the corresponding signals in the spectrum of the peptide SFFFYYS mixed with Everglades peat humic acids have broadened. Peak broadening effects usually indicate the existence of non-covalent binding interactions. These non-bonding interactions may be from charge-charge or Coulombic interactions between peptide SFFFYYS and Everglades peat humic acids. In addition to the broadened peaks, there are three new nitrogen-proton correlated signals observed in Figure 3.4, at 8.26, 8.35 and 8.24 ppm on the ^{1}H frequency and 119.4, 119.9 and 120.3 ppm on the ^{15}N frequency, respectively. The new peaks in the NMR spectrum represent a change of the chemical environment around amide groups suggesting the formation of covalent bonds. In previous studies (Chapter 2), we suggested that quinone structures in humic acids are

responsible for these covalent bonds, perhaps involving the Michael reaction. The chemical shifts of the new peaks observed in Figure 3.4 also suggest covalent bonding with quinone structures, because the aromatic nature of the quinones can induce a downfield shift in the nitrogen-proton correlated peak. The 2D ^{15}N-^{1}H NMR results, therefore, support the existence of both covalent and non-covalent binding interactions between SFFFYYS, a peptide with high aromaticity, and a typical humic acid.

The 2D ^{15}N-^{1}H HSQC NMR spectrum for peptide SFFFYYS mixed with a predominantly aromatic humic acid, Mt. Rainier, is shown in Figure 3.5 (red) overlain by the spectrum of peptide SFFFYYS (black). Several new peaks emerge from the reaction

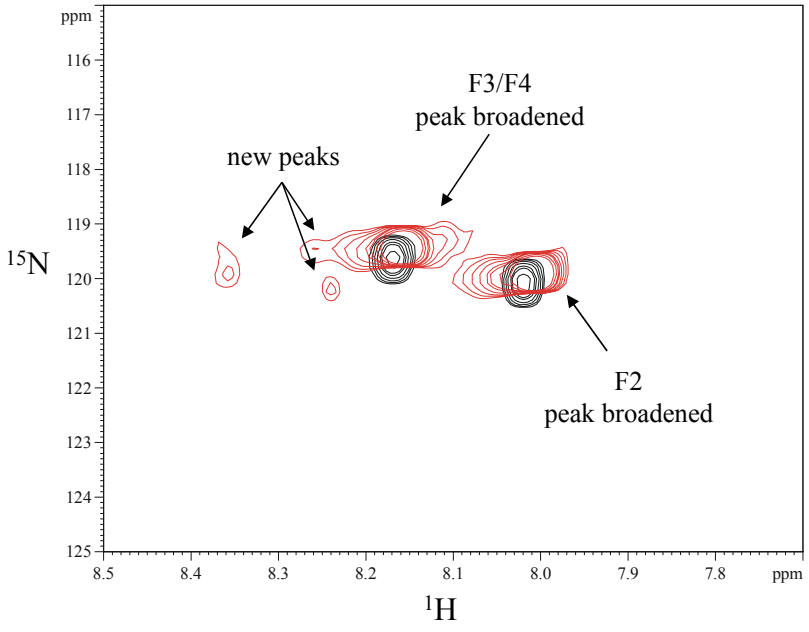


Figure 3.4: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide SFFFYYS and Everglades peat humic acids mixture (red) and an overlay spectrum of ^{15}N-labeled peptide SFFFYYS (black).

indicating that numerous covalent bonding products exist when the peptide SFFFYYS is mixed with Mt. Rainier humic acids. Broadening of the peaks is also observed in Figure 3.5, indicating the formation of non-covalent bonding interactions. Mt. Rainier humic acids, like Everglades peat humic acids, have significant amounts of aromatic functional groups. Two of the new peaks show almost identical chemical shifts as is observed in the reaction of the peptide with Everglades peat humic acids (Figure 3.5). These peaks are at 8.25 ppm and 8.36 ppm on the ^{1}H frequency and around 120 ppm on the ^{15}N. The peak shifts and peak broadening patterns of the two humic acids are very similar, indicating the presence of a common interaction mechanism with peptide SFFFYYS, perhaps formation of Michael adducts with quinonoid groups in the two humic acids.

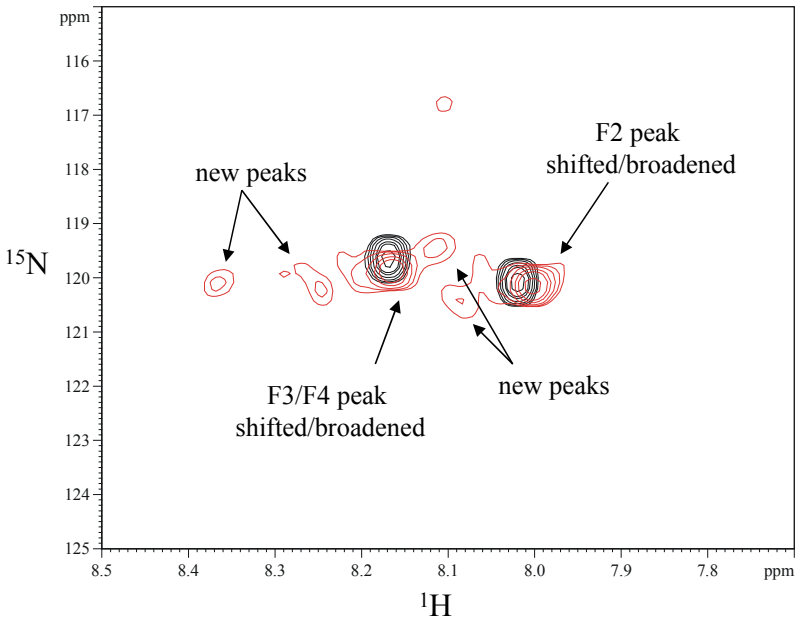


Figure 3.5: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide SFFFYYS and Mt. Rainier humic acids mixture (red), overlain by the spectrum of ^{15}N-labeled peptide SFFFYYS (black).

Comparing the NMR results for peptide SFFFYYS mixed with humic acids abundant in aromatic functional groups and that for the peptide mixed with humic acids that are predominantly aliphatic, Mangrove Lake humic acids, one observes a different nitrogen-proton correlated pattern shift in the 2D ^{15}N-^{1}H HSQC NMR spectrum (Figure 3.6). There is no peak broadening effect nor new N-H cross peaks in the spectrum (red), indicating the lack of both covalent and non-covalent interactions between the peptide SFFFYYS and Mangrove Lake humic acids. Overlaying the spectrum for the peptide SFFFYYS (black), a slight change in chemical shifts in the nitrogen-proton correlation peaks are observed. The two nitrogen-proton correlated peaks shift in almost the same direction. This chemical shift difference could be due to a weak non-covalent binding mechanism that may slightly change the chemical environments around the amide groups to induce the peaks shifts. These NMR results suggest that there is no covalent binding interaction observed between the peptide SFFFYYS and Mangrove Lake humic acids. Clearly, aromatic groups in humic substances play an important role in forming both covalent and non-covalent bonding interactions with proteinaceous materials. Mangrove Lake humic acids are significantly depleted of aromatic functional groups compared with the other two humic acids. Moreover, the aromatic functional groups in humic acids are most likely interacting with the aromatic side chains of the aromatic amino acids in SFFFYYS.

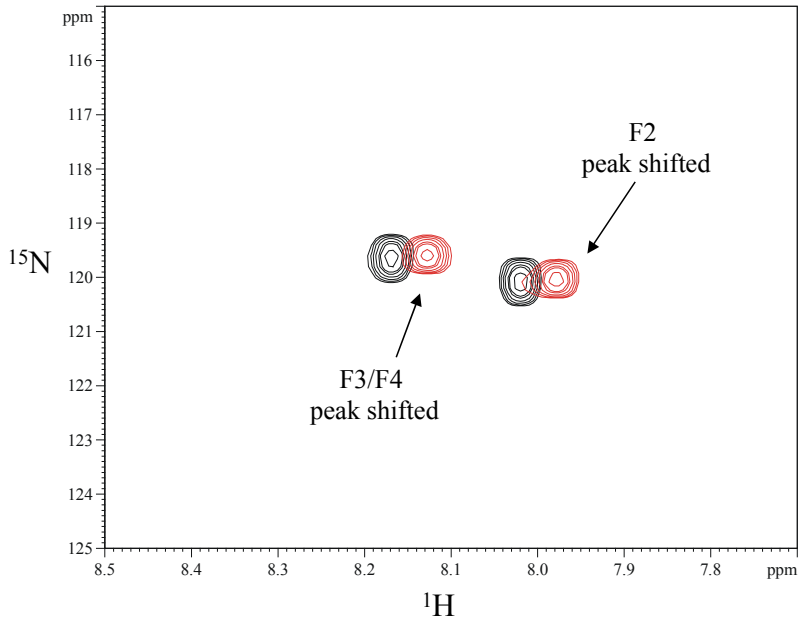


Figure 3.6: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide SFFFYYS and Mangrove Lake humic acids mixture (red) and overlain spectrum of ^{15}N-labeled peptide SFFFYYS (black).

3.3.4 Liquid-state ^{15}N-^{1}H HSQC NMR of peptide SLLLVIS

The ^{15}N-labeled peptide SLLLVIS possessing only aliphatic properties is also investigated by the 2D ^{15}N-^{1}H HSQC NMR and the spectrum is shown in Figure 3.7. Only two nitrogen-proton correlated signals are observed in the NMR spectrum, even though SLLLVIS has three leucine residues labeled with ^{15}N. The signal pattern is very similar to that observed for the peptide SFFFYYS (Figure 3.2). Two of the ^{15}N-labeled leucine residues in peptide SLLLVIS, therefore, have similar chemical environments. However, in the case of the spectrum for peptide SLLLVIS, the nitrogen-proton cross peak at 8.30 ppm on the ^{1}H frequency and 124.6 ppm on the ^{15}N frequency shows higher

intensity than the other signal which is observed at 8.25 ppm on the ^{1}H frequency and 122.5 ppm on the ^{15}N frequency. The signal intensity ratio of these two N-H cross peaks is approximate 2 indicating that two amide groups of ^{15}N-labeled leucines in the SLLLVIS have almost identical chemical environments in solution.

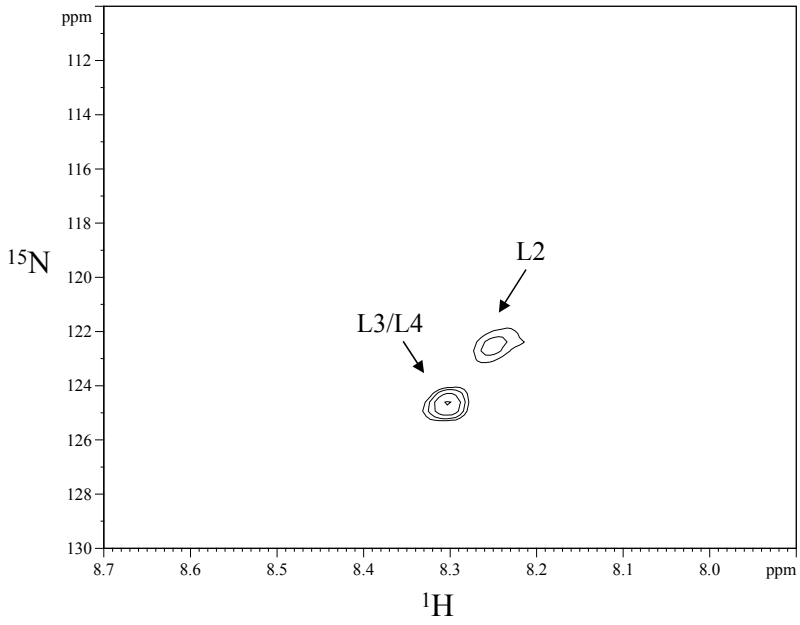


Figure 3.7: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide SLLLVIS.

An energy minimized 3D structure of peptide SLLLVIS is calculated by Chem3D. Most likely, there is no fixed 3D structural conformation for the peptide SLLLVIS because it only has 7 amino acids in its sequence and it is generally known that a larger number of amino acids are required for assumption of a 3D structural configuration. However, the energy minimization yields a spherical shape conformation for SLLLVIS (data not shown). Due to the hydrophobic properties provided by the aliphatic side chains

of the peptide SLLLVIS, the spherical conformation is consistent with these results. The third leucine (L3) and fourth leucine (L4) residues are embedded in the hydrophobic structure. However, the amide group in the second leucine (L2) is less hydrophobic due to the neighboring serine (S1) residue. Thus, the 3D structural model provides a possible explanation for why there are only two nitrogen-proton correlated peaks observed. The predicted conformation also explains why peptide SLLLVIS could only be dissolved, at low solubility, in 50 % CD_3CN/50 % H_2O and not water alone.

3.3.5 Liquid-state ^{15}N-^{1}H HSQC NMR for peptide SLLLVIS mixed with humic acids

When the peptide SLLLVIS is mixed with Everglades peat humic acids, there are four nitrogen-proton correlation signals in the ^{15}N-^{1}H HSQC NMR spectrum, each with different peak intensities (red, Figure 3.8). When compared with the ^{15}N-^{1}H HSQC NMR spectrum of SLLLVIS (black, Figure 3.8), two new nitrogen-proton correlated peaks are observed at 8.30, 8.04 ppm on the ^{1}H frequency and 123.4, 122.8 ppm on the ^{15}N frequency, respectively. The emergence of these two new signals indicate that the chemical environment around the ^{15}N in leucine amide groups changed, indicating the presence of covalent linkages between the peptide and humic acid molecules. However, there is no additional information that helps identify which amide group is involved in the formation of the covalent bonding with humic acid molecules, because we cannot assign the new peaks to any specific residue. In addition, the peak broadening that was so clearly observed in Figure 3.4 for the SFFFYYS peptide and attributed to non-covalent interactions is not present in Figure 3.8. Nevertheless, the nitrogen-proton cross peaks

shift when the SLLLVIS is mixed with Everglades peat humic acids, and this may be from weak non-covalent bonding interactions or from bonding interactions at sites remote from the label. At the pH of the reaction, the N-terminal amino acid, serine, is likely to be in its amine form and could react with the humic acid. Such a reaction could cause a weak inductive shift that is consistent with the observed shift of the two cross peaks.

