JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Adsorption of Peanut (*Arachis hypogaea*, Leguminosae) Proteins by Activated Charcoal

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The binding of peanut protein allergens to activated charcoal (AC), used medically for gastric decontamination following the ingestion of toxic substances, was investigated for potential clinical application. Crude peanut extract (CPE) or purified peanut protein allergens Ara h 1 and 2 were co-incubated with AC under a variety of conditions followed by centrifugation to remove the AC and adsorbed protein. The resulting supernatant solution was analyzed for unadsorbed protein by gel electrophoresis and quantitative protein assay. The extent of protein adsorption by a known amount of AC was determined. Protein binding to AC was rapid and irreversible. The extent of adsorption was unaffected by pH, but was optimal near physiological salt concentrations. Denatured proteins, or those of larger molecular weight, required more AC than smaller or native proteins. The extent of protein binding increased with temperature, supporting the concept that protein molecules diffuse into vacant pores of appropriate size on the charcoal surface.

KEYWORDS: Peanut; protein; activated charcoal; adsorption

INTRODUCTION

Food is the single most common cause of anaphylaxis seen in hospital emergency rooms (*1*). Approximately 30,000 food-induced anaphylactic events are seen in American emergency departments each year, 200 of which are fatal (*2*). Peanuts or tree nuts cause more than 80% of these reactions (*3*). Peanut allergy is one of the most serious of the immediate hypersensitivity reactions to foods (*4*). This derives from its prevalence, persistence, and severity. Approximately 1.1% of the U.S. population, or over 3 million people, are allergic to peanuts or tree nuts (*5*). The incidence of peanut allergies in young American children has doubled during a recent five-year period (*6*). This has been suggested to be due to the increased popularity and use of peanut products by the population and the introduction of peanut products to children's diets at an early age (*7, 8*). It is therefore increasingly common for the public to be exposed to this abundantly utilized and often disguised food. This has led to increasing rates of sensitization, accidental ingestion, anaphylaxis, and even death in peanut-allergic individuals.

Even small amounts of protein allergen can elicit an allergic response. Peanut allergen levels as low as 0.1 to 2 mg have been established by Hourihane et al. to cause significant symptoms in allergic individuals (*9*). In an oral provocation study (*10*), 25% of the peanut-allergic participants responded to less than 100 mg of peanut seeds, a considerably lower dose than that for many other common allergens. If these relatively small amounts of protein allergens could be securely adsorbed onto activated charcoal (AC), they might innocuously traverse the gastrointestinal tract without eliciting an allergic response.

Despite the severity of peanut-induced allergic reactions and the increasing number of people affected, there are no effective treatments other than the immediate administration of epinephrine and emergency care. Avoidance of the allergenic food is currently the only available method for sensitized patients to prevent further allergic responses (*11*). However, hidden allergen sources in unlabeled foods pose a continuous potential threat to sensitized individuals. AC is the most common form of gastric decontamination given to potentially poisoned children in U.S. emergency departments (*12*). It nonspecifically binds most organic molecules by hydrophobic interaction. While not currently considered as a treatment option for the accidental ingestion of peanut, treatment with AC could potentially reduce prolonged allergen exposure in the stomach. This might then be a safe and effective method of rendering these allergens unavailable to initiate additional IgE-mediated allergic responses such as the biphasic reaction in which residual allergen triggers a second response after the effects of an initial epinephrine treatment have worn off. Removal of allergens from the GI tract by AC would then provide an established, simple, safe, and inexpensive treatment to supplement the use of self-injectable epinephrine (prescribed Epi-Pen use) to treat symptoms, which is currently the standard of care for food allergy. This would be especially important if medical assistance would be delayed.

A previous report from this laboratory (*13*) demonstrated that while the roasting process reduces the solubility of peanut proteins, this insoluble material could be resolubilized under standard GI tract conditions. These solubilized proteins were

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10.1021/jf801731n CCC: $40.75
Published on Web 11/05/2008

highly allergenic. The presence of peanut allergens in the insoluble roasted peanut material provides a continuous source of major allergens to the gastrointestinal mucosal immune system. A related study (*14*) showed that insoluble particulate material, present in both the stomach and small intestine, continuously released IgE-reactive peanut allergens as a peanut meal passed through the gastrointestinal tract. This extended release could provide a prolonged source of allergen and be largely responsible for the biphasic anaphylactic reactions observed in some patients. If shown to be effective, the early administration of AC could potentially adsorb initially soluble allergens and continue to bind allergens progressively released during digestion throughout the gastrointestinal tract.

