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**pH-dependent changes in structural stabilities of Bt Cry1Ac toxin and contrasting model proteins following adsorption on montmorillonite**

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4 1 **pH-dependent changes in structural stabilities of Bt Cry1Ac toxin and contrasting**  
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6 2 **model proteins following adsorption on montmorillonite**  
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9 3 Wenqiang Zhao<sup>a,b</sup>, Céline Poncet-Legrand<sup>c</sup>, Siobhán Staunton<sup>a,\*</sup>, Hervé Quiquampoix<sup>a,\*</sup>  
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3 14 **ABSTRACT**  
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5 15 The environmental fate of insecticidal Cry proteins, including time-dependent conservation of  
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7 16 biological properties, results from their structural stability in soils. The complex cascade of  
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10 17 reactions involved in biological action requires Cry proteins to be in solution. However, the pH-  
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12 18 dependent changes in conformational stability and the adsorption-desorption mechanisms of  
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14 19 Cry protein on soil mineral remain unclear. We used DLVO calculation and differential  
15  
16 20 scanning calorimetry to interpret the driving forces and structural stabilities of Cry1Ac and two  
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18 21 contrasting model proteins adsorbed by montmorillonite. The structural stability of Cry1Ac is  
19  
20 22 closer to the “hard” protein,  $\alpha$ -chymotrypsin, relative to the “soft” bovine serum albumin (BSA).  
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22 23 The pH-dependent adsorption of Cry1Ac and  $\alpha$ -chymotrypsin could be explained by DLVO  
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24 24 theory, whereas the BSA adsorption deviated from it. Patch controlled electrostatic attraction,  
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26 25 hydrophobic effects and entropy changes following protein unfolding on mineral surface could  
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28 26 contribute to Cry1Ac adsorption. Cry1Ac, like chymotrypsin, was partly denatured on  
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30 27 montmorillonite and its structural stability decreased with increasing pH. Moreover, small  
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32 28 changes in conformational heterogeneity of both Cry1Ac and chymotrypsin were observed  
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34 29 following adsorption. Conversely, adsorbed BSA was completely denatured regardless of  
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36 30 solution pH. The moderate conformational rearrangement of adsorbed Cry1Ac may partially  
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38 31 explain why the insecticidal activity of Bt toxin appears to be conserved in soils, albeit for a  
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40 32 relatively short time period.  
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48 33 *Keywords:* Cry1Ac protein,  $\alpha$ -chymotrypsin, bovine serum albumin (BSA), adsorption,  
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50 34 structural stability, differential scanning calorimetry (DSC)  
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## 1. Introduction

Transgenic *Bacillus thuringiensis* (Bt) crops have been widely planted in the past two decades due to their benefits for agriculture <sup>1,2</sup>. Although their use is restricted in Europe, they account for a large proportion of some crops worldwide (e.g., 80% for cotton and 32% for maize), with almost 100% uptake rates for some countries <sup>3</sup>. Bt crops release insecticidal Cry proteins into agricultural soils through root exudates and decomposition of residues <sup>4,5</sup>. They are rapidly and somewhat irreversibly adsorbed on soil mineral surfaces, and this adsorption largely determines their environmental fate <sup>6-9</sup>. Soil is known to be an important long-term reservoir for another class of proteins of environmental concern, namely prions, which are responsible for spongiform encephalopathy diseases with a remanence of infectivity of several years <sup>10,11</sup>. In contrast to prion proteins, Cry proteins appear to lose their biological activity more rapidly, within days or weeks, following adsorption <sup>12-17</sup>. This has important implications for monitoring programs, the non-lethal exposure of target insects, and the possible exposure of non-target soil-dwelling organisms <sup>18-20</sup>.

Desorption of Cry protein from ingested soil and enzymatic cleavage within an insect mid-gut is the limiting factor for toxicity. The insecticidal properties of Cry proteins involve a complex cascade of reactions, and require the protein to be in solution <sup>21,22</sup>. The temperature effects on protein adsorption and persistence in soil have led to the hypothesis that the decline in toxicity is due to physicochemical processes, which cause conformational changes of the adsorbed protein and increase its fixation, rather than microbial breakdown <sup>15,16</sup>. Because of the importance of interfacial interaction in regulating Cry protein persistence and toxicity in soil, a few previous studies have tried to characterize the conformational stability of adsorbed Cry proteins on surfaces. For instance, Sander et al. <sup>6</sup> observed high conformational stability of Cry1Ab as demonstrated by the absence of any supply rate dependence of the extent of Cry1Ab adsorption on apolar gold-coated QCM-D sensors. Madliger et al. <sup>23</sup> reported that Cry1Ab

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3 60 adsorption on and desorption from SiO<sub>2</sub> particles retained bioactivity, indicating high protein  
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5 61 conformational stability during adsorption-desorption cycles. Helassa et al.<sup>8</sup> monitored the  
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7 62 NH/ND exchange kinetics of Cry1Aa protein by FTIR spectroscopy and found that the  
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9 63 monomeric Cry1Aa toxin was adsorbed on montmorillonite without significant change of  
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11 64 secondary structure. However, the reported high structural stability of Cry toxin was based on  
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13 65 indirect evidences or partial quantification of molecular-level secondary structure (including  
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15 66 only  $\alpha$ -helix and  $\beta$ -sheets), which could not provide sufficient explanation for the rapid and  
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17 67 marked decline of insecticidal activity of soil-adsorbed Cry proteins within days or weeks<sup>12, 16,</sup>  
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19 68 <sup>17</sup>. To date, we still lack a macroscopic-level quantification of the overall structural stability of  
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21 69 adsorbed Cry protein, which may be useful for the better interpretation of its relatively short-  
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23 70 term persistence and decreased bioactivity in soil environment. The conformational changes of  
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25 71 various model proteins adsorbed by different surfaces have been widely reported<sup>24-28</sup>. Both the  
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27 72 extent of adsorption and the resulting conformation changes are strongly dependent on pH<sup>7, 29,</sup>  
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29 73 <sup>30</sup>. However, we are unaware of any research that has examined the pH-dependent structural  
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31 74 changes of Cry protein regulated by adsorption.  
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38 75 Differential scanning calorimetry (DSC) provides a sensitive probe for protein-surface  
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40 76 interaction<sup>31</sup>, which has been used in this work for the first time to compare the structural  
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42 77 stability of free and surface bound Cry proteins. The information gained from DSC is on a  
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44 78 macroscopic level, which enables the evaluation of the overall protein conformation<sup>32</sup>. Valid  
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46 79 parameters on protein stability and structural heterogeneity can be obtained from the  
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48 80 thermogram of protein unfolding process with increasing temperature, which are characterized  
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50 81 in terms of enthalpy, denaturation temperature, and the width of transition<sup>33</sup>. A few studies  
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52 82 have discussed the possible forces contributing to Cry protein adsorption on SiO<sub>2</sub> and humic  
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54 83 substances<sup>6, 34-36</sup>. However, their conclusions were based on the indirect observations of the  
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56 84 different adsorption or desorption phenomena of Cry proteins on several surfaces varying in  
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3 85 polarity and charge. Predictive models of protein-surface interactions, including the classical  
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5 86 Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, may also be of use<sup>37-39</sup> To date, the pH-  
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7 87 dependent DLVO interaction energies of surface-bound Cry toxin have not been quantitatively  
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9 88 interpreted. The comparison of DLVO prediction with experimental adsorption capacity may  
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11 89 be an effective method to evaluate if non-DLVO interactions contribute to structural  
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13 90 rearrangements in adsorbed proteins.