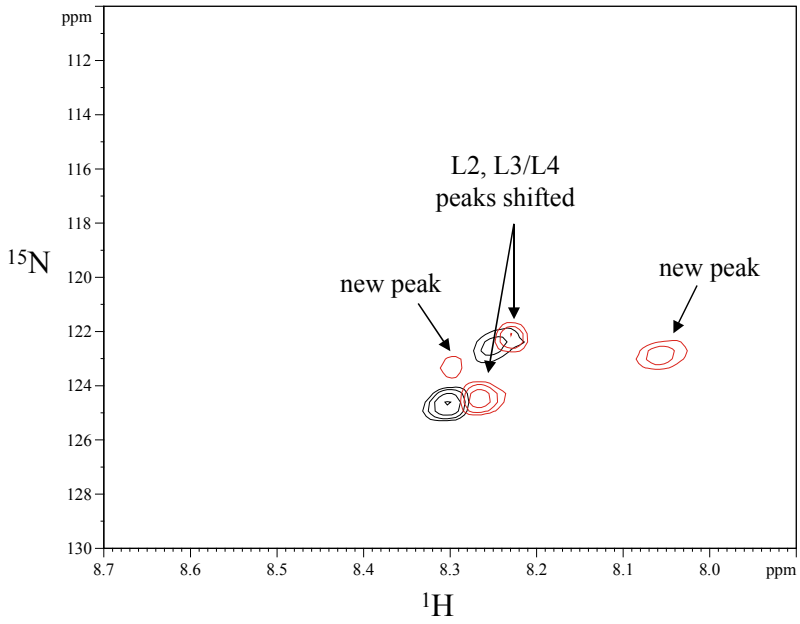


Figure 3.8: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide SLLLVIS and Everglades peat humic acids mixture (red) and overlain spectra of ^{15}N-labeled peptide SLLLVIS (black).

Figure 3.9 shows the ^{15}N-^{1}H HSQC NMR spectrum (red) of SLLLVIS mixed with Mt. Rainier humic acids, which contains mostly aromatic lignin-derived components. When compared with the spectrum of free SLLLVIS (black), one of the peaks disappears, three new nitrogen-proton correlated signals are observed, one at 8.61 ppm on the ^{1}H frequency and 117.3 ppm on the ^{15}N frequency, another at 8.29 ppm on the ^{1}H frequency and 123.5ppm on the ^{15}N frequency, and the third at 8.38 ppm (^{1}H) and 122.2 ppm (^{15}N). As observed above, significant shifts in cross peaks is clear indication of covalent linkages being established between the peptide and the humic acids.

Figure 3.10(red) shows the HSQC NMR spectrum for peptide SLLLVIS mixed

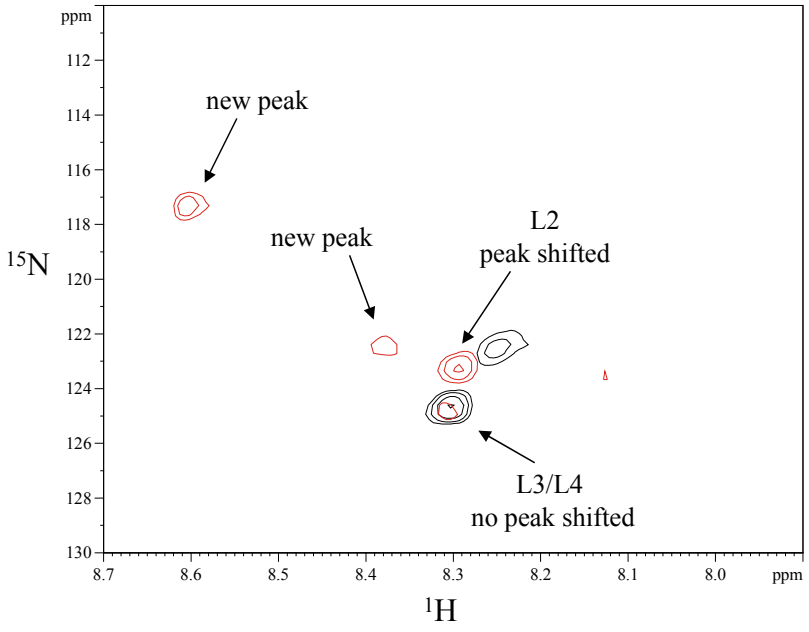


Figure 3.9: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide SLLLVIS and Mt. Rainier humic acids mixture (red) and overlain spectrum of ^{15}N-labeled peptide SLLLVIS (black).

with Mangrove Lake humic acids. A new nitrogen-proton correlated peak with high intensity is observed at 8.05 ppm on the ^{1}H frequency and 122.8 ppm on the ^{15}N frequency. As in the case of all humic acid samples, formation of a new peak suggests the presence of covalent interactions between the peptide and the humic acids. This same cross peak is observed in the spectrum of Everglades peat humic acids reacted with SLLLVIS but not in the one where Mt. Rainier is reacted. The obvious common

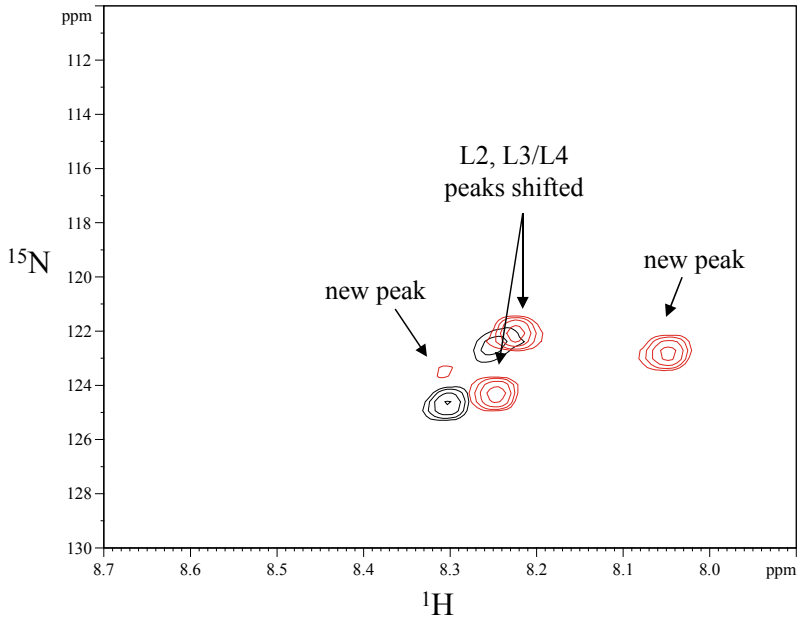


Figure 3.10: ^{15}N-^{1}H HSQC NMR spectrum of ^{15}N-labeled peptide SLLLVIS and Mangrove Lake humic acids mixture (red) and overlain spectrum of ^{15}N-labeled peptide SLLLVIS (black).

structural character between the Everglades and Mangrove Lake samples is the aliphatic structures and these are generally absent in the Mt. Rainier humic acids. Also, there seems to be a correlation between the intensity of this cross peak and the relative intensity of paraffinic structures in the two humic acids. Therefore, it seems reasonable to suggest that the new and intense cross peak observed represents some covalent linkage formed between the aliphatic peptide and the paraffinic structures in humic acids. Another observation from the spectrum of Mangrove Lake humic acids reacted with SLLLVIS is the shifting of positions for the two signals observed in the free peptide. These could be attributed to non-covalent interactions, the inductive effects caused by binding that induces the formation of a new intense cross peak, or reactions at unlabeled sites on the peptide.

3.4 Conclusions

The 2D ^{15}N-^{1}H HSQC NMR techniques provide direct spectroscopic evidence for the development of strong covalent and non-covalent interactions of humic acids with peptides, showing that proteinaceous materials can be incorporated into humic substances. Since ^{15}N-^{1}H HSQC NMR spectra highlight specifically nitrogen-proton correlations, the formation of covalent bonds between ^{15}N-labeled peptides and humic substances results in the evolution of new cross peaks or leads to peak shifting. Non-covalent binding or charge-charge interactions between ^{15}N-labeled peptides and humic acid molecules are expressed by the broadening of N-H correlation peaks.

In this study, two different peptides with ^{15}N-labeled amino acids residues are used to investigate the influence of aromaticity on the interaction between peptides and

humic molecules. Peptide SFFFYYS, with three phenylalanines ^{15}N-labeled, demonstrates a significant amount of aromatic character. The second peptide SLLLVIS, which has three leucines ^{15}N-labeled, and is considered to represent aliphatic peptides, because leucine, valine, and isoleucine residues contain aliphatic side chain structures. Based on the aromatic and aliphatic character of these peptides we demonstrate significant differences in reactivity with a set of humic acids that also vary in extent of aromatic and aliphatic character. Based on the combination of mixing two different ^{15}N-labeled peptides and three humic acids that vary from low to high aromaticity, the functional effect of the molecular interaction between peptides and humic acids is examined by using ^{15}N-^{1}H HSQC NMR techniques.

When the aromatic ^{15}N-labeled peptide SFFFYYS is mixed with humic acids containing aromatic components, the formation of covalent bonds between is confirmed by ^{15}N-^{1}H HSQC NMR experiments. Not only are new nitrogen-proton correlated peaks observed but also peak broadening occurs, indicating the presence of both covalent and non-covalent binding mechanisms. The effect on the predominantly aliphatic humic acid is different, involving changes that are probably due to weak non-covalent interactions. We suggest that the aromatic components in humic substances play an important role in determining the type of interaction when they are mixed with proteinaceous materials. Quinone structures in these humic acids are thought to form covalent bonds with amide groups in proteinaceous materials, as shown in previous studies; however, additional NMR experiments need to be designed to further confirm this hypothesis. Reaction of the labeled peptide with quinones would be appropriate, as was done in previous studies (Chapter 2).

Another important finding in this study regards molecular interactions between peptides and aliphatic components of humic substances. The ^{15}N-^{1}H HSQC NMR results for the aliphatic ^{15}N-labeled peptide SLLLVIS that was mixed with the three humic acids shows the preferential formation of covalent bonds with aliphatic humic acids. The two humic acids exhibiting significant aliphatic character show new nitrogen-proton correlated signals that characterize some unknown covalent interaction with aliphatic structures. Weak non-covalent binding interactions are also probable since the nitrogen-proton correlated peaks are slightly shifted. To our knowledge, this is the first direct observation that aliphatic structures in humic acids can enter into covalent bonding with aliphatic peptides; however, no additional information can be gleaned about what chemical structures are involved in these interactions.

It is clear that the aliphatic and aromatic compositions of both peptides and humic acids play an important role in determining the type of bonding interactions favored by the system. Thus, we can expect that aliphatic humic acids will selectively covalently bind peptides that exhibit significant aliphatic character. Likewise, aromatic humic acids will tend to form strong covalent bonds with peptides that have aromatic character. This selectivity should be observed in the distribution of amino acids associated with aromatic and aliphatic humic acids. To our knowledge, previous investigations on distributions of different amino acids in humic substances have not focused on correlations with aromatic or aliphatic character of the humic substances. We might anticipate a correlation, but there are numerous factors involved in the process of incorporation of peptides into humic acids (extent of degradation of proteins, selectivity for certain biomass

components, microbial inputs, etc.) and these might obscure any correlations we might anticipate.

The covalent bonding that is observed between aliphatic peptides and aliphatic structures in humic acids is of great significance in marine systems. Numerous investigations have shown that the humic acids in most marine sediments are predominantly aliphatic (Hatcher, 1980; Hatcher, 1981; Hatcher et al., 1983; Poutanen, 1986; Sardessai and Wahidullah, 1998; Graguglia et al., 1995). Thus, as proteinaceous organic matter from autochthonous production in the oceans is subjected to biodegradation and becomes incorporated in sedimentary materials, peptides formed as the result of this process may become associated with humified organic matter produced at the same time. We can envision that aliphatic-rich peptides can become covalently linked to the humic materials and that this process can serve to protect them from further enzymatic degradation. Nguyen and Harvey (1998) showed that peptides could be incorporated into high molecular weight material during degradation and that these appear to be preserved from further degradation. The numerous explanations proposed for this phenomenon include, aggregation of proteins, condensation reactions with sugars, and cross-linking of proteins and peptides to non-proteinaceous material. Later studies (Nguyen and Harvey, 2001) showed that condensation with sugars is of minor importance, similar to the findings of Nguyen et al. (2001). Our results indicate that the cross-linking pathway is a plausible mechanism but that non-covalent associations are also important. The aliphatic structures in humic acid extracts, some of which may themselves be proteinaceous, play an important role in binding aliphatic peptide fragments.

CHAPTER 4

ENCAPSULATION OF PEPTIDES IN HUMIC ACIDS AS A MEANS FOR PRESERVATION FROM ENZYMATIC DEGRADATION

Abstract

By simulating the interaction of proteinaceous materials with two different humic substances we conclusively demonstrate that a model peptide, covalent and non-covalently bound to humic acids can be protected from enzymatic degradation. Such a process may be responsible for preservation of proteinaceous materials in sediments for extended periods of geologic time. A peptide containing an ^{15}N-labeled glycine residue, with the sequence of RGFFYTPKA, was mixed with two structurally different humic acids to induce covalent and non-covalent binding and was subjected to enzymatic hydrolysis with pepsin. We found that 5 % to 11 % of the ^{15}N-labeled peptide survived enzyme degradation and we attribute this mainly to formation of covalent bonds to humic acid molecules. A higher recovery of the ^{15}N-labeled peptide comprised of aromatic amino acids was observed when it interacted with the aromatic humic acid, compared with the aliphatic humic acid. This finding is consistent with previous studies that suggest that aromatic components in humic acids play a more important role in binding peptides that are aromatic in nature. The results presented herein show that binding to humic

materials in sedimentary systems can offer significant protection for labile proteinaceous substances in the environment.

4.1 Introduction

Complete mineralization of proteins is an essential component of the nitrogen cycle, especially if one expects to maintain biological productivity for extended periods of time. However, a small percentage of the nitrogen tied up proteinaceous materials in organisms does escape mineralization to become incorporated in sediments (Tanoue, 1995; Tanoue et al., 1996; Nguyen and Harvey, 1997; Pantoja and Lee, 1999; Knicker and Hatcher, 2001) and soils (Stevenson, 1994; Schulten and Schnitzer, 1998).

While several preservation mechanisms involving protection of proteinaceous materials within mineral components of soils and sediments have been proposed (Mayer, 1995; Mayer, 1994; Salmon et al., 1998), they all essentially involve the basic concept that protection is afforded by physical exclusion of hydrolyzing enzymes. Macromolecular organic matter in soils and sediments can also provide a physical barrier to enzyme accessibility, especially if the organic matter is generally hydrophobic and possesses sufficient micro or mesoporosity to entrap proteinaceous materials and exclude hydrophilic enzymes. Knicker and Hatcher (1997) and Knicker et al. (1996) proposed that proteinaceous materials survive via such a process in a algal-dominated sedimentary system that is predominantly organic rich (Mangrove Lake, Bermuda). Nguyen and Harvey (1998; 2001) observed that proteinaceous materials appeared to be stabilized towards degradation when associated with a high molecular weight fraction these predominantly organic rich sediments. Schulten and Schnitzer (1998) and Schulten et al.

(1997) suggested that a portion of the proteinaceous material in soil is trapped in the three-dimensional structure of humic acids but a fraction of these compounds are either physically or chemically preserved on the surface of humic acids. Zang et al. (2000) found that ^{15}N-labeled protein extracts from fresh algae can be physically encapsulated within humic acid structures and can survive 6 M HCl hydrolysis.

In addition to these physical processes, the formation of chemical bonds with natural organic matter (humic materials, membrane protein residues) is also thought to induce preservation (Nagata et al., 1998; Tanoue et al., 1995; Chapter 2). Saxena and Bartha (1983) showed that quinone groups in humic substances are the major reaction sites for nucleophilic addition, especially for reactive amines. Recent studies (Briggs et al., 2003) indicate that amino acids and peptides readily undergo Michael addition reactions with quinones. Considering the fact that quinonoid structures are thought to be important components of humic acids (Stevenson, 1994) it is not too surprising that they can readily bind peptides, thus, providing a mechanism linking these peptides into a molecular framework that has been shown to offer protection from enzymatic attack.