The feasibility of using AC to bind peanut proteins and form an insoluble complex was recently investigated by Vadas and Perelman (*15*). This is the only published study of this concept. They found that AC removed IgE-binding peanut proteins from solution and suggested that it may be useful as an adjunct to the present standard management of peanut anaphylaxis. However, before this form of treatment can be generally recommended, much more about this phenomenon remains to be investigated. The study by Vadas and Perelman was not performed at physiological pH values or physiologically relevant temperature. Their protein−AC mixtures were incubated for only two different time periods, and the reversibility of the protein binding by AC was not studied. This paper introduced a very promising potential method of treatment for a serious medical problem with few other treatment options. The research presented in this investigation fully describes the factors affecting protein adsorption onto AC and suggests the feasibility of this novel form of supplemental treatment for hypersensitivity reactions to peanuts.

AC may be a useful adjunctive treatment to slow or prevent absorption of peanut protein from the gut after accidental ingestion by persons with peanut allergy. All that remains is to ascertain whether adsorption onto AC can be applied to allergenic proteins as a safe, practical, and efficient method of gastric decontamination after ingestion of protein allergens. The present study describes the adsorption of protein onto AC, and the biochemical and physical factors affecting the binding of peanut protein allergens to AC under different conditions. Although clinical efficacy still needs to be determined, these in vitro results demonstrate the feasibility of further in vivo animal studies to determine the potential efficacy of this as a therapeutic technique.

MATERIALS AND METHODS

Reagents. Raw shelled peanut seeds (*Arachis hypogaea*, Leguminosae, Florunner cultivar) were purchased from Red Flower Online (Linden, NJ). Activated charcoal (AC) powder and bovine serum albumin of 99+% purity used as a standard for protein determinations were obtained from Sigma-Aldrich (St. Louis, MO). If not otherwise stated, all chemicals were of analytical grade.

Protein Samples. Peanut seeds were finely ground and defatted by extraction with diethyl ether. Total peanut protein extract was produced by suspending the resulting dry, defatted peanut flour in phosphate buffer (20 mM sodium phosphate, 100 mM NaCl, pH 7.5) and stirring for 30 min at room temperature. After centrifugation, the supernatant solution was recovered as crude peanut extract (CPE). Enriched Ara h 1 protein was purified by ammonium sulfate precipitation and cation exchange column chromatography as previously described (*16*). Enriched Ara h 2 protein was purified by ammonium sulfate fractionation, anion exchange and hydrophobic interaction column chromatography as previously described (*17*).

Protein Adsorption to AC Experiments. Protein samples were co-incubated with weighed amounts of AC under various conditions in 1.5 mL microcentrifuge tubes. Unless otherwise indicated the incubation mixtures contained 100 mM NaCl to approximate the salt concentration present throughout the gastrointestinal tract (*18*). The tubes were incubated in a water bath at a physiological temperature of 37 °C with agitation by vortexing every 30 s. A 2 min incubation period was experimentally determined to be sufficient for protein binding to be complete. Protein samples incubated with sufficient activated charcoal showed decreasing concentrations of soluble protein with time up to 2 min, after which the protein concentration was zero. This was true for both crude peanut extract and purified proteins. At the end of the incubation period, the tubes were centrifuged for 1 min to sediment the AC along with any adsorbed protein. The resulting supernatant solution was assessed for the presence of protein by SDS−PAGE or Bradford protein assay.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Denaturing SDS−PAGE was carried out by the method of Laemmli (*19*) on 12% Tris-glycine gels in a vertical cell unit at a constant voltage of 100 V. Protein bands were visualized using Coomassie Brilliant Blue R-250 staining. The gels were photographed with a Kodak EDAS 290 digital gel photodocumentation system.

Protein Determination. Protein concentrations were determined according to the method of Bradford (*20*). Bovine serum albumin standards and controls were used in the appropriate range of expected protein concentrations.

