Release of Soluble Protein from Peanut (*Arachis hypogaea*, Leguminosae) and Its Adsorption by Activated Charcoal

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Peanut (*Arachis hypogaea*, Leguminosae) allergy is a major cause of food-induced anaphylaxis. The potential use of activated charcoal (AC) to adsorb and reduce the bioavailability of peanut protein allergens for use in the moderation of hypersensitivity reactions was investigated. The rate and extent of protein release from peanut and the adsorption of the solubilized protein by AC was determined under physiological pH values and confirmed in vivo using a porcine animal model system. Peanut proteins were adsorbed with equal efficiency at pH 2 and 7 and are completely removed from solution by an AC/protein ratio of approximately 80:1. This suggests that AC can bind protein under gastric (pH 2) or intestinal (pH 7) conditions. The rapid adsorption of soluble peanut allergens and the continuous binding of allergens released from peanut particulate material suggest the potential efficacy of using AC for gastric decontamination and possible elimination of a biphasic allergic reaction.

KEYWORDS: Peanut; *Arachis hypogaea*; protein; solubilization; activated charcoal; adsorption

INTRODUCTION

Peanut (*Arachis hypogaea*, Leguminosae) allergy is one of the most serious of the immediate hypersensitivity reactions to foods (*1*). Approximately 1.1% of the U.S. population, over 3 million people, are allergic to peanuts or tree nuts (*2*). These numbers appear to be increasing. The incidence of peanut allergies in young American children has doubled during a recent 5-year period (*3*). This increasing prevalence has also been noted worldwide (*4*). These high numbers of allergic individuals have a huge medical consequence. An adverse reaction to food is the most common cause of anaphylaxis seen in hospital emergency rooms (*5*). About 200 of the 30000 food-induced anaphylactic events seen in American emergency departments each year are fatal (*6*), and > 80% are caused by peanuts or tree nuts (*7*). Strict avoidance of the allergenic food is currently the only available method to prevent further reactions (*8*). This is increasingly difficult and can be compromised by the frequent use of peanuts in food preparations. Despite precautions, > 50% of individuals who are allergic to peanuts can be expected to have an accidental exposure and reaction over a 2-year period (*2*).

Activated charcoal (AC) is the most common form of gastric decontamination given to potentially poisoned children in U.S. emergency departments (*9*). A recent policy statement from the Committee on Injury, Violence, and Poison Prevention of the American Academy of Pediatrics indicated that AC "is the most effective intervention for reducing the bioavailability of ingested substances" (*10*). Although not typically considered as a treatment option for the accidental ingestion of peanut, AC may be a safe and effective method of rendering these allergens unavailable

for absorption in the gastrointestinal tract. Its clinical use could then be considered. This is particularly relevant because even small amounts of the allergen can cause a reaction. In an oral challenge study, 25% of the peanut-allergic participants responded to < 100 mg of peanut seeds (*11*). Peanut allergen levels as low as 0.1−2 mg have been established by Hourihane et al. to cause significant symptoms in allergic individuals (*12*). Therefore, if these relatively small amounts of protein allergens could be securely adsorbed onto AC, they might innocuously traverse the gastrointestinal tract without eliciting an allergic response. Results from this investigation suggest that AC may provide an established, simple, safe, and inexpensive treatment as an adjunct to epinephrine.

Previous studies from this laboratory (*13*) have shown that following the introduction of chopped peanut into the porcine stomach, insoluble peanut particulate material continuously released IgE-reactive proteins. This extended release provides a prolonged source of allergen to the gastrointestinal mucosal immune system. This phenomenon could be largely responsible for biphasic anaphylactic reactions. Biphasic reactions have been observed in up to one-third of patients with fatal or near-fatal reactions (*1*). The intensity of the late-phase reaction is typically more severe and can even be fatal, with the secondary response occurring as long as 72 h after resolution of the primary event (*14*, *15*). A recent study of Australian children found a significantly lower biphasic reaction frequency of only 11%, but almost half had a more serious biphasic anaphylactic reaction (*16*). Jarvinen et al. documented a 19% rate of use of more than one dose of epinephrine for a food-allergic reaction among children using any epinephrine (*17*). Multiple doses were provided by health care professionals in 94% of the reactions (*18*). Early administration of AC may scavenge latent peanut allergens and thus prevent biphasic anaphylactic reactions.

