



Recombinant glutathione-S-transferase a major allergen from *Alternaria alternata* for clinical use in allergy patients

Jay Shankar^{a,b}, Bhanu P. Singh^a, Shailendra N. Gaur^c, Naveen Arora^{a,*}

^a Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India

^b Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007, India

^c V.P. Chest Institute, University of Delhi, Delhi 110007, India

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Abstract

Recombinant proteins are used for vaccines, therapy and diagnosis of many diseases. Biological activity of these may differ from native counterpart and needs investigation. The present study aimed to compare recombinant (r) and native (n) glutathione-S-transferase (GST) from *Alternaria alternata*. Glutathione-S-transferase sequence showed an ORF of 696 bp encoding 26-kDa protein with N-terminus conserved domain. Secondary structure of both forms was comparable with melting temperature of 57 and 59 °C, respectively. rGST and nGST showed similar enzymatic activity, allergenicity and potency by ELISA inhibition. Histamine release was comparable in 14/17 patients for both the GSTs. rGST and nGST induced proliferation in PBMC at different concentration. Cell supernatant revealed higher IL-4 and IL-5 levels with low levels of IFN- γ . In summary, recombinant and native GST demonstrated similar physio-chemical, biological and immunological properties and induced comparable cell mediated and humoral response to be used for diagnosis and specific immunotherapy for the fungal allergy cases.

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1. Introduction

Fungi are involved in hypersensitive reactions in about 20–30% of the atopic cases worldwide (Gioulekas et al., 2004). *Alternaria alternata* is known to induce asthma and respiratory diseases (Achatz et al., 1995). Previous studies have identified Alt a 1–3 (heat shock protein), 6 (60 s ribosomal P2 phosphoprotein), 7 (flavodoxin 1), 10 (aldehyde dehydrogenase), 11 (enolase), 12 (60S acidic ribosomal protein P1) as allergenic proteins from *A. alternata* [FAARP database]. Further studies have shown common allergens between *A. alternata*, *Curvularia lunata* and *Epicoccum purpurascens* (Gupta et al., 2002; Bisht et al., 2002). Nuclear transport factor 2 and enolase are

cross-reactive allergens from *Alternaria* (Wagner et al., 2000; Weichel et al., 2003) while 32 kDa, 60S ribosomal protein P2 and MnSOD are from *Penicillium chrysogenum*, *Fusarium*, *Venenum* and *Aspergillus fumigatus* (Wagner et al., 2001; Hoff et al., 2003; Shen et al., 2003). The use of such proteins can minimize allergens for diagnosis/treatment without compromising efficacy (Chapman et al., 2000). Recently, glutathione-S-transferase (GST) was demonstrated as cross-reactive allergen among fungi (Shankar et al., 2005). It has been shown as allergen in *Blatella germanica*, *Dermatophagoides pteronyssinus* and arthropods (O'Neill et al., 1994; Arruda et al., 1997; Galindo et al., 2001). In recent times the knowledge of such proteins is essential to distinguish between co-exposure, co-sensitization and cross-reactivity.

Recombinant allergens have shown potential for diagnosis, immunotherapy, synthesis, study of hypoallergenic variants, epitope mapping, etc. (Lorenz et al., 2001; Hiller et al., 2002). However, recombinant proteins to be used in clinic should have similar physio-chemical, biological and immunological property with the native protein. Therefore, the present study was aimed to clone, sequence and express a major allergenic protein

Abbreviations: CD, circular dichroism; GST, glutathione-S-transferase; IDT, intradermal test; n, native; r, recombinant

* Corresponding author. Present address: Room 509, Allergy and Immunology Section, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road (Near Jubilee Hall), Delhi 110007, India.

Tel.: +91 11 27666156/7; fax: +91 11 27667471.

E-mail address: naveen@igib.res.in (N. Arora).

from *A. alternata*. Further, biochemical, physio-chemical and immunologic properties of recombinant and native proteins were compared.

2. Experimental

2.1. Materials

General cell culture reagents and antibodies were purchased from either Sigma–Aldrich (USA) or Amersham (UK). EZ-RNA isolation kit was from Biological Industries (Israel). mRNA kit was from Gibco-BRL (Invitrogen, USA). cDNA synthesis kit was from Stratgene (La Jolla, USA). Cytokine assay kits were from BD Pharmingen (San Jose, USA).