RESULTS

Quantity of AC Required To Adsorb CPE Protein from Solution. The minimum amount of AC required to adsorb all protein from solution was determined. 0.5 mL of CPE diluted to 1 mg/mL in 20 mM phosphate buffer, pH 7.5 with 100 mM NaCl was incubated in separate microcentrifuge tubes with various amounts of AC at 37 °C for 2 min. The tubes were mixed by vortexing every 30 s. The tubes were then centrifuged to pellet the AC and any adsorbed protein, and the supernatant solutions were analyzed by SDS−PAGE and quantitative protein assay. **Figure 1A** shows the Coomassie stained gel in which successive lanes represent samples incubated with increasing amounts of AC. As the AC:protein ratio increases from 0 in lane 1 to 80:1 in lane 9, the amount of soluble protein remaining in solution steadily decreases until protein is no longer visible at a 90:1 ratio in lane 10. Panel **B** shows the corresponding Bradford quantitative protein assay values of aliquots of the same solutions run on the gel. AC amounts are expressed as a ratio of mg AC per mg protein in the original incubation mixture. The concentration of unbound protein can be seen to progressively decrease as the AC:protein ratio increases. After a 80:1 AC:protein ratio, the protein remaining in solution was not detectable on the gel by protein staining or by the Bradford quantitative protein assay at a detection limit of 1 μg/mL (*21*).

Quantity of AC Required To Adsorb Purified Peanut Proteins from Solution. The experiment described in **Figure 1** above was repeated using purified peanut protein allergens Ara h 1 and Ara h 2. The proteins were co-incubated with increasing amounts of AC under the same conditions and analyzed in the same manner. The resulting gel is shown in **Figure 2A**, and the quantitative protein assay in **Figure 2B**. The amount of AC, expressed as the AC:protein ratio, increases from lane 1 to 10. The significantly larger Ara h 1 molecule (MW of Ara h 1 trimer = 200 kDa) required a slightly larger AC:protein ratio for complete adsorption of the protein onto the AC than did the smaller Ara h 2 protein (MW of Ara h 2 = 18 kDa). Protein was still visible on the gel and detectable by protein assay up to a AC:protein ratio of 100:1 in the case of the large Ara h 1 molecule (lane 6 in panels A and B), and an AC:protein ratio of 80:1 for the smaller Ara h 2 (lane 5 in panels C and D). This

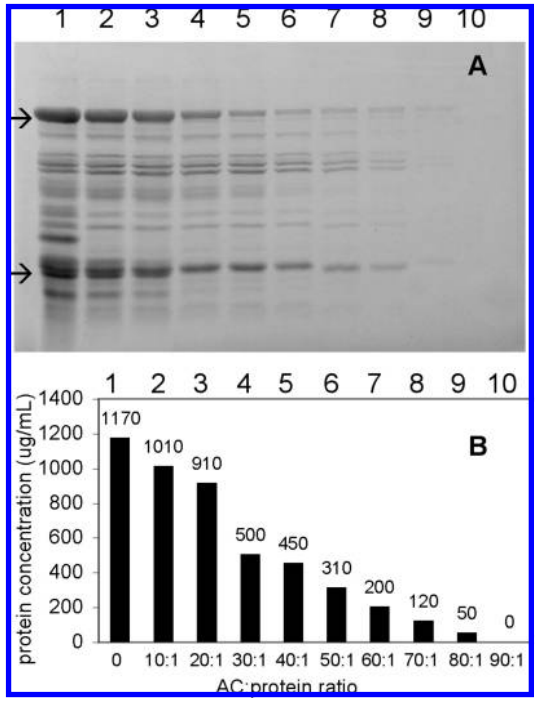

Figure 1. Adsorption of CPE protein upon incubation with increasing amounts of AC. Aliquots of CPE were incubated in separate tubes with increasing amounts of AC for 2 min at 37 °C. The supernatant liquid was then analyzed by SDS−PAGE (**A**) and Bradford quantitative protein assay (**B**). AC amounts are expressed as AC:protein ratios by weight. Arrows indicate the location of the major allergens Ara h 1 (top) and Ara h 2 (bottom).

could be due to the fact that the larger Ara h 1 protein molecules can diffuse into only the largest pores on the porous surface of the AC particles, whereas the smaller Ara h 2 proteins can be accommodated in the same large pores as well as much smaller ones.

