Proteolysis of Iron Oxide-Associated Bovine Serum Albumin

Zhaomo Tian, Tao Wang, Anders Tunlid, and Per Persson

ABSTRACT: Proteins are a substantial nitrogen source in soils provided that they can be hydrolyzed into bioavailable small peptides or amino acids. However, the strong associations between proteins and soil minerals restrict such proteolytic reactions. This study focused on how an extracellular fungal protease (Rhizopus sp.) hydrolyzed iron oxide-associated bovine serum albumin (BSA) and the factors that affected the proteolysis. We combined batch experiments with size-exclusion and reversed phase liquid chromatography and in situ infrared spectroscopic measurements to monitor the generation of proteolytic products in solution as well as the real-time changes of the adsorbed BSA during 24 h. Results showed that protease hydrolyzed the iron oxide-associated BSA directly at the surface without an initial desorption of BSA. Concurrently, the protease was adsorbed to vacant surface sites at the iron oxides, which significantly slowed down the rate of proteolysis. This inhibiting effect was counteracted by the presence of preadsorbed phosphate or by increasing the BSA coverage, which prevented protease adsorption. Fast initial rates of iron oxide-associated BSA proteolysis, comparable to proteolysis of BSA in solution, and very slow rates at prolonged proteolysis suggest a large variability in mineral-associated proteins as a nitrogen source in soils and that only a fraction of the protein is bioavailable.

INTRODUCTION

The primary production in boreal forest ecosystems is limited by the availability of nitrogen (N). Proteinaceous substances are one of the most abundant N sources in the soils of these ecosystems, but in general, plants have limited ability to take up N in the form of intact proteins, although a few examples of direct protein uptake exist. In any case, the hydrolysis of proteins by microbial extracellular enzymes is a critical process for the generation of bioavailable N from complex organic molecules to support plant growth. This process may also be complemented with abiotic hydrolysis catalyzed by reactive metal oxides. Proteinaceous compounds often exhibit a strong affinity to soil mineral surfaces, and typically a major fraction of soil proteins occurs as mineral associations. These associations limit the accessibility of soil extracellular enzymes to the substrate proteins, and accordingly adsorbed proteins can have much longer turnover times than proteins in soil solutions or in particulate organic matter. This is why mineral-associated proteins rarely have been considered as an important source of bioavailable N. At the same time, there are studies indicating that at least a fraction of the mineral-associated proteins is available for microbial degradation. Given the large size of the mineral-associated protein pool, proteolysis of even a small fraction could provide a substantial contribution to bioavailable soil N.

Desorption of mineral-associated organic matter is often considered as the only pathway to generate organic molecules accessible for microbial degradation, although direct degradation of mineral-associated organic matter has also been suggested. In the case of proteins, adsorption to mineral surfaces has been shown to be largely an irreversible process, suggesting that direct proteolysis of the mineral-associated proteins may be the only way to access this N pool. Previous studies of engineered systems have shown that proteolytic enzymes hydrolyze multilayered solid substrates, and that these reactions are sensitive to properties of the water–solid interface. However, experimental evidence for proteolysis of proteins adsorbed onto reactive soil minerals such as secondary (hydr)oxides of aluminum and iron is scarce. Such minerals are ubiquitous in soils and play disproportionately large roles in stabilization of proteins and other forms of soil organic matter. Moreover, it has been shown that adsorption of proteases by minerals decreases the proteolytic activity, and that activity is further decreased if the substrate is also adsorbed on the minerals. This is the result of a physical disconnection between the substrate and the enzyme, leading to a low rate of formation of the enzyme–substrate (ES) complexes, which are necessary for proteolysis.
In the present study, we have addressed two fundamental research questions that are directly related to the possible role of mineral-associated proteins as N sources in soils. These were: (1) can a protease hydrolyze iron oxide-associated proteins? (2) If so, are the ES complexes mainly formed at the iron oxide-associated proteins or in solution, that is, via desorption of the proteins? In addition, we have investigated factors that suppress the enzyme adsorption and possibly promote the formation of ES complexes. To address these research questions, we examined the proteolysis of iron oxide-associated bovine serum albumin (BSA) by an aspartic protease isolated from the filamentous fungus *Rhizopus* sp. that is omnipresent in soils. Aspartic proteases are also key enzymes for protein decomposition by ectomycorrhizal fungi, which are abundant in boreal forest ecosystems. BSA was chosen as a model protein because of its solubility in water and strong affinity to mineral surfaces. It is also a well-characterized protein facilitating the interpretations of our experimental data. Ferrhydrite and goethite included in this study are common soil minerals that differ in surface reactivity toward the soil organic matter. Both were investigated in order to assess the general nature of the results in light of the two main research questions addressed. All experiments were performed at pH 4.0, which is a representative pH value of boreal forest soils, and at different enzyme concentrations and different concentrations of vacant surface sites. The variation in vacant surface sites was accomplished by either increasing the surface coverage of BSA or by coadsorbing phosphate or oxalate. Size exclusion chromatography (SEC) is sensitive to changes in molecular size and was employed to monitor BSA hydrolysis and the generation of smaller peptides. SEC was complemented with reversed-phase high-performance liquid chromatography (RP-HPLC) that provides increased analytical separation between BSA and the protease. An *in situ* infrared (IR) spectroscopic technique was used to capture the real-time changes of IR spectra of iron oxide-associated BSA during proteolysis.

