

Allergen motifs and the prediction of allergenicity

Pamela Marti^a, Renato Truffer^a, Michael B. Stadler^b, Elsbeth Keller-Gautschi^a,
Reto Cramer^c, Adriano Mari^{d,e}, Peter Schmid-Grendelmeier^f,
Sylvia M. Miescher^g, Beda M. Stadler^a, Monique Vogel^{a,*}

^a Institute of Immunology, Inselspital, 3010 Bern, Switzerland

^b Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland

^c Swiss Institute of Allergy and Asthma Research (SIAF), 7270 Davos, Switzerland

^d Center for Clinical and Experimental Allergology, Instituto Dermatologico dell'Immacolata, IDI-IRCCS, I-00167 Rome, Italy

^e Allergy Data Laboratories sc, I-04100 Latina, Italy

^f Department of Dermatology, University Hospital Zürich, Gloriastrasse 31, 8091 Zürich, Switzerland

^g CSL Behring AG, Wankdorfstrasse 10, 3000 Bern 22, Switzerland

Received 28 September 2006; received in revised form 4 January 2007; accepted 10 January 2007

Available online 30 January 2007

Abstract

We have recently shown that the majority of allergens can be represented by allergen motifs. This observation prompted us to experimentally investigate the synthesized peptides corresponding to the *in silico* motifs with regard to potential IgE binding and cross-reactions with allergens. Two motifs were selected as examples to conduct *in vitro* studies. From the first motif, derived from allergenic MnSOD sequences, the motif stretch of the allergen Asp f 6 was selected and synthesized as a peptide (MnSOD Mot). The corresponding full-length MnSOD was also expressed in *Escherichia coli* and both were compared for IgE reactivity with sera of patients reacting to the MnSOD of *Aspergillus fumigatus* or *Malassezia sympodialis*. For the second motif, the invertebrate tropomyosin sequences were aligned and a motif consensus sequence was expressed as a recombinant protein (Trop Mot). The IgE reactivity of Trop Mot was analyzed in ELISA and compared to that of recombinant tropomyosin from the shrimp *Penaeus aztecus* (rPen a 1) in ImmunoCAP. MnSOD Mot was weakly recognized by some of the tested sera, suggesting that the IgE binding epitopes of a multimeric globular protein such as MnSOD cannot be fully represented by a motif peptide. In contrast, the motif Trop Mot showed the same IgE reactivity as shrimp full-length tropomyosin, indicating that the major allergenic reactivity of a repetitive structure such as tropomyosin can be covered by a motif peptide. Our results suggest that the motif-generating algorithm may be used for identifying major IgE binding structures of coiled-coil proteins.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Motif; Allergenicity prediction; Cross-reactivity; IgE binding structure; MnSOD; Tropomyosin

1. Introduction

A large number of allergens of clinical relevance are known today and their complete cDNA sequence has been determined [1,2]. Recent data indicate that a small number of structural protein families contain most of these allergens [3–6]. Nonetheless, the molecular basis for allergenicity of proteins remains largely unknown.

We have recently proposed a bioinformatic approach to assess allergenicity of proteins and to predict cross-reactions by the identification of shared motifs in the amino acid sequences of allergenic proteins [7]. For our initial allergen motif analysis, we used an allergen database which was generated in our institute [7]. Since then the continuously updated Allergome database (<http://www.allergome.org> [8]) has been used for further analysis. This motif search using the 1102 allergen sequences contained in the Allergome database at the time (April 2005) resulted in the assignment of 912 sequences to 69 statistically relevant allergen motifs [9]. Motifs are defined as sequence patterns that occur repeatedly in a group of related protein sequences. Motifs as such exist only as a sequence profile whereby a number of amino acid residues are strictly predicted

Abbreviations: MnSOD, manganese superoxide dismutase; Trop, tropomyosin; Mot, motif; ABPA, allergic bronchopulmonary aspergillosis; AD, atopic dermatitis

* Corresponding author. Tel.: +41 31 632 03 34; fax: +41 31 381 57 35.

E-mail address: monique.vogel@iib.unibe.ch (M. Vogel).

while others leave a freedom of choice with a statistical preference. In the current study, we synthesized allergen motifs by two different approaches. In the case of the manganese superoxide dismutase (MnSOD) motif, we used the amino acid sequence corresponding to the motif stretch of *Aspergillus fumigatus* MnSOD. For the second motif, derived from tropomyosins, we chose the consensus sequence and produced a so far non-existing peptide.

The enzyme MnSOD has been identified as an allergen (Asp f 6) of the pathogenic fungus *Aspergillus fumigatus* (*A. fumigatus*) [10]. It is of clinical relevance especially in individuals who are allergic to *A. fumigatus* and who additionally suffer from allergic bronchopulmonary aspergillosis (ABPA), an intense inflammatory response to *A. fumigatus* in the lung. Furthermore, it has been shown that MnSODs of phylogenetically distant organisms are recognized by IgE antibodies from individuals reacting to Asp f 6 [11].

