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Biological Safety Assessment of Mutant Variant of *Allium sativum* Leaf Agglutinin (mASAL), a Novel Antifungal Protein for Future Transgenic Application

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Supporting Information

ABSTRACT: Genetic engineering has established itself to be an important tool for crop improvement. Despite the success, there is always a risk of food allergy induced by alien gene products. The present study assessed the biosafety of mutant *Allium sativum* leaf agglutinin (mASAL), a potent antifungal protein generated by site directed mutagenesis of *Allium sativum* leaf agglutinin (ASAL). mASAL was cloned in pET28a+ and expressed in *E. coli*, and the safety assessment was carried out according to the FAO/WHO guideline (2001). Bioinformatics analysis, pepsin digestion, and thermal stability assay showed the protein to be nonallergenic. Targeted sera screening revealed no significant IgE affinity of mASAL. Furthermore, mASAL sensitized Balb/c mice showed normal histopathology of lung and gut tissue. All results indicated the least possibility of mASAL being an allergen. Thus, mASAL appears to be a promising antifungal candidate protein suitable for agronomical biotechnology.

KEYWORDS: *genetic engineering, antifungal proteins, mutant Allium sativum leaf agglutinin, allergenicity, biosafety*

INTRODUCTION

Plants are exposed to various biotic and abiotic stress factors such as drought and temperature, as well as attack by various pathogens and pests, which results in huge loss of crop yield worldwide amounting to 30−50 billion USD annually.[1,2] Fungal pathogens causes 70% of the major crop diseases.[3] Fungicide application is the most widely practiced method to control yield loss due to fungal attack.[4] Development of resistance in pathogens against conventional antifungal agents as well as growing concerns regarding the hazardous impact of fungicide on the environment necessitates the development of novel methods to control fungal pathogens. Genetic engineering has proved to be providing a good solution to this issue by transgenic expression of antifungal genes that confer resistance to fungal pathogens and enhance crop yield.[5−7] As a result, antifungal proteins are gaining importance and becoming an integral part of crop management programs against fungal pathogens. Despite several benefits of transgenic technologies, there are social concerns about the possible health hazards of genetically transformed food, including the risk of allergenicity.[8] The past few decades have witnessed a significant rise in food allergies with 3−4% of adults and 5% children affected globally, [9] thereby necessitating the thorough safety assessment of a foreign protein before introducing it in a food crop.

Mutant form of *Allium sativum* leaf agglutinin (mASAL), a member of MMBL (Monocot Mannose Binding Lectin) superfamily, was reported as a potential antifungal candidate protein generated by mutagenesis of the amino acid residues responsible for dimerization of ASAL.[10−15] This mutant form exhibited antifungal property against the pathogenic fungi *Rhizoctonia solani*, *Fusarium oxysporum*, and *Alternaria brassicicola*.[15] The unique antifungal activity of mASAL makes it a promising candidate for engineering agronomically important crop plants with fungal resistance. Nevertheless, when a new protein is being introduced in an ecosystem, it becomes essential to monitor the biosafety aspect of the same. Unfortunately, there is no single experiment that can evaluate the biosafety of any candidate protein. Thus, the best current approach is to follow the recommendation of "Decision Tree Approach" by FAO/WHO (2001).[16]

In the present study, mASAL was subcloned in pET28a+ vector, expressed in the *Escherichia coli* BL21 cell line and purified by affinity column. Bioinformatics analysis, pepsin digestibility assay, thermal stability, immunoscreening, and *in vivo* studies in mice were performed to assess the allergenic potential of mASAL through a weight of evidence approach.

MATERIALS AND METHODS

Sequence Homology of mASAL. The amino acid sequence of mASAL was used as the query sequence (Supporting Information, Figure 1). Structural Database of Allergenic Proteins of the University of Texas Medical Branch (SDAP; 737 allergen sequences; http://fermi.utmb.edu/SDAP),[17] the Allergen Database for Food Safety (ADFS; 2108 registered allergens; http://allergen.nihs.go.jp/ADFS/index.jsp), and the AlgPred database (AlgPred; 578 allergens www.imtech.res.in/raghava/algpred/)[18] are commonly used to determine the potential allergenicity of mASAL according to FAO/WHO guidelines . The sequence of mASAL was searched in the SDAP and ADFS databases with full FASTA alignment on an 80 amino acid window frame with a threshold sequence identity of >35% to be referred as allergen. In addition we studied the sequence identity of eight contiguous amino acids with known allergic protein. Mapping of IgE specific epitopes was done through the AlgPred database.