**MATERIALS AND METHODS**

Below follows brief descriptions of the materials and methods while more details are provided in the Supporting Information under the same sub-headings.

**Materials.** BSA (purity ≥98%) and a protease from *Rhizopus* sp. were purchased from Sigma-Aldrich (now Merck KGaA, Darmstadt, Germany). This protease is an aspartic endopeptidase with optimum activity in acidic environments, and its isoelectric point is suggested to be in the pH range 4–6, similar to that of BSA at ca. pH 4.7. The timecourse of the protease in solution was minor within the timeframe of our experiments (Supporting Information Figure S1). Goethite and 6-line ferrhydrite were synthesized according to the methods described by Hiemstra et al., and Schwertmann and Cornell, respectively. The surface areas of the ferrhydrite and goethite used to normalize the surface concentrations (see below) were 300 and 62 m² g⁻¹, respectively (see Supporting Information S1.1).

**Batch Adsorption Experiments.** The adsorption isotherms of BSA on ferrhydrite and goethite and desorption were investigated at pH 4.0 in 0.01 M NaCl solution (see Supporting Information S1.2). BSA solutions at various concentrations were mixed with a ferrhydrite or a goethite suspension and reacted for 24 h. After centrifugation, the BSA concentration in the supernatant was determined from the absorbance at 214 nm using a UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Desorption was studied by equilibrating the mineral residues with a 0.01 M NaCl solution for 24 h, and the supernatant was analyzed for desorbed BSA.

**Proteolysis of Iron Oxide-Associated BSA.** A 200 mg L⁻¹ protease stock solution was prepared at pH 4.0 and filtered through a 0.22 μm sterile PES filter (Millipore Inc., Bedford, MA) each time before use. Proteolysis was initiated by adding an aliquot of the protease stock solution into 1 mL iron oxide-BSA suspension. For each iron oxide, two BSA surface concentrations (0.7 and 1.4 mg m⁻²) were studied; the detailed procedures for making these iron oxide-BSA suspensions are described in the Supporting Information S1.3. Note that, the final solid concentrations of ferrhydrite and goethite were 0.5 and 1 g L⁻¹, respectively. Thus, the total surface area in the ferrhydrite experiments was 2.5 times larger than that in the goethite experiments. The total enzyme concentrations ranged from 0 to 0.28 mg m⁻³, corresponding to a solution concentration ranging from 0 to 40 mg L⁻¹ or from 0 to 20 mg L⁻¹ in the ferrhydrite and goethite experiments, respectively. The proteolysis experiment was conducted for 3 h during continuous shaking (PTR-35, Grant Instruments, Cambridge, UK). After the reaction, the solid and the liquid phases were immediately separated by mixing 1 mL of iron oxide-BSA suspension with 0.2 mL of 5 M NaCl at pH 4.0 (to induce aggregation and facilitate solid–liquid separation) and centrifuged at 13,793 g for 5 min (Heraeus Biofuge 13 centrifuge, Thermo Fisher Scientific, Waltham, MA). The supernatant was mixed with a phosphate buffer (0.5 M Na₂HPO₄ and 1.5 M NaCl at pH 7.5) to quench the enzyme activity and filtered before SEC analysis. The mineral residue was redispersed in 1 mL water at pH 4.0 and mixed with 0.11 mL of the same phosphate buffer and shaken for 30 min. The addition of the phosphate buffer to the iron oxides after BSA proteolysis served two purposes: (i) to inhibit the proteolytic activity by the rapid pH increase to 7.5 in order to assure that all degradation products were generated during the predetermined reaction time between the protease and the iron oxide-BSA suspension (Supporting Information Figure S2); (ii) to displace the BSA degradation products that were adsorbed by the ferrhydrite and goethite surfaces. Phosphate adsors strongly to these iron oxide minerals and has been shown to outcompete soil organic matter. Note, however, that the efficiency of phosphate to displace the various degradation products is unknown and may vary between these products, and consequently, the results should only be used for relative comparisons among reaction conditions and not as absolute quantitative measures of BSA proteolysis. The phosphate-desorbed BSA proteolysis products were separated from the iron oxide particles by centrifugation and subsequently analyzed using SEC. Control samples at *t = 0* were prepared by adding iron oxide-BSA suspension and an aliquot of enzyme stock solution sequentially into the phosphate buffer and reacted for 30 min. The liquid phase was analyzed using SEC.