Effect of pH on Protein Binding to AC. To determine whether peanut protein adsorption onto AC is affected by pH, protein-binding experiments were conducted at two physiologically relevant pH values: pH 7 (oral and intestinal pH) and pH 2 (gastric pH) (*14*). CPE was adjusted to the appropriate pH with HCl and incubated with increasing amounts of AC in separate microcentrifuge tubes at 37 °C for 2 min. The samples were then processed and analyzed by SDS−PAGE and protein assessed as previously described. The results are shown in **Figure 3**. Increasing amounts of AC bound progressively more protein at both pH 7 (shown in lanes 1−7) and at pH 2 (shown in lanes 9−15). The ratio of AC to protein required for complete adsorption of the protein was essentially the same at both pH values. No detectable protein remained in solution at AC:protein ratios above 80:1.

Effect of NaCl on Protein Binding to AC. The effect of salt concentration on peanut protein adsorption onto AC was determined by incubating CPE with a fixed, limiting amount of AC in the presence of increasing concentrations of NaCl. A constant 40:1 AC:protein ratio was used since that was known to bind approximately half of the protein so that any differences in binding due to the salt could be readily observed. After incubation with AC, the protein samples were processed and analyzed by electrophoresis and protein assay as usual. The results in **Figure 4** indicate that the relative adsorption of peanut proteins onto AC is optimal at the intermediate salt concentrations tested, roughly around physiological values. Relatively little protein is adsorbed, and consequently much protein remains

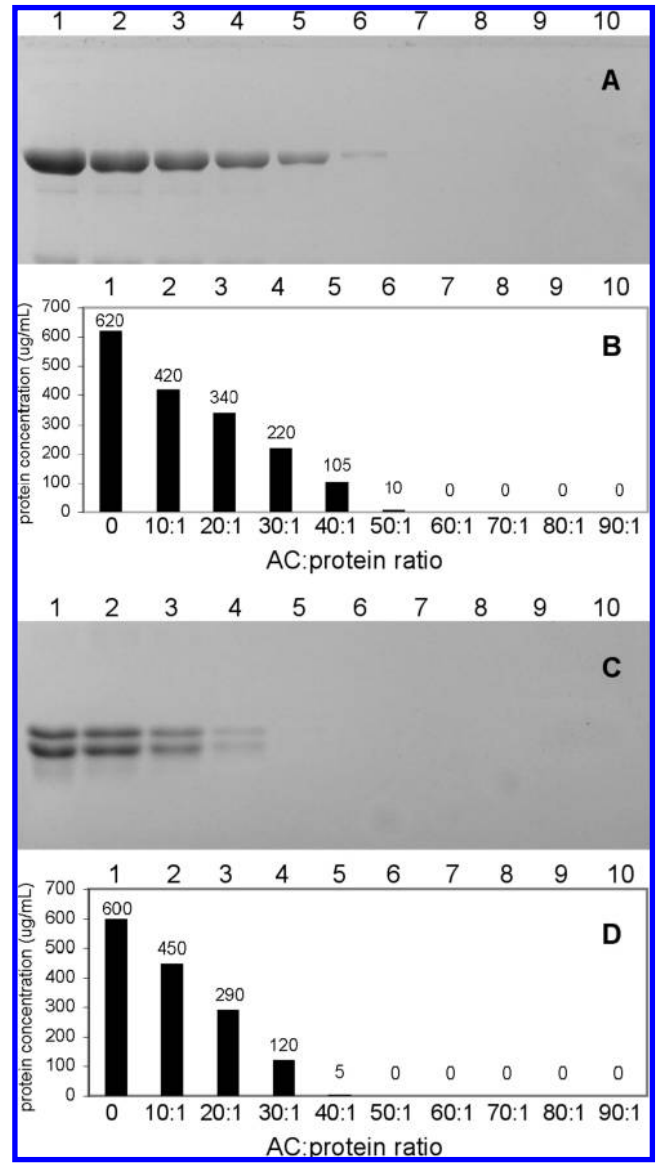

Figure 2. Adsorption of purified peanut proteins upon incubation with increasing amounts of AC. Aliquots of Ara h 1 (panels **A** and **B**) or Ara h 2 (panels **C** and **D**) were incubated in separate tubes with increasing amounts of AC for 2 min at 37 °C. The supernatant liquid was then analyzed by SDS−PAGE (**A**, **C**) and Bradford quantitative protein assay (**B**, **D**). AC amounts are expressed as AC:protein ratios by weight.