In previous studies (Chapter 2 and 3), we employed a 2D NMR technique to investigate the interactions of peptide molecules with humic acids. The presence of covalent and non-covalent bonding was demonstrated to readily occur at room temperature and by simply mixing ^{15}N-labeled model peptides with various humic acids. The 2D NMR studies (Chapter 2) also provided evidence for formation of covalent bonds between amine/amide groups of the peptides and 1,4-naphthoquinone, supporting the formation of chemical linkages with quinone structures in humic acids as one of the possible reaction pathways for proteinaceous materials. Moreover, the 2D NMR

techniques demonstrated that covalent bonding between model peptides and paraffinic structures in aliphatic-rich humic acids was possible (Chapter 3).

It is clear that a covalent binding pathway between humic substances and peptides is possible, but we can only speculate that such adducts would be protected from enzymatic hydrolysis in the environment. The studies reported by Zang et al. (2000) showed that humic acids can trap peptides and protect them from strong acid hydrolysis, but we do not know whether this also protects the peptides from enzymatic hydrolysis. We designed an experiment to test whether peptides bound to humic acids can be protected from enzymatic attack. A small ^{15}N-labeled peptide was reacted with two humic acids that have vastly different structures, one being predominantly aromatic and the other predominantly paraffinic in nature. The peptide with sequence arginine-glycine-phenylalanine-phenylalanine-tyrosine-tryptophan-proline-lysine-alanine (RGFFYTPKA), part of the sequence in human insulin chain β, is chosen as the model peptide. Adducts were then subjected to enzymatic hydrolysis with pepsin, an enzyme that operates at low pH, to evaluate the recalcitrance of the peptide to hydrolysis. The presence of ^{15}N labels allowed us to track the quantitative effectiveness of binding and resistance to hydrolysis by nitrogen isotopic analysis.

4.2 Materials and Methods

4.2.1 Preparation of ^{15}N-labeled peptide and humic acids

The amino acid sequence for the ^{15}N-labeled peptide used in the simulated peptide preservation experiment (described below) was RGFFYTPKA which has glycine

^{15}N-labeled (represented by G). A non-labeled peptide with the same sequence is commercially available (Sigma-Aldrich, St. Louis, MO) and we used it to test the enzyme reaction conditions in advance of utilizing the more expensive ^{15}N-labeled peptide. The ^{15}N-labeled peptide was purchased from Genemed Synthesis, Inc. (South San Francisco, CA) certified at 90% purity. No additional purification was employed for the ^{15}N-labeled peptide before it's use. A peptide stock aqueous solution of approximately 1 mg/mL was prepared by dissolving the powdered peptide in deionized water and adjusting to pH 7 by dropwise addition of 0.1 M NaOH.

Two humic acids sampled from different areas were used in this research. Mt. Rainier humic acids was extracted from degraded wood samples collected on the slopes of Mt. Rainier, Washington (Hatcher, 1987). Mangrove Lake humic acids was extracted from a sediment core sample which was collected by using a 12.7 cm diameter piston core to depths of 4 meters and sampled at depth of 370 to 390 cm in 1982, Mangrove Lake, Bermuda (Hatcher et al., 1983). The humic acid samples were prepared by the standard procedure involving extraction with 0.5 M NaOH, removal of sodium by treating with cation exchange resin, and precipitation after adjusting the solution to pH 2. The humic acids were then washed several times with dilute HCl and freeze-dried. Weighed amounts of the dried humic acids were redissolved in water whose pH was adjusted to 10 by dropwise addition of 0.5 M NaOH to assist in dissolution. The final solution concentrations of humic acids were 5 mg/mL and the solution pH's were adjusted to pH 8 by dropwise addition of 0.1 M HCl.

4.2.2 Peptide preservation simulation

We designed an experiment, diagrammatically shown in Figure 4.1, that would test the efficacy for preservation of our labeled peptide in association with the two humic acids. The intent of this experiment is to simulate the protection of peptides, protein degradation fragments, incorporated into humic substances from enzymatic degradation in natural systems. The peptide RGFFYTPKA is preferred in this experiment because the length is adequate for reaction with pepsin and there is at least one position in the amino acid sequence available for pepsin degradation. Commercial availability of the peptide RGFFYTPKA and low cost for synthesizing this peptide with an ^{15}N-labeled amino acid residue also make this a peptide of choice. Because our analytical scheme requires soluble fractions, we chose humic acids rather than insoluble macromolecular organic matter that is most dominant in sedimentary systems. Accordingly, we were required to conduct an experiment at a pH that was not characteristic of most sedimentary systems but would provide us with a mechanism to physically encapsulate/bind our model peptide with organic matter that undergoes a conformation change with pH. Generally, humic acids molecules have an extended chain-like structure at high aqueous pH and are soluble. However, reducing the pH to 2 induces the aggregation of humic structures and precipitation. Presence of a model peptide in the system effectively encapsulates the peptide with the precipitated humic acid (Zang et al., 2000). We hypothesize that proteinaceous materials can be protected in these aggregated humic structures by forming covalent or non-covalent linkages or are trapped due to a physical adsorption interaction (shown as step 1: preparation, Figure 4.1).

In step 2 of the diagram (Figure 4.1), those peptide molecules that are physically adsorbed on the surface of aggregated humic acid structures are removed by washings. We assume that only the peptides that form covalent bonds with humic substances will remain in the solid phase after the wash procedure. Non-covalently bonded peptides are expected to not exist after washing due to the high hydrophilicity of the peptide. We cannot rule out the fact that washing may not have removed peptides that were encapsulated.

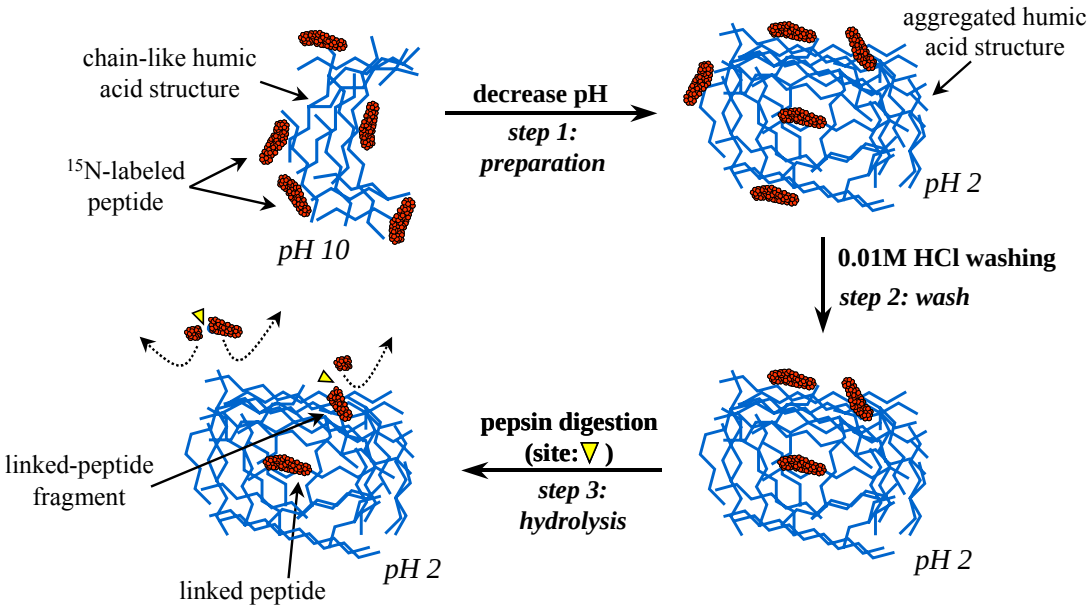


Figure 4.1: An illustrated diagram of the simulated proteinaceous material preservation experiment.

The humic acids with bonded, or encapsulated, peptides are then treated with pepsin, an enzyme that functions at pH 2 and cleaves peptides by attacking the amide

linkage between two phenylalanine residues, and the digested peptide fragments are removed after enzyme hydrolysis (step 3: hydrolysis, Figure 4.1). Peptides or peptide fragments remaining in the solid phase are expected to have covalent linkages with humic acids molecules or be encapsulated. Using nitrogen isotope ratio measurements, the quantity of the residual peptide trapped or bound to the humic acids can be determined, and the preservation efficiency of the peptide in humic substances can be estimated. Because only the glycine is labeled, we can only evaluate fragment residues that are bound to humic acids in this portion of the peptide.

A solution containing 0.1 mg ^{15}N-labeled peptide (R<u>G</u>FFYTPKA, 100 μL of peptide stock solution) was mixed with 2 mg humic acids (400 μL of humic acids stock solution) and made to a volume of 600 μL by addition of H_2O. The peptide and humic acid solutions were adjusted to pH 8 by dropwise addition of 0.1 M NaOH or 0.1M HCl. The mixtures were then vortexed (VWR MV-1 mini vortexer, VWR, PA) at room temperature for at least 48 h. Concentrated HCl was used to adjust the solution pH to 2 to precipitate the peptide and humic acid mixture. After centrifugation and decantation of the supernatant, the peptide/humic acid precipitate was washed with 0.01 M HCl (pH 2) three times. The washed precipitates were then re-suspended in 0.01 M HCl solution (pH 2). A 10 μL aliquot of a solution, containing pepsin that is tethered to agarose beads (Sigma-Aldrich, St. Louis, MO), was added to each sample to enzymatically attack the peptide/humic acid adduct. However, we used this pepsin-agarose bead system in order to limit the autodigestion of pepsin and minimize the interaction between enzyme molecules and humic substances. The solutions were vortexed at 37^{o}C for 2 h. Normally, pepsin reacts with peptides in a matter of minutes. We extended the reaction time to 2 h

to allow for complete digestion of the small peptide and to counteract the possible deactivation of the pepsin by the humic acids. Samples were then centrifuged at carefully controlled speeds to selectively spin-down suspended particles composed of residual humic material. The pellet was washed with 0.01 M HCl solution three times to further aid in the removal of pepsin-agarose and digested peptide fragments. The washings were combined and both these and the residual pellet were subsequently freeze-dried and transferred to tin capsules for nitrogen isotope analysis.

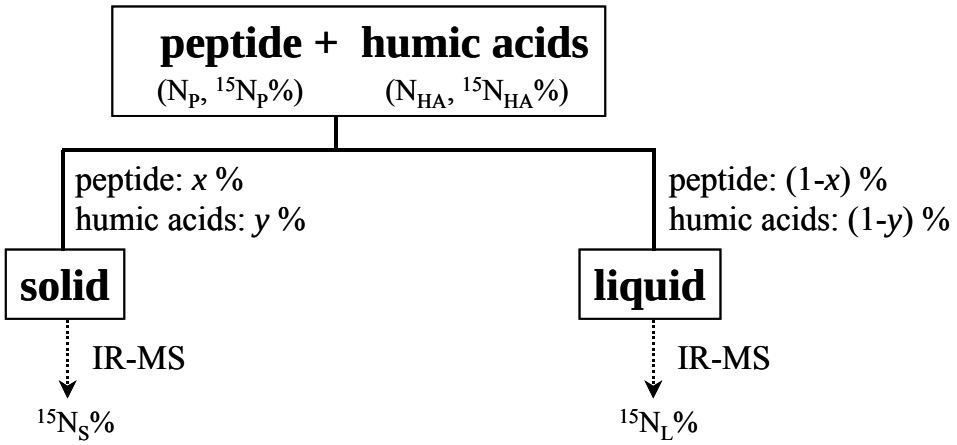
4.2.3 Nitrogen isotopic analysis

The atom percent ^{15}N was measured on an isotope ratio mass spectrometer (ESD 100, InProcess Instruments, Gesellschaft fur Prozessanalytik, Germany) operated in the direct inlet continuous flow mode after combustion of the samples in an elemental analyzer (Flash EA 1112, Thermo Finnigan, USA). Aspartic acid was utilized as the external standard to calibrate the natural abundance atom percent of ^{15}N. The signal response of the isotope ratio measurement was calibrated by analyzing a certain amount of aspartic acid mixed with different amounts of ^{15}N-labeled glycine. A linear response curve was obtained and used for calibration. Calibration using a known amount of ^{15}N-labeled glycine was performed in every 10 samples to ensure the consistency of the signal response of the instrument.

Since the amount of ^{15}N-labeled peptides in each step of the simulated proteinaceous materials preservation experiment needs to be determined, a model was developed to calculate the ^{15}N content of samples. Data acquired from isotope ratio analysis is given in ^{15}N atom percent, which is defined by Eq. 4.1.

$$^{15}N_{atom}\% = \frac{(^{15}N_{sample} + ^{15}N_{std})}{(^{15}N_{sample} + ^{15}N_{std} + ^{14}N_{sample} + ^{14}N_{std})} \cdot 100\% \qquad \text{(Eq. 4.1)}$$

Figure 4.2 shows a model for the separation scheme employed for determining the ^{15}N content of samples in each step in the simulated peptide preservation experiment.



N_P and N_{HA}: total nitrogen content of peptide and humic acids
$^{15}N_P\%$ and $^{15}N_{HA}\%$: the atom percentages of ^{15}N of peptide and humic acids
$^{15}N_S\%$ and $^{15}N_L\%$: the atom percentages of ^{15}N of solid and liquid phase.

Figure 4.2: The separation scheme for determining ^{15}N content in samples.

In the model, no matter how peptide and humic acid mixtures are treated (e.g., washing or hydrolysis), there is a centrifugation process to separate liquid and solid phase, each containing different proportions of peptide and humic acid molecules. The total

nitrogen contents in ^{15}N-labeled peptide and in the unlabeled humic acids are defined as N_P and N_H respectively before they are separated. In addition, the atom percentages of ^{15}N in ^{15}N-labeled peptide and humic acids are described as $^{15}N_P$% and $^{15}N_H$% before the separation. Following each treatment of the peptide/humic acid mixture, *x*% of peptide and *y*% of humic substance are assumed to remain in the solid phase, and (1-*x*)% of peptide and (1-*y*)% of humic substance are washed out and transferred to the liquid phase. Furthermore, the atom percentages of ^{15}N in solid and liquid phase are defined as $^{15}N_S$% and $^{15}N_L$% respectively. Based on the definition of atom percent in Eq. 4.1, Eq. 4.2 and 4.3 can be derived.

$$^{15}N_S\% = \frac{(x)N_P(^{15}N_P\%) + (y)N_{HA}(^{15}N_{HA}\%)}{(x)N_P + (y)N_{HA}} \qquad \text{(Eq. 4.2)}$$

$$^{15}N_L\% = \frac{(1-x)N_P(^{15}N_P\%) + (1-y)N_{HA}(^{15}N_{HA}\%)}{(1-x)N_P + (1-y)N_{HA}} \qquad \text{(Eq. 4.3)}$$

In Eq. 4.2 and 4.3, the $^{15}N_S$% and $^{15}N_L$% are determined from the isotope ratio analysis. Only *x*% and *y*% are the two unknown variables that need to be calculated. Therefore, by solving the Eq. 4.2 and 4.3, the percentage of peptide (*x*) and humic acids (*y*) in the residual solid phase can be determined. Applying this separation model to all three steps in the simulated proteinaceous material preservation experiment, the distribution of peptide and humic acids in both solid and liquid phases of each step can be determined.