For comparison and to facilitate the interpretations of the results from the proteolysis of the iron oxide-associated BSA, several control experiments were performed. These were: (1) proteolysis of BSA in solution; (2) hydrolysis of BSA by the iron oxides; (3) auto-proteolysis of the protease in solution; (4) hydrolysis of the protease by the iron oxides; and (5) competitive adsorption between BSA and the protease. Details...
of these experiments are provided in the Supporting Information S1.3.

**Proteolysis of Iron Oxide-Associated BSA in the Presence of Coadsorbed Phosphate or Oxalate.** The effects of coadsorbed phosphate or oxalate on the proteolysis of iron oxide-associated BSA were investigated by adsorbing phosphate or oxalate to the iron oxide-associated BSA for 1.5 h prior to the addition of the enzyme (see Supporting Information S1.4).

**Proteolysis as a Function of Time.** The time-dependent proteolysis of goethite-associated BSA at 1.4 mg BSA m$^{-2}$, in the absence or presence coadsorbed phosphate, was investigated in batch experiments. The experimental conditions were the same as described above for the proteolysis experiments, except that the added protease concentration were the same as described above for the proteolysis experiments. Also, the time-dependent proteolysis of BSA in solution at a comparable BSA concentration (100 mg L$^{-1}$) was examined.

**Analytical Techniques.** SEC was used to monitor concentration changes of BSA and its degradation products in solution at 214 nm. Although SEC is sensitive to the generation of low molecular weight (LMW) products from hydrolysis of BSA, complete separation between BSA and the protease cannot be achieved. Therefore, we complemented SEC with RP-HPLC measurements at 220 nm in experiments where it was crucial to separate the signals from BSA and the protease. The adsorption, desorption, and proteolysis reactions at the iron oxide surfaces were studied in situ by IR spectroscopy using an attenuated total reflectance (ATR) accessory. In the case of ferrihydrite, we only investigated BSA adsorption because of instabilities of the ferrihydrite overlayer.

**RESULTS AND DISCUSSION**

**Adsorption of BSA on Ferrihydrite and Goethite.** Adsorption isotherms of BSA on ferrihydrite and goethite showed an initial slope of 1, indicating a high affinity of BSA for both ferrihydrite and goethite (Figure 1). Moreover, the adsorption isotherms showed that BSA was completely adsorbed to both iron oxides at the two BSA surface coverages (0.7 and 1.4 mg m$^{-2}$) studied in the proteolysis experiments. BSA has a prolate ellipsoidal shape, and from dimensions, reported in the literature, the maximum area occupied by one BSA molecule can be estimated to be 59.2 nm$^2$. In a closed packed system, this corresponds to 1.9 mg m$^{-2}$ (assuming a BSA molecular weight of 67 kDa), thus our experiments were performed below this theoretical monolayer concentration. Interestingly, the isotherms changed the slope around 2 mg m$^{-2}$ (Figure 1), which may indicate a switch from the monolayer to multilayer adsorption. We detected no desorption from iron oxide-associated BSA into 0.01 M NaCl after 24 h at BSA concentrations \( \leq 1.4 \text{ mg m}^{-2} \).

The adsorption of BSA on goethite and ferrihydrite was also investigated by IR spectroscopy. The spectrum of adsorbed BSA to goethite displayed two main characteristic protein bands: amide I (~1650 cm$^{-1}$) and amide II (~1545 cm$^{-1}$) (Supporting Information Figure S3A). These bands were in agreement with IR spectra of aqueous and adsorbed BSA reported in literature, and the band positions indicated no major distortion of the goethite-associated BSA. We further analyzed the area-normalized amide I band obtained at 0.7 and 1.4 mg BSA m$^{-2}$ and these completely overlapped in the case of goethite (Supporting Information Figure S4A) and displayed only small differences in the ferrihydrite experiments (Supporting Information Figure S4B). This indicated no or small structural changes of BSA as a function of surface coverage. The amide II band has previously been used to quantify the concentration of adsorbed proteins. Finally, we used the intensity of amide II, which is a good indicator of adsorbed proteins, to investigate desorption of goethite-associated BSA in 0.01 M NaCl solution. In agreement with the batch desorption experiments, the invariant band intensity showed that no detectable desorption occurred (Supporting Information Figure S3B).

