



Sta c 3 epitopes and their application as biomarkers to detect specific IgE

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ABSTRACT

Sta c 3 was determined to be one of the major allergens from *Stachybotrys chartarum sensu lato*. This is among the most common fungi on wet building materials. Various cognizant authorities have found that mold and dampness are associated with exacerbation of asthma and increased upper respiratory disease. The primary amino-acid sequence of this allergen was reported, however the IgE-binding epitopes were unknown. Using SPOT-synthesis techniques (SPOTs), one main linear epitope located between V91 and G105 was discovered by Western blot with atopic human sera. This was confirmed by inhibition ELISA assays with both synthesized epitopes and natural Sta c 3. Alanine scanning also revealed R100 and K101 are the critical amino acids for IgE binding. Using the binding-enhanced and alanine substituted epitopes as potential biomarkers, a reverse ELISA method was developed to detect the Sta c 3 sIgE in atopic human sera. This method employed streptavidin-biotinylated mutated epitopes as the capture matrix, probing the possibility of using small peptides for allergen/species specific IgE assays.

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1. Introduction

Mold and dampness in public buildings is an important public health issue worldwide. Fungi that grow on damp building materials comprise numerous species, colonizing a large range of moisture range levels. This comprise species that have low material water activities (a_w) to species that grow at high a_w values and species with intermediate optimal a_w values. *Stachybotrys chartarum sensu lato* is among the most common fungi on wet building materials, especially those with higher water activity levels (Miller et al., 2003, 2008; Flannigan and Miller, 2001). This leads to exposures to fungal particles containing allergens, triple helical glucans and co-occurring toxins (Miller et al., 2010; Rand et al., 2010), which in turn is associated with exacerbation of asthma as well as increased upper respiratory disease (NAS, 2000; WHO, 2009). Recent expert panels have found that the remediation of moisture and mold was justified in terms of improving public health (Krieger et al., 2010; WHO, 2009). *S. chartarum sensu lato* produces allergens and some potent toxins including a number of macrocyclic trichothecenes and atranones. Very high exposure to spore and mycelia fragments can also lead to lung damage from the toxins (Miller et al., 2003).

A number of proteins from *S. chartarum* are recognized by antibodies in sera from atopic patients (Shi et al., 2011; Barnes et al., 2002; Kärkkäinen et al., 2004; Raunio et al., 2001). One known allergen, Sta c 3 is a 144 AA secretory, glycosylated (15%, w/w) alkaline, Mg-dependent exodeoxyribonuclease (Shi et al., 2011; Xu et al., 2007). Antibodies to Sta c 3 and another identified allergen, SchS34, have been successfully used for the development of immunological methods for detecting *S. chartarum* in environmental samples (Xu et al., 2008). In this paper, we report the use of the SPOT-synthesis technique to scan IgE binding epitopes of Sta c 3 and determine the critical amino acids in the interaction between the epitope and the sIgE. The signal-enhanced mutated epitopes were also probed for the potential application of reverse ELISA (rELISA) for Sta c 3/*S. chartarum* specific IgE assay in atopic human sera.

2. Materials and methods

2.1. Material

Natural Sta c 3 was purified from *S. chartarum* filtrate as reported by Xu et al. (2007). Peptides were synthesized by Kinexus Bioinformatics Corporation, Vancouver, BC with >90% purity by MALDI-TOF MS after synthesis. Atopic human sera were provided by IBT Laboratories (Kansas, USA), and non-atopic human sera were purchased from Sigma (S2145).

2.2. Epitope mapping

Epitope mapping was done with synthesized peptides using the spot-synthesis technique (Frank, 2002; hereafter the

Abbreviations: sIgE, specific IgE; HSA, human serum albumin; PBS, phosphate buffer saline; PBST, PBS containing 0.05% Tween 20; TBS, Tris-buffered saline; TBST, TBS containing 0.05% Tween 20; HRP, horseradish peroxidase.

