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Treatment of cashew extracts with Aspergillopepsin reduces IgE binding to cashew allergens

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ABSTRACT

Enzymes from *Aspergillus* fungal species are used in many industrial and pharmaceutical applications. *Aspergillus niger* and *Aspergillus oryzae* were cultured on media containing cashew nut flour to identify secreted proteins that may be useful as future food allergen processing enzymes. Mass-spectrometric analysis of secreted proteins and protein bands from SDS-PAGE gels indicated the presence of at least 63 proteins. The majority of these proteins were involved in carbohydrate metabolism, but there were also enzymes involved in lipid and protein metabolism. It is likely that some of these enzymes are specifically upregulated in response to cashew nut protein, and study of these enzymes could aid our understanding of cashew nut metabolism. Aspergillopepsin from *A. niger* was one of the proteolytic enzymes identified, and 6 distinct peptides were matched to this protein providing 22% coverage of the protein. Cashew extracts were incubated with a commercially available preparation of Aspergillopepsin (Acid Stable Protease, ASP) using simulated gastric fluid conditions to determine if ASP could degrade the protein and lower antibody binding to cashew allergens. Following treatment of cashew extract with ASP, a significant reduction was observed in cashew allergen binding to rabbit anti-cashew IgG using an immunoblot assay and serum IgE antibodies from cashew allergic individuals using competitive ELISA. These findings highlight the possible application of Aspergillopepsin/ASP from *A. niger* in food processing steps to attenuate cashew nut and other tree nut or peanut allergens.

1. INTRODUCTION

The incidence of food allergies in industrialized nations appears to be increasing, and there are no widely accepted therapies for food allergy sufferers. Up to 8% of children in the United States suffer from food allergy [1], and the self-reported frequency of peanut and tree nut allergies has increased over the past ten years [2]. Reactions to peanut and tree nuts are often severe and are rarely outgrown in adulthood [3] and can result in fatalities [4,5]. Very small amounts of a food allergen can cause a reaction [6, 7], and estimates indicate that food allergy reactions are a significant cause of emergency room visits due to anaphylaxis [8, 9]. The constant threat of accidental exposure places significant emotional, financial, and social burdens upon food allergy patients and their families [10, 11]. The overall economic burden associated with food allergy has been estimated at $25 billion a year [12]. Development of new methods to disrupt the structure or sequence of the allergens present in tree nuts like cashews in order to reduce or eliminate the allergenic response would be beneficial. Cashew nuts can be part of a healthy and diverse diet, but may also be a food allergen in susceptible individuals. Food allergy, a Type I hypersensitivity mediated by immunoglobulin E (IgE) binding, can be a serious medical condition [13]. IgE binding to food allergens can result in various symptoms, and some studies have indicated that reactions to cashews are frequently severe [14, 15]. Cashews, like other nuts, contain 3 conserved seed storage proteins that have been characterized as food allergens by the International Union of Immunological Societies (IUIS). These include the 7S vicilin Ana o 1 [16], 11S legumin Ana o 2 [17], and the 2S albumin Ana o 3 [18]. Cashew allergens such as Ana o 1, Ana o 2, and Ana o 3 are resistant to physical and thermal processing methods [19, 20]. Several lines of evidence indicate that food preparation steps such as roasting can effect changes in nut allergens that modulate their ability to cause allergy.

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Enzymatic processing methods targeting food allergens have also been useful in lowering IgE binding to nut allergens, and several studies demonstrate the utility of enzymatic attenuation of nut allergens. For example, IgE binding to allergens from whole roasted peanuts and peanut flour has been effectively reduced following treatment with proteases [21-23]. Similarly, cashew proteins treated with pepsin have reduced IgE binding and have been used in cashew allergy immunotherapy using a mouse model [24, 25]. While protease treatment of cashew allergens has proven useful, the application of these enzymes on a mass scale may be limited by the costs associated with production. As a consequence, the identification of novel proteases produced by microorganisms could be of great use given the applicability of the organisms to large-scale production [26]. Microorganisms that use plant seeds and nuts for sustenance have evolved specialized proteolytic enzymes that allow them to degrade plant proteins. Some of these enzymes have been utilized as food processing reagents for many decades, and fungal enzymes, particularly those from *Aspergillus niger* and *Aspergillus oryzae*, have proven to be some of the most important and long-standing reagents in the biotechnology field. *A. niger* and *A. oryzae* are both on the Food and Drug Administrations' (FDAs) Generally Regarded As Safe (GRAS) list. The Food and Agricultural Organization (FAO) and the World Health Organization (WHO) have repeatedly reviewed and accepted enzyme preparations from these fungi for food related applications and the fungi are used as hosts for the overproduction of recombinant food enzymes. Both *A. niger* and *oryzae* have been isolated as naturally occurring contaminants of cashew nuts [27, 28]. Previous work has shown that changes in growth media can have large effects on the profile of proteins that are secreted during fungal growth [29, 30]. For example, growth of *Aspergillus* species in the presence of collagen has been shown to induce expression of an alkaline protease [31], a fibrinogenolytic enzyme in the presence of peptone [32], and an esterase in the presence of sugar-beet pulp [33]. There are likely many as yet undiscovered applications for enzymes produced by *Aspergillus* and other microbial species awaiting discovery.