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17 91 The objectives of this work were to elucidate directly the adsorption-induced changes of  
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19 92 structural stabilities of Cry protein adsorption on a representative soil clay mineral  
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21 93 (montmorillonite) at various pH, and to deduce the driving forces using DSC, physico-chemical  
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23 94 characterization techniques and DLVO theory. Two well-studied model proteins, bovine serum  
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25 95 albumin (BSA) and  $\alpha$ -chymotrypsin, were used as reference proteins to compare with the  
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27 96 conformational flexibility of a Cry toxin. BSA has a large conformational adaptability (“soft”  
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29 97 protein), while chymotrypsin exhibits a much stronger internal coherence (“hard” protein)<sup>40</sup>.  
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31 98 <sup>41</sup>. Montmorillonite was chosen as a model mineral because of its large adsorption capacity for  
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33 99 Bt proteins in comparison to other soil minerals (for example goethite, kaolinite, hematite,  
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35 100 gibbsite and silica)<sup>7, 42, 43</sup>, and its negative pH-independent surface charge that facilitates the  
36  
37 101 investigation of pH-dependent adsorption phenomena<sup>8</sup>.

## 102 **2. Materials and methods**

### 103 *2.1. Sample preparation*

104 A natural strain of *Bacillus thuringiensis* subsp. Kurstaki HD73 that produces Cry1Ac  
105 protein was used. Cry1Ac toxin was chosen because of its expression in an important Bt crop  
106 (cotton) around the world<sup>44</sup>. This bacterial strain was cultivated in a fermentor, and activated  
107 and purified as described previously<sup>7</sup>. Due to the small concentrations of Cry proteins detected  
108 in soils (0.1 to 100 ng of toxin g<sup>-1</sup>)<sup>45,46</sup>, the protein would probably be present as monomers<sup>47</sup>.  
109 A pH above 6.5 and an ionic strength (IS) of at least 150 mM NaCl were necessary to keep the

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3 110 protein in a monomeric state <sup>7</sup>. To prevent polymerization, the purified toxin was stored in 10  
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5 111 mM CAPS (3-(Cyclohexylamino)-1-propanesulfonic acid), 350 mM NaCl, and pH 10.4 at -20  
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7 112 °C before use <sup>8</sup>. When required, the protein was slowly defrosted at 4 °C, and then the electrolyte  
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10 113 solution was exchanged by repeated centrifugation in an Amicon tube followed by dilution in  
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12 114 10 mM MOPS solution (3-(N-morpholino)propanesulfonic acid) containing 150 mM NaCl (pH  
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14 115 7.0, 8.0 or 9.0). The monomeric Cry1Ac has a molecular weight of 67 kDa and an isoelectric  
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16 116 point (IEP) of 6.4.

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19 117 Alpha-chymotrypsin (C4129, type II, from bovine pancreas) and bovine serum albumin  
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21 118 (BSA, CAS No. 9048-46-8) were both purchased from Sigma-Aldrich, and were used without  
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23 119 further treatment. The molecular dimension of chymotrypsin is  $5 \times 6 \times 6 \text{ nm}^3$ . It consists of 3  
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25 120 polypeptide chains bound by 2 inter strands disulfide bridges. Its IEP and molecular weight are  
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27 121 8.1 and 25 kDa, respectively <sup>48</sup>. Chymotrypsin was dissolved in buffer solution of 10 mM  
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29 122  $\text{NaH}_2\text{PO}_4$  at pH 7.0, 8.0 or 9.0.

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33 123 BSA is a globular, heart shaped ( $8 \times 3 \times 3 \text{ nm}$ ), flexible protein with an IEP of 4.8 and  
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35 124 molecular weight of 66.4 kDa. It is composed of 100 acidic and 99 basic side acids <sup>49</sup>. BSA was  
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37 125 dissolved in 10 mM  $\text{NaH}_2\text{PO}_4$  and 10 mM  $\text{CH}_3\text{COONa}$  solution (pH 4.0, 4.6 or 5.2) for  
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39 126 subsequent experiments. The above buffer solutions have been used in previous studies <sup>6-8, 36,</sup>  
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41 127 <sup>50-52</sup> to ensure that these proteins were always in monomeric forms and carried different net  
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43 128 surface charges at pH values above or below their IEPs (shown in Fig. 1) for the comprehensive  
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45 129 comparison of their structural stabilities.

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49 130 The clay mineral used was SWy-1 Wyoming montmorillonite obtained from the Source  
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51 131 Clays Repository of the Clay Minerals Society (Columbia, MO) <sup>53</sup>. The clay-sized fraction (<2  
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53 132  $\mu\text{m}$ ) was obtained by sedimentation and then saturated in sodium by repeated washings in 1 M  
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55 133 NaCl followed by washing in water and dialysis to provide a salt-free suspension, as previously  
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57 134 described <sup>7</sup>. The specific surface area of this montmorillonite is  $800 \text{ m}^2 \text{ g}^{-1}$  <sup>54, 55</sup>. The surface is  
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3 135 negatively charged over the tested pH range. All solutions were prepared using deionised water  
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5 136 from a Milli-Q purification system.  
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## 8 9 137 *2.2. Characterization of proteins and mineral*

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11 138 The hydrodynamic diameters and electrophoretic mobilities of proteins and  
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13 139 montmorillonite were determined using a Zetasizer (Nano ZS, Malvern Instruments Ltd., UK).

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15 140 Size distribution (% number) was measured by dynamic light scattering (DLS) and analyzed  
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18 141 by the Malvern software. The light scattered by the sample was measured at a backscatter angle  
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20 142 of 173° and the correlation functions were analyzed with a non-linear least squares fit. All DLS  
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22 143 measurements were conducted at 20 °C and repeated three times at different IS and pH values  
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24 144 (Table S1), and the experiment concentrations for Cry1Ac, chymotrypsin and BSA were 0.4,  
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26 145 0.5 and 0.5 mg mL<sup>-1</sup>, respectively.  
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29 146 The zeta potential was then calculated from the electrophoretic mobility using the  
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31 147 Smoluchowski equation. Solution pH values were chosen to encompass the IEP of each protein.  
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33 148 Thus different net surface charges of proteins could be observed and discussed in this study.  
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36 149 Hydrophobicity analysis was conducted by the adhesion to hydrocarbon test<sup>56, 57</sup>. Briefly,  
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38 150 1 mL of *n*-dodecane (laboratory grade) was added to 4 mL of protein solution or mineral  
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40 151 suspension (OD<sub>initial</sub>) in 10 mL test tube. The mixture was vortexed at full speed for 2 min,  
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42 152 followed by a 45 min rest period to obtain phase separation. The aqueous phase was pipetted  
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44 153 into another tube and its optical density (OD<sub>final</sub>) was determined by uv-visible  
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46 154 spectrophotometry. The hydrophobicity (%) was quantified as the percent of total proteins or  
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48 155 montmorillonite partitioned into the hydrocarbon phase: hydrophobicity = [(OD<sub>initial</sub> - OD<sub>final</sub>)  
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50 156 / OD<sub>initial</sub>] × 100. The mean percentage of hydrophobicity was calculated using duplicate or  
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52 157 triplicate samples across a range of IS and pH values.  
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## 58 158 *2.3. Adsorption measurement*