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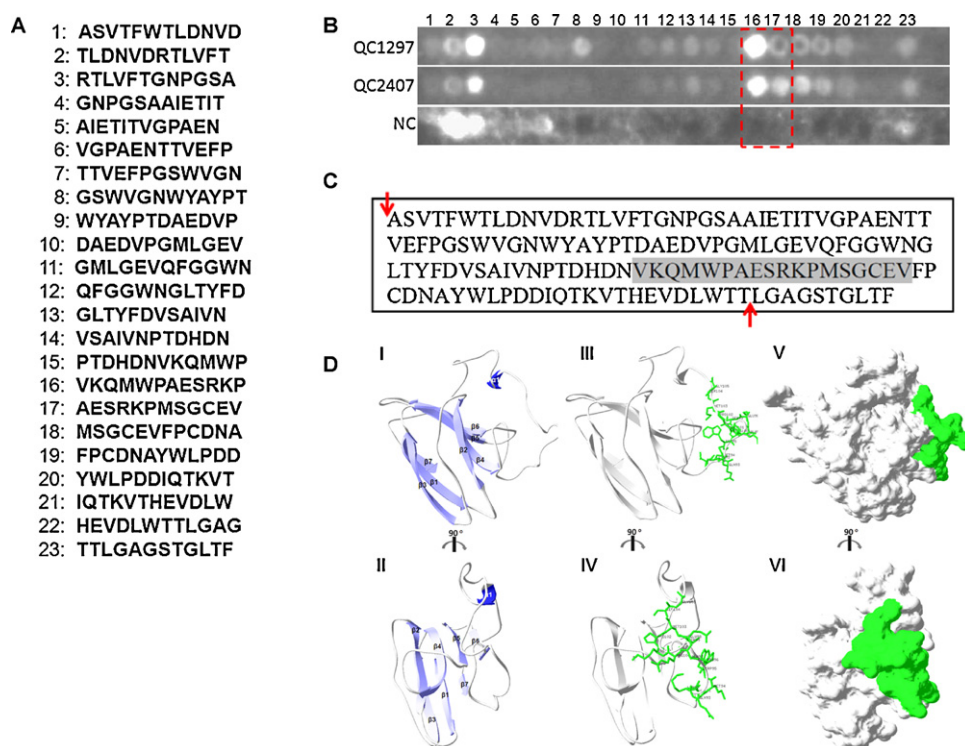


Fig. 1. IgE binding epitopes of Sta c 3. Panel A, the peptide sequences printed on the surface of SPOT-synthesis membrane for epitope screening performed in Panel B. Panel B, representative examples of Spots results obtained with Sta c 3 using human sera and anti-human IgE as a probe. Non-atopic human sera (NC) were included as the negative control. The sequences for each peptide are found in Table 1. Panel C, IgE-binding epitopes deduced from the stained spots are highlighted along the amino acid sequences of Sta c 3. The arrows show the beginning and the end of the Sta c 3 model. Panel D, mapping of the immunogenic IgE binding epitopes of Sta c 3. (I and II) Ribbon diagram of Sta c 3 showing the overall organization of α -helices (α 1) and the bundle of β -strands (β 1– β 7) from the view of 90° rotation respectively; (III and IV) localization of exposed IgE-binding epitopic regions on the ribbon diagram of Sta c 3; (V and VI) IgE-binding-epitopes identified along the amino acid sequence exposed on the molecular surface of Sta c 3.

SPOT-synthesis method). A total of 23 peptides, covering the entire region of Sta c 3 were synthesized on a modified cellulose membrane (Kinexus Bioinformatics Corporation, Vancouver, BC). Each peptide was 12 amino acids long, overlapping by six amino acids with a six amino acids offset between two adjacent peptides (Fig. 1A). Using the alanine scanning method (Hoess et al., 1993), a total of 13 peptides for each 12 amino acid epitope were synthesized on a modified cellulose membrane (Kinexus Bioinformatics Corporation, Vancouver, BC; Supplemental Table 1). Epitope mapping was conducted following the method of Shi and Miller (2011).

2.3. Homology-model of Sta c 3 protein

The three-dimensional structure of an allergenic thaumatin-like protein (2AHN, Protein Database Bank) (Dall'Antonia et al., 2005) was used as the template to build the homology model of the three-dimensional structure of Sta c 3 (<http://www.pdb.org/pdb/home/home.do>). The homology model of Sta c 3 was generated using ESyPred3D (<http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/>) (Lambert et al., 2002) and viewed by Swiss-PdbViewer (<http://spdbv.vital-it.ch/>). The energy minimization of Sta c 3 three-dimension model was performed *in vacuo* with GROMOS96 43B1 set without a reaction field (Schuler et al., 1996). The stereochemistry and overall quality of the homology model was checked using the Procheck (Laskowski et al., 1993). The final model of Sta c 3 included residues 1–134.

2.4. Inhibition ELISA

Sera with IgE reactive to *S. chartarum* or negative control sera (obtained from IBT Laboratories and Sigma (S2145)) were diluted 1/10 in PBST with 1% (w/v) BSA incubated with 0, 25, 50 and 100 μ g/mL of natural Sta c 3 or 5 μ g/mL synthesized peptides, at room temperature on a shaker for 30 min. For the epitope inhibition ELISA, microtiter plates were coated with 2 μ g/mL of Sta c 3 per well, washed and blocked with 1% (w/v) BSA at 4 °C. Then sera mixed with synthesized peptides were incubated overnight at 4 °C. For the Sta c 3 inhibition ELISA, 200 ng/well synthesized peptides in 100 μ L PBST were applied to a high binding streptavidin coated plate (Pierce, #15500), with 1% (w/v) BSA. This was washed and mixed with natural Sta c 3 pre-mixed human sera for overnight at 4 °C. The remaining steps of the inhibition ELISA were prepared as previously reported (Shi et al., 2011). All experiments were performed at least four times in duplicate.