In the present study, cashew flour was incorporated into growth media to identify and characterize enzymes secreted by *Aspergillus oryzae/niger* during growth on cashew protein. The results indicated that Aspergillopepsin I/acid stable protease (ASP) is secreted by *Aspergillus* growing on media containing cashew flour and treatment of cashew extracts with ASP *in vitro* can reduce IgE binding. Continued study of aspergillopepsin and other microbiological enzymes or enzyme combinations may identify new applications for these enzymes in the food allergen processing arena and lead to methods to attenuate the allergenicity of nut proteins.

2. MATERIALS AND METHODS

2.1 Materials

Cashew nuts were purchased from Nuts Online (http://www.nuts.com, Cranford, NJ, USA). Purified porcine pepsin was purchased from Sigma Aldrich (St. Louis, MS, USA). Precast 10-20% Tris-glycine SDS-PAGE gels were from Life Technologies (Carlsbad, CA, USA). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA). Biotinylated anti-human IgE antibody was purchased from Southern Biotech (Birmingham, AL, USA), and the IRdye-800CW labeled strepavidin from LI-COR (Lincoln, NE, USA). Maxisorp 96-well Nunc microtiter plates were purchased from ThermoScientific (Rochester, NY, USA). Defatted cashew flour was generated as described in Mattison *et. al*. [34]. Acid Stable Protease was donated from Bio-Cat Incorporated. (Troy, VA, USA). Human sera from cashew-allergic patients with a documented history of clinical cashew allergy were collected at Dallas-Allergy Immunology (Dallas, TX, USA). The study protocol was approved by the North Texas Institutional Review Board.

2.2 *Aspergillus* culture

Culture of A. oryzae and A. niger and secreted protein sample preparation

Fungal conidia were collected with a solution of Triton X-100 (0.01%) from the surface of colonies following 7 days growth at 30°C on potato dextrose agar (PDA, Difco). Conidia (approximately 10^6/ml) were used to inoculate 250 ml peptone minimal salts broth (PMS; per liter: peptone, 50 g; $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 10 g; $MgSO_4{\cdot}7H_2O$, 2 g; 1 ml micronutrient solution; pH 5.2) in 2 liter baffled flasks containing 5 g glass beads. Cultures were incubated for 17 h at 30°C in the dark with agitation at 200 rpm. Mycelia were collected by vacuum filtration through sterile miracloth and washed 1X with sterile water. Mycelia (1 g) were transferred to 50 ml cashew flour broths (per 100 ml: 0.5 g defatted cashew flour; 0.2 g $MgSO_4{\cdot}H_2O$; 0.1 ml micronutrient solution; pH 5.2) and 50 ml glucose broths (cashew flour media with 5 g glucose replacing cashew flour/100 ml) in 250 ml baffle flasks with 1 g glass beads. One aliquot of the cashew flour broth remained uninoculated as a control. Cultures were incubated in the dark at 30°C with agitation at 200 rpm for 52 h. Mycelia were collected by vacuum filtration through sterile miracloth, frozen in liquid nitrogen and stored at -80°C. Culture broths were collected and lyophilized for 72 h then resuspended in 2-3 ml sterile ddH_20 and stored at -80°C.

2.3 Sodium docecyl sulfate-polyacarylamide gel electrophoresis

Secreted proteins were resolved on precast 10-20% Tris-glycine SDS-PAGE gels using a Novex Mini Cell apparatus (Life Technologies) and prestained Precision Plus molecular weight standards (Bio-Rad) were used as migration markers. NuPAGE LDS 4X sample buffer (Life Technologies) was added to the protein samples using a 1:4 (v/v) ratio, and samples were heated at 65 °C for 15 min prior to electrophoresis. Protein bands were visualized with Safe Stain (Invitrogen, Grand Island, NY, USA), and gel images were captured using the IRdye 680 channel of an Odyssey CLX infrared imaging system (LI-COR, NE, USA).

2.4 Liquid chromatography coupled-mass spectrometry/mass-spectrometry

Samples of resuspended culture broths were analyzed by LC/MS/MS following precipitation with TCA. Equal amounts of secreted protein (30 μg) from each culture were precipitated by the addition of ice-cold trichloroacetic acid to a final concentration of 10% and incubated on ice for 10 minutes. Samples were centrifuged at 20,000g for 30 minutes, and the resulting pellet was washed with acetone. Protein samples were reduced by the addition of 4 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate at 37 °C for 15 minutes and then alkylated by the addition of 15 mM iodoacetamide for 30 minutes at room temperature in the dark. Protein pellets were dried under vacuum and then resuspended in 100 μl of trypsin digestion buffer (100 mM ammonium bicarbonate, 1mM calcium chloride) containing 0.2 μg trypsin per sample.