**Proteolysis of BSA in Solution.** The SEC data of a BSA solution at pH 4.0 in 0.01 M NaCl displayed a broad peak centered at 75 kDa (Figure 2A), which we assigned to the BSA monomer, in reasonable agreement with the reported molecular weight of ca. 67 kDa. A shoulder at shorter retention time indicated compounds of molecular mass larger than 75 kDa likely originating from dimers or larger aggregates of BSA. The protease also showed a broad SEC peak at a retention time of ca. 0.2 min greater than BSA (Supporting Information Figure S1), corresponding to a molecular weight of ca. 70 kDa, which may indicate a dimer. After a 3 h reaction between the protease and BSA, the SEC peak associated with BSA decreased, whereas peak intensities indicative of compounds of lower molecular weights increased (Figure 2A). Given the facts that the contribution of protease...
bands represent standard deviations (horizontal dotted lines in the insets indicate the 0-level. The shaded lines.

The molecular masses (in Da) of peptide standards are represented by the vertical dotted lines.

The experiments were performed at a BSA surface coverage of 1.4 mg m$^{-2}$, in aqueous solution (A) and the phosphate-desorbed fraction of ferrihydrite-associated BSA (B) and goethite-associated BSA (C), before (T0) and after 3 h proteolysis reaction (T3h). The limits show the difference between the chromatograms at T3h and T0. The horizontal dotted lines in the insets indicate the 0-level. The shaded bands represent standard deviations (n = 2). The molecular masses (in Da) of peptide standards are represented by the vertical dotted lines.

to the total SEC intensity was very low (Supporting Information Figure S5A), and autoproteolysis of the protease was negligible (Supporting Information Figure S1), these results showed that BSA was hydrolyzed by the protease into LMW products.

**Proteolysis of Iron Oxide-Associated BSA.** The reaction between the protease and ferrihydrite- or goethite-associated BSA at 1.4 mg BSA m$^{-2}$ generated compounds in the size range 13.7–43 kDa (Figure 2B,C). Concomitantly, we observed a decrease in the concentration of the BSA monomer indicated by the loss of intensity at ca. 75 kDa (Figure 2B,C).

Note that, in the absence of protease, SEC did not detect any abiotic BSA degradation catalyzed by the iron oxides (Supporting Information Figure S6), still contribution from such a process cannot be ruled out completely (see IR results below). A crucial question is whether hydrolysis products from the protease itself contribute to the observed results. Comparison between experiments performed in the absence and presence of BSA clearly showed, however, that contribution from protease products to the LMW compounds was negligible (Supporting Information Figure S5). Accordingly, the detection of compounds with molecular weights between 13.7 and 43 kDa (Figure 2B,C) demonstrated that iron oxide-associated BSA indeed was hydrolyzed by the protease. Thus, adsorption to the iron oxide surface did not protect BSA from proteolysis, in agreement with some previous literature, suggesting that the mineral-associated organic matter is partially decomposed by microbes.39,40

Increasing the protease concentration resulted in an expected increase in proteolysis of the iron oxide-associated BSA (Supporting Information Figure S7). Higher protease concentrations may also cause desorption of BSA via a ligand-exchange mechanism. This was investigated by adding protease that was deactivated by the inhibitor pepstatin A to suspensions containing ferrihydrite- or goethite-associated BSA. The supernatants were analyzed by means of RP-HPLC, which separates BSA and the protease (Figure 3A), and these results showed that desorbed BSA was below the detection limit of the technique, corresponding to <1% BSA desorption under all experimental conditions analyzed herein (Figure 3B,C). The supernatants from these experiments were also analyzed by means of SEC, and a peak at 70 kDa indicated the presence of the protease (cf. Supporting Information Figures S1 and S8). This peak decreased when iron-oxide associated BSA was added to a solution of deactivated protease, which indicated coadsorption between BSA and a fraction of the protease (Supporting Information Figure S8). Moreover, in the absence of pepstatin A, the peak intensity decreased even further (Supporting Information Figure S8), which likely was a result of increased protease adsorption caused by proteolysis of iron oxide-associated BSA that created vacant surface sites.

The facts that proteolysis occurred when all BSA was adsorbed (Figure 1), and that the iron oxide-associated BSA was resistant toward desorption both in the absence (Figure S3B) and presence of the protease (Figure 3B,C), showing that the ES complexes, necessary for the proteolysis,41 were formed at the iron oxide surfaces. Hence, our results are in contrast to the notion that desorption of the substrate is a prerequisite for enzymatic decomposition of mineral-associated organic matter.42,43

The ES complexes form at the iron oxide surfaces either via direct interaction between aqueous proteases and the adsorbed BSA or via adsorption of the protease to the iron oxide and a subsequent surface diffusion. Generally, the latter process results in a slower and less efficient proteolysis because of: (1) the physical disconnection between the enzyme and the substrate in combination with the limited diffusion of the adsorbed enzyme to the substrate;45 (2) the altered structure of the adsorbed enzyme and substrate that may lower the likelihood of favorable ES interactions.46 Based on our results, however, we cannot determine whether a mechanism involving formation of the ES complex from aqueous proteases directly
or one where the protease first adsorbs to the iron oxide dominates but likely it is a combination of both, and the relative importance depends on the experimental conditions as will be discussed.