Previous studies have identified tropomyosin as the major crustacean allergen including shrimp, crawfish, lobster and crab [12–15]. Shellfish allergy is of significant clinical concern, since it can cause fatal anaphylaxis.

In the present study, we analyzed the IgE reactivity of two selected motifs. Only the tropomyosin motif showed IgE binding comparable to the corresponding full-length allergen, indicating that the allergenicity of proteins with coiled-coil structure such as tropomyosin can be represented by a motif peptide. Our motif search analysis may thus serve as a valuable method to identify IgE binding structures.

2. Methods

2.1. Antigens, allergens and allergen extracts

The MnSOD Mot was synthesized as a peptide preparation containing at least 36% peptide fragments having the full-length size of 50 amino acids by Thermo Electron GmbH (Ulm, Germany) and was characterized by mass spectrometry and analytical HPLC. The first two amino acids of the motif (QQ) were omitted because of stability problems linked to the presence of QQ.

Tropomyosin isolated from chicken gizzard was bought from Sigma (Fluka Chemie GmbH, Buchs, Switzerland).

ImmunoCAPs carrying *A. fumigatus* (m3), crab (*C. pagurus*; f23) or shrimp (*P. borealis*; f24) extract, recombinant *A. fumigatus* MnSOD (rAsp f 6; m222) or recombinant shrimp tropomyosin (rPen a 1; Rf351) [16] were purchased from Pharmacia (Uppsala, Sweden).

2.2. Patients sera

For the studies on the *A. fumigatus* MnSOD (Asp f 6), sera from four groups of donors were analyzed: patients suffering from allergic bronchopulmonary aspergillosis (ABPA) or atopic dermatitis (AD), patients reacting to *A. fumigatus* extract and control subjects. Patients suffering from ABPA fulfilled the diagnostic criteria given by Greenberger and Patterson [17]. Individuals with AD were diagnosed according to the criteria

of Hanifin and Rajka [18]. Reactivity to *A. fumigatus* extract was determined using the Pharmacia CAP system. For control purposes, sera from donors who had no specific serum IgE to rAsp f 6, as determined by ImmunoCAP, were included.

The sera analyzed for the studies on the tropomyosin consensus motif included individuals showing strong IgE reactivity to crab or shrimp extract, as determined by ImmunoCAP.

The study design was approved by the ethical committee of the University of Berne, as part of ongoing efforts to validate in house laboratory diagnostics.

2.3. Generation of motifs

Motifs were generated in an iterative way starting with allergen sequences of two Allergome databases, namely the original one from February 2003 (779 sequences) and the follow up one from April 2005 (1102 sequences) as described in ref. [7]. In the iterative procedure, the algorithm MEME (Multiple EM for Motif Elicitation) [19] was used for identifying motifs in the allergen sequences. Briefly, in the first step, the allergen database was analyzed with MEME to determine a motif representing a set of related sequences. These sequences were removed from the allergen database. The remaining sequences were subjected to MEME analysis for the generation of a motif representing a new set of related sequences. This procedure was repeated until no more statistically relevant motifs (*E*-value less than 0.01) were detected. The motif size was defined as 50 amino acids in order to represent the mean length of a protein domain and to prevent the generation of multi-domain motifs [7].

2.4. Expression and purification of Asp f 6

The *Escherichia coli* M15 clone encoding the full-length Asp f 6 was described earlier [20] and was used to produce hexahistidine-tagged recombinant MnSOD. The fusion protein was purified in two steps using a BD TALON™ Metal Affinity Resin (BD Biosciences, San Jose, CA, USA) followed by size exclusion chromatography using a HiLoad 26/60 Superdex 75 column (Amersham Pharmacia, Piscataway, NJ, USA). Purity and molecular size of the recombinant MnSOD were analyzed by SDS-polyacrylamide gel electrophoresis (15% acrylamide) and Western blotting under denaturing conditions as described elsewhere [21]. Western blots were incubated with mouse anti-histidine antibody (Amersham Biosciences Europe, Otelfingen, Switzerland) at 1:1000 overnight at 4 °C followed by incubation with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (DakoCytomation, Baar, Switzerland) at 1:1000 for 4 h at RT.