in solution, at very low salt concentrations (0 mM in lane 1) and at very high salt concentrations (1100 mM in lane 10). Protein adsorption increases to a maximum at intermediate salt concentrations or 200−250 mM (lanes 5 and 6). At very high and low salt concentrations, the adsorption is less efficient. This could be explained by the fact that most proteins fold into their native, compact conformation at intermediate salt concentrations. At high or low ionic strength they progressively denature (*22*), unfolding to occupy larger molecular volumes thus limiting their ability to occupy the smaller pores on the AC surface restricting them, therefore, to only the larger pores.

Effect of Protein Denaturation on Protein Binding to AC. The adsorption of both CPE and purified Ara h 1 onto AC was determined in the presence of increasing concentrations of the protein denaturing agent urea. Samples of either CPE or Ara h 1 were co-incubated with a fixed, limiting amount of AC in the presence of increasing concentrations of urea. Under these conditions some, but not all, of the protein will be adsorbed so

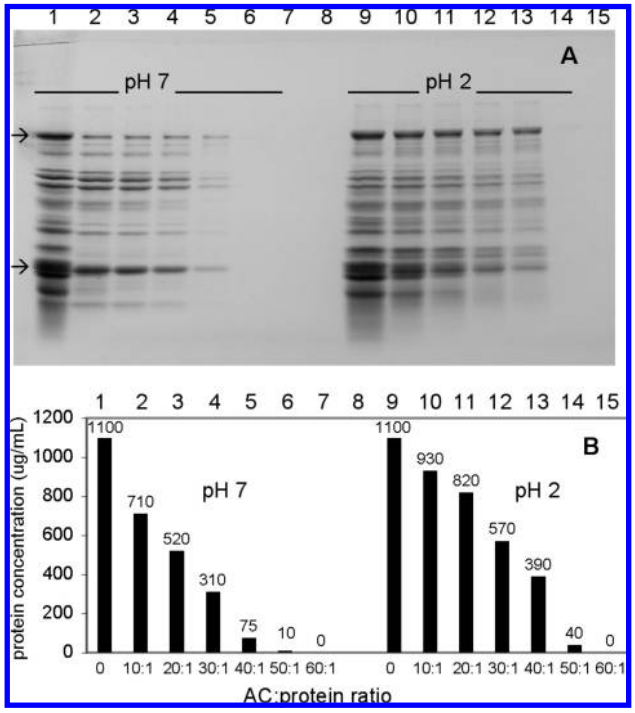

Figure 3. Effect of pH on protein binding to AC. Aliquots of CPE were incubated in separate tubes with increasing amounts of AC at either pH 7 (lanes 1−7) or pH 2 (lanes 9−15). The supernatant liquid was then analyzed by SDS−PAGE (**A**) and Bradford quantitative protein assay (**B**). AC amounts are expressed as AC:protein ratios by weight. Arrows indicate the location of the major allergens Ara h 1 (top) and Ara h 2 (bottom).