Following incubation at 37 °C for 24 hours, 1 μl of formic acid was added to acidify the samples prior to LC-MS/MS analysis. Protein samples excised from SDS-PAGE gels were prepared and analyzed as described in Mattison *et. al.* [25]. Briefly, peptides extracted from gel slices were resuspended in 5% formic acid and analyzed via liquid chromatography (LC) on an Agilent 1200 LC system, an Agilent Chip Cube interface, and an Agilent 6520 Q-TOF tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Raw data files were extracted, sequenced, and searched against the SWISSPROT database to identify matching peptides using Spectrum Mill software (Agilent Technologies, Santa Clara, CA, USA).

2.5 Cashew extract digestion

Cashew extract samples (10 μg) were subjected to digestion with the indicated amounts of acid stable protease (5 μg, 4 SAPU/mg) or pepsin (0.02 ug, 4000 U/mg) in simulated gastric digestion buffer described in the United States Pharmacopeial Convention (35 mM NaCl, 84 mM HCl, pH 1.2) [35]. Samples were removed at the indicated time points and digestion was stopped by adding Tris pH 8.5 to 100 mM, PMSF to 1 mM, and placing the tubes on ice. For SDS-PAGE analysis 4X loading buffer was added to a final 1X concentration prior to gel loading.

2.6 Immunoblotting

Enzymatically treated cashew extract samples resolved on SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane, and membranes blocked for 1 h at room temperature in phosphate buffered saline with 0.2% Tween-20 (PBST) with 2% (w/v) nonfat dry milk. Rabbit anti-cashew antibody [34] was diluted 1:1000 in PBST and incubated for 1 h at room temperature.

The membrane was washed four times for 5 min in PBST and then incubated for 30 min with anti-rabbit IRdye-800 (1:10000 in PBST) at room temperature. The membrane was washed as above and visualized using an Odyssey CLx (LI-COR, NE, USA) infrared imaging system. The data are plotted as the average of 3 independent experiments with standard deviation error bars.

2.7 Competitive enzyme-linked immunosorbent assay

Competitive ELISA to evaluate IgE binding in human sera with enzymatically treated or control cashew extracts was performed essentially as described in Mattison *et. al.* [25]. Enzymatically treated or control cashew extracts were serially diluted 10-fold in 100 mM Tris, pH 8.3, and samples were made using 25 μL of treated cashew sample, 25 μL of pooled sera, and 50 μL of 100 mM Tris, pH 8.3. Mixed samples were incubated at 37 °C for 2 hrs in microtiter plates pre-coated with 1 μg of cashew extract and blocked with PBST containing 1% BSA. Plates were washed 4 times with 200 μL of PBST, and biotinylated anti-IgE (1:1000 in PBST) was added to each well and then incubated at 37 °C for 1 hr.

Plates were washed with PBST, and 50 μL of streptavidin-labeled IRdye-680 (1:5000 in PBST) was added to each well. Plates were incubated at 37 °C for 30 min, washed, and then imaged with the Odyssey CLx instrument. For figure 3A, the data in the plot are representative of duplicate independent experiments and are presented as percent of IgE inhibition using the following formula:

$1 - A/B$

where A equals the IR800 value of inhibited sample, and B equals the IR800 value of uninhibited control with values expressed as a percentage rounded to the nearest integer. For Figure 3B, the data are plotted as the average of 4 independent experiments with the 0.001 mg/ml sample of digested cashew extract. Standard variation in IgE binding percentage is included as error bars in Figure 3B.

3. RESULTS

3.1 LC-MS/MS analysis of bulk secreted proteins from *Aspergillus* cultured on media containing cashew nut flour

In this study we tested whether fungi in the genus *Aspergillus* were capable of producing enzymes that hydrolyze cashew allergen proteins following exposure to cashew nut flour. Proteins secreted by each of the fungi after 24 hours of growth on media containing cashew nut flour were surveyed by LC/MS/MS. As shown in Table 1, following precipitation with TCA and sample treatment with trypsin we observed peptides that could be matched to 62 proteins involved in carbohydrate, lipid, and protein metabolism. This bulk analysis of proteins secreted into the growth media indicated the presence of 31 proteins involved in carbohydrate metabolism, 5 proteins involved in lipid metabolism, 14 proteolytic enzymes, and a single ribonuclease. There were also 11 proteins with various predicted functions including a glycosyl hydrolase, glucanase, glucanosyltransferase, glutaminase, ribonuclease, N-acetylglucosaminidase, S10 peptidase, and serine carboxypeptidase.

Table 1: Mass-spectrometric identification of bulk secreted proteins from *Aspergillus* species cultured on cashew nut flour containing media.