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The first suggested use of AC to bind peanut proteins and form an insoluble complex was recently proposed by Vadas and Perelman (*19*). They found that AC removed IgE-binding peanut proteins from solution in vitro and suggested that it may be useful as an adjunct to the present standard management of peanut anaphylaxis. However, these studies were performed under non-physiological conditions that have subsequently been shown to significantly affect the amount of AC required for complete adsorption of soluble protein (*20*). There have as yet been no studies of protein adsorption by AC under actual physiological conditions in vivo.

MATERIALS AND METHODS

Human Subjects and Experimental Animals. Human serum from peanut-allergic volunteers was used in accordance with policies established by the University of Arkansas for Medical Sciences (UAMS) Institutional Review Board. Piglets (Oak Hill Genetic, Ewing, IL) were used in accordance with UAMS Institutional Animal Care and Use Committee guidelines and the Arkansas Children's Hospital Research Institute Policy on Humane Utilization and Care of Laboratory Animals. All procedures were performed under the supervision of UAMS veterinary personnel.

Reagents. Raw shelled peanut seeds (*A. hypogaea*, Leguminosae, Florunner cultivar) were purchased from Red Flower Online (Linden, NJ). AC powder and bovine serum albumin used as a standard for protein determinations were obtained from Sigma-Aldrich (St. Louis, MO).

SDS-PAGE. Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*21*) on 12% Tris–glycine gels in a vertical cell unit at a constant voltage of 100 V. Equal volumes of all samples were loaded so protein concentrations could be compared. 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 Quantification. Protein concentrations were determined spectrophotometrically according to the method of Bradford (*22*) using bovine serum albumin standards.

Western Immunoblotting. SDS-PAGE gels were electrophoretically transferred to nitrocellulose membranes using an XCell blot module from Invitrogen Life Technologies (Carlsbad, CA) according to the manufacturer's instructions. The membrane was blocked and probed with a 1:15 dilution of pooled sera from two highly peanut-allergic individuals who were shown to be reactive toward all major peanut allergens in immunoblotting experiments with purified proteins and crude peanut extract. The bound IgE was detected using a 1:1000 dilution of alkaline phosphatase conjugated mouse anti-human IgE and visualized using a 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium substrate system (Sigma-Aldrich, St. Louis, MO).

In Vitro Release of Soluble Protein from Peanut. Raw shelled peanut seeds were finely chopped using a commercial coffee grinder. One milliliter of buffer (20 mM sodium phosphate, 100 mM NaCl adjusted to either pH 2 or 7) was added to microcentrifuge tubes each containing 10 mg of chopped peanut (1:100 w/v). The tubes were incubated at 37 °C with periodic mixing for the appropriate time. After incubation, tubes were microfuged at 15000*g* for 30 s to pellet the particulate matter. Aliquots of the supernatant liquid were then removed for analysis by SDS-PAGE or Bradford quantitative protein assay.

Adsorption of Peanut Protein by AC. The protein solutions prepared above, typically 1 mg/mL protein, were co-incubated with AC in 1.5 mL microcentrifuge tubes at 37 °C for 5 min at either pH 2 or 7 and centrifuged at 15000*g* for 1 min to sediment the AC along with any adsorbed protein. The resulting supernatant was assayed for protein by SDS-PAGE or Bradford assay. In one experiment, the results of which appear in **Figure 3**, 10 mg of chopped peanut was incubated in 1 mL of buffer along with various amounts of AC for 30 min. Aliquots were removed for analysis and compared with aliquots from an identical extraction mixture containing no AC, but which had the AC added after the aliquots were collected.

In Vivo AC Adsorption Experiments. Piglets (20–30 lb) were fasted overnight and the following morning suspended in a harness. A slurry of 0.25 g of chopped peanut in 10 mL of water was delivered into the pig's stomach via a gastric tube. Five minutes later, either 30 mL of water
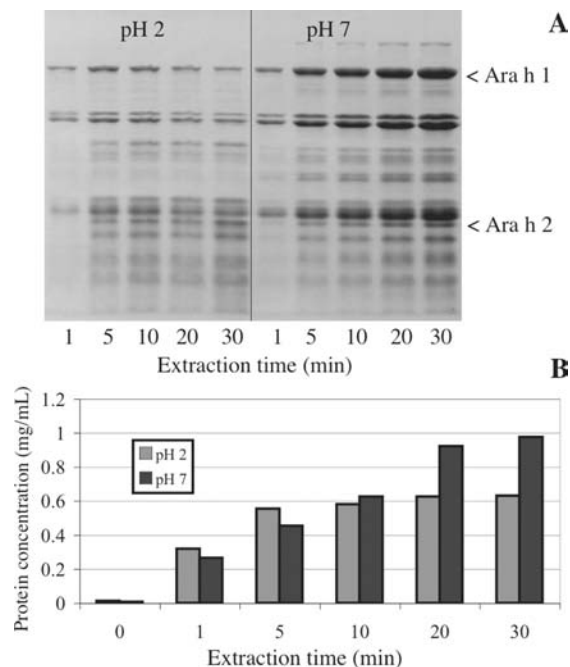