2.5. Specific IgE assay

Initially, 200 ng/well of synthesized peptides in 100 μ L PBST with 1% (w/v) BSA were applied to high binding streptavidin coated plates and incubated at room temperature for 30 min. Atopic human sera were diluted to different concentrations in PBST with 1% (w/v) BSA and incubated overnight at 4 °C. The remaining portion of the sIgE assay was completed as previously reported by Shi et al. (2011). *S. chartarum* specific IgE levels in the human sera were determined using ImmunoCap (Phadia Diagnostics, Uppsala, Sweden) by IBT Laboratories, and compared with the

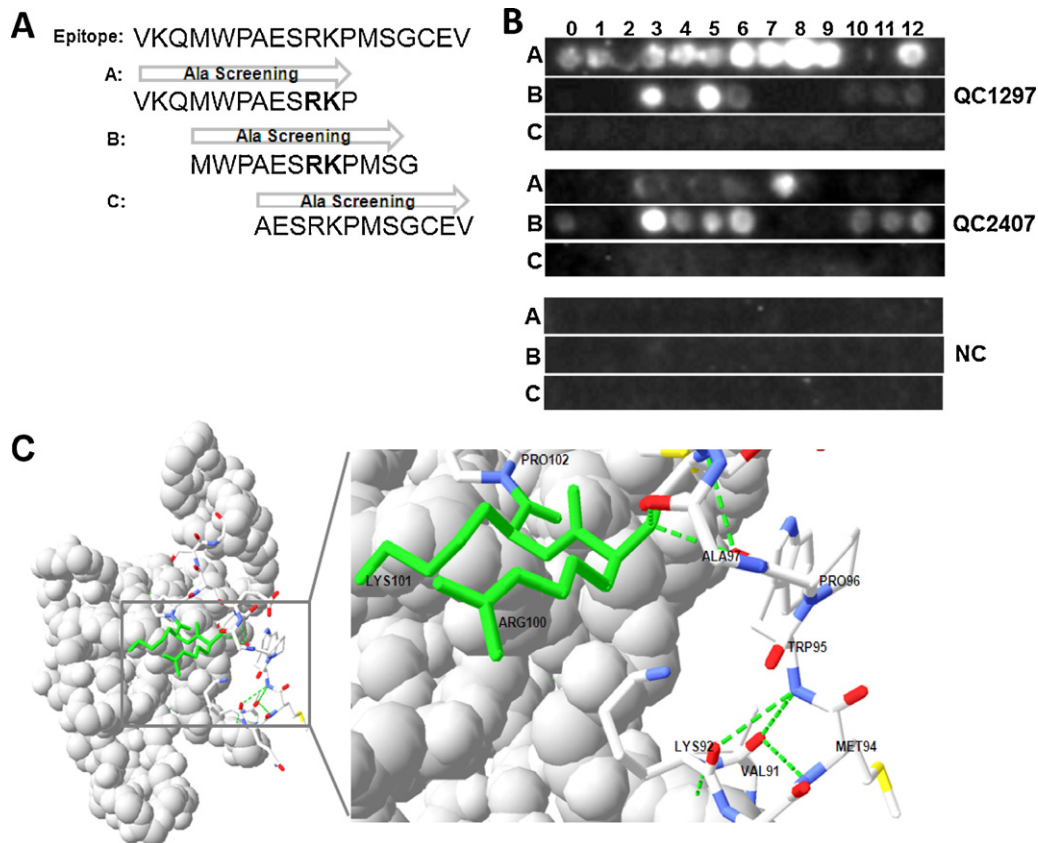


Fig. 2. Alanine scanning of a Sta c 3 epitope. Panel A: diagrammatic representation of Alanine screening of a Sta c 3 epitope. Panel B: representative atopic human sera screened by SPOT-synthesis membrane. The peptide sequences are listed in [supplemental Table 1](#). Non-atopic human serum was used as the negative control. Panel C indicates the critical amino acids, R100 and K101, required to interact with sIgE in the space filled model structure of Sta c 3.

sIgE concentration values determined by epitope methods in the present study. The equal mix of these *S. chartarum* specific human sera was applied as the standard with known *S. chartarum* specific IgE to calculate the known/unknown *S. chartarum* specific IgE value.

2.6. Statistical analyses

The rELISA limit of quantification was calculated using ANOVA (SYSTAT v12 software p -value <0.05). Correlations between ImmunoCap values and our rELISA results or the rELISA results with different peptides were assessed using Pearson's correlation coefficient (Excel). A four parameter logistic nonlinear regression analysis for the rELISA $F(x) = ((A - D) / (1 + ((x/C)^B))) + D$ was calculated using Softmax Pro 4 software.