Accession #NCBI/ASPGD	Matching peptide #	Functional group	Protein [predicted]
113496	4	carbohydrate metabolism	alpha-galactosidase A
217807	5	carbohydrate metabolism	alpha-amylase
1160313	6	carbohydrate metabolism	glucoamylase
3023267	4	carbohydrate metabolism	alpha-glucosidase
13810441	2	carbohydrate metabolism	alpha-L-arabinofuranosidase A
74698498	4	carbohydrate metabolism	1,4-beta-D-glucan cellobiohydrolase B
94706960	13	carbohydrate metabolism	1,4-alpha-D-glucan glucanohydrolase
114794116	9	carbohydrate metabolism	alpha-Amylase
121939500	3	carbohydrate metabolism	mannosyl-oligosaccharide alpha-1,2-mannosidase
134080612	2	carbohydrate metabolism	exo-inulinase inu1
159023686	3	carbohydrate metabolism	fructosyltransferase
350630290	3	carbohydrate metabolism	alpha-galactosidase extracellular
350631148	4	carbohydrate metabolism	alpha-amylase A
350631594	2	carbohydrate metabolism	1,3-beta-glucanosyltransferase
350633017	4	carbohydrate metabolism	1,4-alpha-D-glucan glucohydrolase
350634958	2	carbohydrate metabolism	arabinan endo-1,5-alpha-L-arabinosidase
350635004	2	carbohydrate metabolism	alpha-galactosidase
350636225	3	carbohydrate metabolism	alpha-L-arabinofuranosidase B
350638468	4	carbohydrate metabolism	exo-1, 4-beta-xylosidase xlnD
350638529	2	carbohydrate metabolism	alpha-glucosidase
350638537	2	carbohydrate metabolism	glycosidase
350638658	3	carbohydrate metabolism	alpha-mannosidase
350638806	2	carbohydrate metabolism	glucooligosaccharide oxidase
A2QAN3	3	carbohydrate metabolism	beta-galactosidase A
A2RAL4.1	2	carbohydrate metabolism	beta-glucosidase A
AFL2G_01841	5	carbohydrate metabolism	alpha-amylase A type-3
P56271	5	carbohydrate metabolism	acid alpha-amylase
P56526	2	carbohydrate metabolism	alpha-glucosidase
P69327.1	5	carbohydrate metabolism	glucoamylase
P87076	2	carbohydrate metabolism	beta-glucosidase A
Q4AEG8.1	3	carbohydrate metabolism	exo-1,4-beta-xylosidase
134077929	2	hypothetical protein	1,3-beta-glucanosyltransferase
350630620	2	hypothetical protein	hypothetical protein
350631889	2	hypothetical protein	DUF1237 domain protein
350632039	2	hypothetical protein	hypothetical protein
350632918	2	hypothetical protein	serine carboxypeptidase
350632924	2	hypothetical protein	exo-beta-1,3-glucanase
350633230	2	hypothetical protein	S10 peptidase
350635197	2	hypothetical protein	glutaminase
350635684	2	hypothetical protein	hypothetical protein
350638503	2	hypothetical protein	ribonuclease T2
350639912	2	hypothetical protein	N-acetylglucosaminidase
350633018	3	lipid metabolism	extracellular lipase
350633410	3	lipid metabolism	triacylglycerol lipase precusor
350633910	2	lipid metabolism	GDSL lipase/acylhydrolase
350638842	2	lipid metabolism	phospholipase C
350639908	2	lipid metabolism	triacylglycerol lipase
133241	2	nucleic acid metabolism	ribonuclease T2
129235	5	proteolysis	alkaline protease
3123246	2	proteolysis	serine-type carboxypeptidase
121798407	4	proteolysis	leucine aminopeptidase
121802887	3	proteolysis	dipeptidyl peptidase 4
121804190	5	proteolysis	leucine aminopeptidase 2
350629684	3	proteolysis	S28 peptidase
350630288	3	proteolysis	tripeptidyl-peptidase
350632613	4	proteolysis	aorsin/S53 peptidase
350637380	2	proteolysis	aspartic protease
350637842	2	proteolysis	S8-S53 peptidase
350639368	3	proteolysis	tripeptidyl peptidase
350639535	4	proteolysis	aspergillopepsin A
666429641	2	proteolysis	secreted dipeptidyl peptidase
AFL2G_01995	3	proteolysis	oryzin precursor

3.2 Comparison of secreted proteins from the *A. oryzae* and *A. niger* cultures

A comparison of the secreted proteins from the two *Aspergillus* cultures grown with media containing cashew flour indicated that many of the visible proteins secreted from the two fungi were unique to either *A. oryzae* or *A. niger* (Figure 1). Following SDS-PAGE there were 19 bands that appeared unique between the two cultures, and that were not visible in the uninoculated control. Secreted proteins were analyzed by LC/MS/MS following excision of discrete protein bands from the