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3 159 The prepared protein solutions (methods shown in 2.1. *Sample preparation*) were mixed  
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5 160 with 2 mg of montmorillonite to reach a final volume of 2 mL. Protein-mineral mixtures were  
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7 161 statically incubated at 20 °C for 1 h to minimize protein denaturation at the water-air interface  
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10 162 <sup>58</sup>. Preliminary experiments showed that 1 h was sufficient for the reactions to reach an  
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12 163 equilibrium. After incubation, phases were separated by centrifugation at 20000 × g for 30 min.  
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14 164 The concentration of non-adsorbed protein (mg mL<sup>-1</sup>) in the supernatant solution as well as the  
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16 165 calibration curve were quantified by uv-visible spectrophotometry (Cry1Ac – wavelength 280  
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18 166 nm; chymotrypsin – 280 nm; BSA – 279 nm). Control experiments were performed in the  
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20 167 absence of proteins. The amount of adsorbed protein was calculated from the difference in  
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22 168 concentration before and after centrifugation, and the adsorption percentage (%) of protein was  
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24 169 also calculated. The experimental conditions of adsorption tests for each protein (Cry1Ac,  
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26 170 chymotrypsin and BSA) are indicated in Table S1. The adsorption of each protein with a fixed  
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28 171 initial concentration was measured at 3 different pH values. These conditions were chosen to  
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30 172 ensure that sufficient amounts of protein were adsorbed on montmorillonite, and that the  
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32 173 proteins were always monomers (determined by dynamic light scattering). *Previous studies*  
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34 174 *used comparable solution conditions* (protein concentration of 0.02–1.6 mg mL<sup>-1</sup>; pH 3–8; ionic  
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36 175 strength of 3–50 mM) to avoid protein-protein interactions that may result in bi- to multilayer  
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38 176 adsorption <sup>6, 36, 50-52</sup>. All adsorption experiments were performed in triplicate. Statistical  
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40 177 differences among adsorption or characterization data were analyzed using a Student's t test in  
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42 178 SPSS 17.0 software (SPSS Inc., Chicago, USA).

#### 43 179 *2.4. Calculation of protein-mineral DLVO interaction energy*

44 180 The DLVO theory was used to explain the adsorption of protein on solid surfaces as a  
45 181 function of separation distance by calculating the interaction energies between colloidal  
46 182 particles <sup>59</sup>. Total interaction energies were quantified as the sum of van der Waals and  
47 183 electrostatic interactions, which were calculated from the particle-substrate <sup>21</sup> potentials based

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3 184 on the DLVO theory <sup>60, 61</sup>. Positive interaction energy represents a repulsive force, while  
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5 185 negative energy indicates an attraction <sup>62</sup>. Further details on the calculation of electrostatic  
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7 186 interaction energy ( $\Phi_{EDL}$ ) <sup>63, 64</sup> and van der Waals attractive interaction energy ( $\Phi_{VDW}$ ) <sup>63, 65</sup> are  
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9 187 provided in the Supporting Information.

### 13 188 *2.5. Differential scanning calorimetry*

15 189 DSC experiments were carried out using a microcalorimeter (VP DSC, MicroCal Inc.,  
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17 Northampton, MA) to measure the excess heat capacity ( $C_p$ , kcal/mol/°C) of protein as a  
18 190 function of temperature <sup>33</sup>. The same procedure and buffer solution for adsorption measurement  
19  
20 191 were used to prepare individual protein suspensions and protein-mineral mixtures for DSC  
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22 192 analysis. Proteins were mixed with various buffers or montmorillonite and gently injected into  
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24 193 the sample-ampoule (0.5 mL) via a syringe. Corresponding buffers or montmorillonite  
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26 194 suspensions were used as references. The ampoules were allowed to stabilize at 20 °C for 10  
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28 195 min prior to the up-scanning experiment. Up-scan and down-scan rates of 60 °C/h were used  
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30 196 <sup>66</sup>. Released  $C_p$  value of the sample-ampoule was recorded relative to the reference ampoule in  
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32 197 the temperature range of 20–85 °C. Protein concentrations used in this study (0.4–0.5 mg mL<sup>-1</sup>)  
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34 198 generated satisfactory transition signals during the DSC measurements, and avoided  
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36 199 aggregation phenomena of proteins. The montmorillonite particles showed negligible  
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38 200 endothermic or exothermic transitions over the temperature range examined.  
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40 201

42 202 To obtain the heat capacity derived from the adsorbed protein, the heat capacity of the  
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44 203 mineral particles in the buffer and supernatant protein in adsorption mixture (after  
45  
46 204 centrifugation) were measured and subtracted from the protein-mineral adsorption mixture  
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48 205 measurement <sup>67</sup>. The DSC curves of free and adsorbed proteins were corrected for the baselines  
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50 206 by subtracting reference scan, and then analyzed with MicroCal Origin 7.0 software <sup>31</sup>. The  
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52 207 transition process of protein was recognized as a sharp endothermic peak. The denaturation  
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54 208 temperature  $T_m$  (°C) is defined as the temperature at which the maximum  $C_p$  occurs <sup>24</sup>. The  
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3 209 calorimetric enthalpy ( $\Delta H$ , kcal/mol) is calculated from the area of the transition peak, which  
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5 210 is correlated with the content of ordered secondary structure in protein. The sharpness of the  
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7 211 transition peak can be measured as the full width of transition at half maximum of the transition  
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9 212 peak (FWHM, °C). It is an index of the cooperative nature of the transition from native to  
10  
11 213 denatured state <sup>67</sup>.

### 15 214 **3. Results and discussion**

#### 17 215 *3.1. Protein and mineral properties*

19 216 Zeta potential reflects the net charge on colloidal surface. Fig. 1 shows that the zeta  
20  
21 217 potentials of Cry1Ac (from -8.8 mV to -15.1 mV) and BSA (from 16.9 mV to -3.1 mV)  
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23 218 became more negative with increased pH, which was ascribed to the deprotonation of functional  
24  
25 219 groups with the addition of base <sup>68</sup>. Chymotrypsin possessed a small amount of surface charge  
26  
27 220 (from 0.4 mV to -3.6 mV). Montmorillonite was the most negatively charged, and the zeta  
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29 221 potential values were significantly higher at IS of 10 mM MOPS+150 mM NaCl (from -38.8  
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31 222 mV to -43.8 mV) than those at low IS of 10 mM (from -48.9 mV to -55.1 mV) ( $P < 0.05$ ).  
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33 223 This result was attributed to the charge screening by counter ions and double layer compression  
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35 224 outside the mineral surfaces <sup>34</sup>.

36 225 The hydrodynamic diameters of Cry1Ac (~8 nm), chymotrypsin (~6 nm), BSA (~10 nm)  
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38 226 as well as their size distributions confirmed that proteins were all in monomeric states under  
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40 227 the tested conditions (Table S2 and Fig. S1). These values were comparable to the data reported  
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42 228 by previous literature <sup>69-71</sup>. [Different extents of electrostatic energies and self-associations](#)  
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44 229 [among protein molecules as well as interactions between protein and buffer molecules could](#)  
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46 230 [occur at various pH, and thus affect the hydrodynamic diameters of proteins and diffusion](#)  
47  
48 231 [coefficients](#) <sup>70,71</sup>. Montmorillonite showed a bimodal particle size distribution at low pH of 4.0–  
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50 232 [5.2](#). The sizes of montmorillonite platelets (>60 nm) were much larger than those of proteins  
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52 233 (<12 nm) ( $P < 0.05$ ). Generally, the size distribution of each protein showed insignificant

234 variation with pH ( $P > 0.05$ ).

235 The hydrophobicities (%) of proteins and montmorillonite are presented in Table S3. Across  
236 the solution conditions tested, chymotrypsin (42.9%–50.2%) and BSA (21.7%–40.1%) were  
237 relatively more hydrophobic than Cry1Ac (9.3%–11.3%) and montmorillonite (3.0%–13.3%)  
238 ( $P < 0.05$ ). The hydrophobicities of BSA showed large difference at varied pH ( $P < 0.05$ ), while  
239 those of other proteins and montmorillonite were generally consistent over different pH values  
240 ( $P > 0.05$ ).