3. Results

3.1. IgE binding epitope characterization

A total of 23 peptides, spanning the entire coding region of Sta c 3 were synthesized and probed with *S. chartarum* IgE reactive human sera in a SPOT experiment, including QC1294, QC1297, QC2123, QC2401 and QC2407. One main IgE-binding peptide (peptide 16 in [Fig. 1A](#)) was identified by the sera ([Fig. 1B](#), red box, QC1297 and QC 2407 as the representative sera). (For interpretation of the references to color in the text, the reader is referred to the web version of the article.) Peptide 17 ([Fig. 1A](#)) also had weak binding with IgE. The continuous region of peptides 16 and 17 in the amino acid sequence of Sta c 3 were subject to further analysis ([Fig. 1C](#)). Peptide 16 was also an IgG binding epitope ([supplemental Fig. S2](#)). All the amino acids for IgE binding epitopes are well-exposed in

the surface of Sta c 3 molecule, with a loop-like structure from the 3-dimensional model ([Fig. 1D](#) III, IV, V and VI). The amino acids between the arrows in [Fig. 1C](#) were visualized in the structure model, while the C-terminal 10 amino acids were not visualized for the truncated 3D model.

Peptides such as peptide #8 and #23 gave signals too weak to assess the epitopic character of both polypeptide regions ([Fig. 1A](#) and B) and peptide #23 also reacted with the negative control serum, albeit weakly. Peptide #3 strongly reacted with both the atopic and control human sera ([Fig. 1B](#)). Additional control experiments with anti-human IgE without atopic human sera indicated peptide #3 interacted with anti-human-IgE. This suggested the reactions between atopic human sera and peptide #3 might be an artifact of the interaction between peptide #3 and anti-human IgE antibody, but not between peptide #3 and atopic human sera IgE *per se* ([Supplemental Fig. S1](#)).

3.2. Critical amino acids for IgE binding

To identify the amino acids critical for IgE interaction with this epitope from V91 to V108, mutant peptides derived from this peptide for enhanced interaction with IgE were generated. Three 12-mer peptides with three amino acids shifted from the continuous part of peptide 16 and peptide 17 ([Fig. 1A](#)) were printed on a SPOT membrane with alanine substitutions at each position ([Fig. 2A](#) and [Supplemental Table 1](#)). Their interactions with IgE were then evaluated with individual atopic sera. Of the sera tested, QC1294, QC2401 and QC2407 showed similar responses to the synthesized peptides, with stronger interaction with peptide B and its alanine mutant peptides ([Fig. 2A](#) and B, QC2407 as the representative sera). Only QC1297 reacted stronger to Peptide A and its alanine mutant