2.5. Cloning, expression and purification of Trop Mot

The synthesis of the DNA fragment encoding the consensus sequence of the tropomyosin motif (Trop Mot) was performed using the following three overlapping oligonucleotides synthesized by Microsynth GmbH (Balgach, Switzerland):

- Oligo #1: 5'-CTTCTGTCTGACGAAGAACGCATGGATGCTTTGGAAAATCAACTGAAAGAGGCAAGAATGCTGGCGGA-3';
- Oligo #2: 5'-CCACCATTGCGAGCTTGCCTGCCACTTCACTATTTACGACTCTGCCTCTTCCGCCAGCATCTTGGCC-3';
- Oligo #3: 5'-GCAAGCTCGCAATGGTGGGAAGCAGACCTGGAACGTGCGGAAGAACGGGCAGAAAGCGCGCAATCCAAG-3'.

After annealing of the three oligonucleotides by PCR filling reactions, the cDNA was amplified in a PCR reaction using the primers:

- forpriNcoI 5'-CGTCGCCATGGTTCTGTCTGACGAAGAA-CG-3' and
- repiXhoI 5'-CGTCGCTCGAGCTTGGATTCGCCGCTTTC-TGC-3'.

The amplification products were subcloned into the pET 22b(+) high-level expression vector (Novagen, Madison, WI, USA) and transformed into *E. coli* strain BL21(DE3)pLysS. Hexahistidine-tagged Trop Mot was produced and purified from the periplasm using a BD TALON™ Metal Affinity Resin (BD Biosciences) according to the manufacturers' instructions. Purity and molecular size of the recombinant Trop Mot were analyzed by SDS-PAGE (15% acrylamide) and Western blotting under denaturing conditions as described above.

2.6. IgE binding and inhibition experiments

The specific IgE to Asp f 6 and MnSOD Mot in patient sera was measured by ELISA. Costar EIA/RIA half-well plates (Costar, Cambridge, MA, USA) were coated overnight at 4 °C with either Asp f 6 or MnSOD Mot. Both proteins were coated at a concentration of 5 µg/ml but taking into account that the peptide preparation contains only 36% of full-length peptide fragment only 1.8 µg/ml of full-length MnSOD Mot was coated.

However, because the molecular weight of MnSOD Mot (5 kD) is four times smaller than full-length Asp f 6 (23 kD) the amount of both proteins coated on the plates were similar. Wells were blocked for 2 h at 37 °C with PBS/0.15% casein and incubated with patients' sera diluted 1:4, 1:8, 1:16 and 1:32 in blocking buffer for 3 h at 37 °C. Bound antibodies were detected by using HRP-conjugated Le27 [22] as the detection system.

For inhibition ELISA, half-well plates were coated and blocked as described. Patients' sera were diluted 1:4 and pre-incubated with 0, 1, 5 and 50 µg/ml of Asp f 6 or MnSOD Mot for 2 h at 37 °C, shaking at 320 rpm. The pre-incubated sera were transferred to the coated plates and incubated for 2 h at 37 °C. Residual IgE-binding capacity was determined as described for ELISA assays.

Specific IgE reactivity to recombinant tropomyosin from *Penaeus aztecus* (rPen a 1) and Trop Mot was measured by ImmunoCAP and ELISA, respectively. ImmunoCAP measurements were carried out with undiluted sera as prescribed by the manufacturer (Pharmacia). For ELISA, Costar EIA/RIA half-well plates were coated overnight at 4 °C with Trop Mot at a concentration of 5 µg/ml. Wells were blocked as described and incubated with patients' sera diluted 1:2 in blocking buffer for 4 h at 37 °C. Bound antibodies were detected by using a HRP-conjugated anti-human IgE antibody (The Binding Site, Schwetzingen, Germany) as the detection system.

For inhibition ELISA, half-well plates were coated and blocked as described. Patients' sera were diluted 1:4, pre-incubated with 0, 0.5, 5, 50 and 100 µg/ml of Trop Mot, chicken tropomyosin or BSA for 2 h at 37 °C and processed as described for MnSOD Mot.

3. Results

3.1. Identification of an allergen motif in allergenic MnSODs

Within the allergen motifs found by *in silico* analysis of the Allergome database of April 2005, we selected motif 22 (*E*-value