4.2.4 Solid-state ^{13}C NMR

Solid-state ^{13}C NMR experiments of humic acids were performed on a Bruker DMX-300 MHz spectrometer (Bruker Biospin Corporation, Billerica, MA) with cross polarization-magic angle spinning (CP-MAS). Approximately 100 mg dry weight per sample was placed in a 4 mm (outside diameter) NMR rotor with a Kel-F cap (3M, Minneapolis, MN). Each sample was spun at a frequency of 13 kHz. Experiments were conducted at 300 K and 8192 scans were accumulated using a contact time of 2 ms and a 1 s recycle delay time, and 2048 data points collected on the free induction decay (FID). The FID was zero-filled to 4096 data points and subjected to exponential multiplication equivalent to 50 Hz line broadening. The chemical shifts were plotted using the carboxyl signal of glycine as the external standard (176.03 ppm) and referenced to tetramethylsilane at 0 ppm.

4.2.5 Mass spectrometry

An ion-trap mass spectrometer (Bruker Esquire, Bruker Daltonics Inc., Billerica, MA) with an electrospray ionization source was utilized to obtain mass spectra. Samples were infused directly using a syringe pump at the rate of 120 μL/min. Mass spectral data were collected and processed by the DataAnalysis program (Bruker Daltonics Inc., Billerica, MA).

4.3 Results and Discussion

4.3.1 Chemical properties of the humic acids

Figure 4.3 shows stacked plots of solid-state ^{13}C NMR spectra for the two humic acids, Mt. Rainier humic acids and Mangrove Lake humic acids. This data was previously shown and discussed and is briefly discussed here (Chapter 3). From the solid-state ^{13}C NMR spectra, the basic chemical structures of humic acids can be examined. Based on the solid-state ^{13}C NMR results, Mt. Rainier humic acid is selected due to the high aromatic content (peaks between 100 and 160 ppm and primarily derived from

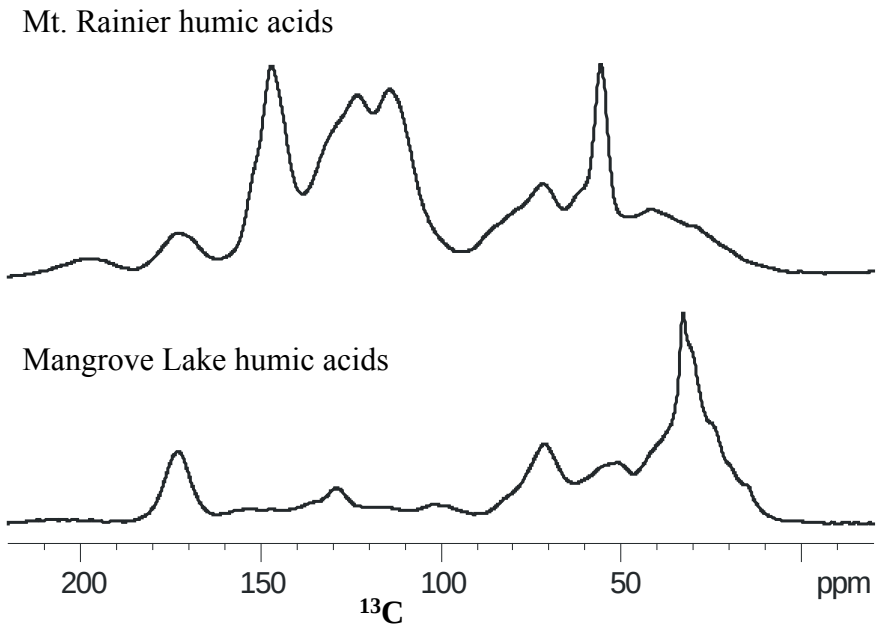


Figure 4.3: Solid-state ^{13}C NMR spectrum of Mt. Rainier humic acids and Mangrove Lake humic acids.

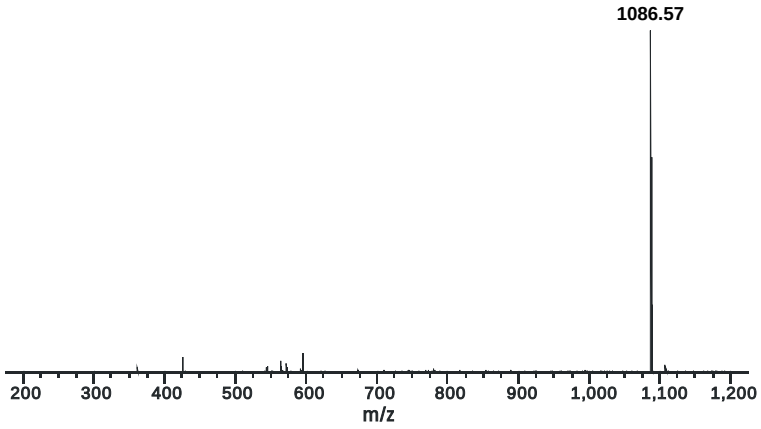
lignin degradation) and, on the other hand, Mangrove Lake humic acid is chosen to represent humic substances with more aliphatic properties. Therefore, Mt. Rainier and Mangrove Lake humic acids, which have vastly different chemical structures, are used in this report to study the functional group influence of the interaction between the model peptide and humic substances.

4.3.2 Reaction of pure Peptide RGFFYTPKA with pepsin

To investigate if the peptide RGFFYTPKA can be recognized by pepsin-agarose and completely digested within 2 h, a control experiment was performed, which uses non-labeled peptide in the pepsin-agarose digestion reaction at 37^{o}C for 2 h. Mass spectrometry was utilized to monitor the enzymatic reaction. Figures 4.4(a) and 4.4(b) show the mass spectra of peptide RGFFYTPKA before and after pepsin digestion. In Figure 4.4(a), the signal at 1086.57 (m/z) is the molecular ion (H^+ adduct) of peptide RGFFYTPKA. Two major signals displayed at 379.08 and 726.23 (m/z) in Figure 4.4(b) represent the peptide fragments RGF and FYTPKA, respectively, demonstrating that the pepsin-agarose digestion was effective as it attacked the site on peptide RGFFYTPKA between two phenylalanines, the 3rd and the 4th amino acids in the sequence. A small peak at 526.12 (m/z), representing the RGFF fragment, is a minor product of pepsin digestion. Pepsin-agarose can hydrolyze peptide RGFFYTPKA between the 4th Phe and the 5th Tyr residue, also. This control experiment confirms that the peptide RGFFYTPKA can be recognized and completely digested by pepsin-agarose within 2 h. Therefore, the ^{15}N-labeled peptide with the same sequence can be used in the simulated peptide

preservation reaction to examine the quantity of peptide being protected by humic substances.

(a)



(b)

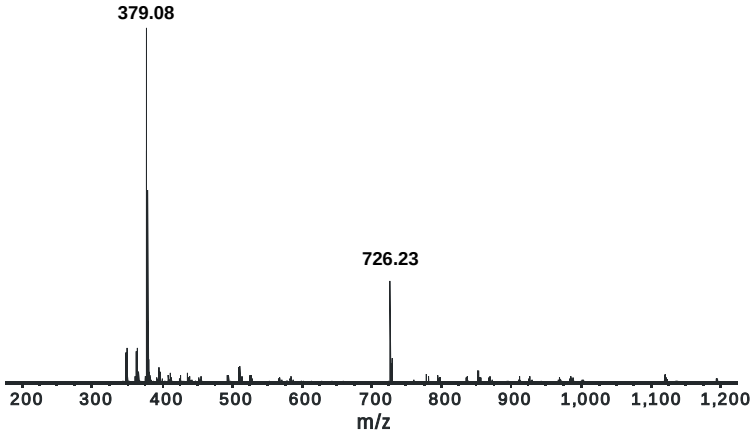


Figure 4.4: Mass spectrum of peptide RGFFYTPKA (a) before, and (b) after the pepsin-agarose digestion.

4.3.3 Isotope ratio results

Since peptide with sequence RGFFYTPKA can be appropriately digested by pepsin-agarose, we subjected the ^{15}N-labeled peptide to the simulated peptide preservation by first binding it to the respective humic acid and then treating the washed residue from this mixture with pepsin-agarose. From step 1, preparation, to step 3, hydrolysis, all liquid and solid phase samples are freeze-dried and subjected to nitrogen isotope analysis. The residual percent of ^{15}N-labeled peptide is calculated serially for each step in the separation scheme and presented in Table 4.1. The total recovery is based on the original amount of ^{15}N being set to 100 %.

	Step 1 preparation	Step 2 wash	Step 3 hydrolysis	total recovery
Mt. Rainier Humic Acids	(85 ± 6) %	(47 ± 24) %	(28 ± 13) %	(11 ± 8) %
Mangrove Lake Humic Acids	(71 ± 2) %	(35 ± 11) %	(18 ± 7) %	(5 ± 2) %

Table 4.1: The remaining percentage of ^{15}N-labeled peptide in the simulated peptide preservation experiment.

In the first preparation step, about 85 % of ^{15}N-labeled peptides are incorporated into the Mt. Rainier humic acid and 71 % into Mangrove Lake humic acid. These results

indicate that the ^{15}N-labeled 9 amino acid peptide is co-precipitated with humic acid molecules when the solution is acidified to pH 2, which results in the aggregation of humic acid molecules. These co-precipitated ^{15}N-labeled peptides are either physically adsorbed or chemically bonded to the humic acid molecules during the conformational change of humic acids molecules induced by the decrease of pH. In the second step where extensive washing of the precipitate is made, 47 % of co-precipitated ^{15}N-labeled peptides remained in the Mt. Rainier humic acids aggregates and 35 % in Mangrove Lake humic acids. The purpose of wash step is to remove those physically adsorbed or trapped ^{15}N-labeled peptides. We, therefore, conclude that the remaining labeled peptides are mainly chemically bonded to the humic acids molecules or strongly physically encapsulated.

In the last hydrolysis step, pepsin-agarose enzyme is added to digest the ^{15}N-labeled peptides that are chemically or physically bonded with humic acid molecules but still available for hydrolysis. The residue represents labeled peptides associated with the humic acids which cannot be hydrolyzed further. According to the results listed in Table 4.1, about 28 % and 18 % of ^{15}N-labeled peptides are found in Mt. Rainier and Mangrove Lake humic acid precipitates, respectively, after the pepsin digestion. Because the pepsin digestion site on the ^{15}N-labeled peptide R<u>G</u>FFTYPKA is mainly between two phenylalanine residues, the isotope ratio analysis results for the remaining ^{15}N-labeled peptides could be from either the whole peptide sequence or the residual fragment R<u>G</u>F. The amount of residual fragment FTYPKA that could possibly be bound to the humic acids cannot be analyzed because there is no ^{15}N-labeled amino acid in this part of sequence. If the digested peptide fragment R<u>G</u>F is detected, it suggests that the fragment

RGF is incorporated with humic structures via formation of a covalent bond or remains encapsulated, though less likely due to the small fragment size. Thus, detection of the label after hydrolysis suggests that the interaction between peptide RGFFTYPKA and the humic substance is through chemical bonding between RGF part of the peptide and the functional groups in the humic substance. The other detected ^{15}N signal in isotope analysis is possibly from undigested whole peptide, RGFFYTPKA, indicating that the enzyme pepsin-agarose does not recognize and hydrolyze it.

Because the ^{15}N-labeled peptides that are physically adsorbed to humic acid molecules have been removed during the washing, the isotope analysis suggests the presence of covalent bonding between the peptide and humic substances. The covalent bonding between peptides and humic substances may either block the pepsin digestion site or affect the peptide conformation to prevent the pepsin digestion. Thus, the ^{15}N signal from the undigested whole peptide can be detected after the enzymatic hydrolysis step.

These isotope analyses provide not only evidence for the presence of covalent bonding interactions between ^{15}N-labeled peptides and humic acid molecules, but also the determinations of the amount of ^{15}N-labeled peptides that have been protected from enzyme hydrolysis in humic substances. When the residual percentages at each step in the simulated peptide preservation experiment are multiplied together, the quantities of total recovery of labeled peptide can be determined. The total recovery data shown in Table 4.1 of undigested ^{15}N-labeled peptide indicate that about 5 % to 11 % of the peptide can survive enzyme degradation, due most apparently to the formation of covalent bonds with humic acid molecules, as shown previously (Chapter 2 and 3). Note

that the percentage of peptides remaining after hydrolysis is greater with Mt. Rainier humic acids than with Mangrove Lake humic acids. This suggests that the aromatic components in Mt. Rainier humic acids play an important role in the covalent bonding between the peptide and humic acids.

4.4 Conclusions

In this study, we designed an experiment to evaluate the preservation of proteinaceous materials that covalently bond with organic substances commonly encountered in sedimentary systems. Our previous studies had suggested that peptides can interact with certain humic acids by formation of covalent bonds, we designed an experiment to test if this effect could protect the peptide from enzymatic attack. The simulation, involving use of a nitrogen-labeled peptide bound to humic acids followed by enzymatic digestion and nitrogen isotope measurements, shows conclusively that the labeled peptide bound to humic acids survives enzyme hydrolysis, a primary mechanism involved in its degradation in the environment.

Based on the amounts of recovered ^{15}N in hydrolysis residues, we calculate that 5 to 11 % of the peptide, that is bound by both covalent and non-covalent bonds, survives. Simple mixing of labeled peptide with humic acids at room temperature, followed by precipitation co-associates approximately 71 – 85 % of the peptide with humic acids. Moreover, of these associated peptides, we propose that about 40 % are incorporated into humic acids via covalent bonds or very strong non-covalent bonds associated with some sort of encapsulation process, because they are not removed from the humic acids by washing. Peptides removed via multiple washings in the simulated preservation

experiment are mainly physically sorbed. When the peptides that are chemically bonded to humic acids are hydrolyzed by the pepsin-agarose enzyme system, 18 % to 28 % remain in the humic acids. The presence of these undigested peptides, labeled with ^{15}N-glycine, indicates that the binding interactions either block the enzyme accessibility to the peptide or modify the peptide conformation to retard or terminate enzyme degradation. Another possibility is that the humic acids modified the pepsin-agarose activity, although we extended the reaction time with agarose to account for this effect. Studies presented elsewhere (Chapter 5) show that this process occurs but is minor.

When comparing the results obtained from two different humic acids used in the simulated preservation experiment, we observe that the aromatic humic acid provides better enzyme attack protection for the peptide, more than twice the preservation efficiency (11 % vs 5 %). This conclusion is consistent with our previous findings showing that aromatic humic acids more effectively bind aromatic peptides by a covalent bonding mechanism than do aliphatic humic acids (Chapter 3). The peptide used in the current study is predominantly classed as an aromatic peptide and we, thus, expect it to bind more effectively to aromatic-rich humic acids.