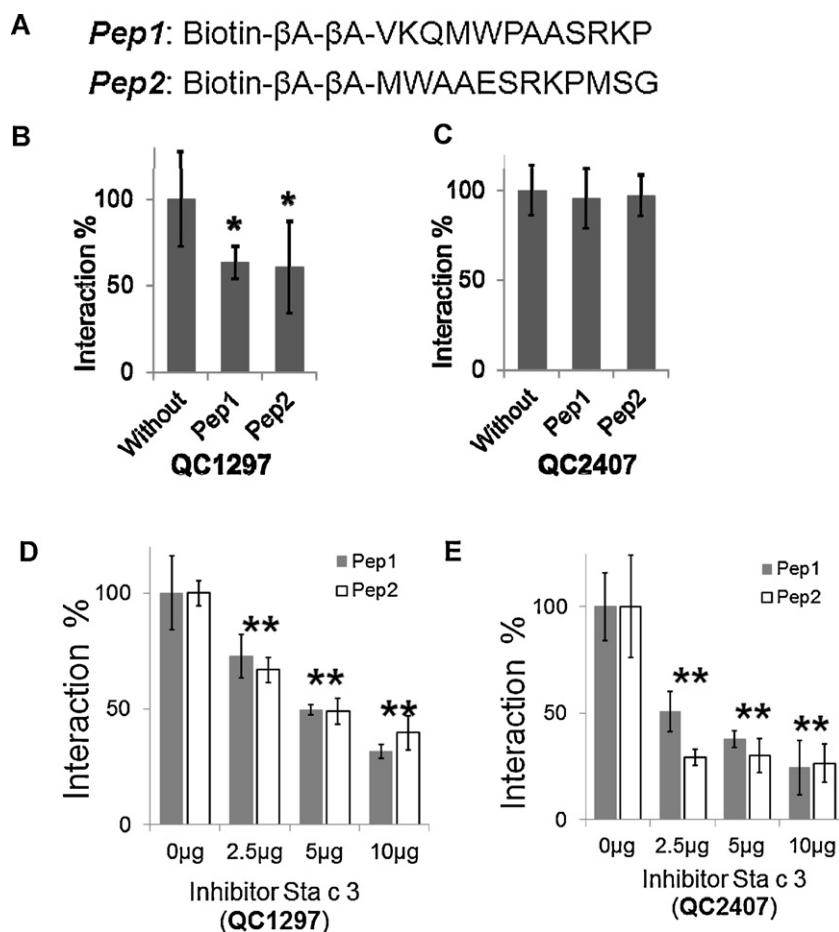


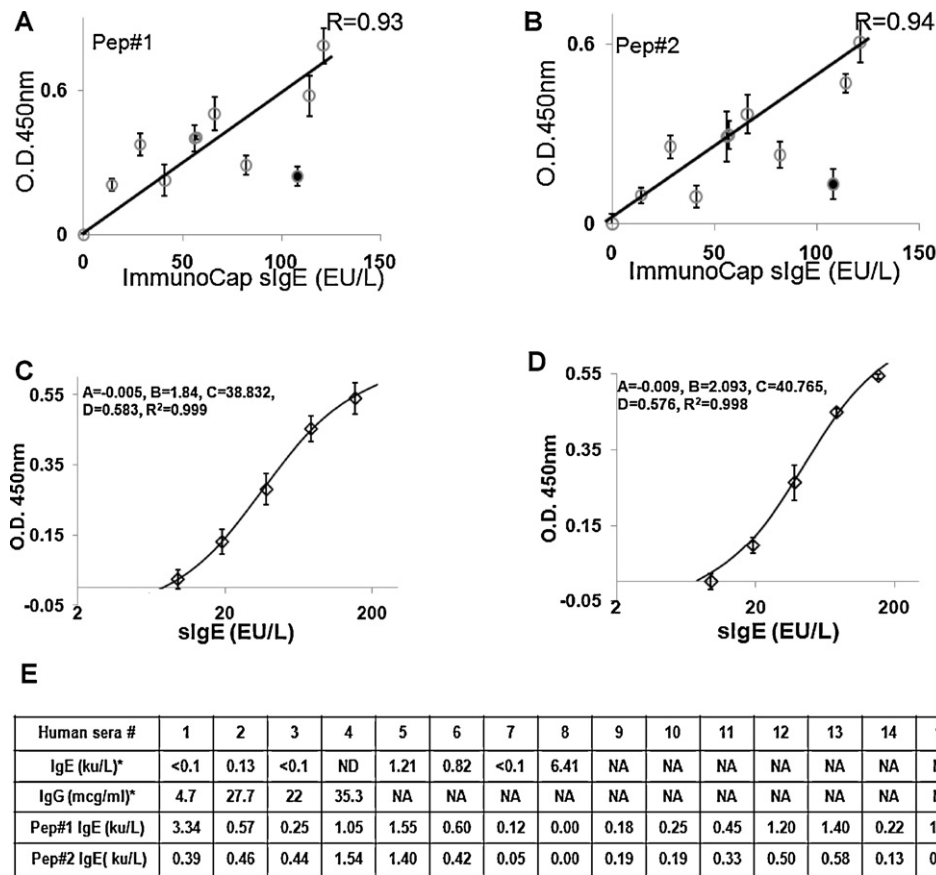
Fig. 3. The competitive interaction between natural Sta c 3 and synthesized epitopes. Panel A sequences of the synthesized peptides used for inhibition ELISA in this study. Panels B and C indicate the percent interaction from an inhibition ELISA for 2 different sera when synthesized peptides were used to inhibit the interaction of Sta c 3/IgE where Sta c 3 was coated on the plates. Panels D and E present the interaction from an inhibition ELISA of 2 sera using different concentrations of natural Sta c 3 as inhibitor of Pep1/IgE and Pep2/IgE interaction when peptides were captured using streptavidin coated on the plate. * indicated the *p*-value of panel B and C were less than 0.05 and ** indicated the *p*-value of panels D and E comparing to the control without inhibition matrix Sta c 3 were <0.000. At least four times duplicates were performed in all the experiments.

peptides (Fig. 2A and B). Substitution of E98 and P96 with alanine resulted in improved IgE binding signals in both peptide A and B. Substitution of alanine at the position of P102, W95 and M94 on the peptide B decreased signal in IgE binding, while not in peptide A binding. A significant loss of IgE binding was observed after R100 and K101 were substituted by alanine in both peptide A and B of both sera (Fig. 2A and B), suggesting both amino acids play a critical role in the interaction between the epitopes and IgE. Peptide C did not demonstrate a clear interaction with IgE. Thus, the central IgE binding region of this peptide is limited to that between V91 and G105. The critical amino acids R100 and K101, were also well exposed in the Sta c 3 space filled structure model (Fig. 2C).

3.3. Inhibition ELISA between Sta c 3 and synthesized peptides

Two peptides (A8 and B3 in supplemental Table 1) with significant signal improvement (Fig. 2) were synthesized and used in an inhibition ELISA. The use of biotin in N-terminus was intended to capture peptides in an ELISA plate pre-coated with streptavidin. Two β-alanine additional amino acids were inserted in their N-terminus after biotin for the purpose of providing space between streptavidin and IgE and to mimic a similar environment of peptides on a SPOT membrane (Fig. 3A).