### 241 3.2. Protein adsorption on montmorillonite

242 As shown in Fig. S2, the adsorption percentages followed the sequence of: BSA (99.5%–  
243 99.7%) > Cry1Ac (78.9%–87.2%) > chymotrypsin (62.1%–68.6%). BSA had the lowest energy  
244 barrier ( $0.19 k_B T$ ), which yielded the weakest repulsive interaction and improved its adsorption  
245 on montmorillonite (Table 1). This trend was consistent with the DLVO theory and  
246 demonstrated that van der Waals and electrostatic forces played a significant role in protein  
247 adsorption<sup>72</sup>. The energy barriers of Cry1Ac ( $0.14$ – $1.11 k_B T$ ) were stronger than those of  
248 chymotrypsin ( $< 0.52 k_B T$ ), revealing the stronger repulsive interactions between Cry1Ac and  
249 mineral. However, the percentages of Cry1Ac adsorbed were found to be larger than  
250 chymotrypsin. Several mechanisms might be involved in the Cry1Ac adsorption process to  
251 provide additional adsorption energy. Firstly, previous work confirmed that a similar Cry  
252 protein (Cry1Ab) had nonuniform surface charge distribution because domain II (IEP of 9.4)  
253 and III (IEP of 9.6) carried positive charges below pH 9, suggesting the patch controlled  
254 electrostatic attraction (PCEA) determined Cry1Ab adsorption on SiO<sub>2</sub><sup>23,34</sup>. In this study, the  
255 Cry1Ac molecules (88% identical sequence to Cry1Ab) tended to be oriented with positive  
256 patches towards montmorillonite especially at high IS<sup>36,73</sup>, which caused greater adsorption  
257 than that predicted by electrostatic energy. Secondly, after Cry1Ac adsorption on the mineral  
258 surface at pH 7.0–9.0, its losses of  $\Delta H$  percentages (62.5%–67.5%) were slightly greater than

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2  
3 259 the “hard” protein – chymotrypsin (55.1%–61.6%) (Tables 2 and 3). Hence, the contribution of  
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5 260 entropy resulted from the greater unfolding processes of adsorbed Cry1Ac was likely to  
6  
7 261 enhance its adsorption phenomenon <sup>67</sup>. Finally, the hydrophobic residues at the protein surface  
8  
9 262 can play a crucial role in adsorption when both surface and protein are negatively charged <sup>72</sup>. It  
10  
11 263 has been reported that the interactions between the residues of  $\alpha 7$  helix and the residues of  $\alpha 6$   
12  
13 264 helix together constitute a hydrophobic network in Cry1Ac protein <sup>74</sup>, and the hydrophobic  
14  
15 265 residues also exist in the loop regions of Cry1Ac domain II <sup>75</sup>. The above hydrophobic residues  
16  
17 266 were closely related to the correct folding of the molecule and the toxin-inclusion formation,  
18  
19 267 which would affect Cry protein stability and adsorption on surfaces <sup>8, 74</sup>. The montmorillonite  
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21 268 surface also exhibits some degree of hydrophobicity (Table S3). Thus, the hydrophobic effect  
22  
23 269 could contribute to Cry1Ac adsorption on the relatively apolar siloxane surface of  
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25 270 montmorillonite. Overall, the main driving forces of Cry1Ac adsorption were DLVO forces,  
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27 271 PCEA, and hydrophobic interaction as well as the configurational entropy gained upon the  
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29 272 structural rearrangements in proteins.  
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35 273 The adsorption of Cry1Ac and chymotrypsin on the mineral generally decreased with the  
36  
37 274 increase of solution pH ( $P < 0.05$ ). This trend was in agreement with the electrostatic and total  
38  
39 275 DLVO energies shown in Figs. 2a and 2b. High pH values generated larger electrostatic/DLVO  
40  
41 276 repulsions or lower attractions. In contrast, although the electrostatic and DLVO energies of  
42  
43 277 BSA-montmorillonite system revealed large changes with increasing pH (from attraction to  
44  
45 278 repulsion), the BSA molecules were almost completely adsorbed by montmorillonite regardless  
46  
47 279 of pH and differences in adsorption were not significant ( $P > 0.05$ ). This unexpected result  
48  
49 280 suggested that BSA adsorption phenomenon at different pH partially deviated from DLVO  
50  
51 281 theory, and other non-DLVO mechanisms may be involved. As unfolding of BSA on  
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53 282 montmorillonite has been shown by various methods <sup>49, 76</sup>, a loss of conformational entropy is  
54  
55 283 probably the secondary process responsible for the loss of the Gibbs energy of the system and  
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3 284 its adsorption behavior <sup>40</sup>.

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6 285 *3.3. DSC thermograms and thermodynamic parameters of proteins*

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8 286 The DSC thermogram for Cry1Ac or chymotrypsin showed a single endothermic peak at  
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10 287 varied pH (Figs. 3a and 3b), whereas transition peaks with shoulders at the left and right sides  
11  
12 288 were visible for BSA in Fig. 3c. A previous study also observed two transition peaks for BSA  
13  
14 289 adsorption on nanomaterials <sup>77</sup>. It suggested that the denaturation process did not follow a  
15  
16 290 simple two-state transition. The BSA molecule is folded into three domains in the native state,  
17  
18 291 and these domains may unfold at different temperature ranges <sup>78</sup>. The shapes and heights of the  
19  
20 292 transition peaks changed with the solution pH as well as the protein states (free or adsorbed),  
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22 293 indicating that the conformations of proteins were disturbed and some of the secondary  
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24 294 structures were unfolded after protein adsorption on montmorillonite.

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26 295 The DSC thermograms were evaluated with respect to the parameters of  $T_m$ ,  $\Delta H$ , and  
27  
28 296 FWHM. As seen in Table 2, compared to free Cry1Ac, the decrease in  $T_m$  (1.1–3.3 °C) and  
29  
30 297 reduction in  $\Delta H$  values (236.0–330.9 kcal/mol) for adsorbed Cry1Ac were found at pH 7.0–9.0.  
31  
32 298 It suggested that the surface bound-state Cry1Ac was partially denatured, and adsorbed Cry1Ac  
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34 299 was obviously less stable and structured than free Cry1Ac. As indicated by FWHM values, the  
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36 300 denaturation peaks for the adsorbed proteins were broader than for the free ones. This was an  
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38 301 indication of a weakened cooperativity in the melting process and a larger conformational  
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40 302 heterogeneity in the adsorbed state <sup>79</sup>. Our previous study observed that Cry1Aa adsorption on  
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42 303 montmorillonite had no effect on the  $\beta$ -sheets, but the  $\alpha$ -helical component decreased from 42%  
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44 304 to 38% after contact with the clay in the presence of 10 mM CAPS and 350 mM NaCl buffer <sup>8</sup>.  
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46 305 In the vicinity of the electronegative clay, the local higher concentration of counter ions could  
47  
48 306 protect the  $\alpha$ -helical domain from change <sup>8</sup>. Since Cry1Aa and Cry1Ac share a similar three-  
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50 307 dimensional structure consisting of three domains <sup>80, 81</sup>, it is reasonable to expect that the  
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52 308 relatively lower buffer concentration in this study (10 mM MOPS and 150 mM NaCl) may  
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3 309 induce greater extent of unfolding of the  $\alpha$ -helices in adsorbed Cry1Ac and lead to a reduction  
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5 310 in  $\Delta H$  value. In addition, the  $T_m$  and  $\Delta H$  values of free and adsorbed Cry1Ac decreased with  
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7 311 the increase of pH, suggesting lower Cry1Ac structural stability at higher pH. Cry1Ac was more  
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9 312 negatively charged at higher pH, and the electrostatic repulsions between Cry1Ac and mineral  
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11 313 became stronger, causing fewer Cry1Ac molecules to be adsorbed on montmorillonite with  
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13 314 lower surface coverage of proteins. Considering kinetic dynamics, the rate of Cry1Ac  
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15 315 molecules arriving at the mineral surface would be slower under these conditions, and therefore  
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17 316 the molecules would have more time and enough space to adjust their structure to the new  
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19 317 environment before a neighboring site became occupied by subsequently arriving molecules <sup>78</sup>.  
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22  
23 318 <sup>82</sup>. Hence, Cry1Ac structural rearrangement was more pronounced under unfavorable  
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25 319 adsorption conditions, which probably modified its biological properties. This finding also  
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27 320 provides a coherent explanation for the more rapid decline in immunologically detectable Bt  
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29 321 toxin in sandy soils (lower surface coverage of protein) than clay textured soils reported by a  
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31 322 previous study <sup>15</sup>.