Figure 1. Time course of protein solubilization from chopped peanut into buffer (1:100 w/v) at pH 2 and 7: (**A**) SDS-PAGE of extraction mixture (15 μL/lane) sampled at various times; (**B**) Bradford protein assay of the same samples. Soluble protein concentration is plotted as a function of time for extractions performed at pH 2 and 7.

(control) or a weighed amount of AC (2–6 g) suspended in 30 mL of water was administered via the tube. At timed intervals, 2–3 mL samples of stomach contents were withdrawn by means of the gastric tube. Each milliliter of gastric fluid was treated with 1 mL of 1 mM pepstatin A, a pepsin inhibitor, to prevent any further peptic digestion of the peanut proteins. The samples were immediately frozen on dry ice and kept frozen until analyzed by SDS-PAGE, Bradford quantitative protein assay, and Western immunoblotting.

RESULTS

Assessment of Soluble Protein Release from Peanut. The rate and extent of chopped peanut protein solubilization were compared at two physiological pH values, pH 2 (gastric pH) and pH 7 (intestinal pH). At timed intervals, aliquots were removed and centrifuged. The soluble protein in the supernatant was analyzed by SDS-PAGE and Bradford assay. **Figure 1A** presents the SDS-PAGE of soluble protein profile with increasing extraction times at pH 2 and 7. The protein concentrations as determined by Bradford assay are presented in **Figure 1B**. The results indicate that protein is released from chopped peanut more gradually, but more completely, at pH 7 than at pH 2. Auxiliary experiments showed that protein initially solubilized at pH 7 is partially precipitated when acidified to pH 2. After initial solubilization of protein at pH 2, additional protein was released from the insoluble material after adjustment to pH 7 (results not shown). This indicates that potentially allergenic protein may be solubilized as digesta moves from the stomach (at pH 2) into the intestine (at pH 7).

In Vitro Assessment of Protein Adsorption by AC. The ability of AC to adsorb soluble protein and remove it from solution was investigated at pH 2 and 7. **Figure 2A** shows the progressive removal of soluble protein from solution by increasing amounts of AC. Equal sample volumes were loaded into adjacent wells of an SDS-PAGE gel. The amount of soluble protein remaining in solution can be seen to diminish in a progressive manner until no protein remains at a AC/protein ratio of 80:1 (by mass). The same
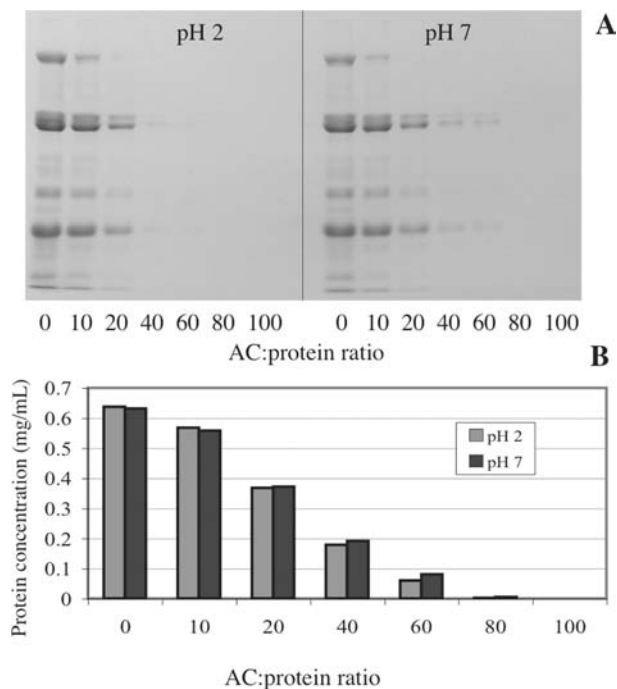
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Figure 2. Adsorption of soluble peanut protein by AC at pH 2 and 7: (**A**) SDS-PAGE of protein samples incubated with increasing amounts of AC (expressed as AC/protein ratio) at pH 2 and 7; (**B**) Bradford protein assay of the same samples. Soluble protein concentration is plotted against the amount of AC added (expressed as AC/protein ratio).
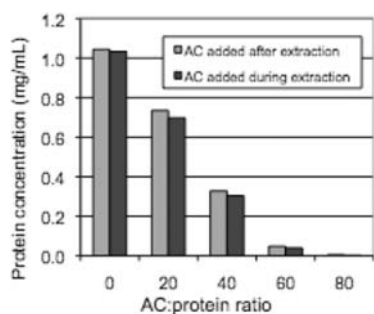