Both ferrihydrite- and goethite-associated BSA displayed a strong reduction in proteolytic products as the BSA surface coverage decreased from 1.4 to 0.7 mg m\(^{-2}\) (cf. Figures 2 and S9). This decrease was particularly dramatic in the case of ferrihydrite, which can be explained by differences in the experimental conditions between the ferrihydrite and goethite experiments. Although the experiments were conducted at identical total protease concentration and BSA surface coverage, differences in the solid-to-liquid ratio and the specific surface areas resulted in a 2.5 times larger total surface area in the ferrihydrite experiments (see Materials and Methods). Hence, at low BSA surface coverage, more adsorption sites were available for the protease on ferrihydrite than on goethite, causing more extensive protease adsorption on ferrihydrite (Supporting Information Table S1 and Figure S10) and a reduced proteolysis. Likely, aqueous protease promotes faster proteolysis by increasing the likelihood of favorable interactions with the iron oxide-associated BSA. Hence, the effect of increasing the BSA surface coverage is twofold; it increases the probability of favorable interaction between BSA and the protease, and it decreases protease adsorption to the iron oxides, which otherwise leads to slower proteolysis or deactivation.

The \textit{in situ} IR spectroscopic measurements performed at 0.7 and 1.4 mg BSA m\(^{-2}\) on goethite revealed only minor changes (Supporting Information Figure S11). The total protease concentration was 10 or 5% of the total BSA concentration added in the low and the high surface coverage treatments, respectively. This difference in concentrations between BSA and the protease implies corresponding large differences in IR intensities if both species are completely adsorbed to goethite (Supporting Information Figure S12). Moreover, only a fraction of the protease was adsorbed in the presence of BSA as indicated above, and in line with this large predominance of BSA, we were not able to resolve any increased signal from adsorbed protease in the initial spectra collected immediately after protease addition. Instead, we observed a decrease in the intensity of the amide I and amide II bands, and this decrease was most pronounced at the high BSA surface coverage. These IR spectral changes suggested that goethite-associated BSA was degraded with time, in agreement with the SEC data. Comparison of proteolysis rates obtained from batch SEC data and from IR spectroscopy using the change in intensity of amide II revealed a lower rate obtained from the IR data (Figure 4). The difference in rate may be caused by differences in aggregation between the goethite particles in the batch experiments and those in the film on the ATR crystal, and/or that adsorbed BSA degradation products have IR bands that coincide with the amide II of intact BSA; this will be further discussed below. In any case, the discrepancy between the rates

Figure 3. RP-HPLC chromatograms of BSA and protease aqueous solutions at pH 7.5 (A) and of BSA desorbed from ferrihydrite-associated BSA (B) and goethite-associated BSA (C) by the addition of deactivated protease after 24 h equilibration. The protease was deactivated by elevating pH to 7.5 using phosphate (in panel A) or by adding pepstatin A (in panels B,C). In (B,C), the protease concentration was 40 mg L\(^{-1}\) for ferrihydrite and 20 mg L\(^{-1}\) for goethite. Two surface coverages were investigated for each iron oxide: 0.7 (low coverage, corresponding to a total BSA concentration of 100 and 50 mg L\(^{-1}\) for ferrihydrite and goethite, respectively) and 1.4 mg BSA m\(^{-2}\) (high coverage, corresponding to a total BSA concentration of 200 and 100 mg L\(^{-1}\) for ferrihydrite and goethite, respectively). In (B,C), the peak of the protease is not shown because it overlaps with a peak from an impurity contained in pepstatin A. The dotted lines are chromatograms of 2 and 5 mg L\(^{-1}\) BSA, and the detection limit of BSA is estimated to 0.5 mg L\(^{-1}\). The shaded bands represent standard deviations (\(n = 2\)).
The proteolysis was measured as the decrease of the intensity of the BSA peak in SEC (cf. Figure 2B,C) and the amide II band in the batch and IR experiment, respectively. All values were normalized against the initial value (t = 0). The proteolysis of aqueous BSA at a concentration of 100 mg L$^{-1}$ is shown for comparison. All experiments were performed at pH 4.0 in 0.01 M NaCl, and the protease concentration was 5 mg L$^{-1}$. Error bars represent standard deviations ($n$ = 3 for goethite-associated BSA and $n$ = 2 for aqueous BSA).

