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Engineered hypoallergenic variants of osmotin demonstrate hypoallergenicity with *in vitro* and *in vivo* methods

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ABSTRACT

The molecular structure of protein and epitope mapping strategies are required to engineer the epitopes of a protein. In the present study, IgE binding regions of osmotin were identified and mutated to obtain hypoallergenic variant. The three dimensional (3-D) model of osmotin obtained by homology modeling comprised of a characteristic thaumatin-like fold. This model was used to predict IgE binding regions of osmotin. These regions were mutated and three mutant proteins with four mutations (Ma, Mb and Mc) and one with six mutations (Mabc) were expressed and purified to homogeneity. IgE binding of the mutant proteins was evaluated by *in vitro* studies using patients' sera. Ma, Mb and Mc demonstrated reduction in IgE binding of 73%, 83% and 77%, respectively, whereas Mabc showed complete abrogation of IgE binding. Ma, Mb and Mc showed inhibition of 48%, 44% and 38%, respectively to osmotin, while Mabc showed 24% inhibition at 10 μg with pooled patients' sera. Osmotin reached effective concentration at 50% inhibition (EC_{50}) at 3 ng and none of the mutant proteins reach the EC_{50} value. The immunological response to mutant proteins was examined in mice. Blood, bronchoalveolar lavage fluid spleen and lung tissue were excised from mice for analysis. The mice treated with mutant proteins showed significant reduction in IgE and IgG1 levels as compared to mice given osmotin ($p < 0.001$). Th2 cytokines level in splenocyte supernatant and BALF of mice given mutant proteins were significantly lower ($p < 0.001$), accompanied with significant reduction in cellular infiltration in lungs ($p < 0.001$). In conclusion, osmotin structure was predicted by homology modeling and IgE binding regions predicted were mutated to obtain a hypoallergenic protein.

1. Introduction

Genetically modified (GM) foods/crops are being used in many countries. It improves the crops by conferring resistance to herbicides, pests, abiotic stress *etc.* thereby enhancing the quantity and quality. GM foods like corn, soya, rice, canola, *etc.* have been developed using genetic engineering (Schwember, 2008). The consumption of GM foods has raised a number of fundamental issues regarding environment and health concerns (Conner et al., 2003; Taylor and Hefle, 2002). Among the health issues, allergenic potential of newly expressed proteins in genetically modified foods is a cause of concern and is required to be assessed as per the prevailing guidelines (CAC, 2003).

Many proteins are expressed in GM crops which include Cry proteins (Pinto et al., 2013), thaumatin like proteins (TLPs) (Mackintosh et al., 2007), chitinases (Oldach et al., 2001), defensins (Portieles et al., 2010), betaine aldehyde dehydrogenase (Wang et al., 2013), β-1,3-glucanase (Amian et al., 2011) *etc.* Cry proteins produced by *Bacillus thuringiensis* are toxic to major insect pests such as the European corn borer, southwestern corn borer, tobacco budworm, cotton bollworm, etc, TLPs and β-1,3-glucanases are pathogenesis-related proteins show broad spectrum resistance against multiple pathogens, chitinases improve the resistance to disease, defensins have been be effective in curbing fungal diseases, glycine betaine improves plant tolerance to various abiotic stresses and lectins serve as defense proteins against many pathogens. *In silico* analysis of some proteins have shown sequence identity of more than 35% with known allergens in different allergen databases (Mishra et al., 2012). Betaine aldehyde dehydrogenase showed sequence identity values of 41.02% and 43.70% with Cla h 10 of *Cladosporium herbarum* and Alt a 10 of *Alternaria alternata*, respectively. Similarly, rice chitinase showed high degree of identity with allergens

Abbreviations: PDB, protein data bank; BLAST, basic local alignment search tool; BALF, bronchoalveolar lavage fluid; GM, genetically modified; OD, optical density.

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from 69% with rubber, 63% with chestnut and 67% with avocado endochitinase (Mishra et al., 2012). A study identified Cry1Ac to be a potent immunogen where mice receiving intraperitonial (*i.p.*) dose of Cry1Ac showed high anti-Cry1Ac IgG antibody levels in the large and small intestine fluids (Vázquez-Padrón et al., 1999). These studies indicate that either to avoid the use of these genes or modify them by genetic engineering to make them hypoallergenic which may help to reduce the risk of adverse reactions.

Osmotin is a mutifunctional pathogenesis related protein isolated from tobacco and used for osmotic stress tolerance and antifungal activity for developing transgenic crops (Subramanyam et al., 2012; Patade et al., 2013). Many transgenic crops like tobacco, strawberry, wheat, cotton, tomato, mulberry and Soybean contain osmotin. In earlier studies osmotin has been shown as potential allergen (Sharma et al., 2011). Three linear IgE binding epitopes of osmotin were predicted using computational sequence based tools. These regions were further mutated to obtain a mutant having reduced allergenicity (Sharma et al., 2013). Osmotin has potential to develop more transgenic crops. Therefore, the present study was aimed to get an osmotin mutant having no allergenic potential.