Our conclusion that binding of small peptides to humic substances reduces their susceptibility for degradation has some important consequences for nitrogen cycling in the environment. One can expect that environmental processes of degradation will reduce proteins in living systems to smaller and smaller peptides as degradation proceeds. As these peptides encounter humic substances in soils and sediments, they will become incorporated through covalent and non-covalent mechanisms much like those that are active in our simulation experiment. As they do, the hydrolytic enzyme systems of the

soil/sediment medium will be unable to access the peptides so associated, thus, offering further protection to degradation. Perhaps, eventually the peptides will succumb to enzymatic hydrolysis are the humic acid network itself undergoes degradation. However, we expect this to be a long-term process and as the fate of the peptide rests primarily on the rate of humic acid degradation. We also can expect that the aging process might contribute to further stabilization of the peptide. Continued binding of peptide subunits at multiple sites to humic acids can occur well beyond the timeframe for the initial contact made in this study, reinforcing the extend to which the peptide becomes preserved.

As a final comment, we point out that covalent bonding of peptides does not lead to major structural rearrangement of the peptide units, as has commonly been supposed by those who suggest rearrangements to form heterocyclic nitrogen (Schulten and Schnitzer, 1998). We observe that the peptide bond is preserved when reacting with humic acids, suggesting a reaction perhaps analogous to the Michael reaction where adduction to reactive quinonoid structures in humic acids can readily occur. This is completely consistent with observations that stable nitrogenous materials in humic acids show mainly amide signals in ^{15}N NMR spectra (Knicker and Hatcer, 1997). The results are also consistent with the fact that these NMR signals survive strong acid hydrolysis (Zang et al., 2000). They do so because the covalent linkage to humic acids provides a degree of protection.

CHAPTER 5

PROTEIN PRESERVATION IN HUMIC ACIDS STUDIED BY GEL ELECTROPHORESIS

Abstract

The interactions between cytochrome c and three different humic acids, Everglades peat humic acid, Mt. Rainier humic acid, and Mangrove Lake humic acid, have been examined using gel electrophoresis coupled with silver staining and immunoblotting detection methods. In an enzymatic digestion experiment, cytochrome c was completely degraded by trypsin-agarose in the absence of humic acid molecules. However, in the same enzymatic digestion reactions, a portion of cytochrome c is detected in the presence of humic acid. The decrease in enzyme reaction efficiency may be due to conformational changes of cytochrome c by the formation of covalent interactions with humic acid molecules. In a time-dependent enzyme reaction experiment, lower reaction rates are observed in the cytochrome c and humic acid mixtures indicating that the cytochrome c is structurally modified or changed by humic substances. In addition, the influence of the different functional groups in humic acid on enzyme degradation was also studied. Under the same enzyme reaction conditions, a higher amount of the undigested cytochrome c is found when it is mixed with the humic acids containing mainly aromatic structures. This finding suggests that the aromatic functional

groups in humic acids may be the key components for interacting and preserving proteins in the environment. These results provide the first evidence that utilizes gel electrophoretic separation to analyze and identify the presence of covalent bonding between proteins and humic acids. The simulated enzyme reaction experiment may also provide a model to better understand protein degradation mechanisms in the environment.

5.1 Introduction

Proteins are generally believed to be quickly mineralized during early diagenesis in environmental systems because they are known to be highly labile to microbial degradation and chemical hydrolysis. However, evidence in recent research demonstrated that some fraction of proteinaceous material is preserved in marine environments (Tanoue, 1995; Tanoue et al., 1996), sediments (Nguyen and Harvey, 1997; Nguyen and Harvey, 1998; Pantoja and Lee, 1999; Knicker and Hatcher, 2001), and soils (Stevenson, 1994; Schulten and Schnitzer, 1998). Several preservation mechanisms involving protection of proteinaceous materials within mineral components of soils and sediments have been proposed (Mayer, 1995; Mayer, 1994; Salmon et al., 1998), and involve the concept that protection is derived from the physical exclusion of hydrolyzing enzymes. Besides the concept of physical protection, the formation of chemical bonds with natural organic matter (e.g., humic materials) is also thought to be involved in preserving proteinaceous molecules (Nagata et al., 1998; Tanoue et al., 1995).

Some studies provided evidence for the hypothesis that proteins are preserved by either physical or chemical protection. Nguyen and Harvey (1998; 2001) observed that proteinaceous materials appeared to be stabilized towards degradation when associated

with a high molecular weight fraction of organic-rich sediments. Schulten and Schnitzer (1998) suggested that a portion of the proteinaceous material in soil was trapped in the three-dimensional structure of humic acids, but a fraction of these compounds were either physically or chemically preserved on the surface of humic acids. Zang et al. (2000) observed that ^{15}N-labeled proteins extracted from fresh algae can survive after 6 M HCl hydrolysis likely due to physical encapsulation within humic acid structures. Knicker and Hatcher (1997) and Knicker et al. (1996) suggested that proteinaceous materials survive in an organic-rich sedimentary system by trapping in the mesopores. Keil and Kirchman (1993) found that the condensation reaction between hydrolyzed protein units, amino acids, and sugars, formed a refractory protein which was less degradable by bacteria and appeared to be resistant to natural degradation.

In addition to this evidence that proteins were preserved by either physical or chemical mechanisms, other spectroscopic data were also obtained to demonstrate the presence of amide structures, which is the basic structural unit for constructing protein molecules in humic substances, using both solid-state ^{15}N NMR (Knicker et al., 1993; Knicker et al., 1996; Knicker and Hatcher, 1997; Knicker et al., 2000; Knicker and Hatcher, 2001) and X-ray photoelectron spectroscopy (XPS) (Patience et al., 1992; Yuan, et al. 1998; Zubritsky, 2001; Abe, 2004). The existence of amide groups within the humic fraction implies the covalent binding of peptides to the humic material. However, there is, as yet, no evidence to confirm covalent interactions between protein molecules and dissolved organic matter. To elucidate the interactions between proteins and dissolved organic matter, the protein cytochrome c was mixed with humic materials, and the

reaction products are separated using gel electrophoresis. Both silver staining and immunoblotting are used for detection.

Applying polyacrylamide gel electrophoresis (PAGE) to the separation of humic substances was first reported by Stepanov and Pakhonov (1969). The presence of sodium dodecyl sulfate (SDS) in the gel electrolyte buffer disaggregates humic substances, thus increasing the number of separated humic substance bands (Klocking, 1973). Non-covalent interactions between proteins and humic substances can be destroyed by the addition of SDS, since SDS molecules can bind strongly to the proteins to form SDS-protein complexes (Gersten, 1996). Therefore, SDS-PAGE can be used to distinguish between protein molecules chemically bonded to humic substances and proteins associated with, but not bound to, humic materials.

To observe proteins that have been separated electrophoretically on a gel, a method is needed to visualize the proteins. Two detection techniques are used in this research, silver staining (Gottlieb and Chavko, 1987; Gersten, 1996) and immunoblotting (Harlow and Lane, 1988; Bollag, et al. 1996) (also called Western blotting). Silver staining is a fast, inexpensive, and sensitive tool for detection protein bands on the gel. However, silver staining is non-specific, and may stain other molecules besides proteins. Immunoblotting provides highly specific detection results with very high sensitivity, but requires expensive materials (specific antibodies) and much longer experiment times. The immunoblotting method uses an antibody to specifically recognize and bind proteins of interest, followed by a secondary antibody that produces a fluorescent signal. Therefore, the immunoblotting provides a clearer signal with higher sensitivity and much lower background signal than the conventional silver stain method.

5.2 Materials and Methods

5.2.1 Preparation of humic acids

Three humic acids with different chemical properties were used in this study: 1) Everglades peat humic acids, 2) Mt. Rainier humic acids, and 3) Mangrove Lake humic acids. Everglades peat humic acid was from a peat collected in The Everglades National Park, near Alligator Alley, west of Hialeah, Florida (Hatcher et al., 1986). Mt. Rainier humic acid were extracted from a wood sample that had undergone degradation by brown rot fungi, and was collected on the slopes of Mt. Rainier, Washington (Hatcher, 1987). Mangrove Lake humic acid was extracted from a lacustrine sediment sample, which was collected in Mangrove Lake, Bermuda, at a depth of 370 to 390 cm (Hatcher et al., 1983). All humic acid samples were prepared by the standard procedure involving extraction with 0.5 M NaOH, removal of sodium by cation exchange resin, and precipitation by acidification of the solution to pH 2. The humic acid samples were then washed several times with dilute HCl and freeze-dried. Weighed humic acid samples were redissolved in water adjusted to pH 10 by the dropwise addition of 0.5 M NaOH. The final concentrations of humic acid solutions were 5 mg/mL and the pH of the solutions were adjusted to pH 8 by dropwise addition of 0.1 M HCl.

5.2.2 Solid-state ^{13}C NMR

Solid-state ^{13}C NMR spectra of humic acids were obtained on a Bruker DMX-300 MHz spectrometer (Bruker Biospin Corporation, Billerica, MA) with cross polarization-magic angle spinning (CP-MAS). Approximately 100 mg dry weight of each sample was

placed in a 4 mm (outside diameter) NMR rotor with a Kel-F cap (3M, Minneapolis, MN). Each sample was spun at a frequency of 13 kHz. Experiments were conducted at 300 K and 8192 scans were accumulated using a contact time of 2 ms and a 1 s recycle delay time, and 2048 data points collected for the free induction decay (FID). The FID was zero-filled to 4096 data points and subjected to exponential multiplication equivalent to 50 Hz line broadening. The chemical shifts were plotted using the carboxyl signal of glycine as the external standard (176.03 ppm) and referenced to tetramethylsilane at 0 ppm.

5.2.3 Trypsin digestion of cytochrome c/humic acid mixtures

Mt. Rainier humic acid, Everglades peat humic acid and Mangrove Lake humic acid were dissolved in NaOH solution (5 mg/mL) and adjusted to pH 8 before use. Cytochrome c (1 mg, purchased from Sigma-Aldrich, St. Louis, MO) was vortexed with the three humic acids (400 μL) for 48 h at room temperature. Cytochrome c was then digested by addition of 10 μL trypsin-agarose (17.3 U/mL, Sigma-Aldrich, St. Louis, MO) for 18 h at 37 ^{o}C. When the trypsin digestion was complete, the sample was prepared for electrophoresis by mixing one part of the sample solution with two parts sample loading buffer (200 mM Tris-HCl, pH 6.8, 2 % sodium dodecyl sulfate (SDS), 40 % Glycerol, 0.04 % coomassie blue G-250, 2 % β-mercaptoethanol).

5.2.4 Gel electrophoresis with silver staining

For silver staining, the cytochrome c and humic acid reaction mixtures were electrophoretically separated on a 16.5 % Ready Gel Tris-Tricine gel (Bio-Rad, Hercules,

CA), using a Ready Gel Precast System (Bio-Rad) and the sample running buffer (100 mM Tris-base, pH 8.3, 100 mM tricine, 0.1 % SDS). Samples were separated by running for 90 minutes at 100 V, in constant voltage mode. The separated protein bands were visualized using the Silver Stain Plus Kit (Bio-Rad) and optical density of the bands were measured and integrated by the Gel-Pro Analyzer (version 4.0, Media Cybernetics, San Diego, CA). Analyses were conducted in triplicate for each set of samples.

5.2.5 Gel electrophoresis with immunoblotting

For immunoblotting, samples were taken after the trypsin digestion and separated on a 15 % SDS-PAGE gel, using an SDS running buffer (25 mM Tris-base, pH 8.3, 192 mM glycine, 0.1 % SDS). Samples were separated by running for 90 minutes at 90 V. Protein bands were then transferred onto nitrocellulose membranes (Millipore, Billerica, MA) using a Trans-Blot semi-dry transfer cell (Bio-Rad). To prevent non-specific binding of the antibody to the membrane, the trans-blotted membrane was washed twice with Tris-buffered saline containing 0.1 % Tween 20 (TBST) and then blocked for 40 min with 5 % nonfat milk in TBST. The membrane was incubated at 4 °C overnight with the primary antibody, mouse anti-cytochrome c monoclonal antibody (BD Biosciences Pharmingen, San Jose, CA), which was diluted 1:1000 in TBST with 1 % nonfat milk. After treatment with the primary antibody, the membrane was washed three times with TBST for 15 min. Following the wash step, a solution of the secondary antibody, anti-mouse IgG-horseradish peroxidase (IgG-HRP) conjugate (diluted 1:4000, purchased from Jackson Labs, Bar Harbor, ME), was added and incubated for 1 h at room temperature. The excess secondary antibody was then removed by three successive 1 hour washes

with TBST. The immunoblotted membranes were visualized by enhanced chemiluminescence using the ECL Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ). Briefly, this ECL kit contains the substrate and reagent for the HRP enzyme. The product of this enzymatic reaction emits light, which is detected by exposing the membrane to x-ray film (Amersham Biosciences). The optical density of the resulting bands was measured and integrated by the Gel-Pro Analyzer. Triplicate gel analyses were obtained.

5.2.6 Time-dependent trypsin digestion experiments

Cytochrome c (0.1 mg) was vortexed with Everglades peat humic acid (2 mg) for 48 h at room temperature. Samples were then digested by adding 10 μL trypsin-agarose at 37 ^{o}C. During the digestion reaction, a 30 μL aliquot of the cytochrome c/Everglades peat humic acids mixture was removed at 0, 20, 40, 60, 90, 120, and 180 mins. To stop the trypsin reaction, the aliquot was mixed with 60 μL of the sample loading buffer, followed by heating at 95 ^{o}C for 5 min. The cytochrome c and Everglades peat humic acid mixtures were then separated and detected by the tricine SDS-PAGE with silver staining, and by SDS-PAGE gels with immunoblotting. The silver-stained gels and the immunoblotted x-ray films were then analyzed by the Gel-Pro Analyzer.

5.3 Results and Discussion

5.3.1 Solid-state ^{13}C NMR

Solid-state ^{13}C NMR was used to demonstrate the chemical properties of the three humic acids used in this study. Figure 5.1 shows the stacked plots of solid-state ^{13}C NMR spectra of Everglades peat humic acid, Mt. Rainier humic acid, and Mangrove Lake humic acid. This data was previously shown and discussed in detail (Chapter 3). These three humic acids were selected because they are representative of different types of humic acids. Based on the solid-state ^{13}C NMR results, it can be seen that Mt. Rainier humic acid has a high aromatic content (peaks between 100 and 160 ppm and primarily derived from lignin degradation). In contrast, Mangrove Lake humic acid contains primarily aliphatic components. Therefore, Mt. Rainier and Mangrove Lake humic acids, which have vastly different chemical structures, were chosen to study the functional group influence on the peptide-humic acid interaction. The third humic acid, Everglades peat humic acid, was selected because it represents a more typical humic acid with both aromatic and aliphatic chemical properties.

5.3.2 Gel electrophoresis with silver staining

Tricine SDS-PAGE was used to separate the trypsin-digests of the mixtures of cytochrome c with the three humic acids, and the gel image is shown in Figure 5.2. These 14 samples include cytochrome c with and without humic acids, and also the mixtures treated with and without the enzyme, trypsin-agarose. The detailed composition of samples loaded in each lane is listed in Table 5.1.