SDS polyacrylamide gels. Several bands from specific regions of the gel were removed from each lane (Figure 1) and the contents were reduced, alkylated, and trypsinized. Proteins having at least 3 matched peptides within the excised band are listed in Table 2. In some cases, such as the bands in between boxes 16 and 17, we were unable to identify proteins from the gel samples. Two proteins secreted from *A. oryzae*, migrated above the 100 kDa marker that were not visible in the *A. niger* sample. One protein migrating near the 50 kDa marker was particularly intense from the *A. oryzae* secreted sample, but was absent, or drastically reduced in intensity, from the *A. niger* sample. The *A. niger* sample contained a cluster of proteins migrating around the 75 kDa marker that were not as visibly intense in the *A. oryzae* sample. From the *A. niger* culture, there were several enzymes involved in carbohydrate metabolism including alpha-amylase A, 1,4-alpha-D-glucan glucohydrolase, alpha-galactosidase A, exo-inulinase, and alpha-L-arabinofuranosidase B (Table 2). Many of the higher molecular weight bands in the *A. oryzae* culture were also involved in carbohydrate metabolism including beta-galactosidase, fructosyltransferase, glucoamylase, 1,4-alpha-D-glucan glucohydrolase, and alpha-amylase (Table 2). We also matched peptides indicating the presence of an extracellular lipase in slice 5. Peptides matching lipid metabolism proteins such as triacylglycerol were also present in gel slices 7, 8, and 9. We were interested in identifying secreted fungal proteases that could degrade cashew nut seed storage proteins and limit the allergic potential of these proteins. Peptides were matched to 4 proteases; aspergillopepsin (GI 350639535)/acid stable protease (ASP), didpeptidyl peptidase 4 (121802887), leucine aminopeptidase (121798407), and an alkaline protease (129235) within slices 7, 13, 17, and 19. Notably, there were 5 good matches to aspergillopepsin I/ASP (GI 350639535) peptides in gel slice number 7 migrating just below the 37 kDa marker from the *A. niger* sample (Figure 1 and Table 2).

Analysis of several samples by SDS-PAGE and LC/MS/MS to confirm the presence of aspergillopepsin I/ASP from the corresponding band showed matches to 6 distinct aspergillopepsin I/ASP peptides covering 22% of the protein (Table 3). A search of the protein databases failed to identify matches for gel slice samples 10, 11 and 18. None of the proteins identified in this analysis were present in the corresponding segment of the control lane.

Table 2: Mass-spectrometric identification of gel isolated secreted proteins from *Aspergillus* species cultured on cashew nut flour containing media.

Gel Spot #	Accession #NCBI/ASPGD	Peptide #	Function [predicted]	Protein [predicted]	MW
1	350631148	7	carbohydrate metabolism	alpha-amylase A	65230
1	350633017	5	carbohydrate metabolism	1,4-alpha-D-glucan glucohydrolase	70400
1	P28351.1	4	carbohydrate metabolism	alpha-galactosidase A	60148
2	350631148	7	carbohydrate metabolism	alpha-amylase A	65230
2	350633017	5	carbohydrate metabolism	1,4-alpha-D-glucan glucohydrolase	70400
2	134080612	4	carbohydrate metabolism	exo-inulinase inu1	59070
2	350630290	4	carbohydrate metabolism	alpha-galactosidase extracellular	59950
3	350631148	8	carbohydrate metabolism	alpha-amylase A	65230
3	350633017	7	carbohydrate metabolism	1,4-alpha-D-glucan glucohydrolase	70400
3	350630290	6	carbohydrate metabolism	alpha-galactosidase extracellular	59950
3	113496	6	carbohydrate metabolism	alpha-galactosidase A	59950
4	350631148	4	carbohydrate metabolism	alpha-amylase A	65230
4	350633017	6	carbohydrate metabolism	1,4-alpha-D-glucan glucohydrolase	70400
5	350631148	6	carbohydrate metabolism	alpha-amylase A	65230
5	350633018	4	lipid metabolism	extracellular lipase	61820
5	350636225	4	carbohydrate metabolism	alpha-L-arabinofuranosidase B	54890
6	AFL2G_01841	6	carbohydrate metabolism	alpha-amylase A type-3	54780
6	350630290	4	carbohydrate metabolism	alpha-galactosidase extracellular	59950
7	350633410	5	lipid metabolism	triacylglycerol lipase precusor	32670
7	350639535	5	proteolysis	aspergillopepsin	43340
8	350633410	4	lipid metabolism	triacylglycerol lipase precusor	32670
9	350633410	4	lipid metabolism	triacylglycerol lipase precusor	32670
10			no match		
11			no match		
12	528082121	3	carbohydrate metabolism	beta-galactosidase	110179
13	121802887	3	proteolysis	dipeptidyl peptidase 4	86875
14	159023686	3	carbohydrate metabolism	fructosyltransferase	65230
15	1160313	7	carbohydrate metabolism	glucoamylase	67320
16	94706960	25	carbohydrate metabolism	1,4-alpha-D-glucan glucohydrolase	54890
16	114794116	24	carbohydrate metabolism	alpha-amylase	52580
17	121798407	4	proteolysis	leucine aminopeptidase	41470
18			no match		
19	129235	3	proteolysis	alkaline protease	44330

Table 3: *Aspergillus niger* Aspergillopepsin peptides identified by mass-spectrometry.