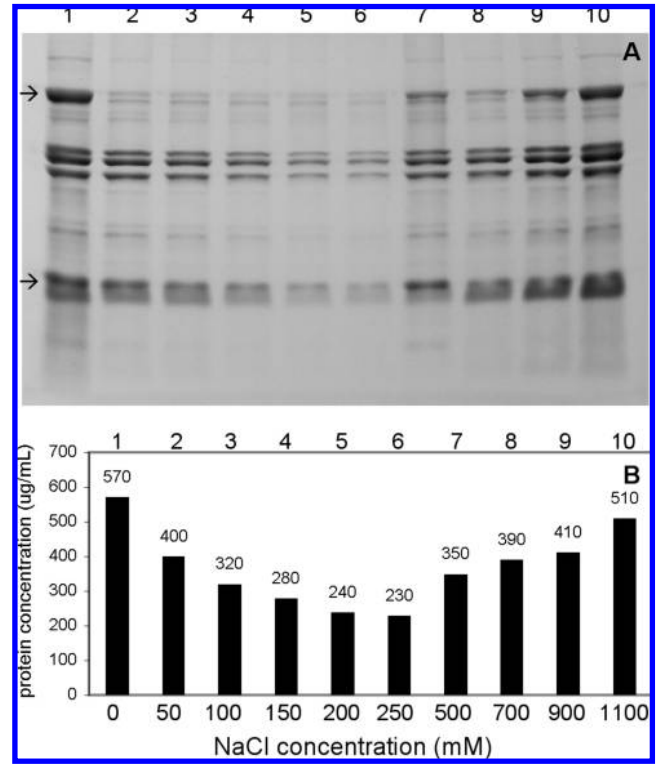

Figure 4. Effect of salt on protein binding to AC. Aliquots of CPE were incubated in separate tubes containing a fixed, limiting amount of AC with increasing amounts of NaCl. The supernatant liquid was then analyzed by SDS−PAGE (**A**) and Bradford quantitative protein assay (**B**). The concentration of unadsorbed protein at each NaCl concentration is given in μg protein/mL. Arrows indicate the location of the major allergens Ara h 1 (top) and Ara h 2 (bottom).

any differences in binding due to the urea could be readily observed. After incubation with AC, the protein samples were processed and analyzed as usual. The results are shown in **Figure 5**. Panel **A** shows the effect of urea on the binding of CPE to AC. Lane 1 represents a control sample of the 2 mg/mL CPE solution used for the experiment in which no AC was added. No protein was adsorbed under these conditions, so the entire sample remained in solution. With increasing concentrations of urea in lanes 2−10, the CPE proteins are partially adsorbed by the limiting amount of AC with a loss of efficiency as the urea begins to partially denature the proteins. Upon complete denaturation of the proteins, at 4 M urea and above, the amount of protein bound by the AC decreases significantly by visual assessment (lanes 9 and 10). The larger, unfolded protein molecules are presumably able to occupy only the larger pores on the AC surface, reducing the number of potential binding sites for the proteins. A similar trend was observed when a single purified protein, Ara h 1, was used in a similar experiment (shown in panel **B**). Results with another protein denaturant, SDS (results not shown), were obtained when the same protein samples were progressively denatured. In this experiment, the protein binding was significantly reduced upon full denaturation of the proteins at a concentration of 1% SDS.

Adsorption of Additional Protein onto Protein-Saturated AC. The theory that large proteins can occupy only large pores on the surface of AC, but smaller proteins can occupy both relatively large and small pores on the AC surface, was tested. The results are shown in **Figure 6**. Lane 2 contains a sample of purified Ara h 1, a relatively large protein (MW of Ara h 1 trimer = 200 kD). AC was saturated by co-incubation with an excess of purified Ara h 1. Excess, unadsorbed Ara h 1 remained in solution after this saturation step as seen in lane 3 indicating that the AC was indeed saturated. The Ara h 1 saturated AC was then washed repeatedly, and an aliquot of the third wash, shown in lane 4, contained no free protein in solution detectable by SDS−PAGE. This indicates that all unbound or weakly bound protein has been removed. Aliquots of the Ara h 1 saturated AC were then incubated with solutions of either Ara h 1 or the smaller Ara h 2 (MW of Ara h 2 = 18 kDa). Two different concentrations of each purified protein were used. After incubation, the AC along with any adsorbed protein was removed by centrifugation and the resulting supernatant solutions were analyzed by SDS−PAGE. Lanes 6 and 7 show aliquots of 0.25 mg/mL and 0.5 mg/mL Ara h 1 before the addition of AC. Lanes 8 and 9 show the resulting supernatant solution after 1 mL portions of these solutions were added to 50 mg of Ara h 1 saturated AC. No additional adsorption of Ara h 1 protein by the Ara h 1 saturated AC was observed. This is presumably because all of the pores on the AC surface large enough to accommodate Ara h 1 were already occupied. Lanes 11 and 12 show aliquots of 0.25 mg/mL and 0.5 mg/mL Ara h 2 before the addition of AC. Lanes 13 and 14 show the resulting supernatant solution after 1 mL portions of these solutions were added to 50 mg of Ara h 1 saturated AC. In this case, all of the Ara h 2 protein in the 0.25 mg/mL solution was adsorbed, and most of the protein in the 0.50 mg/mL solution was adsorbed by the Ara h 1 saturated AC. This is presumably because the smaller pores on the AC surface that could not accommodate the Ara h 1 protein during the initial saturation step were unoccupied and could subsequently accept the significantly smaller Ara h 2 molecule.