2.2. Methods

2.2.1. Purification of native GST (nGST)

A. alternata (4789) obtained from IARI, Delhi and extract was prepared as described previously (Shankar et al., 2005).

A. alternata extract (80 mg) was loaded on DEAE-sephadex G50. The column was washed and protein eluted with 0.5 M NaCl. Fractions containing GST activity were pooled, dialyzed, lyophilized and incubated with glutathione Sepharose 4B column (Amersham) at 4 °C. The fraction containing GST activity was pooled, dialyzed, lyophilized, protein estimated by modified Lowry's method (Singh et al., 1992) and analyzed on SDS-PAGE and Western blotted with anti-GST peroxidase (1:2500, v/v; Sigma).

2.2.2. Construction and screening of cDNA library

cDNA library was prepared from 5 µg mRNA isolated (Gibco-BRL) from total RNA using EZ-RNA kit (Biological industries) in λ-ZAP vector (Stratagene) as per manufacturer's protocol. The radioactive probe used for screening was 5'-ATG GCT CAT AAA CTT GTT GTC TAT TTT TCC-3'. Positive clone was sequenced using T3 and T7 primers (ABI-377, DNA Sequencer, Applied Biosystems).

2.2.3. Expression and purification of rGST

PCR amplified cDNA was ligated into pET22b+ vector and protein expressed in *E. coli* BL-21 cells, induced with 1 mM IPTG for 4 h. GST was purified using affinity column and activity assayed with 1-chloro 2,4-dinitrobenzene.

2.2.4. Skin testing and sera collection

Intradermal tests were performed with *A. alternata* extract (1:500, w/v), nGST (10 µg/ml) and rGST (10 µg/ml) on respiratory allergy patients at V.P. Chest Institute, Delhi (Malling, 1985). Phosphate buffer saline and histamine diphosphate (100 µg/ml) were used as negative and positive controls, respectively. Ten controls were also skin tested with protein and extract. Informed consent was obtained for participation in the study and Institutional Ethics Committee has approved the protocol.

2.2.5. Circular dichroism (CD)

CD was done in far-UV range (190–300 nm) for rGST or nGST in phosphate buffer (Jasco 715). Thermal scans between 10 and 100 °C were performed to detect the T_m of proteins.

2.2.6. IgE reactivity of rGST and nGST

SDS-PAGE resolved rGST and nGST (10 µg) were transferred to a nitrocellulose membrane and developed as described previously (Shankar et al., 2005).

To determine specific IgE, ELISA plates (Nunc, Denmark) were coated with 100 ng of rGST, nGST or 1 µg of *A. alternata* extract (Voller et al., 1976). The plates were washed, blocked with 3% defatted milk and incubated with patients' sera (1:10) at 4 °C overnight. The wells were incubated with anti-human IgE peroxidase (1:1000) for 3 h, color developed and read at 492 nm (Gupta et al., 2002).

For ELISA inhibition, ELISA plates were coated with antigen (100 ng of rGST, nGST or 1 µg of *A. alternata* extract) and kept overnight with pre-incubated sera containing different concentrations of rGST or nGST and processed as described above.

2.2.7. Histamine release on allergen challenge

For histamine release (Verma et al., 2003) (Immunotech), heparinized blood from 17 patients were challenged with 1 or 5 ng extract, rGST or nGST for 30 min at 37 °C. Histamine standards (0–100 nM) and released histamine from the samples were acylated and measured by ELISA. Spontaneous histamine release in unchallenged sample and total histamine was measured. Five non-allergic individuals were used as negative control.

2.2.8. Cytokine analysis and PBMC proliferation on antigen challenge

For lymphoproliferation assays, 10⁵ cells/well isolated from heparinized blood by Ficoll hypaque (Sigma) were cultured in RPMI media and challenged with 1 or 10 µg of extract, rGST or nGST. PHA (5 µg) was used as positive control and no stimulant was added to negative control. Plates were incubated for 72 h and supernatant collected for cytokine assay. Cells were washed, incubated for 2 h with MTT (100 µg/ml), lysed in acidic isopropanol and read at 570 nm.

IL-4, IL-5 and IFN-γ were estimated by ELISA kits following manufacturer's protocol (BD Pharmingen OptEIA kits). Standards (5–500 ng), 100 µl of sample (1:2) or controls were added to wells and incubated at 37 °C for 3 h. The plates were washed, incubated with biotinylated detector antibody and color developed.