#	peptide sequence	peptide start amino acid number	m/z measured [Da]	MH+ matched [Da]	MH+ error [ppm]
1	[K]GSAVTTPQNNDEEYLTPVTVGK[S]	3	774.047	2320.13	-1.7
2	[R]DTVTVGGVTTNK[Q]	87	596.316	1191.622	2.7
3	[K]AQTTFFDTVK[S]	135	579.3016	1157.584	10.5
4	[K]SQLDSPLFAVQLK[H]	145	482.6051	1445.8	0.6
5	[K]HDAPGVYDFGYIDDSK[Y]	158	600.2645	1798.792	-7.2
6	[K]SQYVVFNSEGPK[L]	307	677.8345	1354.664	-1.5

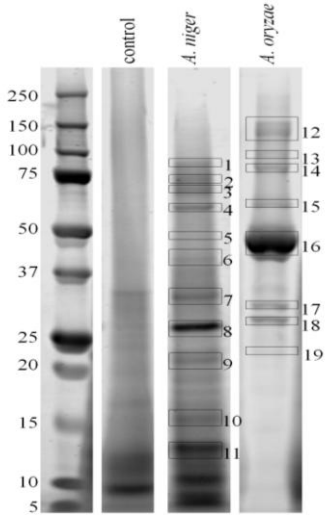

Fig. 1: Protein expression patterns observed in *A. niger* and *A. oryzae* following exposure to cashew nut flour during growth. *A. niger* and *A. oryzae* samples were grown in the presence of cashew nut flour for 24 hrs, the control is media with cashew nut flour that was not inoculated with fungi. Gel slices [indicated by numbered boxes] of specific protein bands from the secreted proteins of *Aspergillus* cultures were harvested for mass-spectrometric analysis.

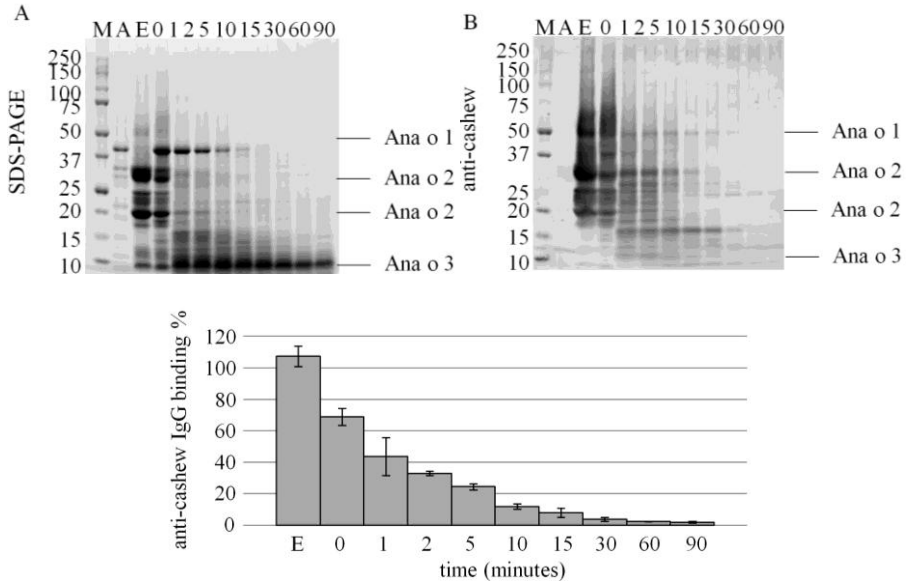

Fig. 2: Treatment of cashew extracts with ASP reduces antibody binding of cashew allergens. Cashew extract samples [10 µg] were subjected to digestion with ASP [5 µg, 4 SAPU/mg] and samples of extract harvested and cashew allergen proteins examined by SDS-PAGE [A] and western blot using rabbit antibodies to cashew proteins [B]. Quantitation of binding of rabbit IgG to cashew allergens was performed at the time points illustrated with the mean values of 3 independent tests +/- standard deviation plotted [C]. Lanes: molecular weight markers [M], ASP [A], cashew extract [E], cashew extract with ASP incubation times [0-90] in minutes.

3.3 Proteolytic treatment of cashew nut flour

Enzymatic processing has been used to lower IgE binding to several food allergens. Commercially available aspergillopepsin I/ASP is described as having a pH optimum of 2.0-3.5 and is marketed as a dietary supplement. We were interested in the ability of this enzyme to degrade cashew proteins at low pH, so we evaluated the ability of ASP to digest cashew nut proteins *in vitro* under conditions of simulated gastric fluid. Cashew extracts were treated with ASP (76 SAPU/ml) for varying amounts of time and evaluated by SDS-PAGE and immunoblot with rabbit anti-cashew antibody to characterize cashew protein degradation. A significant reduction in the intensity of the ASP

band during the time course suggested autolysis of the ASP protein, migrating between the 50 and 37 kDa markers, during the assay. Treatment of cashew nut extract with ASP visibly reduced the intensity of the Ana o 1 and Ana o 2 bands on an SDS-PAGE gel (Figure 2A). After only 1 minute, the intensity of Ana o 1 and Ana o 2 bands was decreased, and by 30 minutes they were drastically reduced. Quantification indicated a drop in the intensity of the 50 kDa Ana o 1 band of 70% by 1 minute and 95% by 30 minutes. Likewise, the intensity of the 32 kDa and 20 kDa Ana o 2 bands were decreased 70% at 1 minute and 97% at 30 minutes. During this time there was a corresponding increase in the size and intensity of a diffuse band migrating near the 10 kDa marker. This band is presumably composed of peptide fragments from larger cashew proteins, and it obscured visualization of the Ana o 3 band also migrating near the 10 kDa marker. To verify proteolysis of the Ana o proteins, we probed the ASP treated cashew extracts with rabbit anti-cashew antibodies on a western blot and found a corresponding decrease in immunoreactivity with cashew proteins (Figure 2B). We quantified rabbit IgG binding to the cashew allergens. Following 10 minutes of treatment of cashew allergens with ASP, the IgG binding of cashew allergens had been reduced to less than 20%. By 30 minutes of treatment with ASP, the binding of IgG to cashew allergens was lowered to less than 5% (Figure 2C).