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Cloning of mASAL. The mASAL gene was subcloned using forward primer (5′ AGCTGGATCCATGGCCAGCAACCTAC-TGACGAAC 3′) and reverse primer (5′ AATGAGCTCCTAG-GTACCAGTAGACCAAAT 3′) containing the *Bam*HI and *SacI* sites (underlined) respectively. The PCR amplified product was purified using a PCR purification system (Qiagen, Germany). The PCR purified product and the pET28a+ vector (Novagen, WI, USA) were independently digested with *Bam*HI and *SacI* enzymes, ligated at 16 °C overnight, and transformed in to *E. coli* DH5α cells. The transformants were selected by plating in LB agar plates containing kanamycin. The recombinant plasmid was screened by restriction digestion and confirmed by DNA sequencing (data not shown), and the positive clones were transformed into the *E. coli* BL21 cell line (Invitrogen, CA, USA).

Expression and Purification of mASAL. The recombinant plasmid pET28a+ vector harboring the mASAL gene under the control of the T7 promoter was used for the expression and purification of mASAL protein. Ten milliliters of bacterial culture was grown in 500 mL of Luria broth (LB) medium until an OD (λ = 600 nm) of 0.5−0.8 was attained. The culture was induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated with constant shaking for 16 h at 16 °C. The recombinant cells were pelleted by centrifugation at 5000*g* at 4 °C for 30 min, resuspended in lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole, pH 7.4), and sonicated. Cell suspension was centrifuged at 10,000*g* for 30 min at 4 °C, and the supernatant was incubated for 2 h in 2 mL of Ni-NTA (nickel nitrilotriacetic acid) column equilibrated with lysis buffer. The column was washed with wash buffer (50 mM NaH_2PO4, 300 mM NaCl, 20 mM imidazole, pH 7.4) to remove nonspecific proteins, and finally the target fusion protein was eluted with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 7.4).

The expression and purification of the recombinant protein was analyzed in 15% SDS-PAGE stained with Coomassie brilliant blue. The expression of mutant ASAL was further confirmed by Western blot analysis using anti-mASAL polyclonal antibody (1:8,000) and anti-rabbit IgG-horse radish peroxidase (HRP) conjugate as secondary antibody (1:20,000). All purification steps were carried out at 4 °C.

Analysis of Purified mASAL by MALDI-TOF Mass Spectrometry. Approximately 0.5 μL of purified mASAL was mixed with the same volume of presonicated sinapinic acid (SA) matrix and loaded on to the 384-well MALDI target steel plate (Bruker Daltonik, GmbH). The plate was dried at room temperature to form crystals. The protein mass fingerprinting (PMF) of the purified protein was determined by MALDI-TOF mass spectrometry using an Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, GmbH) in linear mode using a 337-nm N_2 laser at 54% power in the positive ion mode. The final data was obtained by averaging 200 spectra, each of which was the composite of 20 laser firings, and analyzed by Flex analysis 2.4 software (Bruker Daltonik, GmbH).

***In Vitro* Digestion of mASAL.** The simulated gastric fluid (SGF) reaction buffer was prepared by adding 122.8 mg of NaCl to 59.2 mL of distilled water and adjusting the pH to 1.2 using 6 M HCl. Pepsin (Sigma, USA) was added at a concentration of 3.2 mg/mL (approximately 3460 U activity/mg) in freshly prepared SGF. mASAL was digested in SGF at a concentration of 0.25 mg/mL to each reaction vial, and digestion was carried out at 37 °C for time periods of 0, 2, 5, 15, 30, 60, and 120 min. In a control setup only mASAL was used. The reaction was stopped immediately by adding 5 N NaOH. Laemmli buffer[19] was added followed by heating in a water bath for 5 min. Then each sample was analyzed in 15% SDS−PAGE followed by Western blotting.

Thermal Stability of mASAL. *1. Circular Dichroism (CD) Spectroscopy.* The changes of secondary structures of the purified protein at different temperatures were recorded with a Jasco Corp. J-815 Circular dichroism spectroscopy (CD) spectropolarimeter with a temperature controller within a wavelength range of 200−260 nm. Protein concentration of approximately 0.2 mg/mL in phosphate buffered saline (PBS) (pH 7.4) was measured in a quartz cuvette with a 0.1-cm path length at different temperatures in the range of 35−95 °C at intervals of 10 °C. Spectra were obtained after cooling as an average of 10 scans on a degree ellipticity scale.