Figure 3. Efficiency of protein adsorption at pH 7 by AC when added during protein extraction versus after protein extraction is complete. Soluble protein concentration as determined by Bradford assay is plotted against the amount of AC (expressed as AC/protein ratio) added at the beginning or end of the protein extraction process.

samples were analyzed by the Bradford protein assay to quantify the results seen in the gel. This is shown in **Figure 2B**.

The efficiency of protein binding by AC was compared between the early addition of AC during protein extraction from chopped peanut (experiment 1) and the addition of AC to the final extract (experiment 2). The supernatant solutions were assayed for unbound soluble protein as shown in **Figure 3**. Under either set of conditions, essentially all soluble protein was adsorbed at an 80:1 AC/protein ratio.

The capacity of AC to bind soluble peanut protein as it is released from chopped peanut at pH 7 was further examined. Tubes containing chopped peanut in buffer and identical tubes containing 30 mg of AC (an amount of AC insufficient to bind all of the protein ultimately released) were incubated for various periods of time. At specific times, one tube from each set was centrifuged and the supernatant solution assayed for unbound protein remaining in solution. The results of the Bradford assay
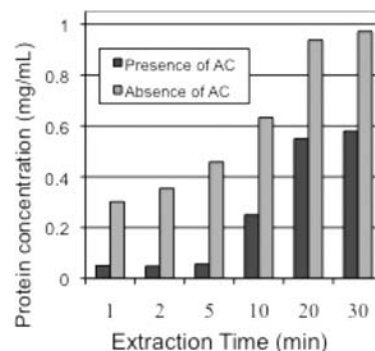

Figure 4. Capacity of limited AC to adsorb protein released during extraction from the peanut. The soluble protein concentration of an extraction mixture in the presence and absence of a limited amount of AC is expressed as a function of extraction time.

are shown in **Figure 4**. The time course of protein extraction from chopped peanut in the absence of AC shows a progressive accumulation of protein in the solution with time. When a limited amount of AC is present in the tubes, the protein is adsorbed and does not accumulate in solution until the available AC becomes saturated with protein. Once the limited amount of AC is saturated, additional protein accumulates in solution.

In Vivo Assessment of Protein Adsorption by AC. The feasibility of using AC to adsorb peanut allergens in vivo was examined in a porcine animal model system. The results are shown in **Figure 5**. SDS-PAGE analysis and the corresponding Western immunoblot of the stomach fluid from a pig that received 2 g of AC (panel **A**) reveals an insufficient amount of AC to adsorb all of the protein released from 0.25 g of chopped peanut. However, the administration of 6 g of AC (panel **B**) resulted in complete adsorption of allergenic protein. Differences in control blots are due to experimental variation between experiments performed on different animals on different days under slightly different conditions. Analysis of the samples by the Bradford method revealed a total protein concentration of 50 μg/mL in the stomach fluid of the pig treated with 2 g of AC and no detectable protein (below the detection limit of 1 μg/mL) in the stomach fluid of the pig treated with 6 g of AC. Therefore, a limited amount of AC adsorbs most of the soluble peanut protein previously introduced into a porcine stomach. A sufficient amount of AC adsorbs all of the peanut protein.