2.2.9. Statistical analysis

Specific IgE, histamine release and cytokine levels to rGST and nGST were compared by a paired *t*-test. The correlation coefficients between rGST and nGST were determined by linear regression. *p*-Values < 0.001 were considered statistically significant.

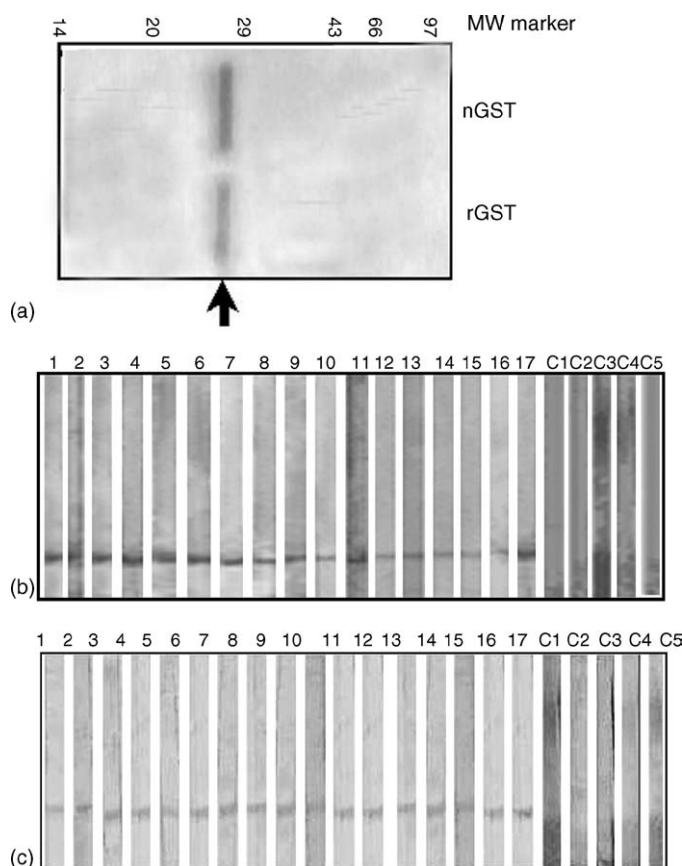


Fig. 1. SDS-PAGE profile and immunoblot of native and recombinant GST. (a) Ten micrograms of protein was resolved and stained with CBB. Lane 1, nGST; lane 2, rGST; (b) rGST and (c) nGST were transferred to membrane and probed with 17 individual *Alternaria* hypersensitive patient's sera (1:10) or normal human serum (C1–C5) as negative control and incubated with anti-human IgE-HRP (1:1000).

2.3. Results

2.3.1. Purification of nGST

nGST was purified from *A. alternata* extract and yield was 1.02 mg/g of fungal culture. It migrated at 26 kDa (Fig. 1a, lane 1) with >95% purity having 0.987 units/min/ μ g enzymatic activity.

2.3.2. Cloning and sequencing of GST

Sequence of cDNA was 801 bp with an ORF of 696 bp encoding 231 aa protein (accession no. AY514673) with predicted mass of 26.5 kDa. The ORF had a conserved N-terminus GST domain and was 27% identical to GSTs from *Candida albicans*, with <20% identical to other GSTs.

2.3.3. Recombinant expression and purification of rGST

The yield of purified rGST was approximately 2.5 mg/l with a molecular weight (Fig. 1a, lane 2) of 26 kDa. Enzymatic activity of rGST was 0.983 units/min/ μ g.

2.3.4. CD spectra

Secondary structure of nGST and rGST showed slight differences at 198, 208, 210, 223 and 232 nm indicating differences

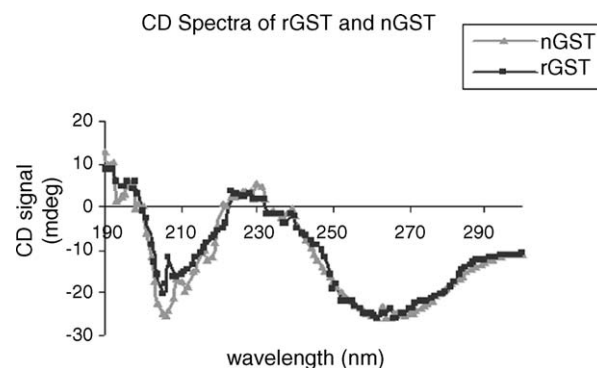


Fig. 2. Circular dichroism. Spectra of rGST (■) and nGST (Δ). Protein was taken at a concentration of 1 mg/ml and spectra recorded in 20 mM PBS in far-UV range (190–300 nm).

in folding. Melting temperature of rGST and nGST was 57 and 59 °C suggesting slight variation in the secondary structure (Fig. 2).