2. Disc Diffusion Assay against the Fungal Pathogen Rhizoctonia solani. The thermal stability assay was performed by incubating aliquots of mASAL (15 μg) in PBS buffer at 25, 37, 55, 75, 85, 95, and 100 °C, respectively, for 30 min in temperature-controlled heating blocks. The assay was terminated by rapid cooling in ice. Each aliquot was subjected to a hyphal extension inhibition assay against *Rhizoctonia solani* as described by Roberts and Selitrennikoff.[20] Phosphate buffer was used as a negative control. The plates were sealed with parafilm and incubated at 28 °C. Each set of experiments was performed in triplicate. Antifungal activity was monitored by the appearance of a crescent-shaped zone of inhibition around the disc.

Specific IgE Estimation. IgE specific ELISA was performed with the sera of 10 allergic patients having a history of food allergy (aged 18−50 years), with symptoms of allergic asthma, rhinitis, and dermatitis. The patients having any two of the symptoms, viz., sneezing, rhinorrhea, nasal blockage, postnasal drip, etc., for the past two years were diagnosed as rhinitis.[21] Sera were collected from a referral allergy clinic (Dan Diagnostic Clinic, Burdwan, India). Patients having chronic illnesses and smokers were excluded from this study. Sera collected from healthy individuals without any history of allergenicity was used as negative control. Blood samples (sera) were collected from patients with their written consent. The entire study was approved by the Human Ethics Committee of the Institute.

The wells of microtiter plate were coated with 10 ng/ μL of pure protein (50 μL/well) in PBS and incubated overnight at 4 °C coated with coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide; pH 9.6). The wells were then washed thrice (15 min each) with phosphate-buffered saline and Tween 20 (0.5% v/v), pH 7.3 (PBST) and blocked with 50 μL of 1% bovine serum albumin (Sigma) in PBST for 3 h at 4 °C. The wells were again washed three times with PBST and incubated with 50 μL of individual patient's sera diluted (1:5) with blocking solution (PBST-BSA) at 37 °C for 16 h. After washing with PBST the wells were incubated with 50 μL of monoclonal mouse anti-human IgE−alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA), diluted 1:1000 times in blocking solution at 37 °C for 3 h. The final wash with PBST was followed by the addition of 50 μL of *p*-nitrophenyl phosphate (pNPP) liquid substrate system for ELISA (Sigma) and incubation in the dark at room temperature for 25−30 min. The reaction was stopped by adding 3 N NaOH after color development. The absorbance was measured at 405 nm with an ELISA reader (ELx 800, Bio-Tek Instruments Inc., Winooski, VT, USA).The P/N value (ratio of average OD of individual patient sera with respect to the control group) of individual patient sera was calculated.[22] The control was the average OD values of sera of all healthy individuals. A P/N value greater than 3.5 for a particular serum was considered to be potentially IgE reactive.[23]

Balb/c Mice. Healthy 8−10-week-old female Balb/c mice (22 ± 2g) were used in this experiment. The mice were kept under standard conditions of controlled temperature (22 ± 2 °C), humidity (55 ± 16), and a 12 h:12 h light:dark cycle. Mice were fed with a commercially available mannose-free pellet diet and water *ad libitium* for one week for acclimatization to laboratory conditions.

Sensitization Protocol. Mice were sensitized according to an earlier described protocol with little modifications.[24] Balb/c mice were sensitized by the intraperitoneal (ip) route, considered to be the most accepted route to study allergic response in animals.[25] Mice were randomly segregated into three groups of five mice each. Group I mice were sensitized with 100 μL of PBS daily by ip injection. Group 2 and group 3 were sensitized with ovalbumin (OVA; purified chicken albumin) and mASAL (100 μg of protein in 100 μL PBS), respectively, by ip route, once a week for seven weeks. On day 60, mice were ip injected with 3, 6, and 8 mg of OVA or mASAL protein in PBS and sacrificed for the collection of lung and gut tissue. OVA was used as a positive control as it is considered to be a potent allergen. The Animal Ethics Committee of Bose Institute approved the study protocol.