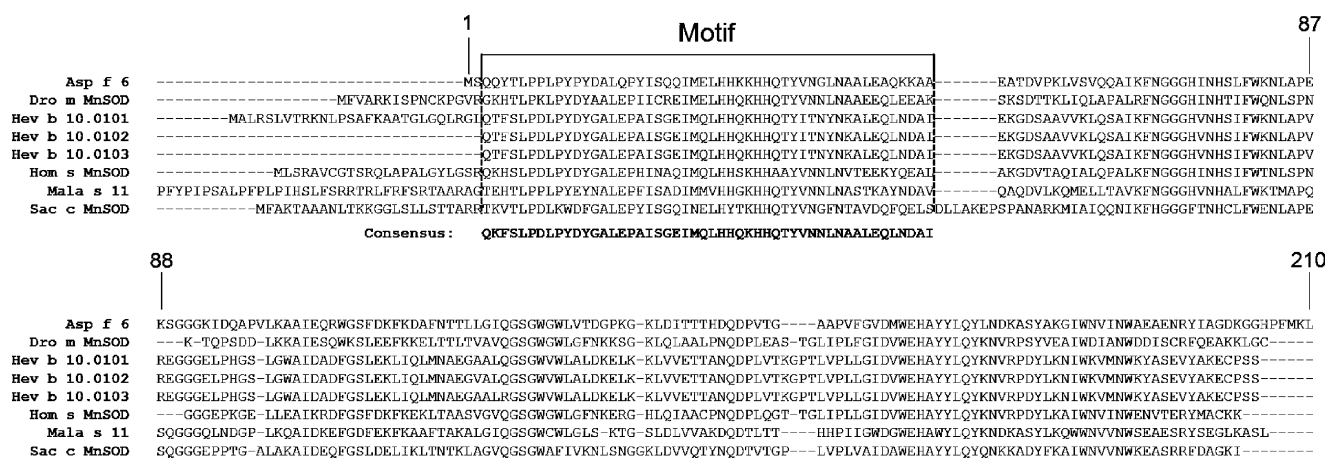


Fig. 1. Amino acid sequence alignment of the MnSOD from *A. fumigatus* (Asp f 6) with other MnSOD allergens within the motif group 22, namely those from *D. melanogaster* (Dro m MnSOD), *H. brasiliensis* (Hev b 10.0101, Hev b 10.0102 and Hev b 10.0103), *H. sapiens* (Hom s MnSOD), *M. sympodialis* (Mala s 11) and *S. cerevisiae* (Sac c MnSOD). The region of the motif and the theoretical consensus sequence of this region are indicated.

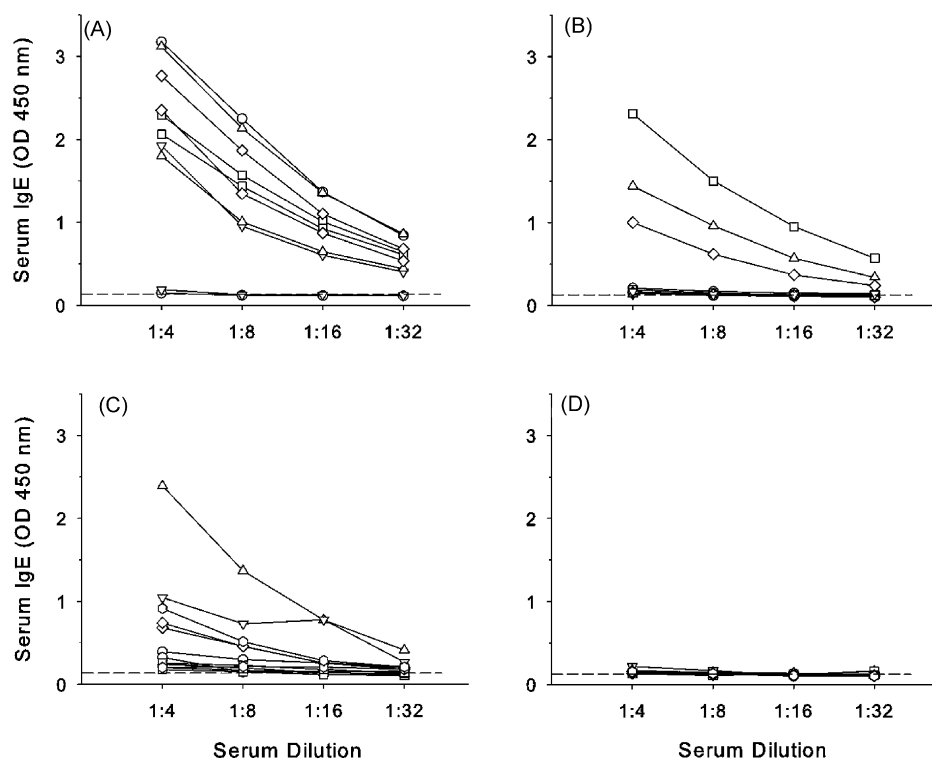


Fig. 2. Binding to MnSOD of serum IgE of three patient groups and control subjects. (A) Patients suffering from allergic bronchopulmonary aspergillosis (ABPA) (10 donors). (B) Patients suffering from atopic dermatitis (AD) (10 donors). (C) Patients tested positive on *A. fumigatus* extract by ImmunoCap (12 donors). (D) Control group (12 donors). The dashed line indicates the background level.