DISCUSSION

Immediately after ingestion under fasted conditions, masticated peanut is essentially incubated at 37 °C in the stomach at approximately pH 2. **Figure 1** shows that the initial release of protein is faster at pH 2 than at pH 7, perhaps because the carbohydrate matrix of the peanut seed is more susceptible to acid-catalyzed hydrolysis at lower pH values where the glycosidic bond is labile. This would allow the release of protein components more readily. This finding is in agreement with a previous report from this laboratory that additional protein is released from insoluble peanut material upon prolonged treatment at pH 2 or with a glycosidase such as amylase (23). **Figure 1** also illustrates that the solubilization of protein at pH 2 is incomplete and suggests that additional protein can be released when the pH is raised to a value of 7. This additional protein has been shown to have a high IgE-binding capacity and could be a factor in sensitization and gastrointestinal allergic responses (23). This might also account for the biphasic reactions observed in many peanut-allergic individuals (1, 14−18). The mechanism of this secondary reaction is unknown, but could likely be due to the
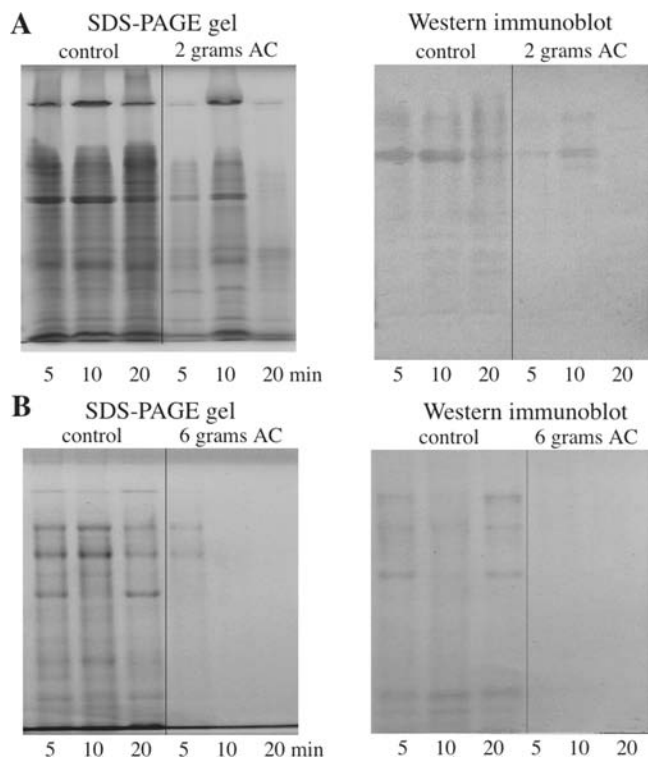
A   SDS-PAGE gel   Western immunoblot
     control   2 grams AC   control   2 grams AC

     5   10   20   5   10   20 min   5   10   20   5   10   20

B   SDS-PAGE gel   Western immunoblot
     control   6 grams AC   control   6 grams AC

     5   10   20   5   10   20 min   5   10   20   5   10   20

Figure 5. Adsorption of peanut protein by AC in vivo. SDS-PAGE and Western immunoblot analysis of gastric samples taken at various times from piglets given chopped peanut followed by (**A**) 2 g of AC or (**B**) 6 g of AC.

additional protein released from the insoluble peanut material as it moves into an environment of pH 7. Because only small amounts of peanut allergen are required to cause significant symptoms in sensitive individuals, if these proteins could be adsorbed onto AC, this would render these allergens unavailable for absorption in the stomach and intestinal tract, thereby reducing the bioavailability of these allergens. AC present in the gastrointestinal tract from an earlier administration could continue to scavenge latent allergens and prevent the initiation of a second-phase reaction.

When increasing amounts of AC are added to peanut extract, the protein is progressively adsorbed and removed from solution. When sufficient AC is added, the soluble protein becomes undetectable. **Figure 2** indicates that peanut proteins are adsorbed with equal efficiency at pH 2 and 7 and are completely removed from solution by an AC/protein ratio of approximately 80:1. These findings corroborate our previously published results using a peanut protein mixture prepared from defatted peanut flour (*20*). This suggests that AC could quantitatively adsorb soluble allergenic peanut protein whether encountered in the stomach (pH 2) or intestine (pH 7).