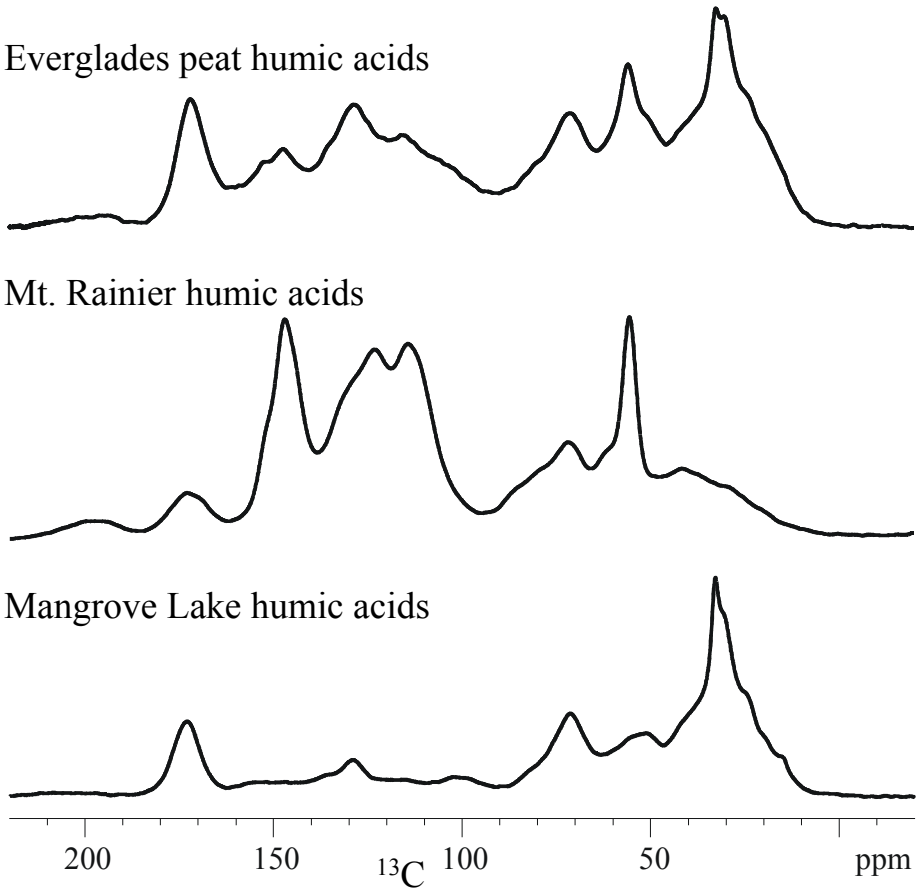


Figure 5.1: Solid-state ^{13}C NMR spectra of Everglades peat humic acids, Mt. Rainier humic acids, and Mangrove Lake humic acids.

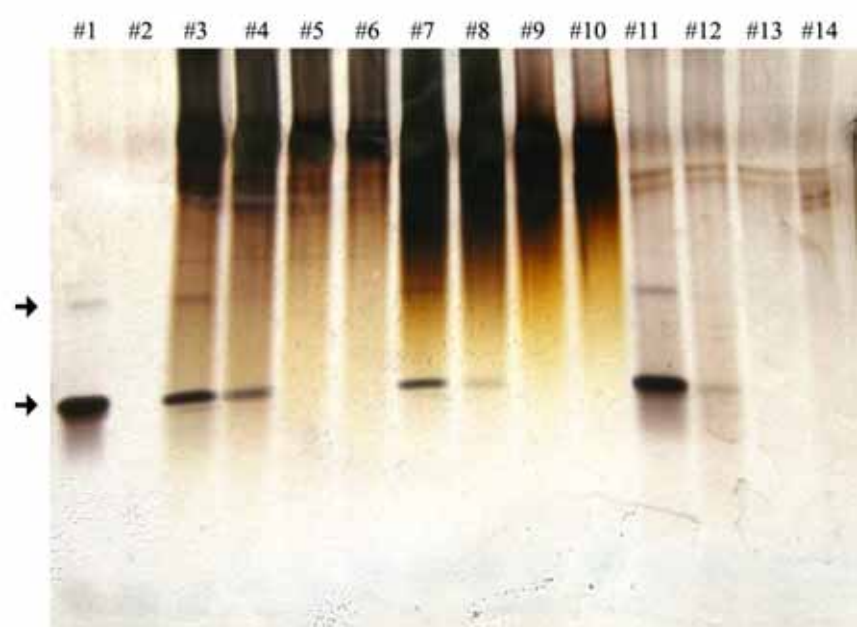


Figure 5.2: Cytochrome c mixed with three different humic acids separated on a tricine SDS-PAGE gel with the silver staining method. The sample composition of each lane is listed in Table 5.1. Upper and lower arrows indicate the position of the cytochrome c dimer and monomer bands on the gel.

Lane #	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cytochrome c	+	+	+	+			+	+			+	+		
Humic acid			E	E	E	E	R	R	R	R	M	M	M	M
Trypsin-agarose		+		+		+		+		+		+		+

Table 5.1: Sample compositions and loading sequence for cytochrome c with different humic acid mixtures with and without trypsin-agarose digestion.

Note: + represents that the component is contained in the sample. Letter codes for humic acids: E: Everglades peat humic acids; R: Mt. Rainier humic acids; M: Mangrove Lake humic acids.

The most significant finding from this experiment was that the presence of humic acid prevented the complete digestion of cytochrome c by trypsin. When comparing the cytochrome c monomer bands in lanes #1 and #2 in Figure 5.2, cytochrome c without humic acids is completely digested by trypsin-agarose within 18 h. However, for those samples of cytochrome c with humic acids (lanes #4, #8, and #12 in Figure 5.2), the cytochrome c bands observed after the trypsin reaction indicate that the trypsin digestion of cytochrome c is retarded in the presence of humic acid molecules. According to our 2D NMR results (Chapter 2 and 3), both covalent and non-covalent interactions between peptides and humic acids have been identified. In addition, covalent linkages were observed between amide groups of peptides and quinone-like structures in humic acids

(Chapter 2); and we therefore postulate that the reduction of trypsin digestion may be due to chemical linkages of cytochrome c to humic acid molecules. Covalent binding could produce structural or conformational changes in cytochrome c, which could influence the trypsin reaction. Alternatively, the decrease of the trypsin reaction could be a result of non-covalent binding of cytochrome c to humic acid molecules, which could also alter the conformation of cytochrome c. Thus, both covalent and non-covalent binding interactions could provide modification of the cytochrome c molecules, and be responsible for the observed decrease in enzyme efficiency.

The cytochrome c signals detected on the tricine SDS-PAGE gel (lower arrow, Figure 5.2) could be either the protein monomers or the covalently-bound humic acid/protein complex. The non-covalently bound cytochrome c may not be observed in Figure 5.2, since SDS in the sample-loading buffer should destroy non-covalent interactions. The molecular weight of cytochrome c (12.1 kDa) is much larger than that expected from most humic acid molecules. Even though there is little consensus on the molecular weight distribution of humic acids, studies in our group have shown, by electrospray ionization mass spectrometry, that humic acids from soils contain a significant proportion of molecules having molecular weights less than 1000 Da (Kujawinski et al., 2002; Kramer et al., 2001). The covalent interaction of cytochrome c with humic acid should produce only a small change in the molecular weight. This modification should result in either the tailing or blurring of the protein band. The cytochrome c bands on the gel appear sharp, however, with no tailing or blurring, which could indicate that these bands represent only free cytochrome c. However, the resolving power of the tricine SDS-PAGE gel may be insufficient to show such tailing, resulting in

the sharp bands observed in Figure 5.2. Additional experiments are required to determine if the observed band represents free or covalently-bound cytochrome c.

Klocking (1973) suggested that the presence of SDS was able to disaggregate humic material, resulting in clear humic bands in PAGE. However, in our experiments, no separation of the humic material was observed. The undifferentiated high optical density (OD) on the upper parts of the gel falls in the region of the stacking gel, which has no separating capability, indicating that the humic components have not been separated. One possible reason that humic acid molecules cannot be separated by the tricine SDS-PAGE gel electrophoresis may be due to high cross-linking of the tricine gel, preventing the humic material from leaving the stacking gel region. SDS is usually added to denature proteins by wrapping the hydrophobic tail around the polypeptide backbone and forming negatively charged SDS-protein complexes for electrophoresis. However, SDS may not have been able to interact with the humic acid components to form charged aggregates since there are negative charges on the humic molecules when they are dissolved in basic solution.

The other finding observed in Figure 5.2 is that the samples containing Mt. Rainier humic acid (lanes #7 to #10) has higher OD in the stacking gel area of the tricine SDS-PAGE gel than the other two humic acids. It is possible that the variation of the amount of silver staining may be due to the structural difference of the three humic acids. As shown in the solid-state ^{13}C NMR results, Mt. Rainier humic acid appears to contain more aromatic character than Everglades peat humic acid, and Mangrove Lake humic acid has the least. The measured OD values of each lane in the tricine SDS-PAGE gel (Figure 5.2) follow the same trend: Mt. Rainier is the highest, followed by Everglades

peat, then Mangrove Lake. Thus, based on our findings, the aromatic components in humic acids may have a higher tendency to bind with silver ions than aliphatic components, resulting in the observation of higher OD bands for the more aromatic humic acids.

sample compositions	cytochrome c	cytochrome c/ Everglades peat HA	cytochrome c/ Mt. Rainier HA	cytochrome c/ Mangrove Lake HA
after trypsin digestion	(0.4 ± 0.5) %	(38.7 ± 6.9) %	(28.3 ± 6.5) %	(9.3 ± 4.8) %

Table 5.2: The percentages of undigested cytochrome c in the presence of three different humic acids after trypsin-agarose digestion followed by tricine SDS-PAGE separation coupled with silver staining detection. The error shown is one standard deviation from triplicate analyses.

When the tricine SDS-PAGE gel image was analyzed by the Gel-Pro Analyzer, the integration of the optical density (IOD) of each protein band was determined. The IOD of each band is theoretically proportional to the protein concentration. In Figure 5.2, the percentages of cytochrome c that remained after reaction with trypsin could be determined by comparing the IOD values of the cytochrome c monomer bands (indicated by the lower arrow, Figure 5.2). For example, in the sample with Everglades peat humic

acid in Figure 5.2, lane #3 represents the amount of cytochrome c in the absence of trypsin, and lane #4 represents the amount remaining after trypsin digestion. The calculated percentages of undigested cytochrome c with different humic acids are listed in Table 5.2. The percentage of undigested cytochrome c is close to 0 in the control experiment (lanes #1 and #2), which contains cytochrome c without any humic acids, and the enzyme digestion is almost complete after 18 h. The presence of humic acid molecules in the cytochrome c samples appears to have reduced the enzymatic digestion, and resulted in the higher percentage of undigested cytochrome c. From Table 5.2, one finding is that, in the protein/humic acid mixtures, the percentages of the undigested cytochrome c with Everglades peat humic acid (38.7 %) and Mt. Rainier humic acid (28.3%) are higher than with Mangrove Lake humic acid (9.3 %). Thus, the two humic acid samples with the higher aromatic character showed lower degree of enzymatic digestion than the primarily aliphatic humic acid.

Since the formation of covalent bonds between peptide molecules and quinone structures in humic acids has been demonstrated in our previous research (Chapter 2), it is possible that such an interaction accounts for the observed reduction in enzymatic digestion in the samples containing Everglades peat humic acid and Mt. Rainier humic acid. Based on solid-state ^{13}C NMR results (Figure 5.1), it was shown that Mangrove Lake humic acids contain the least amount of aromatic components. This result suggests that Mangrove Lake humic acid is the least likely to contain quinone structures. We suggest that without the formation of the chemical linkages to Mangrove Lake humic acid molecules, the conformation of the cytochrome c molecules is not changed significantly, resulting in the lower percentage of undigested cytochrome c. These results

support our hypothesis that the aromatic components in humic acids play an important role in the interaction with proteins. Moreover, according to our previous studies (Chapter 2), we believe that the covalent bonds formed via the quinone structures in humic acids are likely to be the major mechanism for preserving proteins in humic acids.

5.3.3 Gel electrophoresis with immunoblotting

Figure 5.3 is the x-ray film image of cytochrome c with different humic acids separated by SDS-PAGE and detected by the immunoblotting technique. The sample compositions and loading sequences on the SDS-PAGE gel are the same as in Figure 5.2, and are listed in Table 5.1. Since immunoblotting is a very precise and highly sensitive detection method for spotting unique proteins on gels, only the presence of cytochrome c on the gel will be observed on the x-ray film. In Figure 5.3, no signal is detected for those samples containing no cytochrome c (lanes #5, #6, #9, #10, #13, and #14). This result indicates that, as expected, there is no background interference observed from humic acids or other molecules, making the immunoblotting method a better detection technique than the silver staining method. As observed in the silver-stained gel image, the presence of humic acid resulted in incomplete enzymatic digestion of cytochrome c.

Despite the lack of signal from humic acid alone (lanes #5, #6, #9, #10, #13, and #14), lanes containing both humic acid and cytochrome c showed a slight “shadow” above the cytochrome c monomer band (especially visible in lanes #7 and #8). This shadowing effect suggests that cytochrome c is present in the higher molecular weight range of the gel, implying that the molecular weight of the protein has been modified by the addition of humic acid. Since the sample-loading buffer contains SDS, the

cytochrome c/humic acid non-covalent complexes should be dissociated before the electrophoretic separation. Because the humic acid molecules vary in size, we expect that any covalent interactions between humic acid and our protein would result in a diffuse cytochrome c signal—matching the results observed in Figure 5.3. Thus, the molecular weight modifications are likely due to covalent interactions between cytochrome c and humic acid molecules.

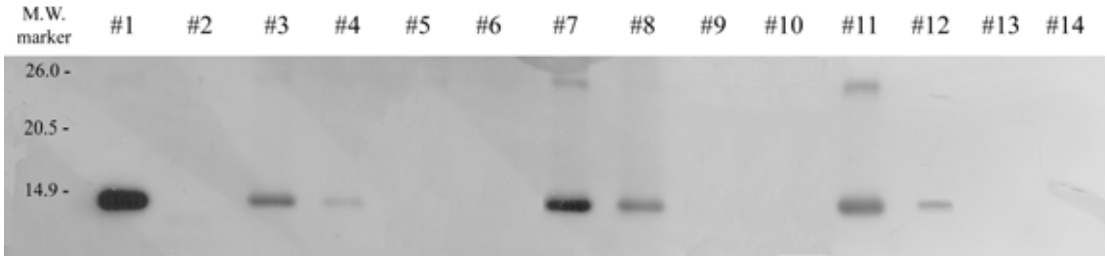


Figure 5.3: The gel image of cytochrome c mixed with different humic acids separated by SDS-PAGE coupling with the immunoblotting detection method. The sample composition of each lane is listed in Table 5.1. Upper band (around 26.0 kDa M.W. marker) and lower band (around 14.9 kDa M.W. marker) indicate the position of the cytochrome c dimer and monomer bands on the gel.