2. Material and methods

2.1. Homology modeling of osmotin and prediction of conformational epitopes

The amino acid sequence of the osmotin protein from *Nicotiana tabacum* was retrieved from the sequence database of NCBI http://www.ncbi.nlm.nih.gov/entrez ID: P14170. A suitable template protein for the modeling of osmotin protein was searched in PDB (Brookhaven Protein Data Bank). BLAST (Basic Local Alignment Search Tool) was used to find the homologues of osmotin. Amino acid sequence alignment of target (osmotin) and template proteins was performed using CLUSTALW. PDB file of the template was obtained from RCSB Protein Data Bank. The 3D model was constructed by alignment of target sequence with template structure using MODELLER 9v7 (Sanchez and Sali, 2000). The best model with the lowest value of the Modeller objective function and DOPE (Discrete Optimization Protein) score from a number of models built by MODELLER was selected. The model selected was energy minimized in Amber force field by steepest descent algorithm implemented in the SYBIL software from Tripos Inc. (St. Louis, MO). The 3D model obtained was validated by PROCHECK (http://nihserver.mbi.ucla.edu/SAVES_3/) and ERRAT (http://nihserver.mbi.ucla.edu/). The selected model was visualized using PyMOL (http://pymol.org/) softwares.

The discontinuous epitopes were predicted on the basis of knowledge of the 3D structure of osmotin. The servers used for prediction of conformational epitopes were conformational epitope prediction (CEP), DiscoTope, SEPPA, ElliPro and Epitopia (Yao et al., 2013). The consensus approach was used where epitopes identified frequently by most of the structure based tools were selected.

2.2. Site directed mutagenesis of osmotin

Four mutants were generated in our previous work (Sharma et al., 2013). The triple point mutant protein, M123 showed maximum reduction in IgE binding. Hence, pET22b+ vector containing the triple point mutant M123 clone was used as a template to generate hypoallergenic variants of osmotin. Each of the three predicted B cell epitopes were mutated at different residues. The clones of the vector pET22b+ containing the three mutants Ma, Mb, and Mc with four mutations (3 of M123 + 1 in each IgE binding region) and a six point mutant with all the above mutations (Mabc) were developed. Mutagenesis was performed using oligonucleotide primers and mutation-specific oligonucleotide primers accommodating each mutation. The first two steps of PCR reaction was carried out with an internal mutant primer and one of the flanking primers. Final products of these two steps were purified from agarose gel, mixed and amplified using flanking primers. The mutant gene Ma was generated using forward primer 5′ACGATTTACTGCAATGGCACGT 3′ and reverse primer 5′ ACGTGCC ATTGCAGTAAATCGT 3′, mutant gene Mb was generated using forward primer 5′ CCGTACTGCTTGTAACTTCAATG 3′ and reverse primer 5′ CATTGAAGTTA CAAGCAGTACGG 3′, mutant gene Mc using forward primer 5′ AACTTGTGGTTC CCGGAGGATGT 3′ and reverse primer 5′ ACATCCTCCGGGAACCACAAGTT 3′. The flanking primers used were 5′GAATTGAATTCGATGGGCAACTTGAGATCTTC TTTTG3′ with *EcoR I* site and 5′GCTCGAGCTCCTTA GCCACTTCATCACTTCCAG 3′ with *Xho I* site. The fourth mutant gene Mabc with all three mutations was generated step wise using mutant Ma as template. The PCR products were digested with *EcoRI* and *XhoI* enzymes, subcloned in pET22b+ vector to generate four plasmids pPS5, pPS6, pPS7 and pPS8. These were further sequenced to confirm the mutations. Constructs containing the mutations in the epitopes were used for the expression of fully modified recombinant osmotin protein mutants.

2.3. Expression and purification of mutants

The plasmids pPS5, pPS6, pPS7 and pPS8 containing Ma, Mb, Mc and Mabc sequences, respectively, were transformed into *Escherichia coli* BL-21 cells. The cells were grown till 0.6 O.D., induced with 1 mM isopropyl-beta-D-thiogalactopyranoside for 3 h, harvested and the pellet was suspended in binding buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (pH 8.0), sonicated and centrifuged. The supernatant was incubated for 2 h with 2 ml Ni-NTA equilibrated with binding buffer. The column was washed with buffer containing 20 mM imidazole, 300 mM NaCl and 50 Mm NaH_2PO_4 (pH 8.0) and bound proteins were eluted using buffer containing 250 mM imidazole, 300 mM NaCl and 50 mM NaH_2PO_4. The purified mutant proteins were resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies raised in mice.

2.4. Peptide synthesis

Total of six peptides, three peptides (P1, P2 and P3) corresponds to the predicted B-cell epitopes, and the other three (P4, P5 and P6) with two amino acid change corresponding to the mutations were synthesized by the manufacturer (Thermo Fischer Scientific GmbH) with a purity of $\geq$90% and their sequences were confirmed by mass spectrometry (MALDITOF) analysis.

2.5. Sera collection

The patients ($n = 15$) showing symptoms of allergic rhinitis and asthma aged 15–50 years were skin prick tested (SPT) with various food extracts to detect sensitization. The diagnosis of asthma and rhinitis was confirmed following American thoracic society guidelines (American Thoracic Society, 1991) and ARIA (Bousquet et al., 2008), respectively. Blood samples of the healthy subjects and patients that showed positive SPT to different food extracts were collected from the outpatient department, V.P. Chest Institute, Delhi, a referral chest hospital. The study protocol was approved by human ethics committee of the Institute of Genomics and Integrative Biology, Delhi.