The IR spectral data sets, shown in the Supporting Information Figure S11, were subjected to multivariate curve resolution-alternating least square (MCR-ALS) analysis. The ability of this method to resolve the spectral components depends on the complexity of the system and the extent of overlap between these components. The IR spectra of the goethite-associated BSA during proteolysis only displayed minor changes of the strong features of adsorbed BSA (Supporting Information Figure S11), and the effects because of increasing concentrations of adsorbed proteolysis products will thus be embedded under the component consisting of intact BSA. Hence, the ability of MCR-ALS to resolve pure components will be limited, and the results should only be used in a qualitative sense considering that the obtained components will not be pure. Two MCR components explained >99.99% of the spectral variation during proteolysis at 0.7 and 1.4 mg BSA m$^{-2}$ (Figure 5). Duplicates showed that the spectral features of these components were reproducible while the absolute contributions were variable, although the trends in the contribution ratios between components 1 and 2 were consistent (cf. Figures 5 and Supporting Information S13). The MCR results from both BSA surface coverages displayed very similar spectra of the dominating component (C1) characterized by strong bands at 1650 and 1546 cm$^{-1}$ in agreement with the amide I and amide II bands of adsorbed BSA. Moreover, the contributions from the C1 components at both surface coverages decreased relative to C2 during proteolysis (Figure 5 insets), which was consistent with proteolysis of the goethite-associated BSA. The second MCR component (C2) showed spectral features very different from those of C1 and indicated the presence of adsorbed products generated from BSA proteolysis. At high BSA surface coverage, the C2 spectrum was characterized by bands at 1670, 1566, 1525, and 1402 cm$^{-1}$ (Figure 5B). The pair of bands at 1566 and 1402 cm$^{-1}$ coincided with typical asymmetric and symmetric stretching vibrations of carboxylate groups, suggesting the generation of carboxylates during proteolysis. This interpretation is supported by a previous study showing that proteolysis of BSA increased the IR absorbance from carboxylate groups. The bands at 1670 and 1525 cm$^{-1}$ were probably shifted amide I and II modes, respectively, caused by structural changes of the partially degraded BSA. The spectrum of C2 obtained at low BSA surface coverage displayed similar bands at 1658, 1560, 1536, and 1403 cm$^{-1}$ (Figure 5A), but the resolution of these bands was worse. As shown, the proteolysis of the goethite-associated BSA was more extensive at the high BSA surface coverage (cf. Figures 2 inset and S9), and thus we attribute the lower spectral resolution of C2 in Figure 5A to a lower concentration of proteolysis products at the goethite surfaces and concomitantly smaller changes in the IR spectral data set. A seemingly counterintuitive result was the substantial contribution from the C2 spectra already at the initial time points [Figure 5A,B (insets)]. This indicated the presence of hydrolysis products already in the first data point analyzed, that is, after

Figure 4. Proteolysis of goethite-associated BSA at 1.4 mg BSA m$^{-2}$, with or without coadsorbed phosphate, determined from either batch or IR experiments. The proteolysis was measured as the decrease of the intensity of the BSA peak in SEC (cf. Figure 2B,C) and the amide II band in the batch and IR experiment, respectively. All values were normalized against the initial value (t = 0). The proteolysis of aqueous BSA at a concentration of 100 mg L$^{-1}$ is shown for comparison. All experiments were performed at pH 4.0 in 0.01 M NaCl, and the protease concentration was 5 mg L$^{-1}$. Error bars represent standard deviations ($n$ = 3 for goethite-associated BSA and $n$ = 2 for aqueous BSA).

Figure 5. MCR analysis of IR spectral data sets of goethite-associated BSA during 20 h proteolysis reaction at 0.7 (low coverage, A) and 1.4 mg BSA m$^{-2}$ (high coverage, B) and at pH 4.0 in 0.01 M NaCl. The spectra of components 1 (C1) and 2 (C2) are presented as dotted and solid lines, respectively, and the relative contribution of C1 and C2 during the proteolysis is shown as insets. Note that the spectra of C1 and C2 are presented as the unmodified output from the MCR analysis, that is, they have not been scaled separately after the analysis. The numbers in the plain and bold text indicate the main peak positions of C1 and C2, respectively. The first time point was collected after ca. 1 min.
approximately 1 min of reaction with the protease. It may be the result of an initial, rapid proteolytic reaction but we cannot rule out the contribution from abiotic surface-promoted hydrolysis of BSA as well as desorption of BSA impurities, although SEC analysis indicated that these latter alternatives only played a minor role. The obtained contribution profiles may also suffer from limitations of the MCR method with respect to resolving pure species. Thus, it is important to underline that individual contributions should not be interpreted as absolute concentrations. The trends in C1/C2 ratios indicate, though, that spectral features associated with C2 increases with time relative to those of C1. Despite the uncertainties associated with the MCR analyses, the IR results corroborate the SEC analysis and show that the iron oxide-associated BSA is hydrolyzed by the protease, and that hydrolysis products are coadsorbed with the remaining intact BSA. Moreover, the IR spectral changes identified are in agreement with those expected from partial proteolysis of BSA, that is, the generation of carboxylate groups and distortion of the secondary structure of the modified BSA.