2.3.5. Skin reactivity with rGST and nGST

Intradermal tests performed on 115 allergic patients showed marked positive skin reactivity to *A. alternata* extract in 17 patients, whereas 14 showed with rGST and nGST. Skin reactivity of rGST and nGST differs slightly in patients 8, 10 and 11 (Table 1). Ten normal controls did not show positive reactivity to rGST and nGST or *A. alternata* extract. However, only five normal individuals consented to participate in the study.

2.3.6. IgE binding of rGST and nGST

All the individual patient's sera ($n = 17$) recognized both rGST and nGST (Fig. 1b and c) whereas control sera did not react with either GSTs ($n = 5$). ELISA using hypersensitive patient's sera showed higher specific IgE values for the crude extract as compared to recombinant or nGST (Table 1). Specific IgE values differed for both GSTs (Table 1) but showed a significant correlation ($R = 0.946$ and $p < 0.001$). Specific IgE values of controls ($n = 5$) were from 0.056 to 0.123 for crude extract, rGST and nGST.

EC₅₀ for self-inhibition of rGST and nGST was 2.7 and 2.5 ng, respectively, indicating both have similar potency. EC₅₀ of crude extract with rGST as inhibitor was 6.5 ng while with nGST it was 5 ng. On cross-inhibition of nGST with rGST and vice-versa EC₅₀ was 2.8 and 3.5 ng, respectively, demonstrated close nature of both the GSTs.

2.3.7. Histamine release on challenge with rGST and nGST

Out of 17 hypersensitive patients, 14 showed histamine release between 11 and 48% on challenge with 5 ng of rGST and nGST (Fig. 3) and showed statistically significant correlation ($R = 0.924$ and $p < 0.001$) though individual difference between the patients were observed. Significant correlation was also observed between histamine release and specific IgE for rGST ($R = 0.958$ and $p < 0.001$) and nGST ($R = 0.917$ and $p < 0.001$). Patients 2, 4 and 7 with less than 10% histamine release showed low levels of specific IgE for rGST and nGST (Table 1). The

Table 1

In vivo and in vitro sensitization to *A. alternata*, rGST and nGST

Serial number	Age/sex	Clinical history ^a	Intradermal test report ^b			Sp. IgE values (OD _{492nm})		
			<i>Alternaria</i> extract (100 µg/ml)	rGST (10 µg/ml)	nGST (10 µg/ml)	Crude extract	rGST	nGST
1	40/F	AR	++	++	++	0.389	0.336	0.313
2	28/M	AR	++	+	+	0.286	0.201	0.213
3	28/F	AR	+++	+++	+++	0.789	0.716	0.762
4	25/F	AR, BA	++	+	+	0.278	0.213	0.207
5	23/M	BA	++	++	++	0.601	0.352	0.365
6	22/F	AR	++	++	++	0.458	0.413	0.389
7	22/F	AR, BA	++	+	+	0.282	0.261	0.298
8	42/F	BA	++	+	++	0.413	0.262	0.318
9	35/F	AR	++	++	++	0.354	0.312	0.324
10	60/M	AR	++	++	+++	0.589	0.529	0.513
11	38/F	AR, BA	+++	+++	++	0.613	0.516	0.489
12	18/F	AR	++	++	++	0.689	0.515	0.556
13	23/F	AR	++	++	++	0.613	0.408	0.458
14	29/M	AR	++	++	++	0.484	0.412	0.434
15	18/M	AR	++	+	+	0.402	0.345	0.356
16	23/M	AR	+++	++	++	0.718	0.476	0.51
17	18/F	AR, BA	++	++	++	0.563	0.453	0.467
C1	31/F		–	–	–	0.101	0.098	0.089
C2	27/F		+	–	–	0.112	0.109	0.097
C3	39/F		–	–	–	0.123	0.115	0.118
C4	30/M		–	–	–	0.086	0.056	0.065
C5	21/M		–	–	–	0.073	0.078	0.065