The binding capacity to IgE with synthesized peptide #1 (Pep1) and peptide #2 (Pep2) was evaluated by inhibition ELISA with natural Sta c 3 and Pep1/Pep2. Purified Sta c 3 was used to coat the plates to evaluate Pep1 and Pep2 inhibitions to the interaction between natural Sta c 3 and IgE. Approximately 500 ng/well of Pep1 or Pep2 inhibited the Sta c 3–IgE interaction by 37% and 40% with serum QC1297, respectively ($p < 0.05$; Fig. 3B). In human sera QC2407, 500 ng/well synthetic Pep1 and Pep2 did not show significant inhibition of the Sta c 3–IgE interaction ($p = 0.409$ and 0.486 respectively), possibly because multiple linear/conformational epitopes exist in natural Sta c 3 or high cross-reactivity of this atopic serum prevented the detection of this interaction (Fig. 3C). QC1294 and QC2401 were found to be inhibited (p -value <0.05). Sera QC2123 and QC2407 did not show significant inhibition (data not shown). In the inhibition ELISA, natural Sta c 3 (2.5, 5.0 and 10.0 μg/well) inhibited the Pep1–IgE interaction by 27.2%, 50.3% and 68.4% (p -value <0.001) and the Pep2–IgE interaction by 33.2%, 50.9% and 60.4% (p -value <0.001) respectively in human sera QC1297. In the other serum sample (QC2407), natural Sta c 3 at 2.5, 5.0 and 10.0 μg/well inhibited the Pep1–IgE interaction by 49.2%, 62.2% and 75.6% (p -value <0.001) and the Pep2–IgE interaction by 70.8%, 70.0% and 73.5% (p -value <0.001). The competitive binding to sIgE between the synthesized peptides and natural allergen confirms the interactions of the synthesized peptides with the same population of



Note: * The IgE aIgA IgG reference values assayed with ImmunoCap by IBT Reference Lab; NA: Data not available.

Fig. 4. The potential application of epitopes in specific IgE assay. Panel A and B: comparison of the ImmunoCap determined *S. chartarum* specific IgE with our mutated epitopes #1 (A) and #2 (B). Panels C and D: the equal mix of these *S. chartarum* specific human sera was applied as the standard with known *S. chartarum* specific IgE value from ImmunoCap. E is the table showing some atopic human sera with/without their sIgG and sIgE detected (Row 2 and Row 3) by ImmunoCap and epitopes method in this article.

IgE specific to Sta c 3, excluding the artifactual possibility of linear peptides.

3.4. Reverse ELISA assay for Sta c 3/*S. chartarum* specific IgE

Pep1 and Pep2 were then studied using reverse ELISA (rELISA) to evaluate Sta c 3 specific IgE value in human sera. *S. chartarum* sIgE concentrations from different sera determined were compared with their O.D. 450 nm as measured with rELISA using Pep1 or Pep2. There was a strong correlation between the ImmunoCap and the rELISA assay: there was a Pearson correlation coefficient of $r=0.93$ and $r=0.94$ with Pep1 and Pep2 respectively, for 9 of the 10 *S. chartarum*-positive sera (Fig. 4A and B). The O.D. value from one serum (solid points in Fig. 4A and B) was significantly lower than the result from the ImmunoCap. This difference is likely the result of non-specific cross-reactivity of the allergen extract used to prepare the ImmunoCap reagent.

An equal mixture of the above nine atopic human sera was diluted from 1/5 to 1/320 as the standard reference for *S. chartarum* specific IgE. The detection limits using Pep1 and Pep2 in rELISA were 19 EU/L (p -value=0.001 and p -value <0.001, respectively). Both rELISA standard curves showed similar absorbance values and their Pearson correlation coefficients were 0.99 (Fig. 4C and D). A four parameter logistic nonlinear regression indicated $A=-0.005$, $B=1.84$, $C=38.832$ and $D=0.583$ with $R^2=0.999$ for Pep1 rELISA and $A=-0.009$, $B=2.093$, $C=40.765$ and $D=0.576$ with $R^2=0.998$

for Pep2 rELISA. Using the reference human sera mix (above), *S. chartarum*-specific IgE and IgG human sera were probed and the response to Pep1 or Pep2 rELISA determined (Fig. 4E). This represents the Sta c 3-specific IgE value.

4. Discussion

IgE plays a fundamental role in the expression of allergic disease. IgE-mediated mechanisms are involved in most clinical symptoms (Cox et al., 2008). Identification and characterization of IgE-binding epitopes provides basic information in understanding the properties of the allergens and their interaction with human IgE antibodies at the molecular level (Lin and Sampson, 2009; Robotham et al., 2009). This information can be used for designing immune therapeutics (Thomas et al., 2011; Larché, 2007). Both the epitope characterization and sIgE diagnosis may also facilitate the development of safer and clinically useful strategies in allergy therapeutics (Thomas et al., 2011; Swoboda et al., 2002; Chan et al., 2006).

The Sta c 3 is one of the two main allergens from *S. chartarum*, containing 144 AAs without its signal peptide in its N-terminus (Shi et al., 2011). The incomplete three-dimensional model (residues 1–134) for Sta c 3 was built using the template structure of 2AHN (an allergenic thaumatin-like protein from ripe cherries; Dall'Antonia et al., 2005), which shares 30.4% similarity with Sta c 3 using the LALIGN program. The quality of the three-dimensional

model of Sta c 3 obtained was evaluated by Procheck. The most favored regions of the Ramachandran Plot statistic was 83.0% compared to 91.1% of the template structure of 2AHN from X-Ray diffraction with 1.3 Å resolution (Laskowski et al., 1993). That supports the reliability of the Sta c 3 three-dimension model structure in terms of epitope localization.