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35 323 Chymotrypsin is classified as a structurally “hard” protein <sup>41,83</sup>, and it is expected to undergo  
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37 324 little structural change upon adsorption. However, we found markedly lower structural stability  
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39 325 of adsorbed chymotrypsin in comparison to the free state (i.e.,  $\Delta H$  values decreased by 55.1%–  
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41 326 61.6%) (Table 3). It has been reported that a reduction in transition enthalpy, upon adsorption  
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43 327 onto silica particles, was observed for rigid proteins – lysozyme and RNase only at low IS (10  
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45 328 mM sodium) <sup>84</sup>. On the one hand, this result was ascribed to the low IS of the buffer solution  
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47 329 (10 mM NaH<sub>2</sub>PO<sub>4</sub>). At low IS the intramolecular electrostatic repulsion will dominate, leading  
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49 330 to the proteins being less rigid and thus enhancing their ability to alter steric conformation <sup>85</sup>.  
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51 331 On the other hand, when unexpectedly large amounts (>25%) of chymotrypsin molecules were  
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53 332 adsorbed, hydrophobic interaction was involved in protein binding and chymotrypsin could  
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55 333 undergo extensive denaturation with loss of both tertiary and secondary structure <sup>56</sup>. Using  
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3 334 molecular dynamics simulations, a previous study reported that the presence of negative  
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5 335 (aspartic acid) and hydrophobic (leucine) residues could contribute to the stable adsorption of  
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7 336 chymotrypsin on amorphous silica <sup>51</sup>. FTIR data in our previous work further showed that a  
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10 337 small secondary structure unfolding affected about 15-20 peptide units of chymotrypsin after  
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12 338 its adsorption on montmorillonite <sup>83</sup>. With the partial loss of secondary structure, the unfolding  
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14 339 process of adsorbed chymotrypsin required smaller enthalpy than free protein. It should be also  
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17 340 noted that the  $T_m$  values of adsorbed chymotrypsin were larger than those of free ones,  
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19 341 suggesting that the transition temperatures of chymotrypsin were delayed upon adsorption on  
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21 342 mineral surface. Considering the crowding of chymotrypsin molecules at the mineral surface,  
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23 343 intermolecular association among adsorbed chymotrypsin molecules probably existed on  
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25 344 montmorillonite particles during the DSC heating process. This surface aggregation could give  
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27 345 more thermal stability to the adsorbed chymotrypsin than the free protein, resulting in higher  
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29 346 transition temperatures <sup>86</sup>. The wider FWHM of transition peaks at higher pH also displayed  
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31 347 larger conformational heterogeneity of the adsorbed chymotrypsin, due to the uneven structural  
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33 348 changes of chymotrypsin molecules.

37 349 The  $T_m$  and  $\Delta H$  for free BSA in buffer solutions corresponded well with the values ( $T_m$ :  
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39 350 52.5–67.5 °C;  $\Delta H$ : 130.9–173.7 kcal/mol) reported elsewhere (Table 4) <sup>24, 67, 78</sup>. The  $\Delta H_1$  and  
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41 351  $\Delta H_2$  values for free BSA were the largest at pH 4.6, which was close to the IEP of BSA (4.8).  
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43 352 Under these conditions, the repulsive electrostatic energy (Fig. 2a) between the BSA molecules  
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45 353 was small, which caused weak intermolecular association (indicated by slightly larger  
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47 354 hydrodynamic diameter at pH 4.6, Table S2) and subsequent higher unfolding enthalpy. The  
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49 355 thermograms of adsorbed-state BSA showed negligible calorimetric signals, and the  
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51 356 denaturation enthalpies for adsorbed BSA were strongly reduced relative to those in solutions.  
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53 357 This result indicated that the adsorbed BSA was almost completely denatured, and the contents  
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55 358 of ordered secondary structure components ( $\alpha$ -helix and  $\beta$ -sheet) decreased markedly following  
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3 359 adsorption<sup>49</sup>. In this work, since the BSA adsorption capacity was not mainly governed by  
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5 360 DLVO theory, the large decrease in BSA structural stability and the entropy gain originated  
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7 361 from the rearrangements within “soft” BSA molecule were the major reasons for the highest  
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9 362 BSA adsorption capacity<sup>67, 77</sup>. Hydrophobic interactions may also occur between the  
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11 363 hydrophobic subdomain IIIB of BSA and montmorillonite<sup>72</sup>.

#### 15 364 3.4. Comparing the structural stability of Cry1Ac with model proteins

16 365 The extent of structural change for the adsorbed Cry1Ac on mineral was closer to  
17  
18 366 chymotrypsin than to BSA due to the following reasons: (i) the  $\Delta H$  values declined by 62.5%–  
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20 367 67.5% after the free Cry1Ac adsorbed on montmorillonite, which were comparable to the  
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22 368 percentage  $\Delta H$  decrease of chymotrypsin (55.1%–61.6%); (ii) Cry1Ac and chymotrypsin both  
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24 369 showed small changes of FWHM values (<0.5 °C) following adsorption on the mineral; and  
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26 370 (iii) the pH-dependent adsorption abilities of Cry1Ac or chymotrypsin were consistent with the  
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28 371 patterns predicted by DLVO theory, which may result from similar adsorption mechanisms as  
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30 372 well as comparable degrees of structural changes upon adsorption. In summary, Cry1Ac and  
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32 373 chymotrypsin were both partially denatured in the adsorbed state and their adsorption processes  
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34 374 could be explained by DLVO theory. In contrast, the “soft” BSA was unfolded to a great extent  
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36 375 and was totally denatured at the mineral-solution interface, while its pH-dependent adsorption  
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38 376 deviated from DLVO prediction.