3.4 IgE binding to proteolytically treated cashew nut extract

To test IgE binding to cashew allergens we incubated the ASP treated cashew extracts in a competitive ELISA with a pool of serum obtained from 8 individuals with cashew allergy. We compared IgE binding to the ASP treated extract with both undigested control and porcine pepsin treated extract. Porcine pepsin treated cashew extract has been demonstrated to have lowered IgE binding *in vitro* [24, 25]. Both the pepsin and ASP treated cashew extracts exhibited reduced IgE binding in the competitive ELISA compared to the control extract (Figure 3A). While pepsin treatment of the extracts noticeably shifted the binding curve, the ASP treated extract reduced binding capacity of the extracts over 10 fold at 50% binding as compared to the undigested control. Binding to pooled sera can be misleading due to uneven contributions from dominant serum samples, so we chose 5 of the sera to individually evaluate IgE binding to ASP treated extracts.

In nearly every case, the ASP-induced degradation of cashew proteins resulted in a reduction of IgE binding to the treated sample in comparison to both the pepsin treated and control extract (Figure 3B).

The values for the ASP treated extract varied, but in 3 of the 5 samples (patients 1, 3, and 4) we observed a greater than 20% reduction in IgE binding compared to the control. While the effect was less pronounced, IgE binding to the ASP treated extract from patients 2 and 5 sera was reduced 5% and 15%, respectively. These results using individual serum samples are consistent with IgE binding from pooled serum samples and rabbit anti-cashew IgG binding to cashew proteins. The data collectively suggest that continued study of ASP enzymes as reagents in tree nut and peanut allergen processing steps is warranted. Binding to pooled sera can

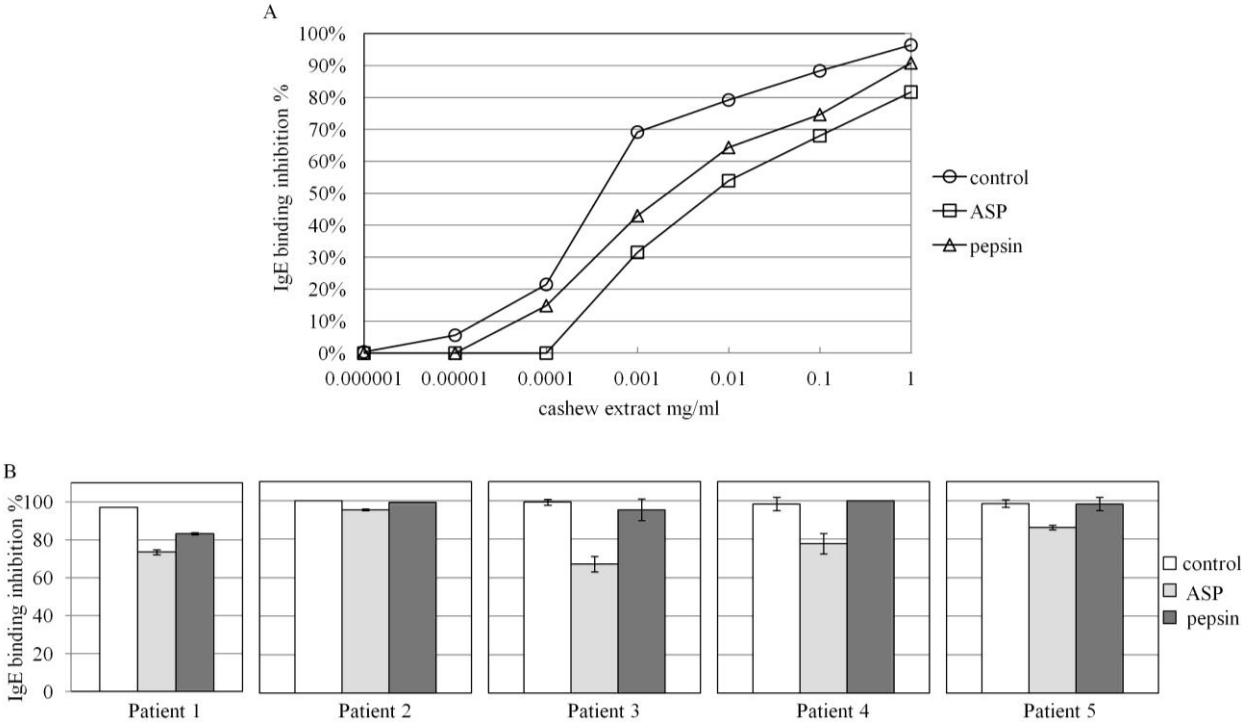

Fig. 3: Treatment of cashew nut extracts with ASP or pepsin reduces IgE binding of cashew nut allergens. Pooled serum from 8 patients with cashew nut allergies was analyzed for competitive IgE binding of cashew allergens following treatment with ASP or pepsin [A] and plotted data are representative of 2 independent repetitions. Five individual patient serum samples were examined for IgE binding using competitive ELISA with 0.1 mg/ml cashew allergen proteins following treatment with ASP or pepsin [B].