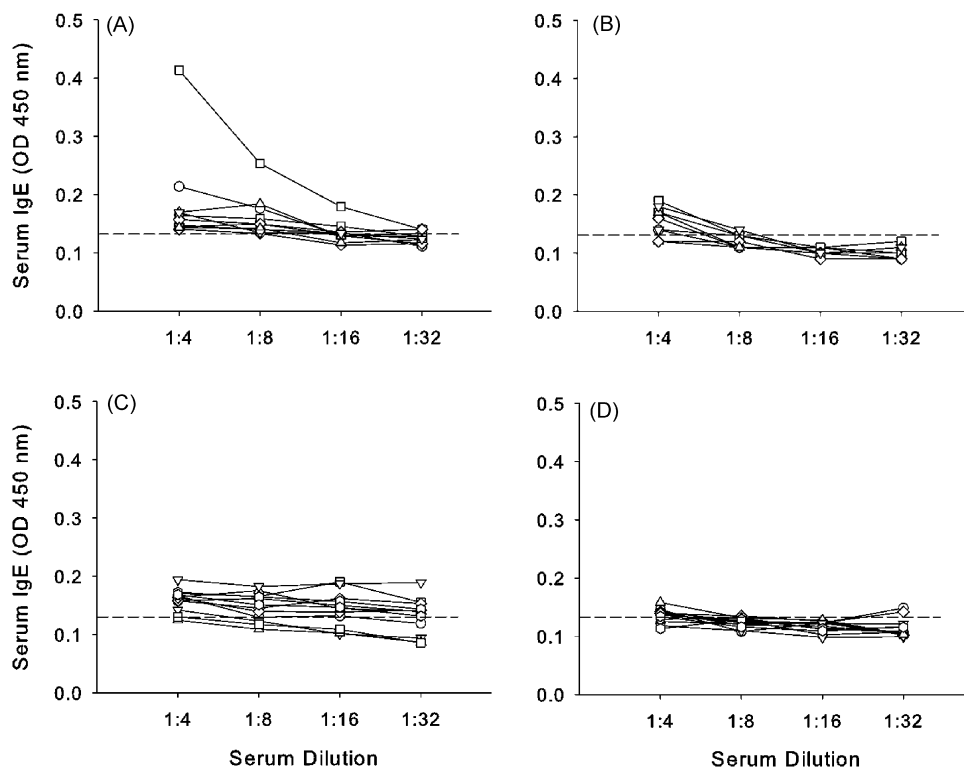


Fig. 3. Binding to MnSOD Motif of serum IgE of three patient groups and control subjects. (A–D) Patient groups and control subjects as indicated for Fig. 2. The dashed line indicates the background level.

2.3×10^{-162}) representing a group of MnSOD sequences of different organisms. This motif was the only found in the sequence and was chosen because the corresponding full-length protein for comparison of IgE reactivity and appropriate sera for testing them were available.

Fig. 1 shows the alignment of Asp f 6, including the localization of the motif, with seven selected MnSOD sequences of other organisms. We have selected the amino acid sequence corresponding to the motif stretch of Asp f 6 and compared the *in vitro* immunogenicity of this 50 amino acid stretch to that of its corresponding full-length protein.

3.2. IgE reactivity to MnSOD and MnSOD Mot

The MnSOD Mot peptide was chemically synthesized, whereas the full-length Asp f 6 was produced in *E. coli* as a recombinant soluble hexahistidine-tagged protein.

To compare the *in vitro* IgE reactivity of the Asp f 6 motif with the whole Asp f 6 protein, both products were tested in ELISA with sera of three groups of patients. In a first group of ABPA patients, 8 of 10 showed a high IgE reactivity to full-length Asp f 6 (Fig. 2A). This result is in agreement with previous reports showing that ABPA patients recognize recombinant Asp f 6 [23] and suggests that our protein was correctly folded.

To verify the cross-reactivity between MnSODs of different organisms predicted by our motif analysis, the full-length Asp f 6 was tested for IgE reactivity with sera of patients with atopic dermatitis (AD). These patients react to the MnSOD of the skin-colonising yeast *Malassezia sympodialis* (*M. sympodialis*) and were reported to recognize the MnSOD of *A. fumigatus* (Asp f 6) [24]. Of 10 AD patients, 3 exhibited strong reactivity with Asp f 6 in our IgE binding studies, confirming the previously seen cross-reactivity between the two fungal MnSODs (Fig. 2B).

The third patient group, which included 12 sera of individuals reacting to *A. fumigatus* extract, showed low reactivity to Asp f 6, with only one serum exhibiting a higher IgE binding reactivity (Fig. 2C). This result is in agreement with the literature, since the majority of individuals allergic to *A. fumigatus* do not react with Asp f 6 [23,24]. None of the control sera showed binding to Asp f 6 (Fig. 2D).