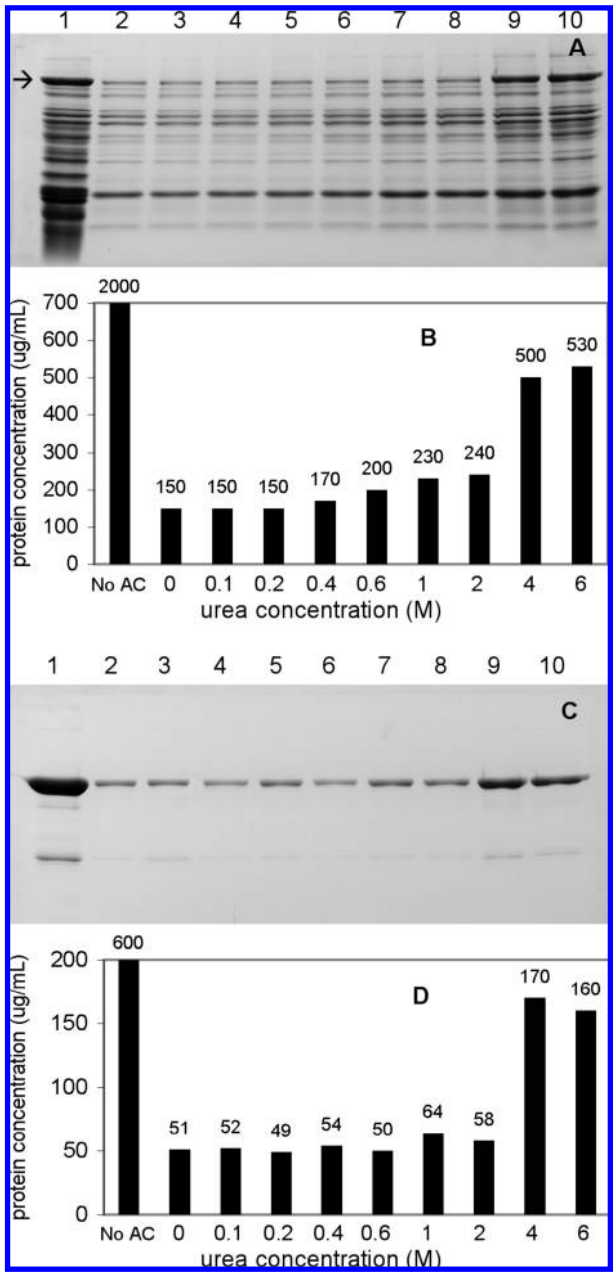

Figure 5. Effect of protein denaturation on binding to AC. Aliquots of CPE (**A**, **B**) or Ara h 1 (**C**, **D**) were incubated in separate tubes containing a fixed, limiting amount of AC with increasing amounts of urea. The supernatant liquid was then analyzed by SDS−PAGE (**A**, **C**) and Bradford quantitative protein assay (**B**, **D**). The concentration of unadsorbed protein at each urea concentration is given in μg of protein/mL. The arrow indicates the location of Ara h 1 in the CPE protein mixture.