A 15 amino acid epitope from V91 to G105 was characterized, with R100 and K101 representing the critical amino acids for binding to sIgE (Figs. 1 and 2). This epitope is not found in Sta c 3-homologous proteins from other fungal extracellular DNases (Fig. S3). This provides additional evidence of the specificity of this epitope. The relevance of this epitope to Sta c 3 was further demonstrated by studying the inhibition of the interaction with IgE in ELISA against the two synthesized peptides (Pep1 and Pep2) and purified Sta c 3 (Fig. 3). The inhibition of Sta c 3 by the peptides excludes the possibility of the non-specific binding of the IgE to the synthesized peptides. Stronger Sta c 3 inhibition was observed with Pep1/Pep2–IgE interaction compared to the Pep1/Pep2 inhibition to Sta c 3–IgE (Fig. 3). This is likely because there are more IgE binding epitopes. Often conformational epitopes and other linear epitopes can be missed in the SPOT screening. The favored conformations of Pep1 and Pep2 may contribute to the enhanced IgE-binding signal compared to the native peptides, but not from the false positive peptides-unspecific IgE interactions (<30% interaction signal was not inhibited by 10 µg Sta c 3 and the sensitivity increases for both peptides are dominant in all the sera tested (Fig. 2)).

The ImmunoCap system (Phadia, Uppsala, Sweden) is one of the most widely used IgE immunoassays. The various other commercial methods may give greater or lesser responses compared to the ImmunoCap. This variation is due to both method and allergen extract variation. This means that different populations of specific IgE antibodies are detected in all these clinical IgE detection methods. Since the antigens on the solid phase matrix are not pure, there is a significant risk of false-positives due to cross-reactivity (Esch, 2004; Sicherer, 2001). All the commercial tests use whole allergen extracts as the binding matrix with the resulting interactions with nonspecific IgE antibodies (Cramer et al., 2009). Purified allergens and purified molecular epitopes can provide lower cross-reactivity and greater clinical utility, but the cost is higher to prepare these molecules (Mari et al., 2010).

The use of one epitope such as our 12mer peptides in this assay may significantly decrease the cross-reactivity problems and reduce the preparation costs compared to other allergen extracts and pure allergens. Using the primary epitope, as opposed to natural Sta c 3, greatly improves the sensitivity of the sIgE assay. The detection limit of 19 EU/L was lower than that of 50 EU/L using ImmunoCap *S. chartarum* assay. It is likely that the epitopes we report here could be adapted for use on the ImmunoCap platform.

In conclusion, a dominant linear IgE epitope of Sta c 3 was characterized in this study. We identified residues V91–G105 on Sta c 3 as a dominant epitope. Lys100 and Arg101 are among critical core amino acid residues of Sta c 3 recognized by atopic human IgE, which are both located on the surface of the 3D model of Sta c 3. This is the first report using mutated epitope for a high sensitivity sIgE assay. After more epitopes in this species, as well as other species are revealed, this approach may be the next generation of high-throughput specific IgE tests with molecular specificity and high sensitivity.

Conflict of interest

The authors have no conflicting financial interests.

Contribution

CS developed the technical approach and drafted parts of the manuscript. JDM contributed to the development of the research strategy and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2012.02.002.