In order to compare the full-length Asp f 6 with our motif, we used the same groups of patients (Fig. 3). The motif showed a good reactivity with one ABPA serum and was weakly recognized by two sera from each ABPA and AD patients, suggesting that the major IgE epitopes of Asp f 6 are not fully represented by our motif peptide. Interestingly, those sera recognizing the motif also showed strong binding to the full-length Asp f 6. These results indicate that the sequence stretch defined by the motif partially covers IgE reactive epitopes of Asp f 6.

3.3. MnSOD Mot cross-reacts with the fungal MnSOD allergens

Two sera reacting with the motif were selected, one from the ABPA and one from the AD patient group, to perform inhibition ELISAs (Fig. 4). Serum IgE binding from the ABPA patient to

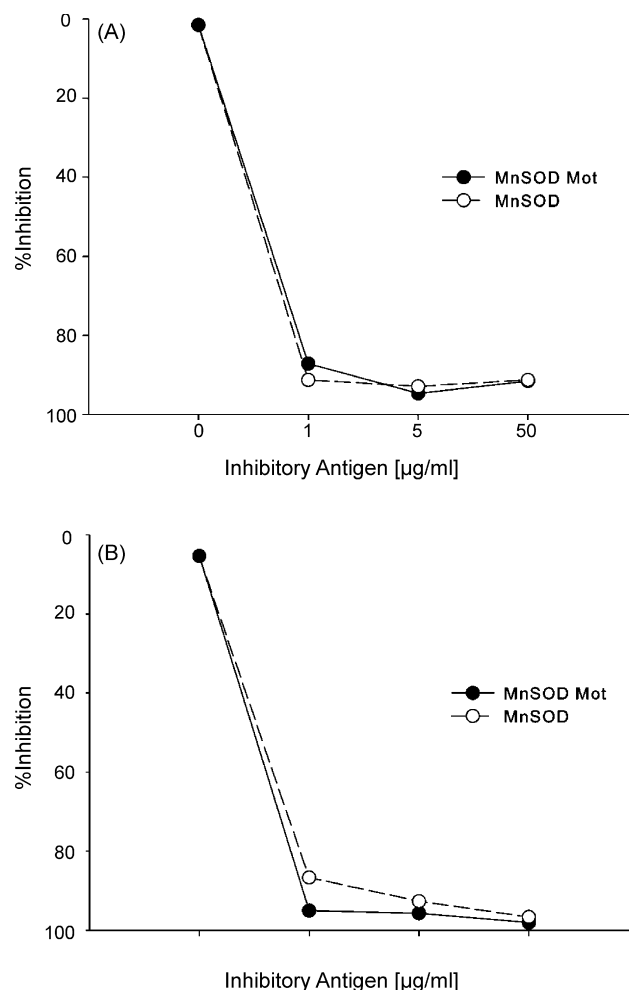


Fig. 4. Inhibition of IgE binding to MnSOD Mot. The patients' serum showing the highest binding signal to MnSOD Mot of the ABPA patient group (A) and the AD patient group (B) were pre-incubated with different concentrations of MnSOD Mot or Asp f 6 and tested for IgE binding to MnSOD Mot.

MnSOD Mot was inhibited by soluble MnSOD Mot, confirming specificity (Fig. 4A). Full-length Asp f 6 inhibited the IgE binding to MnSOD Mot at equimolar concentration indicating that the region defined by the motif includes one of the epitopes of the full-length allergen.

The same results were obtained using AD patients serum (Fig. 4B), suggesting that the motif region contains one of the determinants responsible for cross-reactivity between the MnSODs of *A. fumigatus* and *M. sympodialis*.

3.4. Allergen motif in tropomyosins and designation of consensus sequence

As a second motif, we have chosen motif 11 (E -value 2.0×10^{-356}) of the in-house allergen database analysis [7] representing a group of 10 sequences of highly conserved tropomyosins of different invertebrates. Fig. 5 shows alignment of the 10 tropomyosin sequences and the localization of the motif region in the aligned sequences. The reason for choosing this motif was the same as for the MnSOD motif as described above.