Histological Studies. The challenged mice were sacrificed by cervical dislocation. The gut and lung tissues were removed from

sacrificed mice of each group, fixed immediately in 10% neutral-buffered formaldehyde (v/v) (0.1 M phosphate buffer, pH 7.4), and embedded in paraffin. The tissues were cut into 3−5-μm sections, deparaffinized with xylene and graded ethanol, and stained with hematoxylin and eosin (H&E). Histopathological assessment was done under a light microscope and photographed by using an in-line camera (Leica Microsystem DN1000; Camera DFC450C).

RESULTS

Sequence Homology of mASAL. The potential allergenecity of mASAL was analyzed by amino acid sequence comparison with an allergenic protein database. *In silico* analysis showed that no known allergen was found to be similar with mASAL with full FASTA search or by the criteria of more than 35% identity in the amino acid sequence of the query protein, using windows of 80 amino acids. Even the search for short (eight amino acids) contiguous stretches in the allergen database resulted in no significant match. IgE epitopes search of mASAL using Algpred database showed that the protein sequence does not contain any experimentally proven IgE binding epitopes.

Expression of Purified mASAL. The mASAL gene was subcloned in pET28a+ vector having a histidine tag for purification. The recombinant protein is highly expressed in soluble form, and a clear band of ~12 kDa was observed in 15% SDS-PAGE gel (Figure 1, inset a). The expression was further validated by immunobloting with anti-mASAL polyclonal antibody (Figure 1, inset b). The MALDI-TOF profile authenticated the molecular mass and purity of mASAL (Figure 1).

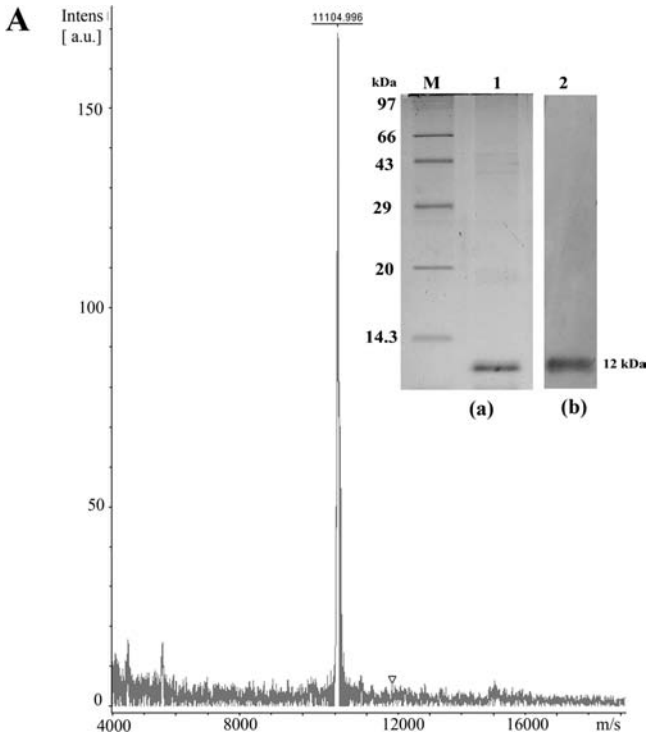

Figure 1. Purification of mASAL. (A) MALDI-TOF mass spectrometry of mASAL. This profile illustrates intact peptide mass that is typical for the mass spectra of 11.1 kDa. Appearance of one peak confirms the quality of purification of mASAL. (Inset) SDS-PAGE elution profile of mASAL. The purified mASAL was resolved in 15% SDS-PAGE. (a) Lane 1, the Coomassie brilliant blue stained purified mASAL. (b) Lane2, Western blotting of mASAL against anti- mASAL polyclonal antibody showing a band at 12 kDa; lane M, a standard molecular weight marker.

Degradation in Simulated Gastric Fluid (SGF). The digestion profile of mASAL in SGF was monitored in 15% SDS-PAGE stained with Coomassie brilliant blue, which determined the purified mASAL was completely digested within 2 min of treatment (Figure 2A). Immunobloting could not detect mASAL in the sample after 2 min of digestion treatment in SGF (Figure 2B).