**Proteolysis of Iron Oxide-Associated BSA in the Presence of Coadsorbed Phosphate or Oxalate.** The effect of coadsorbed phosphate on the proteolysis of iron oxide-associated BSA was investigated by adding a range of phosphate concentrations to the iron oxide-associated BSA prior to the proteolysis reaction. At the lowest phosphate concentration, almost complete adsorption of phosphate was observed while the adsorbed fraction decreased at higher phosphate concentrations and reached a minimum at ca. 40% on ferrihydrite at a total phosphate concentration of 5 μmol m\(^{-2}\) (Supporting Information Figure S14). The observed maximum phosphate surface concentrations on ferrihydrite and goethite in the range of 2–3 μmol m\(^{-2}\) were consistent with previous findings made in the absence of preadsorbed proteins. Note that, the phosphate concentrations added in these experiments did not trigger detectable BSA desorption (Supporting Information Figure S15) as opposed to the phosphate pretreatment step before SEC analysis where a much higher phosphate concentration was used. The SEC data showed that coadsorption of phosphate promoted the proteolysis of the iron oxide-associated BSA (Figure 6). Without treating the iron oxide-associated BSA, containing preadsorbed phosphate, with a concentrated phosphate buffer prior to SEC analysis, we found evidence of aqueous protease in the liquid phase (Supporting Information Table S1 and Figure S16).

The IR spectra of goethite-associated BSA with coadsorbed phosphate displayed characteristic P–O modes in the spectral region between 900 and 1200 cm\(^{-1}\), indicating the presence of phosphate inner sphere complexes. These bands were contained in the C2 spectrum resolved by the MCR analysis (Supporting Information Figure S17). Except for the phosphate bands, the C1 and C2 spectra were similar to those obtained in the absence of phosphate. Thus, the C1 and C2 component spectra represented intact BSA and modified polypeptides and/or amino acids, respectively. The identified phosphate inner sphere surface complexes out-competed the protease and that increased the concentration of aqueous protease (Supporting Information Figure S16), which increased the extent and rate of proteolysis of the iron oxide-associated BSA. Interestingly, the time-resolved SEC data indicated that the initial proteolysis rate of goethite-associated BSA in the presence of coadsorbed phosphate was approaching that of aqueous BSA (Figure 4). One should be cautious though when comparing these proteolysis rates because the former represents the fraction of BSA desorbed by phosphate while the latter represents the total BSA fraction in solution. However, the substantial increase in proteolysis rate in the presence of coadsorbed phosphate points to a predominance of a mechanism involving direct interaction between the aqueous protease and the goethite-associated BSA. The proteolysis of the iron oxide-associated BSA may be further facilitated by the accumulation of negatively charged phosphate ions at the water–iron oxide interface. At pH 4.0, both ferrihydrite and goethite are positively charged, and BSA and protease are indicated to have isoelectric points above pH 4.0. Hence, the reduction of positive surface charge by phosphate may lower the electrostatic repulsion between protease and the surface and thereby promote the formation of ES complexes.

The extent of the response to increasing phosphate concentrations was different for ferrihydrite- and goethite-associated BSA (Figure 6). Although the maximum effect in the goethite system was observed at 1.0 μmol phosphate m\(^{-2}\), which then diminished with further increasing phosphate concentration.
concentrations, the effect was increasing with increasing phosphate concentrations in the case of ferrihydrite. The trend observed for the goethite system coincided with an increasing tendency of particle aggregation (Supporting Information Figure S18), likely induced by charge neutralization from the adsorbed phosphate ions. The smaller enhancement of proteolysis at high phosphate concentrations can be explained by a lower probability of ES complex formation in a more aggregated state, as a result of the slower diffusion of molecules into pores between or within aggregates. This suggested that the effect of aggregation on proteolysis was also consistent with the in situ IR results, which represent the proteolysis in the goethite film on the ATR crystal and thus proteolysis in a completely aggregated state. Accordingly, the considerably lower rates observed by the in situ IR technique as compared to those in dispersed suspensions were likely a consequence of aggregation (Figure 4). However, it is important to note that proteolysis also was observed in the goethite film, which suggests that aggregation is not sufficient to completely protect the adsorbed BSA.