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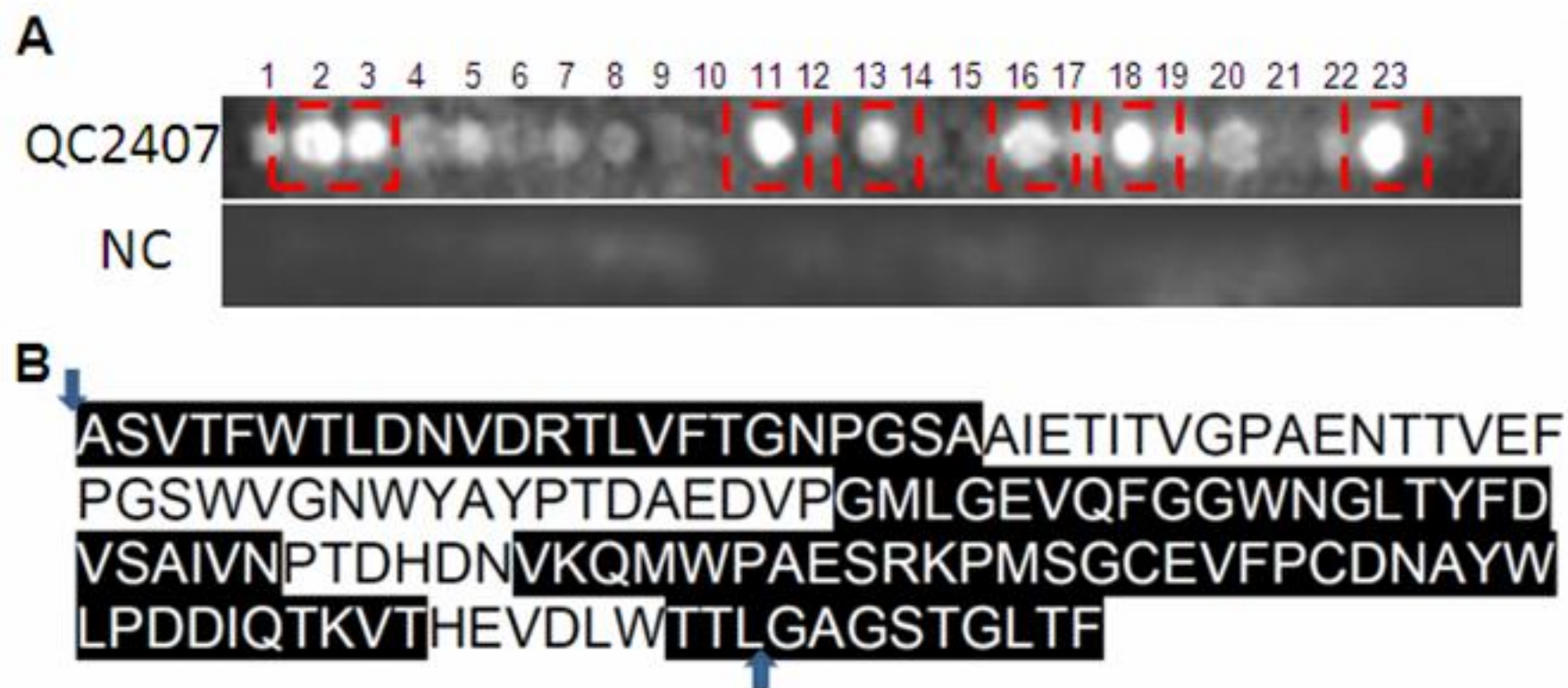
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Supplemental Table 1. The Peptide sequences printed in the surface of SPOTs membrane for Alanine scanning in Figure 2.

A	B	C
0:VKQMWP AESRKP	0:MWP AESRKPM SG	0:AESRKPM SGCEV
1:AKQMWP AESRKP	1:AWP AESRKPM SG	1:AESRKPM SGCEV
2:VAQMWP AESRKP	2:MAP AESRKPM SG	2:AASRKPM SGCEV
3:VKAMWP AESRKP	3:MWAAESRKPM SG	3:AEARKPM SGCEV
4:VKQAWP AESRKP	4:MWP AESRKPM SG	4:AESA KPM SGCEV
5:VKQM A P AESRKP	5:MW P AASRKPM SG	5:AESRAPM SGCEV
6:VKQM WAAESRKP	6:MW P AEARKPM SG	6:AESRKAM SGCEV
7:VKQM WP AESRKP	7:MW P AESAKPM SG	7:AESRKPA SGCEV
8:VKQM W P AASRKP	8:MW P AESRAPM SG	8:AESRKPM AGCEV
9:VKQM W P AEARKP	9:MW P AESRKAM SG	9:AESRKPM SACEV
10:VKQM W P AESAKP	10:MW P AESRKPA SG	10:AESRKPM SGA EV
11:VKQM W P AESRAP	11:MW P AESRKPM AG	11:AESRKPM SGC AV
12:VKQM W P AESRKA	12:MW P AESRKPM SA	12:AESRKPM SGC EA

Supplemental Figure S2. IgG binding epitopes of Sta c 3. Panel A, examples of Spot results obtained with Sta c 3 using human sera and anti-human IgG as a probe. Non-atopic human sera (NC) was included as the negative control. Panel B, IgG-binding epitopes deduced from the stained spots are highlighted along the amino acid sequences of Sta c 3.



Supplemental Figure S3. Alignment of IgE binding epitope of Sta c 3 with its homologous proteins from the listed species, which belongs to a subfamily of putative fungal extracellular secreted DNases. The dashed square indicates critical amino acids R100 and K101 of Sta c 3 IgE binding epitope is not conserved with Sta c 3 homologous proteins.

<i>P. anserina</i>	V	K	M	M	W	P	R	K	S	A	S	V	T	S	G
<i>S. sclerotiorum</i>	V	K	E	L	F	P	L	H	T	G	L	P	V	S	G
<i>B. fuckeliana</i>	V	K	E	M	F	P	L	N	T	N	T	P	L	S	G
<i>N. haematococca</i>	V	K	Q	M	Y	P	A	K	S	M	T	P	D	S	G
<i>M. anisopliae</i>	V	K	Q	M	Y	P	A	S	G	K	S	P	M	S	G
<i>G. zeae</i>	V	K	Q	M	W	P	A	S	S	N	T	P	M	S	G
<i>S. chartarum</i>	V	K	Q	M	W	P	A	E	S	R	K	P	M	S	G
	*	*				:	:	*						*	*