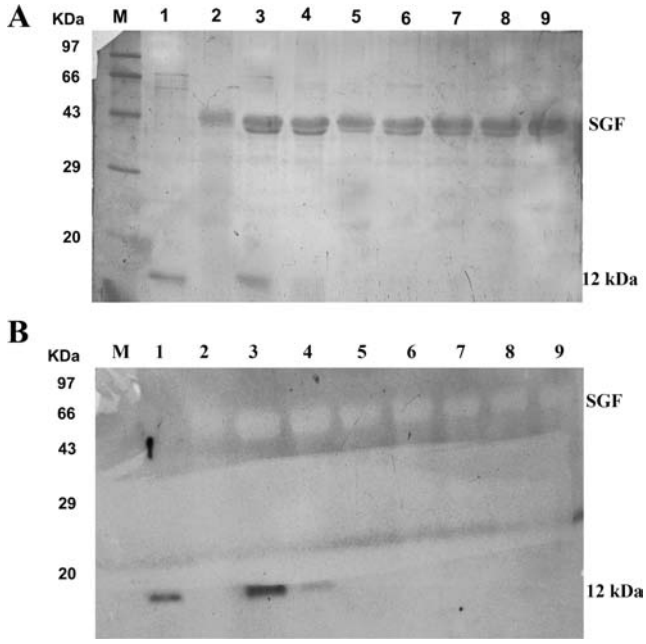

Figure 2. Pepsin digestibility of mASAL. (A) SDS-PAGE profile of SGF treated mASAL. Lane M, molecular weight marker; lane 1, mASAL; lane 2, pepsin in SGF; lanes 3−9, mASAL treated with SGF for 0, 2, 5, 15, 30, 60, and 120 min and resolved in 15% SDS-PAGE and stained with Coomassie brilliant blue. (B) Western blot analysis of the degradation of mASAL in SGF. Lane M, molecular weight marker; lane1, mASAL as positive control; lanes 3−9, incubation of mASAL with SGF for 0, 2, 5, 15, 30, 60, and 120 min. The molecular weights (kDa) of the protein markers are sown at the left. (Label indicates the position of Pepsin and mASAL band).

Thermal Stability Assay. *CD Analysis.* Far UV-CD was used to measure the extent of secondary structural changes induced by high temperature treatment. Monomeric ASAL has a signature negative ellipticity at 228 nm (λ_{max}) like other lectins as studied by Banerjee et al.[15] In the thermal stability assay, temperature-induced loss of structural integrity was evident at 55 °C onward. It was observed that the changes in the negative ellipticity at 228 nm (λ_{max}) began at 55 °C and were complete at 95 °C. After heating to 75 °C and onward, the signature negative ellipticity of mASAL was completely lost, indicating complete loss of secondary structural confirmation of mASAL (Figure 3A,B).

Disc Assay. The antifungal activity of the purified mASAL toward *Rhizoctonia solani* was discernible in the disc assay up to 37 °C. The protein pretreated at 55 °C for 30 min completely lost its biological activity against *Rhizoctonia solani* (Figure 3C).

IgE Specific ELISA. IgE specific ELISA was performed with the sera of 10 allergic patients suffering from asthma, allergic rhinitis, dermatitis or having history of food allergy. In specific IgE ELISA, significantly low IgE level (0.56−1.22) was detected

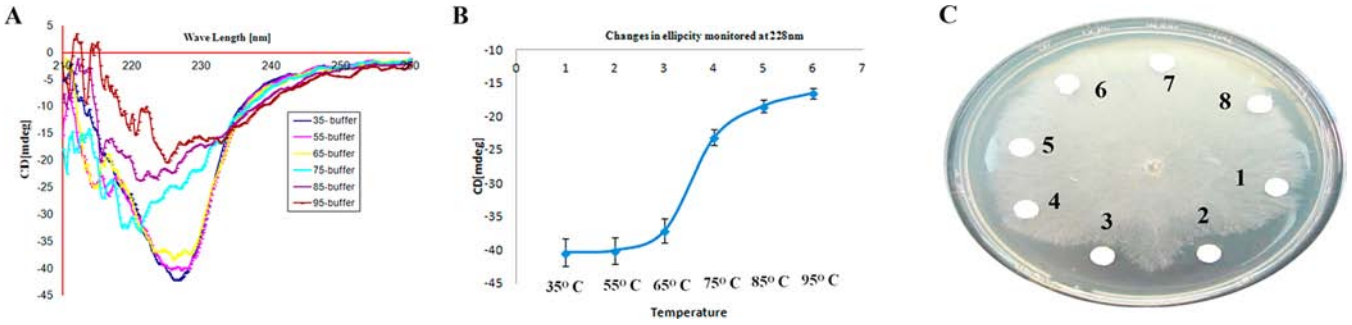

Figure 3. Heat stability of mASAL. (A) CD (circular dichroism) spectra of mASAL were recorded over wavelengths of 200−260 nm at different temperatures: 35, 55, 65, 75, 85, and 95 °C. (B) Graphical representation of changes in the ellipticity at 226 nm (λ_{max}). (C) Effect of temperature on antifungal activity of mASAL against *Rhizoctonia solani*. Disc 1 represents the 10 mM sodium phosphate buffer; discs 2−10 represents mASAL (15 μg) treated at 25, 37, 55, 75, 85, 95, and 100 °C, respectively.

when mASAL was used as probe (Table 1), in contrast to the high P/N ratio (1.97−3.51) observed when probed with ovalbumin, a commonly known allergen (Supporting Information, Table 2).