be misleading due to uneven contributions from dominant serum samples, so we chose 5 of the sera to individually evaluate IgE binding to ASP treated extracts. In nearly every case, the ASP-induced degradation of cashew proteins resulted in a reduction of IgE binding to the treated sample in comparison to both the pepsin treated and control extract (Fig 3B).

4. DISCUSSION

Our analysis indicates that an aspergillopepsin I/acid stable protease (ASP) is secreted by fungi in the genus *Aspergillus* following growth on media containing cashew flour. We have identified an ASP I enzyme produced by *A. niger* that degrades cashew nut proteins and reduced antibody binding of the allergen proteins under simulated gastric fluid conditions *in vitro*. The ASP enzymes have been previously characterized as secreted proteins from *A. niger* [36], *A. fumigatus* [29], and *A. oryzae* [37]. Aspergillopepsins require aspartic and glutamic acid residues for catalytic activity [38, 39] and two forms of ASP are generated ASP I and ASP II [40, 41]. ASP I (GI 134081775) is a pepsin-type aspartic proteinase with a predicted molecular weight of 41 kDa, that is active within a range of pH 2.0 to 4.0, and can be inhibited by pepstatin [26, 42, 43]. In contrast, ASP II (GI 134054586) is a non-pepsin-type acid proteinase, with a predicted molecular weight of 30 kDa, that is active in a pH range between pH 2.0 to 3.0 and is resistant to pepsin-type aspartic proteinases inhibitors such as pepstatin [42, 44, 45]. Despite the differences in size and inhibitor sensitivity, both ASP enzymes can be irreversibly inactivated above pH 6.0 [46]. The stability and activity of ASP enzymes at low pH make the proteins attractive targets for commercial development for the enzymatic processing of cashew allergens.

ASP enzymes are commercially available proteases that have been used in many applications including degradation of haze forming wine proteins [47] and enhancement of gluten degradation within simulated gastric digestion conditions [48]. While we did not purify the ASP I enzyme from *A. niger*, we utilized a commercially available ASP enzyme to determine if ASP I could hydrolyze cashew allergens. Under our assay conditions, the commercial ASP enzyme appeared to be unstable and may have undergone autolysis. We used these conditions to mimic gastric digestion, and future research could be directed towards the development of recombinant proteases such as ASP with increased stability under gastric conditions. Despite this reduction in the amount of active ASP enzyme, we observed hydrolysis of the cashew extract proteins into lower molecular weight peptides. Hydrolysis of the cashew proteins produced a diffuse band that formed at approximately 10 kDa. Degradation of Ana o 1 and Ana o 2 by the ASP I enzyme into lower molecular weight forms was apparent from SDS-PAGE analysis and confirmed by western blot analysis using a rabbit anti-cashew IgG antibody. While our results indicate that ASP enzyme may be utilized for degradation of cashew allergen proteins, the degraded products may still induce an allergic response. As such we performed experiments to determine if the hydrolyzed cashew allergen proteins could be bound by the IgE antibody in serum samples taken from patients with a cashew allergy. Treatment of the pooled serum samples resulted in a greater than 10 fold decrease in IgE binding capacity to cashew extract proteins. We observed a reduction in IgE binding of cashew allergen proteins following incubation with 5 individual patient serum samples. Overall, our *in vitro* assays demonstrated a reduction in IgE to cashew allergens following ASP treatment, and our results are consistent with previous findings demonstrating that proteolysis can attenuate IgE binding to peanut and tree nut allergens [21-25].

5. CONCLUSION

Based upon our findings, continued efforts to characterize ASP proteins as food allergen processing enzymes are warranted for the identification of novel methods of food allergenicity attenuation. Experiments to confirm the direct hydrolysis of cashew allergen proteins and testing the efficacy of the proteins at reducing IgE binding of the allergens using mouse model systems are necessary next steps in validating the usefulness of ASP enzymes in attenuating cashew allergens. Studies investigating the structure-function relationship of the ASPs would also be useful for the design a more robust enzyme that is stable under gastric conditions and exhibits reduced autolysis. Incorporation of ASPs optimized for the gastric environment could have a multitude of nutritional and medical applications.

The global market for food and feed enzymes is a multibillion-dollar industry [49, 50]. Enzymatic processing methods are widely used and collectively comprise an essential tool in the food-processing arena. Members of the *Aspergillus* genus are widely used in the food industry for food processing, are metabolically diverse and known to vary gene expression in response to changes in culture conditions [30]. As such *Aspergilli* are good targets for the identification of novel enzymes and enzyme complexes that can be mass produced and used to modify or degrade nut allergens so that the proteins are no longer able to induce an allergic reaction.

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