AC is effective in the removal of soluble protein as soon as it is released from the peanut. AC present during the extraction of protein from chopped peanut reduces the concentration of soluble protein in proportion to the amount of AC added. This can be seen in **Figure 3**, for which increasing amounts of AC were co-incubated with chopped peanuts during protein extraction. Once sufficient AC was present (80:1 AC/protein ratio), the protein was adsorbed as it was released from the peanut and never accumulated in solution. Essentially the same result was obtained if AC was added to the chopped peanut mixture after protein extraction was complete. This implies that sufficient AC administered either immediately, or after the accidental ingestion of peanut, would be effective for the quantitative adsorption of peanut allergens.

The presence of AC during protein extraction from peanut maintains the level of soluble protein at low concentrations until the AC becomes saturated with protein. **Figure 4** illustrates that although protein was being progressively extracted from the peanut, as indicated by a control mixture that did not contain AC, the presence of AC in the other mixture held the soluble protein concentration at low levels until the limited amount of AC became saturated. After saturation of the available AC with protein, the soluble protein concentration increased as additional protein was solubilized from the peanut but was not adsorbed by the AC. The addition of a sufficient amount of AC (an 80:1 or greater AC/protein ratio by mass) at the beginning of the extraction resulted in the detection of only trace amounts of soluble protein until eventual quantitative adsorption occurred. The presence of AC during the release of protein from peanut particles can effectively scavenge soluble allergens and remove them from solution.

Experiments were designed to assess the ability of AC to effectively remove peanut protein allergens from solution in the gastrointestinal tract under physiological conditions in vivo. The porcine system has been shown to be a valid approximation of the analogous system in humans and has been used extensively to model human digestion and immune response (*24−27*). The results in **Figure 5** indicate that the administration of 2 g of AC following a 0.25 g dose of chopped peanut results in the removal of much, but not all, of the soluble protein in a gastric sample. Under these conditions, approximately 10% of the initial weight of the peanut is released as soluble protein. This would result in an 80:1 AC/protein ratio in the stomach. Because there are likely to be residual proteins, enzymes, and other peptides even in a fasted porcine stomach, and the gastric volume is much greater than in the in vitro situation, it is not unreasonable that the 80:1 ratio was insufficient for complete adsorption of all soluble peanut protein. However, by increasing the amount of AC administered, complete adsorption of the soluble protein was obtained. After the administration of 6 g of AC, no remaining peanut protein could be detected by quantitative protein assay, SDS-PAGE, or immunoblotting. This suggests that the ability of AC to effectively bind protein in vitro is valid under physiological conditions.

A previous in vitro study by Vadas and Perelman (*19*) reported an AC/protein ratio of 200:1 as necessary to achieve complete binding of protein by AC. A pH value of 3.5 was used to mimic gastric conditions, but normal stomach pH is much lower. The protein−AC mixtures were incubated at 22 °C, a nonphysiologically relevant temperature. Subsequent findings from this laboratory have shown that the adsorption of protein onto AC increases progressively with temperature (*20*). Experiments at 37 °C not only represent physiological temperature but also increase protein binding to AC. The AC used by Vadas and Perelman was obtained by centrifuging a commercial suspension of AC in water. Even though excess water was discarded, a large proportion of the mass of the wet AC would be due to water. If the amount of AC is measured via the volume of a suspension, because AC is not soluble and settles quickly, an aliquot can contain significantly less AC. Although AC administered in a clinical setting is in slurry form, the AC in the experiments presented in the current investigation was weighed as a dry powder so its mass could be determined exactly. These factors may explain the apparent discrepancy between the 200:1 AC/protein ratio required for complete adsorption reported by Vadas and Perelman and the 80:1 ratio reported both here and in a previous publication (*20*).

The results of these experiments show that AC may be a useful treatment to slow or prevent absorption of peanut allergens from the gut after accidental ingestion by persons with peanut allergy. The rapid adsorption of soluble protein and the continuous

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binding of protein released from peanut particulate material is a strong indication of the potential efficacy of using AC for initial gastric decontamination and potential elimination of a biphasic reaction. Two factors remain to be addressed: (1) ascertain whether adsorption onto AC can be applied to allergenic proteins as a safe, practical, and efficient method of reducing the bioavailability of these allergens after accidental ingestion and (2) determine any additional matrix effects present when peanut is only one of several components in a food source. If the efficacy of binding peanut allergens with AC can be demonstrated in humans, another clinical tool may become available to expand clinical treatment options for hypersensitivity reactions to peanuts. Although the focus in these studies has been on peanuts, this same concept should be applicable to other food allergies.

ACKNOWLEDGMENT

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