Table 1. Clinical Characteristics of Patients and *in Vitro* IgE Specific ELISA Results[a]

patient no.	age (years)/ sex	symptoms	specific IgE P/N[b] value (mASAL)
1	34/M	AR + BA	1.22
2	28/M	AR	1.21
3	52/F	BA	0.86
4	21/F	BA	0.68
5	27/M	AR + BA + D	0.70
6	29/M	BA	0.62
7	25/M	BA + D	0.92
8	35/F	AR + BA	0.79
9	23/M	AR + BA	1.19
10	39/F	AR	1.08

[a]M, male; F, female; IgE, immunoglobin E; AR, allergic rhinitis; BA, bronchial asthma; D, dermatitis. [b]IgE-reactive proteins shows P/N value >3.5.

Histopathological Analysis of Lungs and Gut. *Lung Histology.* Hematoxylin and eosin-stained (H&E) lung sections of ovalbumin (OVA) sensitized mice showed congested lung structure with peribronchial and perivascular inflammatory cell infiltrate (Figure 4B). In contrast mASAL sensitized mice showed normal lung structure with defined alveoli and bronchioles with no evidence of inflammation (Figure 4C). The lung section of mASAL sensitized mice was similar to that of control mice, sensitized with PBS (Figure 4A).

Gut Histology. The gut histoarchitecture of ovalbumin sensitized Balb/c mice showed the common pathological symptoms encountered during normal food allergic reaction.[26] Prominent distortion in gut lining with abnormalities in ileum, duodenum, and jejunum villi accompanied with destruction of mucosa lining and infiltration of inflammatory cells was observed in ovalbumin challenged mice (Figure 5B), whereas normal histology of gut lining was observed in both mASAL and PBS sensitized mice (Figure 5A,C).

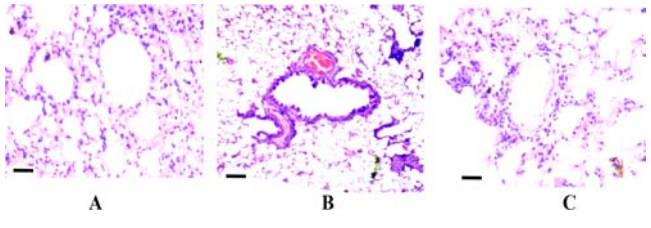

Figure 4. Histopathological illustration of the lung of the sensitized Balb/c mice. (A) PBS contol. (B) Ovalbumin. (C) mASAL. (bar = 1 μm)

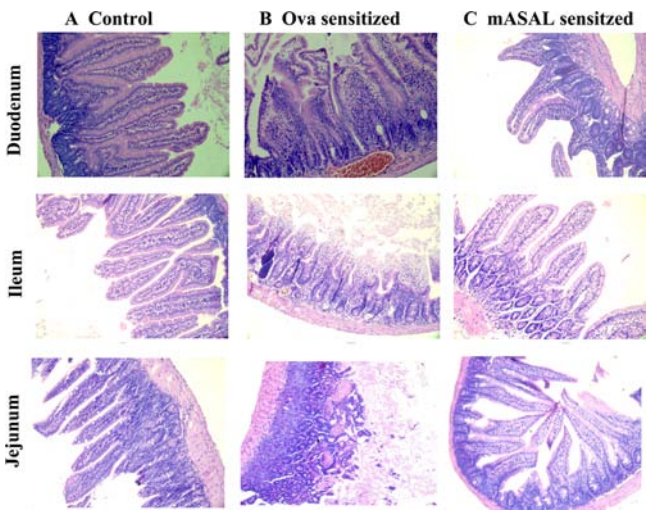

Figure 5. Histolopathological analysis of duodenum, ileum, and jejunum of sensitized Balb/c mice. (A) Control group. (B) OVA sensitized group. (C) mASAL sensitized group.