Phosphate also induced aggregate formation in the ferrihydrite system (Supporting Information Figure S18), but in this case, the proteolysis of ferrihydrite-associated BSA increased with increasing phosphate concentrations. Again, we ascribe this difference between ferrihydrite and goethite to the higher total surface area in the experiment with ferrihydrite. Thus, the capacity to accommodate part of the added protease is higher for ferrihydrite than goethite. Accordingly, the increase of phosphate concentration exerted a larger impact on alleviating the protease adsorption in the ferrihydrite system, which in turn caused an increased proteolysis of the ferrihydrite-associated BSA that out-weighted the adverse effect from particle aggregation. These results exemplify the intricate interplay between the extent of particle aggregation and coadsorption and the competitive adsorption among enzymes, substrates, and other ions that finally determine the decomposition rates of iron oxide-associated substrates.

The complexity of proteolysis of iron oxide-associated proteins was further emphasized by the experiments performed in the presence of coadsorbed oxalate. Oxalate is an important plant root and microbial exudate, especially in the rhizosphere soil where concentrations can exceed 0.5 mmol kg\(^{-1}\) soil. The strong adsorption of oxalate to mineral surfaces implies that oxalate potentially can have a significant impact on the proteolysis of mineral-associated proteins and thus on the bioavailability of N. However, we only observed minor effects of coadsorbed oxalate on proteolysis of iron oxide-associated BSA (Supporting Information Figure S19), despite the fact that oxalate readily adsorbed onto the iron oxide-associated BSA causing detectable protease concentrations in the aqueous phase after the proteolytic reaction (Supporting Information Table S1 and Figure S20). This difference between oxalate and phosphate was attributed to the inhibitory effect of oxalate on BSA proteolysis in solution (Supporting Information Figure S21). Thus, we ascribe the minor effect of coadsorbed oxalate on the proteolysis of iron oxide-associated BSA to a balance between an inhibition similar to what is observed in solution and a promoting effect similar to that of phosphate. We did not explore the details of the inhibition mechanism, but previous studies have suggested two general inhibitory effects of oxalate or oxalic either via chelation of metal enzyme cofactors or via interactions with cationic moieties at the active site of the enzyme. Moreover, aspartic protease activity has been shown to be insensitive to strong metal chelators, for example, EDTA, suggesting that the inhibitory effect we observed was caused by interactions between the active site of the protease and oxalate.

**Environmental Implications.** Aspartic peptidases make a large contribution to the total proteolytic activity in most soils, and they are common among the extracellular peptidases expressed by soil fungi, including ectomycorrhizal plant root symbionts that dominate the boreal system. Given the results of the present study, plants in these boreal soils should have the potential to utilize also proteins immobilized by minerals as a source of N. Thus, adsorption to soil mineral surfaces is not an off-switch for enzymatic protein decomposition and subsequent N acquisition. As shown in our study, the initial rate of proteolysis of iron oxide-associated BSA can be similar to proteolysis of BSA in solution, but the rate is strongly regulated by the environmental conditions and, compared to BSA in solution, rates of prolonged hydrolysis of iron-oxide associated BSA are much slower. Moreover, the very slow rates close to the steady state, observed after 24 h (Figure 4), suggest that a fraction of iron-oxide associated BSA is not susceptible to proteolysis. The observation of both comparatively fast and very slow proteolysis points to a large variability in bioavailability of mineral-associated proteins in the environment. The promotion of proteolysis by coadsorption of other molecules, exemplified by the phosphate results, suggests that microbes can actively facilitate this process by the concomitant secretion of surface-reactive secondary metabolites and extracellular proteases. Such synergetic processes need to be studied together with the roles of other components of dissolved organic matter (e.g., lignin-like molecules and carbohydrates) in order to further unravel the molecular mechanisms behind N acquisition from mineral-associated proteins.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c00860.

Detailed descriptions of materials and methods, table of protease concentrations, figures of SEC chromatograms of BSA and protease solutions from proteolysis experiments, and IR spectra of iron oxide-associated BSA during proteolysis and MCR results and iron oxide-associated BSA suspensions (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Per Persson — Department of Biology, Microbial Ecology Group and Centre for Environmental and Climate Research (CEC), Lund University, SE-223 62 Lund, Sweden; orcid.org/0000-0001-9172-3068; Phone: +46 46-222 17 96; Email: per.persson@biol.lu.se

**Authors**

Zhaomo Tian — Department of Biology, Microbial Ecology Group and Centre for Environmental and Climate Research (CEC), Lund University, SE-223 62 Lund, Sweden

Tao Wang — Department of Biology, Microbial Ecology Group, Lund University, SE-223 62 Lund, Sweden

Anders Tunlid — Department of Biology, Microbial Ecology Group, Lund University, SE-223 62 Lund, Sweden
The authors declare no competing financial interest.

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