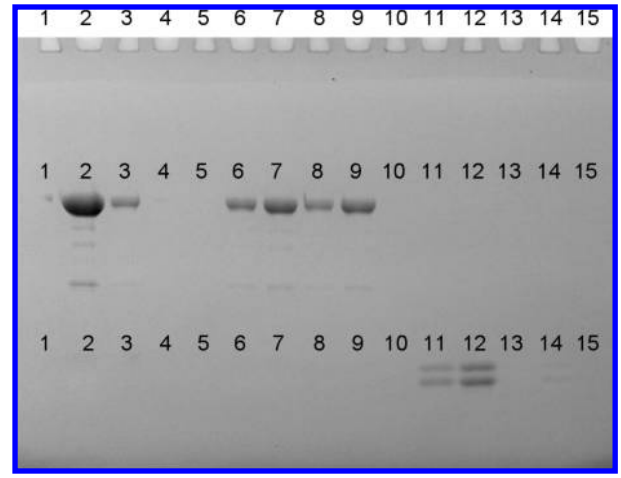

Figure 6. Adsorption of additional Ara h 1 or Ara h 2 onto Ara h 1 saturated AC. Aliquots of AC were saturated with purified Ara h 1 protein (lane 2). The resulting solution (lane 3) contained excess, unbound protein. After washing the AC, the supernatant solution contained no unbound protein (lane 4). Aliquots of 0.25 mg/mL or 0.50 mg/mL Ara h 1 were added to empty tubes (lanes 6 and 7) and tubes containing Ara h 1 saturated AC (lanes 8 and 9). None of the added protein was adsorbed by the previously saturated AC. Aliquots of 0.25 mg/mL or 0.50 mg/mL Ara h 2 were then added to empty tubes (lanes 11 and 12) and tubes containing Ara h 1 saturated AC (lanes 13 and 14). In this case, most of the additional protein was adsorbed by the previously saturated AC.

DISCUSSION

All soluble protein from a peanut extract was found to be completely adsorbed by a 90-fold excess of AC. This result is in contrast with that reported previously by Vadas and Perelman (*15*), in which a 200:1 protein:AC ratio was required. However, their experiments were conducted in 10 mM saline at 22 °C. The experiments described here were performed in 100 mM saline, which is much closer to physiological or stomach salt concentrations, and was shown in **Figure 4** to enhance protein binding to AC. The adsorption of protein onto AC was observed to increase progressively with temperature in these studies (results not shown). Experiments at 37 °C not only represent physiological temperature, but also increase protein binding to AC.

Purified Ara h 1 protein requires more AC for complete adsorption compared with either CPE or the smaller Ara h 2, likely due to its requirement for relatively large pores on the surface of the AC. These studies also show that protein binds to AC with equal efficiency at pH 7 and pH 2. Previous studies in this laboratory (*14*) have shown that stomach content pH rises immediately after a meal from pH 2 to pH 7. It then returns to pH 2 over the course of 30−45 min before again returning to neutral pH in the intestines. It is important that the affinity of AC for protein does not change over this pH range. The total ionic strength of a solution affects the adsorptive capacity of the AC as shown in **Figure 4**. Salt concentrations around physiological values are optimal for binding. The experiments described here used a 100 mM NaCl concentration to simulate physiological conditions.

Protein denaturation by either urea or SDS significantly increases the amount of AC required for complete binding of the protein. Again, this may be due to the unfolding and increase in molecular size that accompanies protein denaturation, requiring more large pores on the AC surface to accommodate the denatured protein molecules. This was confirmed when AC saturated with a large protein (Ara h 1) was found to bind additional small protein (Ara h 2), but not additional Ara h 1.

Protein adsorption onto AC is rapid. Vadas and Perelman tested only two incubation times, but more extensive experimentation confirmed their general result. It is shown here that AC binds 90% of its ultimate capacity in the first 2 min of contact. Protein adsorption is essentially complete after 5 min (results not shown). It was also found that once bound to AC, all attempts to desorb any bound protein were unsuccessful, indicating that the binding process is essentially irreversible.

Future plans include the expansion of this work into experiments conducted in vivo using an animal model system. It is

hoped that the results of this research will determine the efficacy of AC as a supplemental treatment for the accidental ingestion of peanuts by allergic individuals. By preventing continuous exposure of the allergen in the stomach and intestines, it could prevent further IgE-mediated allergic responses and eliminate any biphasic reaction. If successful, this will expand the treatment options available for accidental ingestion by peanut allergic individuals. Although the focus of this research is on peanut allergens, this same concept should be applicable to other food allergies.

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Received for review June 5, 2008. Revised manuscript received September 20, 2008. Accepted October 2, 2008. This project was funded by The Food Allergy & Anaphylaxis Network.

JF801731N