DISCUSSION

Several international organizations have framed specific guidelines to address the biosafety-related issues of a new candidate protein using *in vitro* and *in vivo* model systems.[27−30] Currently, allergenicity assessment of novel (GM) food proteins involves multifactorial and comprehensive approaches such as the source of the gene, bioinformatics analysis using sequence and structural homology comparisons of the candidate protein with known allergens, *in vitro* pepsin digestibility assay, and specific IgE sera screening studies.[31] Following the guidelines of the FAO/WHO, candidate proteins such as *Bacillus thuringiensis* (Bt) toxin,[32−34] 2S albumins,[35] and Q2 and lysine-rich protein[36] have been assessed for their probable allergenicity and toxicity to nontarget organisms. After such investigation some proteins stand out as nonallergenic (i.e., Bt, lysine-rich protein,

etc.), whereas other proteins exhibited toxicity and/or allergenicity to human being (2S albumins of the Brazil nut).

The present study describes the evaluation of monomeric ASAL (mASAL), generated by introduction of five site specific mutations in a homodimeric lectin *Allium sativum* leaf agglutinin (ASAL), which was known for its insecticidal efficacy in native as well as in transgenic conditions.[10−14] This ∼12 kDa antifungal mASAL protein was initially expressed in a pMAL-c2X expression system. As purification of an adequate quantity of protein from the pMal-c2x expression system is cost-intensive and time-consuming, the mASAL was subcloned in the pET28a+ vector to utilize the advantage of a one-step purification method by using an immobilized affinity chromatograpy protocol. Expressed mASAL was purified through a Ni-NTA column, purity was confirmed by SDS-PAGE and MALDI-TOF analysis, and the mASAL was then used for the biosafety assessment.

Biosafety Assessment of mASAL: A Bioinformatic Approach. The initial step recommended for the assessment of allergenic potential of a novel protein is the screening of its amino acid sequence with known allergens. As per FAO/WHO (2001) recommendations, a protein is considered cross reactive with immunoglobin E (IgE) when it shares more than 35% identity in a window of 80 or more amino acids with a known allergen. In 2001, FAO/WHO proposed using a contiguous six amino acids sequence similarity instead of eight between a query protein and recorded allergen in the database. However, that resulted in a great number of false positive predictions.[37] Matching of minimum eight contiguous and identical amino acids between the query protein and allergens is considered to have some relevance in identifying a possible allergen. Bioinformatics analysis of mASAL showed no evidence of any match with any known allergen in the SDAP and ADFS databases. Even mapping of IgE binding epitopes of mASAL using AlgPred showed that the protein sequence does not contain any experimentally proven IgE epitope, which is further validated through targeted serum screening. Thus, mASAL appears to be nonallergenic on the basis of sequence identity.

Stability in Pepsin. Stability to digestion is considered to be a crucial predictive tool for the assessment of allergenic potential of a protein. Generally food proteins are promptly denatured and degraded in the mammalian gastrointestinal tract (GI) by proteolytic enzymes in acidic conditions, whereas allergic proteins are highly stable and resistant to digestive process. This stability enables the allergenic protein to be absorbed through the intestinal mucosa for triggering allergic response. Therefore, the digestive stability of any exogenous protein can be used as an important indicator for assessing the allergenic potential of a candidate protein.[38] SGF mimics the condition of the human stomach and consists of gastric protease pepsin at pH 1.2.[39] It has been reported that many known allergen such as lectins from soybean, Ara h 2 from peanut, Sin a l and Bra j IE from mustard, β-lactoglobin from milk, and ovalbumin and phosvitin from egg are resistant to SGF digestion up to 60 min.[38] In addition, there are reports of some other allergenic proteins such as 2S albumins that showed resistance even after 2 h of gastric digestion.[40] However, the SGF digestibility test showed that mASAL was completely digested within 2 min, indicating its similarity to ASAL concerning stability.[23] Moreover, bioinformatics analysis by ExPasy peptide[41] cutter showed 38 pepsin cleavage sites in mASAL, which matches the result of the pepsin digestibility assay (Supporting Information, Figure 2). Thus, the *in vitro* digestibility of mASAL suggests the protein to be nonallergenic.