39 377 The modelling of protein adsorption on solid surfaces dates back to the 1980s. Arai and  
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41 378 Norde<sup>87</sup> studied the principles governing adsorption by adapting existing theories for polymers  
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43 379 and taking into account the electrostatic interactions and the three-dimensional structures of  
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45 380 proteins. Norde and co-workers<sup>88</sup> deduced from their experimental data that the adsorption of  
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47 381 a protein is related to the stability of its native structure. Hard proteins have since been described  
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49 382 as having a higher unfolding energy than soft proteins (e.g., 60 kJ mol<sup>-1</sup> for the hard lysozyme  
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51 383 and 21 kJ mol<sup>-1</sup> for the soft  $\alpha$ -lactalbumin), and both proteins have a similar molecular weight  
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3 384 <sup>89</sup>. In parallel, the energy required to unfold an  $\alpha$ -helix is lower than that required for a  $\beta$ -sheet,  
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5 385 and soft proteins are characterized by a higher  $\alpha$ -helix/ $\beta$ -sheet ratio than hard proteins <sup>9, 90</sup>. As  
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7 386 indicated by the changes of  $\Delta H$  values, the structural loss of adsorbed Cry1Ac was slightly  
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9 387 higher than chymotrypsin (hard protein) but much lower than BSA (soft protein). It is therefore  
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11 388 expected that the proteins in our study would have the following structures: 1) majority of  $\beta$ -  
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13 389 strands for chymotrypsin and fewer for Cry1 Ac; and 2) majority of  $\alpha$ -helices for BSA and  
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15 390 fewer for Cry1Ac. The Uniprot database allowed us to calculate the following proportions: BSA:  
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17 391 71%  $\alpha$ -helix, 3%  $\beta$ -strand (ratio 24);  $\alpha$ -chymotrypsin: 15%  $\alpha$ -helix, 43%  $\beta$ -strand (ratio 0.35);  
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19 392 Cry1 Ac: 28%  $\alpha$ -helix, 29%  $\beta$ -strand (ratio 1) <sup>91-93</sup>. The Cry1 protein has an intermediate  
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21 393 behavior between the two model proteins studied.  
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#### 27 394 **4. Conclusions and environmental implications**

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29 395 To conclude, this is the first study to evaluate quantitatively the pH-dependent DLVO  
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31 396 theory driving Cry protein-clay mineral interaction, as well as the different structural stabilities  
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33 397 between Cry toxin and model proteins. A combination of electrostatic and van der Waals forces,  
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35 398 PCEA, hydrophobic effect and entropy gained from the protein unfolding process could  
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37 399 contribute to Cry1Ac adsorption on montmorillonite. The structural stability of Cry1Ac protein  
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39 400 was slightly lower than the “hard”  $\alpha$ -chymotrypsin but much higher than the “soft” BSA. With  
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41 401 the increase of pH, the structural stabilities of both free and surface-bound Cry1Ac decreased.  
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45 402 Previous studies have reported high conformational stability of Cry1A proteins that retained  
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47 403 insecticidal activity on mineral surfaces, as evidenced by the supply rate-independent extent of  
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49 404 Cry adsorption on apolar gold or minor molecular-level change of  $\alpha$ -helix and  $\beta$ -sheet contents  
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51 405 in surface-bound protein <sup>6, 8, 23</sup>. It was therefore proposed that the small conformational change  
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53 406 and associated gain in conformational entropy contributed little to Cry1A adsorption <sup>6, 23</sup>.  
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55 407 However, these findings could not explain the rapid and marked decline of protein insecticidal  
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57 408 activity in soils <sup>13, 16, 17</sup>. From the perspective of macroscopic-level quantification and DLVO  
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3 409 prediction, we observed moderate structural loss of adsorbed Cry1Ac with non-DLVO process  
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5 410 involved (e.g., entropy gain during protein unfolding), which could be a coherent explanation  
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7 411 for the fact that their insecticidal properties remain in soils for only days or weeks<sup>13,16,17</sup>. This  
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9 412 can reduce the risk of exposure of non-target soil dwelling organisms, but can also affect the  
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11 413 feasibility of monitoring the introduction of Cry toxins to soil by transgenic Bt crops.  
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#### 15 414 **Declaration of Competing Interest**

16  
17  
18 415 The authors declare that they have no known competing financial interests or personal  
19  
20 416 relationships that could have appeared to influence the work reported in this paper.  
21  
22

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31  
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34 422 subsp. *Kurstaki* HD73.  
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3 423 **List of Tables and Figures**  
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5

6 424 **Table 1**  
7

8 425 Primary energy barriers ( $1^{\circ}$  max), secondary energy minima ( $2^{\circ}$  min) and corresponding  
9  
10 426 separation distances ( $h$ ) between proteins and montmorillonite calculated by DLVO theory as  
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13 427 a function of pH.  
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15 428 **Table 2**  
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18 429 Transition enthalpy ( $\Delta H$ ), transition temperature ( $T_m$ ), and full width at half maximum (FWHM)  
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20 430 of free and adsorbed Cry1Ac at pH 7.0–9.0. Parameters were obtained from the fitting process  
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22 431 in Non-2-State model. Values in parentheses represent standard errors of the fitting parameters  
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24  
25 432 given by MicroCal Origin 7.0 software.  
26

27 433 **Table 3**  
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30 434 Transition enthalpy ( $\Delta H$ ), transition temperature ( $T_m$ ), and full width at half maximum (FWHM)  
31  
32 435 of free and adsorbed chymotrypsin at pH 7.0–9.0. Parameters were obtained from the fitting  
33  
34 436 process in Non-2-State model. Values in parentheses represent standard errors of the fitting  
35  
36  
37 437 parameters given by MicroCal Origin 7.0 software.  
38

39 438 **Table 4**  
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42 439 Transition enthalpy ( $\Delta H$ ), transition temperature ( $T_m$ ), and full width at half maximum (FWHM)  
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44 440 of free and adsorbed BSA at pH 4.0–5.2. Parameters were obtained from the fitting process in  
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46 441 Non-2-State model. Values in parentheses represent standard errors of the fitting parameters  
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49 442 given by MicroCal Origin 7.0 software.  
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3 443 **Figure 1.**  
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5 444 Zeta potentials of Cry1Ac protein, chymotrypsin, bovine serum albumin (BSA), and  
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7 445 montmorillonite as a function of pH. Error bars represent the standard deviations of three  
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9 446 replicates.  
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13 447 **Figure 2.**  
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15 448 Electrostatic energy (a) and DLVO energy profiles (b) of protein-montmorillonite interactions  
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17 449 as a function of separation distance ( $h$ ).  $h$  value is scaled by the radius of protein. Mont stands  
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19 450 for montmorillonite.  
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22 451 **Figure 3.**  
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24 452 DSC thermograms for free and adsorbed (a) Cry1Ac protein, (b) chymotrypsin, and (c) BSA  
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26 453 as a function of pH. The up-scan and down-scan rates were 60 °C/h.  
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3 454 **Supporting Information**  
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6 455 **Table S1** Experimental conditions of protein adsorption test on montmorillonite (1 mg mL<sup>-1</sup>)  
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9 456 and physico-chemical characterization of proteins and montmorillonite. These buffer  
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11 457 solutions were used to ensure that the Cry1Ac protein, as well as BSA and chymotrypsin,  
12  
13 458 were always in monomeric forms and carried different surface charges for the  
14  
15 459 comprehensive comparison of their structural stabilities.

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18 460 **Table S2** Hydrodynamic diameters of Cry1Ac protein, chymotrypsin, bovine serum albumin  
19  
20 461 (BSA) and montmorillonite as a function of solution pH. The data are presented as the means  
21  
22 462 ± standard deviations.

23  
24  
25 463 **Table S3** Hydrophobicities (%) of Cry1Ac protein, chymotrypsin, bovine serum albumin (BSA)  
26  
27 464 and montmorillonite as a function of solution pH. The data are presented as the means ±  
28  
29 465 standard deviations.

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32 466 **Fig. S1** Size distribution of Cry1Ac protein, chymotrypsin, bovine serum albumin (BSA), and  
33  
34 467 montmorillonite measured by dynamic light scattering at 20 °C as a function of pH.