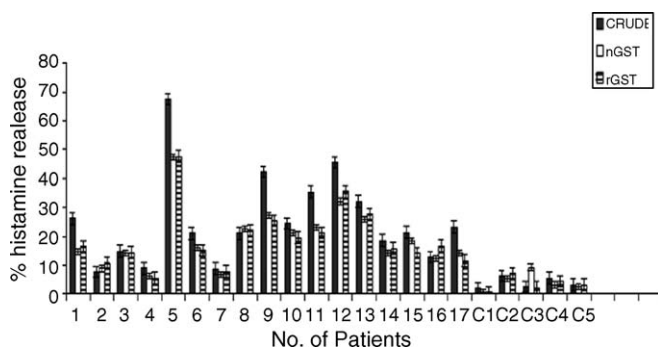
^a AR, allergic rhinitis; BA, bronchial asthma.^b +, mild reactivity; ++, equivalent to histamine release; +++, more than histamine release.

Fig. 3. Histamine release assay. Heparinized blood of patients ($n = 17$) or healthy controls (C1–C5) was challenged with 5 ng of crude extract (■), rGST (▒) or nGST (□). Histamine release was measured by ELISA and calculated as percentage release from the standard dose response curve. S.E.s (bars) is represented.

normal controls ($n = 5$) demonstrated less than 6% histamine release.

2.3.8. rGST and nGST induces PBMC proliferation on antigen stimulation

A dose-dependent proliferation of PBMC in *A. alternata* sensitized patients was observed with higher stimulation at 10 than 1 µg ($R = 0.836$ and $p < 0.001$, data not shown).

Fig. 4 shows IL-4, IL-5 and IFN- γ levels in supernatant with rGST or nGST. PBMC challenged with 1 µg rGST or nGST or *Alternaria* extract demonstrated secretion of IL-4 (60–80 pg/ml) and IL-5 (50–60 pg/ml) but at 10 µg proteins relatively higher secretion was observed for IL-4 (130–500 pg/ml, $R = 0.949$ and

$p < 0.001$) and IL-5 (120–350 pg/ml, $R = 0.987$ and $p < 0.001$). Levels of IFN- γ were, however, low (20–50 pg/ml) for both rGST and nGST at 10 µg protein and minimal secretion was observed at 1 µg. The control subjects showed non-detectable levels of IL-4 and IL-5. IFN- γ level was similar for patient and control ($R = 0.898$ and $p < 0.001$).

3. Discussion

Last decade has seen an unprecedented increase in population suffering from allergy and asthma (Chapman et al., 2000) even with improved drugs. Long-term use of pharmacotherapy induces tissue damage and immunotherapy is the only alternative to prevent tissue damage by changing the immune status of the individuals. Recently recombinant allergens have been derived from various sources such as tree pollen, molds, foods, pet danders, etc. (Chapman et al., 2000). These need to be evaluated with native protein(s) for clinical use. The present study demonstrates that rGST, a major cross-reactive allergen, has similar properties when compared with native GST from *A. alternata*.

Alternaria GST showed a conserved domain at 25–147 aa, a characteristic of GST superfamily proteins (McGoldrick et al., 2005). Homology studies showed <30% similarity with other fungal GSTs suggesting that it belongs to novel class of GST. It also showed >10% similarity with other known GST from allergenic sources such as *B. germanica* and *D. pteronyssis*. CD spectra and melting temperature of rGST and nGST differed slightly, which may be due to differential folding or the reduction of cysteine present at C-terminus. Native and recombinant