Thermolability of mASAL. Heat treatment results in significant alteration in protein structure. Generally the loss of tertiary structure is followed by reversible unfolding and denaturation of secondary structure around 70−80 °C, with further increase in temperature intra/intermolecular interaction occurs and rearrangement of disulfide bond takes place around 80−90 °C, and finally aggregation occurs at 90−100 °C. [42] Thermal treatment may alter the structure of the protein by rendering some conformational changes that could lead to the exposure of few allergic epitopes. Birch-pollen-related allergens Cor a 1.04 and Cor a 2 found in hazelnut are heat-labile allergens causing 90% reduction of immunoreactivity upon heating.[43] However, roasted peanuts have much higher IgE binding capacity in respect to raw peanuts of the same cultivars.[44] Therefore, heat treatment may increase or decrease allergenic potentiality, although in reality it varies from patient to patient. So there is no general correlation between the heat stability and allergenicity of a protein. However, most of the allergenic proteins are resistant to heat, and the probability of a candidate protein being allergenic may be correlated with its structural stability at high temperature. Previously it was detected that ASAL completely lost its biological activity after 30 min of incubation at 50 °C .[23]However, mASAL almost retained its secondary structure up to 55 °C, which was lost with further increase in temperature. In contrast, the functional activity of mASAL was completely missing at 55 °C, indicating that during the onset of structural loss at 55 °C, the active site of mASAL was affected first, which consequently resulted in loss of antifungal activity.

Immunoscreening with Targeted Patient Sera. Allergic proteins are known to induce IgE production. IgE mediated allergic diseases such as asthma, food allergies, rhino conjunctivitis, eczema, and anaphylaxis are predominant in over 20% of the world population. In India, a large amount of population suffers from allergic rhinitis and bronchial asthma.[45] Thus, allergic potential is generally assessed by specific IgE screening with sera from subjects allergic to the identified allergen. Serum IgE measurement is considered to be an encouraging approach to detect food allergens.[46] The presently described IgE specific ELISA of mASAL showed the P/N ratio is quite below the threshold level (<3.5), which suggests that mASAL is potentially IgE non reactive.

Evaluation of Allergenic Potential in Mouse Model. A variety of animal models are proposed for the allergenicity assessment.[47] Mice are widely used animal model, mainly for their high similarity with humans regarding many important immunological mechanisms, such as Th1, Th2, Th17, and regulatory responses.[48,49]Additionally, Balb/c mice are also capable of differentiating between allergenic and nonallergenic proteins when sensitized systematically (ip) in the absence of adjuvant.[50]Histopathological studies are considered to be one of the most pivotal approaches for evaluating allergenic response *in vivo*.[25,51] Prominent histopathological changes specific for allergic inflammation were observed in the jejunum, lungs, and spleen of ip sensitized Balb/c mice with crude chickpea protein extract (CP-CPE).[26] Similar structural changes were also observed in the ileum of mice fed with GM potato expressing Cry 1 gene.[52] There are also reports about the adverse effect of lectins on animals generated from feeding experiments. GM potato expressing *Galanthus nivalis* lectin showed proliferation of gastric mucosa when fed to

mice.[53] PHA (phytohemagglutinin) from red kidney bean has also been documented to induce prominent allergic manifestation in the lung, intestine, and spleen of mice.[45] In the present study mASAL sensitized Balb/c mice displayed normal appearance of lung and gut tissue, similar to PBS treated mice, indicating that mASAL indeed has no detrimental features, whereas ovalbumin sensitized mice showed prominent allergic reactions and resulting loss of normal morphology.

So, considering "weight-of-evidence" approach and the results of all experiments including bioinformatics analysis, pepsin digestion, thermal stability assay, targeted sera screening, and *in vivo* analysis with mouse model, it can be concluded that mASAL is nonallergenic in nature. Hence, mASAL may be considered as a safe candidate antifungal protein for its future application in the arena of plant biotechnology. Incidentally, plants have repeated interactions with several beneficial insects, mycorrhizae, and bacteria involved in root nodule induction during the entire course of their life cycles. In future the effect of mASAL on the above organisms needs to be monitored before field application of this novel protein.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Sequence of mASAL, table showing clinical characteristics of patients and *in vitro* IgE specific ELISA result against ovalbumin, figure showing the cleavage of mASAL with Pepsin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

mASAL, mutant *Allium sativum* leaf agglutinin; MMBL, monocot mannose binding lectin; SDAP, Structural Database of Allergenic Proteins; ADFS, Allergen Database for Food Safety; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, nickel nitrilotriacetic acid; SGF, simulated gastric fluid; IgE, immunoglobulin E; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; OVA, ovalbumin; H&E, hematoxylin-eosin

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