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36 468 **Fig. S2** Adsorption percentages of Cry1Ac protein, chymotrypsin, and bovine serum albumin  
37  
38 469 (BSA) on montmorillonite at 20 °C as a function of pH. Error bars represent the standard  
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40 470 deviations of three replicates.  
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471 **Table 1**

472 Primary energy barriers (1° max), secondary energy minima (2° min) and corresponding  
 473 separation distances ( $h$ ) between proteins and montmorillonite calculated by DLVO theory as  
 474 a function of pH.

Protein	pH	1° max ( $k_B T$ )	$h$	2° min ( $k_B T$ )	$h$
Cry1Ac	7.0	0.14	0.23	-0.07	0.80
	8.0	0.38	0.23	-0.05	0.91
	9.0	1.11	0.11	-0.02	1.14
Chymotrypsin	7.0	–	–	–	–
	8.0	–	–	–	–
	9.0	0.52	0.34	–	–
BSA	4.0	–	–	–	–
	4.6	–	–	–	–
	5.2	0.19	0.46	-0.01	2.96

475 Note: “–”denotes no energy barrier or secondary energy minimum.  
 476



477 **Table 2**

478 Transition enthalpy ( $\Delta H$ ), transition temperature ( $T_m$ ), and full width at half maximum (FWHM)  
479 of free and adsorbed Cry1Ac at pH 7.0–9.0. Parameters were obtained from the fitting process  
480 in Non-2-State model. Values in parentheses represent standard errors of the fitting parameters  
481 given by MicroCal Origin 7.0 software.

Cry1Ac state	$T_m$ (°C)	$\Delta H$ (kcal/mol)	FWHM (°C)
Free Cry1Ac (pH 7.0)	76.5 (0.2)	492.0 (16.9)	9.1
Free Cry1Ac (pH 8.0)	73.0 (0.3)	442.5 (15.3)	7.5
Free Cry1Ac (pH 9.0)	70.9 (0.1)	377.4 (12.0)	7.0
Adsorbed Cry1Ac (pH 7.0)	73.2 (0.3)	161.1 (4.1)	9.1
Adsorbed Cry1Ac (pH 8.0)	70.8 (0.1)	143.7 (2.2)	7.8
Adsorbed Cry1Ac (pH 9.0)	69.8 (0.1)	141.4 (2.4)	7.5

482

483 **Table 3**

484 Transition enthalpy ( $\Delta H$ ), transition temperature ( $T_m$ ), and full width at half maximum (FWHM)  
485 of free and adsorbed chymotrypsin at pH 7.0–9.0. Parameters were obtained from the fitting  
486 process in Non-2-State model. Values in parentheses represent standard errors of the fitting  
487 parameters given by MicroCal Origin 7.0 software.

Chymotrypsin state	$T_m$ (°C)	$\Delta H$ (kcal/mol)	FWHM (°C)
Free chymotrypsin (pH 7.0)	47.2 (0.0)	184.7 (1.1)	10.4
Free chymotrypsin (pH 8.0)	43.5 (0.0)	203.1 (0.9)	10.7
Free chymotrypsin (pH 9.0)	42.7 (0.2)	215.2 (6.9)	10.9
Adsorbed chymotrypsin (pH 7.0)	48.4 (0.1)	71.0 (1.0)	10.1
Adsorbed chymotrypsin (pH 8.0)	43.6 (0.3)	85.6 (1.4)	10.7
Adsorbed chymotrypsin (pH 9.0)	42.8 (0.1)	96.6 (1.8)	10.7

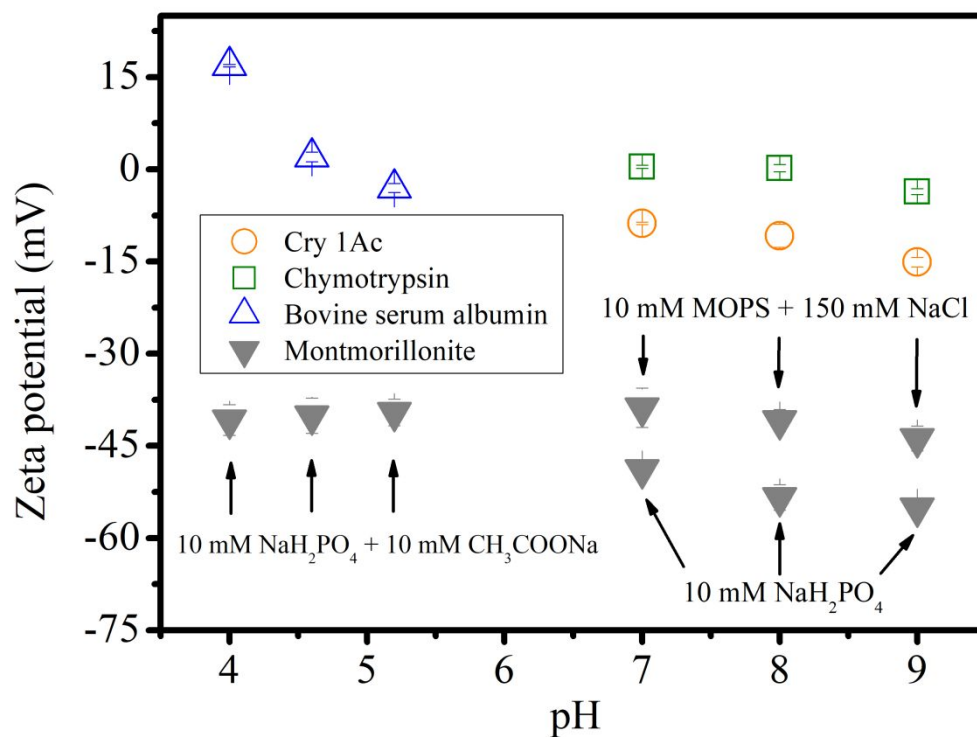
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489 **Table 4**