As in the silver-stained gel, the x-ray film image was analyzed to measure the OD of each protein band, and the percentages of the undigested cytochrome c in each sample were determined. Table 5.3 lists the percentages of the undigested cytochrome c with the three different humic acids. In the control experiment, cytochrome c without the addition

of any humic acid is completely digested after 18 h. However, in the presence of humic acid, there was 10.7 % to 31.6 % of cytochrome c remaining after trypsin digestion. As before, cytochrome c mixed with Mangrove Lake humic acid had the lowest percentage remaining (10.7 %), while cytochrome c mixed with the more aromatic humic acids, Everglades peat and Mt. Rainier, had more protein remaining after the enzyme reaction.

The results obtained by the two detection methods in Tables 5.2 and 5.3 show that the two humic acids with more aromatic character led to the greatest protection of the protein. However, there are minor differences in the percentages of remaining cytochrome c as determined by silver staining and immunoblotting. In Table 5.2, cytochrome c mixed with Everglades peat humic acids has the highest amount of protein remaining. In contrast, the highest percent of undigested cytochrome c in Table 5.3 is found in the sample mixed with Mt. Rainier humic acid. These differences could be explained by intrinsic limitations of the two detection methods. The results obtained by the silver staining detection method could be affected by the interference of background signals caused by the presence of humic acid molecules. Immunoblotting, in contrast, could be an under-estimated measurement since the antibody recognition reactions could be influenced by the protein conformational changes and/or blockage of the antibody recognition site by binding to humic acid molecules. However, both results shown in Tables 5.2 and 5.3 support the finding that highly aromatic humic acids provide better protection from enzymatic degradation than the primarily aliphatic humic acid.

sample composition	cytochrome c	cytochrome c/ Everglades peat HA	cytochrome c/ Mt. Rainier HA	cytochrome c/ Mangrove Lake HA
after trypsin digestion	(0.1 ± 0.1) %	(17.8 ± 3.8) %	(31.6 ± 8.8) %	(10.7 ± 5.9) %

Table 5.3: The percentages of undigested cytochrome c in the presence of three different humic acids after trypsin-agarose digestion followed by SDS-PAGE separation coupled with immunoblotting detection. The error shown is one standard deviation from triplicate analyses.

5.3.4 Time-dependent trypsin digestion experiment

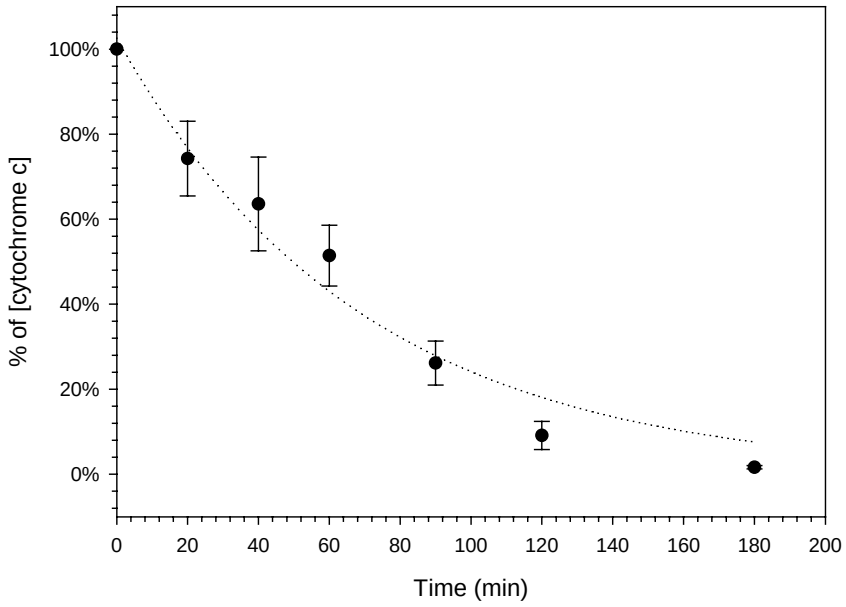
In order to determine whether the presence of humic acid altered the enzyme efficiency, an enzyme digestion experiment at various time periods was performed using cytochrome c and Everglades peat humic acid. After the trypsin-agarose reaction, the residual amounts of undigested cytochrome c were determined by gel electrophoresis with both silver staining and immunoblotting detection methods. The concentrations of the undigested cytochrome c were plotted as the percentage of cytochrome c remaining undigested vs. reaction time (Figures 5.4 and 5.5). Since the trypsin digestion is a pseudo-first-order enzymatic reaction under our experimental conditions, an exponential decay curve (Eq. 5.1) could be fit to the data shown in Figures 5.4 and 5.5.

$$[S] = a \cdot e^{-k \cdot t} \qquad \text{(Eq. 5.1)}$$

In Eq. 5.1, the substrate (cytochrome c) concentration ([S]) exponentially decreases with respect to the reaction time (t), and the coefficient *k* represents the rate constant of the enzymatic reaction. In our experiments, the initial concentration of cytochrome c is a constant, thus making the trypsin digestion rate proportional to the coefficient *k*. By comparing the rate constant coefficient (*k*) obtained with cytochrome c with and without Everglades peat humic acids, the trypsin reaction rates could be evaluated and the influence of humic acid on the enzyme could be examined.

With samples containing only cytochrome c, the trypsin-agarose reaction coefficient *k* is determined to be 0.0145 (min^{-1}) with the silver-stained tricine SDS-PAGE gel (Figure 5.4(a)) and 0.0188 (min^{-1}) with immunoblotted SDS-PAGE technique (Figure 5.4(b)). When Everglades peat humic acid was added to the time-dependent enzyme reaction experiment, the calculated trypsin-agarose reaction coefficient *k* was found by silver staining to be 0.0118 (min^{-1}) (Figure 5.5(a)) and by immunoblotting to be 0.0071 (min^{-1}) (Figure 5.5(b)). The values for the coefficient *k* of the different samples are summarized in Table 5.4. One important finding of these experiments was that the trypsin digestion rate of the cytochrome c/humic acid mixtures was lower than that of cytochrome c samples, as determined by either silver staining (19 % reduction) or immunoblotting (63 % reduction) detection methods.

(a)



(b)

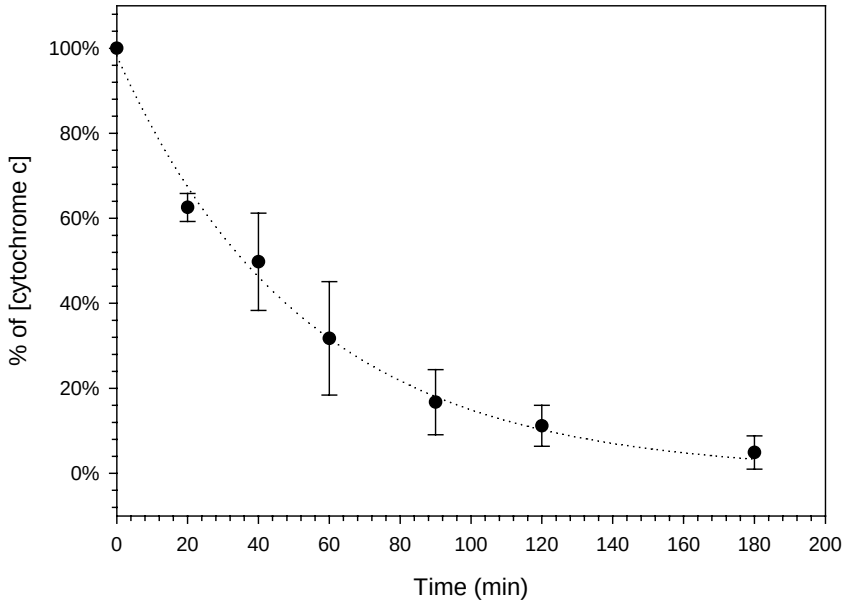
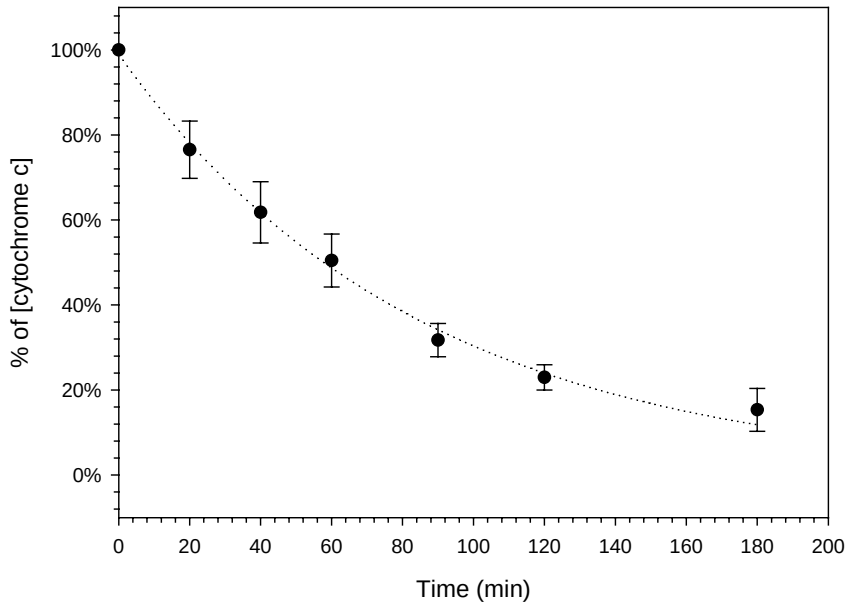


Figure 5.4: Cytochrome c digested by trypsin-agarose at different time periods. Samples were separated by (a) tricine SDS-PAGE gel coupled with the silver staining method; (b) SDS-PAGE gel coupled with the immunoblotting method.

(a)



(b)

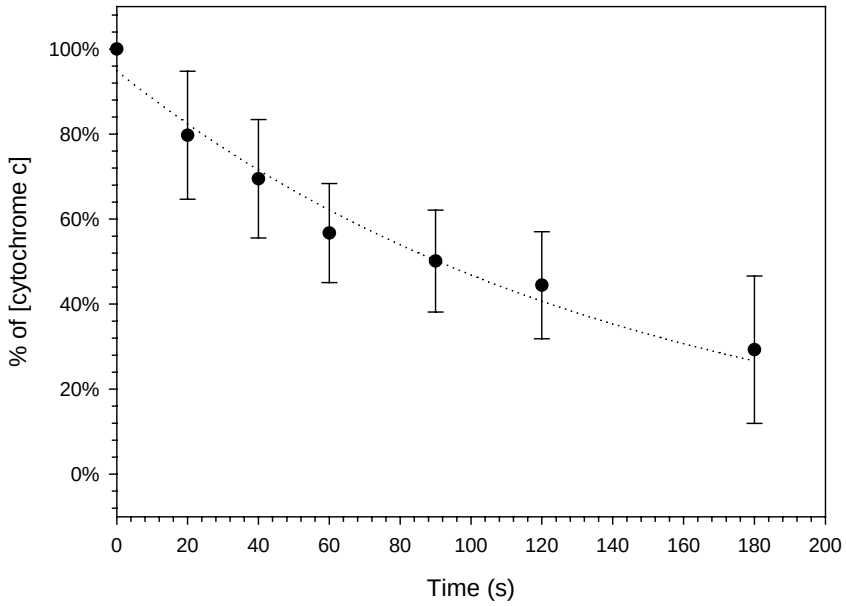


Figure 5.5: Cytochrome c with Everglades peat humic acid digested by trypsin-agarose at different time periods. Samples were separated and determined by (a) tricine SDS-PAGE gel with silver staining; (b) SDS-PAGE gel with immunoblotting.

detection method	cytochrome c	cytochrome c/Everglades humic acids
silver staining	0.0145 (min^{-1})	0.0118 (min^{-1})
immunoblotting	0.0188 (min^{-1})	0.0071 (min^{-1})

Table 5.4: The reaction coefficient *k* of cytochrome c trypsin digestion with and without Everglades peat humic acid, as determined by the silver staining or the immunoblotting detection techniques. The error shown is one standard deviation from triplicate analyses.

There are three possible explanations for the decrease in trypsin reaction rates in the cytochrome c/humic acids mixtures: the humic acid induced changes to the substrate, changes to the enzyme, or both. According to previous studies (Chapters 2 - 4), both covalent and non-covalent interactions between humic acids and proteinaceous molecules have been demonstrated, and both the substrate and enzyme in this reaction are proteins. Modification of the cytochrome c substrate could take the form of physical blockage of the trypsin digestion site or a general alteration in the tertiary structure of cytochrome c, making the digestion less likely to be recognized by the trypsin enzyme. Either of those two modifications could cause the observed decrease in trypsin digestion rates with the addition of Everglades peat humic acid. On the other hand, interactions between humic acid and the trypsin protein itself could alter the trypsin enzyme efficiency, either by full

or partial blockage of the active site, or by an alteration in tertiary structure that would render the enzyme less efficient. Effects on the trypsin were likely to be minimized in these reactions, because the concentration of added trypsin-agarose is relatively low in comparison to the concentration of cytochrome c, and the contact time between humic acids and trypsin-agarose is unlikely to be long enough to allow the humic acid molecules to bind to trypsin-agarose covalently. Therefore, it is most likely that the decrease of the trypsin-agarose reaction rates is the result of the modification of the molecular structure of cytochrome c by humic acid molecules through covalent bonds. In addition, these results imply that the protein structures modified by humic acid molecules have the potential to prevent or delay enzymatic degradation reactions. As a result, the interactions between humic acid and the protein molecules may be an important mechanism for preserving the proteinaceous molecules in soil.

We hypothesize that the influence of humic acids on trypsin digestion efficiency is due to binding interactions between humic acid and cytochrome c, as shown in Figure 5.6. For example, five illustrative trypsin reaction positions on cytochrome c are indicated by yellow arrow (▼) in the schematic diagram. After the addition of humic acid, two of these sites are blocked (no arrow), two are modified by covalent and/or non-covalent interactions with humic acid molecules (orange arrow), leaving only one site unaffected (yellow arrow). The decrease of the number of available trypsin digestion positions on cytochrome c, therefore, impedes the enzyme reaction, as observed in our experiments.

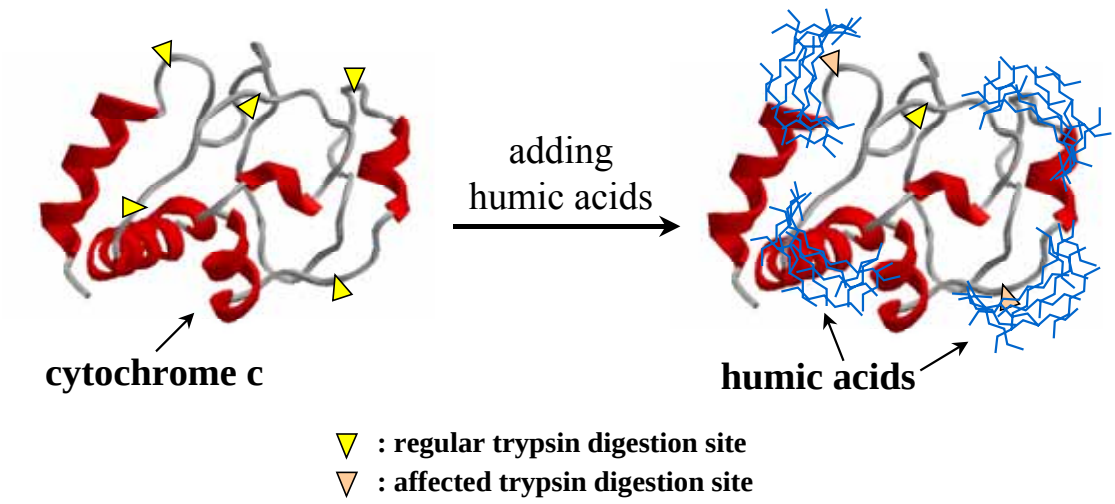


Figure 5.6: The schematic diagram illustrates that the trypsin digestion sites on cytochrome c are influenced by the presence of humic acid molecules.