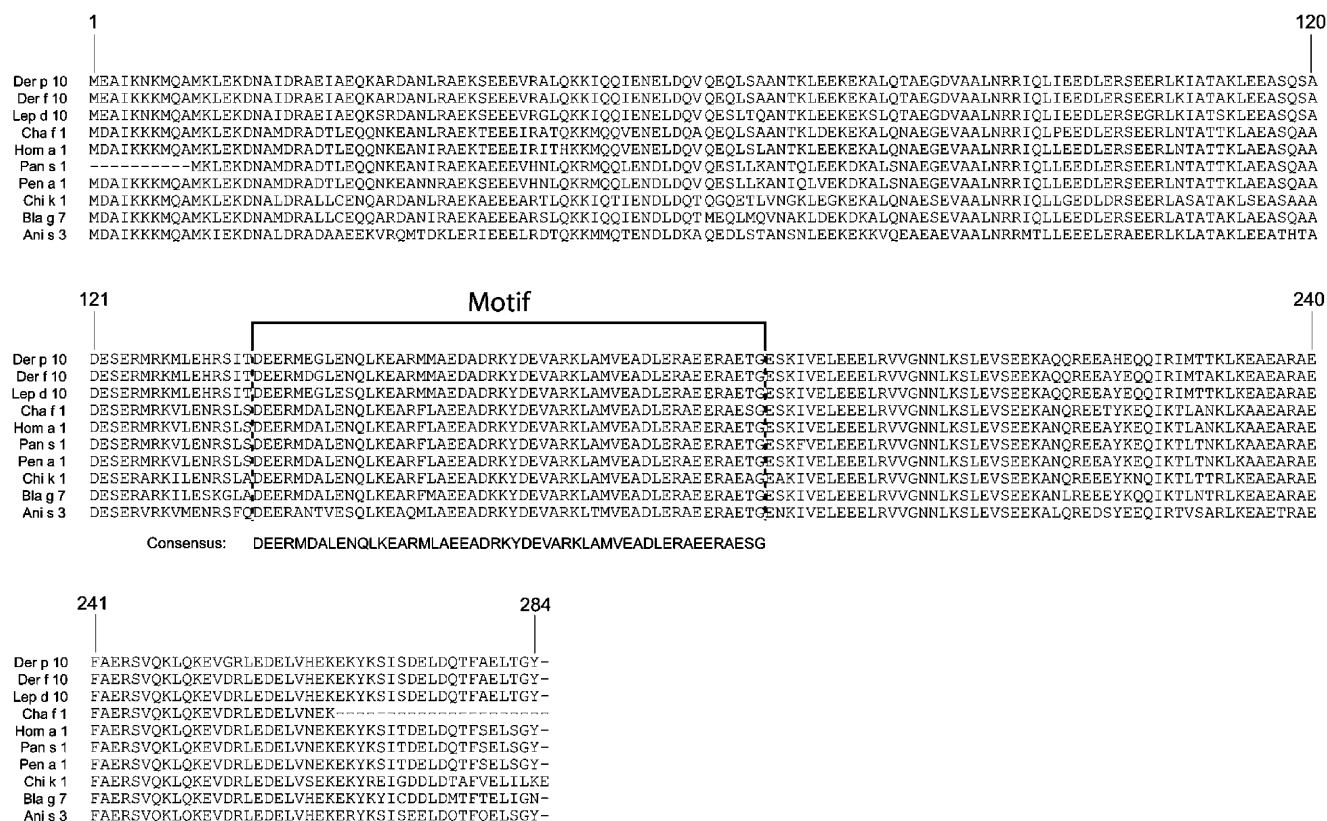


Fig. 5. Amino acid sequence alignment of the tropomyosins from *D. pteronyssinus* (Der p 10), *D. farinae* (Der f 10), *L. destructor* (Lep d 10), *Charybdis feriatius* (Cha f 1), *H. americanus* (Hom a 1), *P. stimpsoni* (Pan s 1), *P. aztecus* (Pen a 1), *C. kiliensis* (Chi k 1), *B. germanica* (Bla g 7) and *A. simplex* (Ani s 3). The motif and the consensus sequence are shown as indicated.

To investigate the biological relevance of this allergen motif, we chose an alternative strategy than the one described for the MnSOD motif: instead of selecting one of the tropomyosin sequences, a motif consensus sequence considering all the aligned sequences was generated by selecting the most abundant amino acids from the aligned sequences. In case of several amino acids occurring at the same frequency at a particular position in the alignment, the one which shows the best fitting to the coiled-coil structure of tropomyosin was chosen.

3.5. Trop Mot represents a major allergenic region of the full-length tropomyosins

In order to test the IgE reactivity of the tropomyosin consensus motif, it was produced as a soluble protein in *E. coli* and purified via the linked His (6) tag epitope.

The IgE binding capacity of Trop Mot was tested in ELISA using sera from donors allergic to shellfish. In order to compare this reactivity to that obtained with a full-length tropomyosin, the same sera were tested for IgE binding to the recombinant shrimp *P. aztecus* tropomyosin (rPen a 1) in ImmunoCAP assay. The same sera contained IgE against both recombinant proteins. As depicted in Fig. 6A, IgE reactivity to rPen a 1 correlates with the IgE binding to Trop Mot, suggesting that the Trop Mot peptide reflects the allergenicity of tropomyosin.