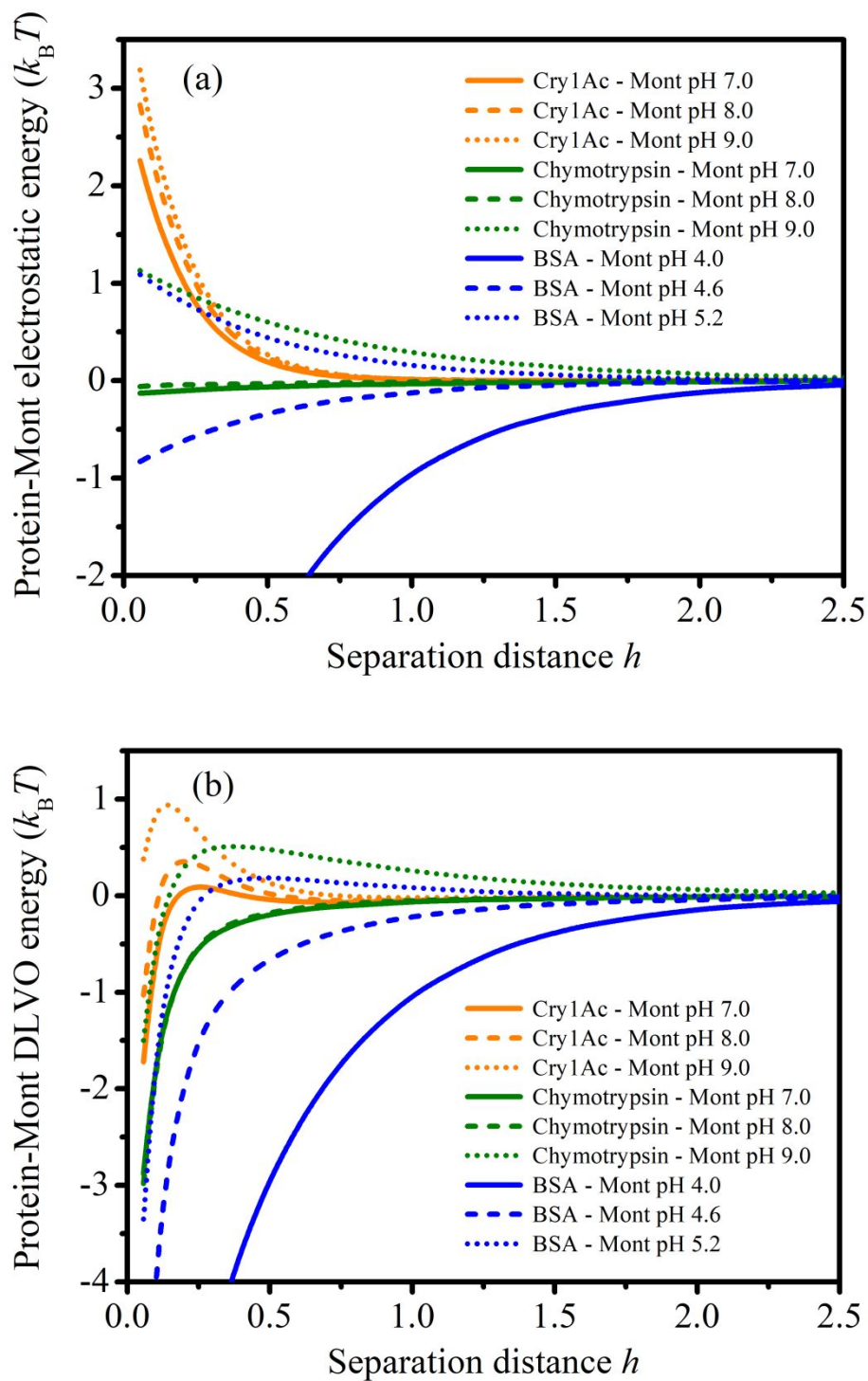
490 Transition enthalpy ( $\Delta H$ ), transition temperature ( $T_m$ ), and full width at half maximum (FWHM)  
 491 of free and adsorbed BSA at pH 4.0–5.2. Parameters were obtained from the fitting process in  
 492 Non-2-State model. Values in parentheses represent standard errors of the fitting parameters  
 493 given by MicroCal Origin 7.0 software.

BSA state	$T_{m1}$ (°C)	$\Delta H_1$ (kcal/mol)	FWHM <sub>1</sub> (°C)	$T_{m2}$ (°C)	$\Delta H_2$ (kcal/mol)	FWHM <sub>2</sub> (°C)
Free BSA (pH 4.0)	44.1 (0.1)	82.0 (1.0)	13.6	67.1 (0.1)	61.3 (0.9)	10.7
Free BSA (pH 4.6)	55.7 (0.1)	121.1 (1.9)	10.7	71.9 (0.1)	87.9 (2.0)	10.1
Free BSA (pH 5.2)	61.8 (0.1)	116.9 (2.3)	9.1	70.5 (0.2)	27.3 (2.5)	7.7
Adsorbed BSA (pH 4.0)	–	–	–	–	–	–
Adsorbed BSA (pH 4.6)	–	–	–	–	–	–
Adsorbed BSA (pH 5.2)	–	–	–	–	–	–

494 Note: The thermograms of adsorbed-state BSA (totally denatured) showed negligible  
 495 calorimetric signals, and no DSC parameters could be obtained by MicroCal Origin 7.0  
 496 software.

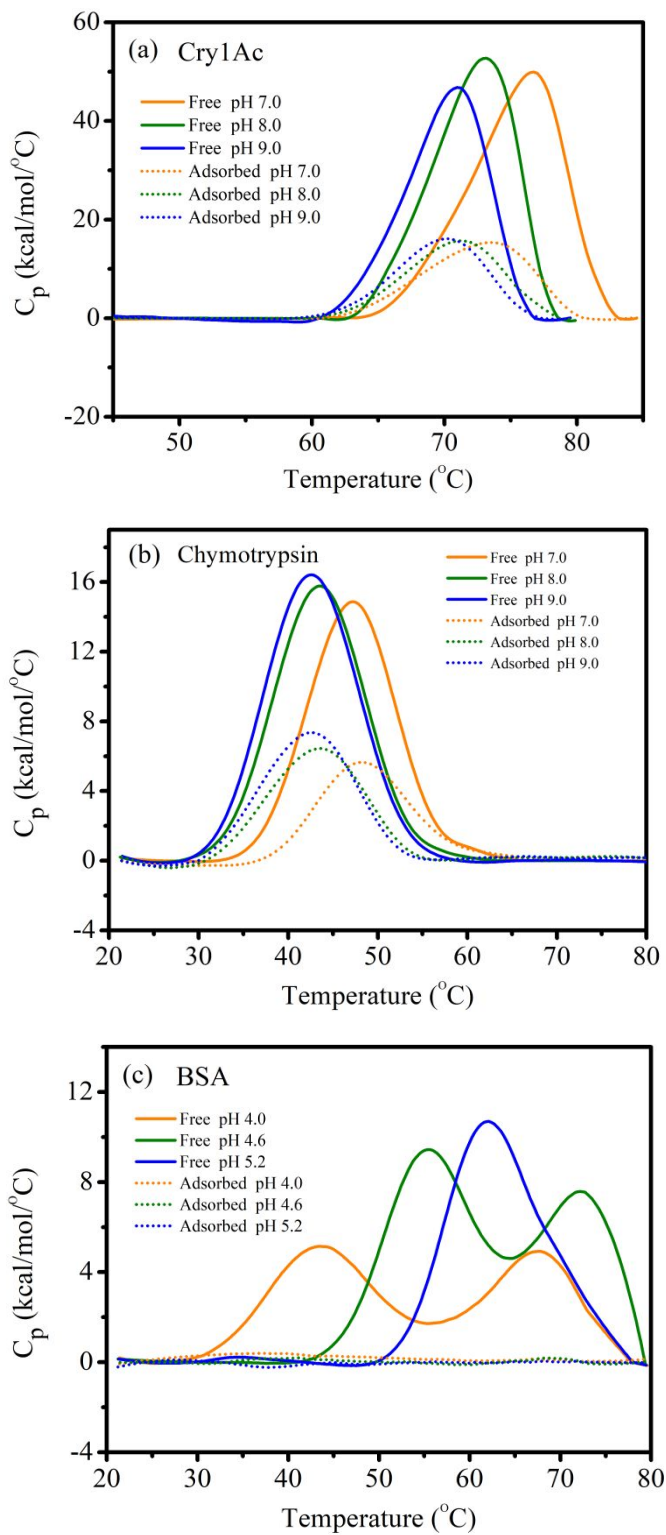
497 **Figures**

498 **Fig. 1.** Zeta potentials of Cry1Ac protein, chymotrypsin, bovine serum albumin (BSA), and  
499 montmorillonite as a function of pH. Error bars represent the standard deviations of three  
500 replicates.  
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503 **Fig. 2.** (a) Electrostatic energy and (b) DLVO energy profiles of protein-montmorillonite  
 504 interactions as a function of separation distance ( $h$ ).  $h$  value is scaled by the radius of protein.  
 505  
 506 Mont stands for montmorillonite.



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510 **Fig. 3.** DSC thermograms for free and adsorbed (a) Cry1Ac protein, (b) chymotrypsin, and (c)

511 BSA as a function of pH. The up-scan and down-scan rates were 60 °C/h.

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