5.4 Conclusions

In this study, SDS-PAGE coupled with two detection methods, silver staining and immunoblotting, were used for the separation and visualization of protein and humic acids mixtures. We observed that the conventional silver staining detection on the tricine SDS-PAGE gel displayed a dark background signal for those samples containing humic acids. In the tricine SDS-PAGE gel image, the dark blurred bands observed in the stacking gel area indicated that the humic acid molecules were not separated efficiently. This effect may be the result of the difficulty in forming SDS and humic acid complexes. The other finding from the tricine SDS-PAGE experiments was that humic acids with higher aromatic components have darker background staining, and this high background may result in signal interference in the measurement of the OD values. In contrast, the

immunoblotting detection method provided highly specific and more sensitive results, and revealed the bands of the target protein molecules on the gel without any interference from the background humic acid signal. However, in comparison to the conventional silver staining method, immunoblotting is expensive and requires longer experimental times.

Cytochrome c was combined with three humic acids containing different chemical components. The three humic acids had different chemical properties: Mt. Rainier humic acid contains primarily aromatic components, Mangrove Lake humic acid has more aliphatic chemical structures, and Everglades peat humic acid represents the typical humic substances, with both aliphatic and aromatic properties. Cytochrome c mixed with the three respective humic acid samples was digested by the enzyme trypsin-agarose to examine the intermolecular interactions between protein and humic acids. Cytochrome c was not completely digested by trypsin-agarose in the presence of humic acids. The incomplete digestion of cytochrome c indicated that the interactions with humic acid molecules influenced the enzyme reactions on cytochrome c. The intermolecular association between cytochrome c and humic acid could be either covalent or non-covalent interactions. Both of these possibilities could change the cytochrome c conformation or modify its molecular structure, thus affecting the enzyme reaction. The percentages of cytochrome c remaining determined by both detection methods, higher residual amounts of cytochrome c were found to correlate with a higher aromatic content in the humic acid. The results obtained from both detection methods are consistent, and support our hypothesis that the aromatic components in humic acids play an important role in interacting with proteins. We believe that the covalent bonds formed

via quinone structures, as demonstrated in Chapter 2, are most likely the major mechanism for preserving proteins in humic acids.

In order to further understand the mechanism of interaction between cytochrome c and humic acid molecules, a time-dependent enzyme digestion experiment was performed. The enzyme reaction coefficients were determined for the trypsin-agarose digestion of cytochrome c with and without Everglades peat humic acid. The results of this experiment indicated that the chemical interaction between humic acid and cytochrome c reduced the rate of the trypsin-agarose digestion reaction. These findings further support our proposed mechanism for the protection of proteinaceous substances by humic acids in the environment.

This study provided a model system to investigate the effects of humic acids in an enzymatic degradation reaction. The results suggested that the interaction between humic acids and proteins impeded enzymatic degradation. Furthermore, the functional groups in humic acids appeared to determine the level of protection of proteinaceous materials from degradation. The aromatic functional groups in humic acid are likely to be the important components for protecting protein molecules. In addition, the time-dependent enzymatic reaction provided an additional line of evidence that the humic acid/protein interaction reduced enzymatic degradation. In summary, proteinaceous substances may be protected from enzymatic reactions through covalent interactions with humic acid. These results demonstrate a possible mechanism for preserving proteinaceous material in the environment.

CHAPTER 6

SUMMARY OF RESULTS AND DIRECTONS OF FUTURE STUDIES

6.1 Summary of results

This research focuses on the formation of covalent and non-covalent interactions between proteinaceous materials and humic acids through a series of simulations with the intended purpose of evaluating an important mechanism for the sequestration and preservation of peptides in environmental systems. We simulated the process of peptide preservation by designing experiments that take advantage of several chemical and bioanalytical techniques to study the molecular-level interactions. A novel approach was used in which peptides with amino acids residues labeled with ^{15}N were incorporated into humic acids and the resulting products examined by 2-dimensional ^{15}N-^{1}H cross correlation NMR experiments. In addition, the survival of the humic acid-peptide adducts to enzymatic attack, as an attempt to mimic their fate in the environment, was tested by nitrogen isotope tracer experiments and gel electrophoresis coupled with enzyme treatment. Significant experimental evidence was gathered concerning 1) the formation of covalent and non-covalent bonding, 2) the influence of functional groups on bond formation, and 3) the preservation efficiency of peptide/humic acid adducts.

In Chapter 2, 2D HSQC NMR techniques are combined with isotope enrichment experiments, to obtain direct spectroscopic evidence for the reaction of nitrogen-

containing molecules (peptides) with macromolecular organic matter in the form of humic acids. A model tetrapeptide, GGGR, with three glycines ^{15}N-labeled was mixed with Everglades peat humic acids. The HSQC NMR spectra clearly revealed shifts in nitrogen-proton or carbon-proton correlated signals that can be interpreted as the formation of covalent and non-covalent bonds between peptides and humic acids. The formation of new peaks and/or loss of peaks in ^{15}N-^{1}H HSQC NMR spectra suggest that the covalent bonds are formed between amide groups and humic acids. The broadening of peaks observed in the NMR spectra also suggest that non-covalent binding interactions between model peptide and humic acids are the most likely interactions, because broadening is maintained at different temperatures.

Previous studies (Mason and Liebler, 2000) have shown that quinone structures, known components of humic acids, form Michael adducts with peptides. 1,4-naphthaquinone was chosen as a model molecule to react with the ^{15}N-labeled peptide to examine the effects of adduct formation on ^{15}N-^{1}H HSQC NMR spectra. ^{15}N-labeled peptide mixed with 1,4-naphthaquinone showed a similar pattern of new peaks and peak broadening as was observed with ^{15}N-labeled peptide mixed with humic acids. This suggests that the quinone structures in humic acids are the most likely functional groups to form covalent bonds with peptides.

After demonstration of the presence of both covalent and non-covalent interactions between peptide and humic acids, an experiment to study the functional group effects to the molecular interactions was employed. In Chapter 3, two different peptides with some of the amino acids residues ^{15}N-labeled were used to investigate the influence of functional properties of peptides and humic acids on each other. Peptide

SFFFYYS with three phenylalanines ^{15}N-labeled, exhibiting mainly aromatic character, and peptide SLLLVIS, having three leucines ^{15}N-labeled, were chosen to represent aromatic and aliphatic peptides, respectively. 2D HSQC NMR spectra were obtained for these two peptides each mixed with three humic acids, which also display variations in aromatic and aliphatic character. New peaks and peak broadening effects were also observed in the NMR spectra, providing additional confirmation for the presence of covalent and non-covalent interactions between peptides and humic acids.

A consistently similar pattern of peak shift/broadening was observed when the aromatic peptide was mixed with humic acids displaying aromatic character. No covalent interaction was observed in the aromatic peptide mixed with aliphatic humic acids. In contrast, the ^{15}N-^{1}H HSQC NMR results show that the aliphatic peptide only showed new nitrogen-proton correlated signals when mixed with two of the humic acids that have significant amounts of aliphatic structures. The presence of these new cross peaks highlight new unknown covalent interactions between aliphatic structures in peptides and humic acids. When the aliphatic peptide was mixed with the predominantly aromatic humic acid, a different new and broadened peak pattern was observed. These overall results provide an important new finding; aromatic structures in humic acids react with peptides containing either aromatic or aliphatic properties, however, the aliphatic humic acid only react with the peptide having mainly an aliphatic nature. We suggest that the aromatic components in humic acids play an important role in reacting with peptides, consistent with previous belief, and the formation of covalent interactions between aliphatic structures in both peptide and humic acids is a process that is also of importance but has not previously been considered important.

An experiment to evaluate whether peptides that are covalently bonded with humic acids can be preserved from enzymatic degradation was demonstrated in Chapter 4. A simulation experiment was designed to test if the covalent interactions between peptides and humic acids could protect the associated peptide from enzymatic degradation. The simulated preservation experiment included use of an ^{15}N-labeled peptide that was reacted with humic acids and then subjected to enzyme degradation. Samples at each step of the preparation were analyzed by nitrogen isotope mass spectrometry to determine the residual content of ^{15}N-labeled peptide. The results showed that 5 to 11 % of the labeled peptide/humic acid adducts survive enzyme degradation, indicating that preservation is effected by formation of covalent and non-covalent bonds. We suggest that the recovery of labeled peptide is due to the fact that binding interactions either block the enzyme accessibility to the peptide or modify the peptide conformation to retard or terminate enzyme degradation. Aromatic humic acids provide better enzyme protection for the predominantly aromatic peptide, more than twice the preservation efficiency (11 % vs 5 %). This conclusion is consistent with previous findings in Chapter 3 showing that aromatic humic acids more effectively bind aromatic peptides by a covalent bonding mechanism than do aliphatic humic acids.

The simulated preservation experiments lead to a conclusion that binding of peptides to humic acids reduces their susceptibility for degradation, and this process has some important consequences for nitrogen cycling in the environment. Peptides, that derive from degraded proteins in the environments and come into contact with humic substances in soils and sediments, become incorporated into humic acids through covalent and non-covalent interactions like those that are observed active in the

simulations conducted in this study. This incorporation of peptides provides protection from enzyme degradation and results in the preservation of peptides for extended periods of time in the environment. This partially explains why some proteinaceous residues can be recovered from ancient sediments.

In Chapter 5, gel electrophoresis coupled with silver staining and immunoblotting visualization tools are used to examine interactions of proteins with humic acids and to assess the degree of protection from enzyme hydrolysis. When a model protein, cytochrome c, is reacted with humic acids it survived enzyme degradation. The diminution of enzyme reaction efficiency is due to the formation of covalent and/or non-covalent bonds between protein and humic acids, which either changes protein conformation or modifies protein structure. About 10 to 30 % of humic acid/protein adducts remained after enzyme degradation based on integration of optical density of each protein band on gel images. Humic acids with aromatic character provided better enzyme protection for cytochrome c, consistent with previous findings that show that aromatic structures are important for covalent bond formation. Enzyme activity was confirmed to be affected by formation of covalent and non-covalent binding to humic acids based on time-dependent enzyme digestion experiments. Therefore, cytochrome c survived in the presence of humic acids because of the modification of the protein and enzyme structure through formation of covalent and non-covalent bonds.

In summary, covalent bonding of proteinaceous materials, from a small tetrapeptide (375 Da) to a protein (12.1 kDa), with different humic acids varying in structural composition readily occurs. It unlikely that covalent interactions lead to the structural rearrangement of the peptide bond, as has been supposed by those who suggest

rearrangements to form heterocyclic nitrogen (Schulten and Schnitzer, 1998). It is also clear that aromatic components in humic acids are important for covalent and non-covalent interaction with peptides and that quinone structures in humic acids are the most likely functional groups to react. A new and important finding is that covalent interactions of peptide and humic acids occur with aliphatic structures. The results of this study explains the observations that stable nitrogenous materials in humic acids show mainly amide signals in ^{15}N NMR spectra (Knicker and Hatcher, 1997) and they were are also consistent with the fact that these NMR signals survive strong acid hydrolysis (Zang et al., 2000). Proteinaceous materials are believed to be protected by humic acid molecules through the covalent linkages and non-covalent interactions they form.

6.2 Directions of future studies

In this study, we used 2D NMR to obtain significant evidence concerning the formation of covalent and non-covalent interactions, and the influence of functional groups on bond formation, using synthesized peptides with ^{15}N-labeled amino acid residues. We designed these peptide sequences (SFFFYYS and SLLLVIS, in Chapter 3) to reduce the complexity of the model system, because of their sequence length and ideal aromatic/aliphatic properties. The purpose of using multiple ^{15}N-labeled amino acid residues in sequences was for studying the positions of interactions with peptides and increasing the ^{15}N-labeled content for enhancing ^{15}N NMR signals. We expected to observe three separated ^{15}N-^{1}H correlated signals for both peptide SFFFYYS and SLLLVIS. However, two amino acid residues with similar chemical environments showed almost identical peaks in ^{15}N-^{1}H HSQC NMR for both peptides, and these may

be due to the short length of the peptide without a fixed conformation. Additionally, the peptide SLLLVIS had low solubility in aqueous systems, preventing the sample preparation at high concentrations to increase the ^{15}N signal. To remedy these complications, new peptide sequences can be developed by 1) extending the length to induce the formation of secondary structures, 2) synthesizing peptides with alternative ^{15}N-labeled residues instead of those in which the ^{15}N-labeled residues are sequentially arranged, and 3) using either arginine in the peptide sequence or coupled with other hydrophilic groups to increase the solubility.

To completely understand the interactions between proteinaceous materials and humic acids, bigger model molecules (large proteins instead of peptides) should be introduced in our experiments. Unlike the ^{15}N-labeled peptides, it is almost impossible to synthesize whole proteins with ^{15}N-labled amino acid residues. Nevertheless, it is feasible to use another method to produce uniformly ^{15}N-labeled proteins. This process involves over-expression and purification of ^{15}N-labeled proteins from bacteria which grow in minimal medium using $^{15}NH_4Cl$ as sole nitrogen source. One of the advantages to using ^{15}N-labeld proteins as model molecules is to fully imitate the natural ones in the environment. Furthermore, since every amino acid is ^{15}N-labeled in the protein model, it is possible to locate the exact position of interaction with humic acid with ^{15}N-^{1}H assignments from 2D HSQC NMR experiments. Based on interactions assigned in NMR, 3D structure simulation can be used to construct the modeled complex structure between protein and humic acid molecules. The information obtained from protein/humic acid adduct model studies will improve our knowledge of covalent and non-covalent interaction between them.

Furthermore, more structural information about covalent interactions between proteinaceous molecules and humic acid can be revealed if both carbon and nitrogen atoms in model molecules are isotopically labeled. The chemical environments around the reacting amide groups can be further examined using the ^{13}C and ^{15}N double-labeled peptides in 2D or even 3D NMR, and the detailed peptide/protein and humic acids adduct structures could be determined.

The use of ^{15}N-labeled model molecules coupled with 2D NMR techniques to study these phenomena is clearly a significant advance in the study of the interactions of proteinaceous materials with humic acid molecules. Thus, we expect that our experimental system will provide a significant contribution and afford us a powerful tool to reveal insights into proteinaceous molecule preservation.

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