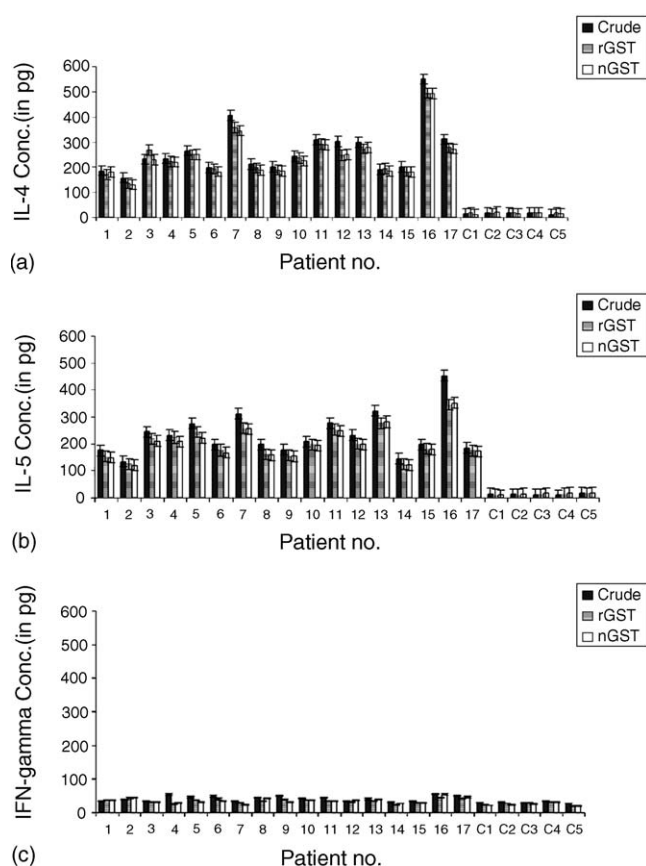


Fig. 4. Cytokine levels on allergen stimulation in PBMC supernatant. (a) IL-4, (b) IL-5 and (c) IFN- γ were measured by coating capture antibody (1:250 dil.) for each cytokine on plates. Standards (5–500 ng), 100 μ l of diluted sample (1:2) or controls were added to wells, incubated and color developed. S.E.s (bars) is represented.

GST migrated at same molecular weight indicating both proteins might not be glycosylated. These findings are supported by similarity in rGST and nGST IgE binding and inhibition. Furthermore, glycoprotein staining of nGST showed no reactivity (data not shown). Earlier, nPhl p 6 reacted with 50% of allergic sera whereas non-glycosylated rPhl p 6 to 21% of the patient's sera (Suck et al., 2003).

Skin reactivity of nArt v 1 and rArt v 1 was significantly different at 10 μ g/ml (Schmid-Grendelmeier et al., 2003). The present study showed nearly identical results for both the GSTs. However, there was little difference in 3/17 patients between the two forms in reactions severity. ELISA inhibition with nGST and rGST showed similar potency at different inhibitors. However, the recombinant tropomyosin expressed in *E. coli* and *Pichia pastoris* demonstrated differences at high concentration compared to native tropomyosin (Jeong et al., 2004). Similar SPT, ELISA inhibition and specific IgE response was similar with native and rAlt a 1 expressed in *E. coli* and yeast (Asturias et al., 2005).

Specific IgE and histamine release showed significant correlation ($p < 0.001$) with rGST and nGST. Both GST induced IL-4 and IL-5 levels showing similar stimulating capacity. Three patients having lower specific IgE showed <10% histamine release and lower cytokine secretion. Pru p 3 (Diaz-Perales et

al., 2003), Blot t 11 (Ramos et al., 2003) and Bet v 1 (Ferreira et al., 1993) induced identical histamine release and T-cell proliferation with native and recombinant proteins.

E. coli expressed recombinant proteins tend to form insoluble aggregates thereby denaturing the protein and during renaturation bulk protein is lost (Ventura, 2005). rGST is a soluble protein and could bind directly to the glutathione for purification indicating protein has folded as per the natural form. Further, the ease of purification may have advantage for using GST in clinical settings.

Physio-chemical, biochemical and immunological property of rGST was similar to nGST, a major cross-reactive allergen. However, recombinant Phl p 5 and Bet v 1 (Flicker et al., 2000; Pauli et al., 2000) differed in the IgE reactivity and biological activity (basophil histamine release/cytokine secretion) with its native counterpart. Three-dimensional structural analysis and identification of epitopes of rGST will further delineate the molecular interaction with IgE/T-cells which can be modulated for synthesis of hypoallergenic variants.

In conclusion, rGST is biologically potent and a clinically relevant major allergen from *A. alternata*. Earlier we have shown it to be a cross-reactive allergen among different allergenic fungi. The cross-reactive nature of GST can be exploited clinically for the diagnosis and therapy of fungal allergy.

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