One of the sera showing IgE reactivity to both rPen a 1 and Trop Mot was tested for its binding to immobilized Trop Mot after pre-incubation with different amounts of soluble Trop Mot. As shown in Fig. 6B, IgE binding to Trop Mot was inhibited by increasing concentrations of the consensus motif, confirming that the reaction to Trop Mot was specific. BSA used as control protein did not interfere with IgE binding to Trop Mot. Interestingly, the binding could not be inhibited by pre-incubation with increasing amounts of soluble chicken tropomyosin, suggesting that this non-allergenic vertebrate tropomyosin, which shows a sequence identity of 62.5% to Trop Mot, does not cross-react with epitopes present in the allergen consensus motif.

4. Discussion

The exact molecular characteristics rendering a protein allergenic are still a matter of debate and intense research. A recent paper, however, has shown that dimerization and oligomerization of proteins could be an important characteristic of allergens [25]. We have recently described the identification of motifs using allergen sequences by an iterative sequence comparison protocol [7]. The majority of allergen sequences could be represented by 69 motifs indicating a limited variability of allergen motifs compared to the total number of allergens contained in the database. This motif-generating algorithm confirmed previous findings suggesting that the majority of allergens can be

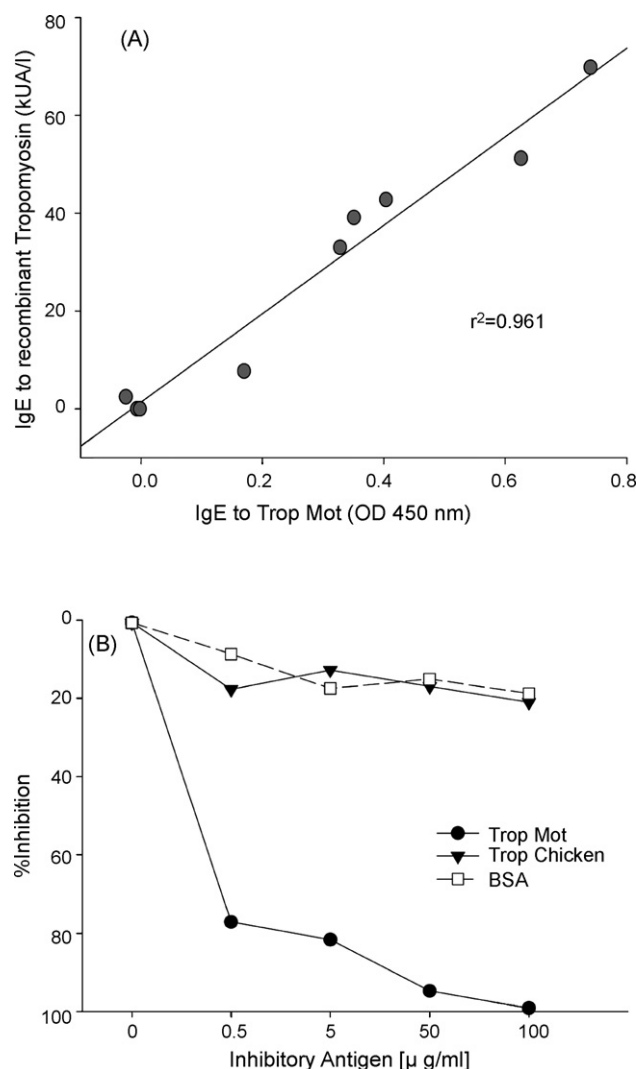


Fig. 6. (A) Correlation of values of specific IgE binding to recombinant Pen a 1 (*P. aztecus*, brown shrimp) analyzed by ImmunoCap and to Trop Mot analyzed by ELISA. (B) Inhibition of IgE binding to Trop Mot. One patient's serum was pre-incubated with different concentrations of Trop Mot, recombinant chicken tropomyosin (Trop Chicken) or BSA and tested for IgE binding to Trop Mot.

grouped into a limited number of structural protein families [3,26,27] and that such an approach may also be used to predict allergenicity.

In this study, we analyzed the IgE antibody binding properties of two allergen motifs in comparison to the corresponding full-length allergens. From a first group containing MnSOD sequences of different organisms and defined by motif 22, the *A. fumigatus* sequence was selected and analyzed for IgE binding activity using different groups of patients sera. Previous studies analyzing the sequence similarities between MnSODs of different organisms aimed at assigning identical residues which are solvent exposed and thus candidates for involvement in IgE binding [28]. A number of solvent exposed conserved amino acids were found and considered to be involved in IgE cross-reactivity of the two proteins. Additionally, sequence alignment of the MnSODs of *A. fumigatus*, *H. sapiens*, *S. cerevisiae*, *D. melanogaster* and *E. coli* revealed 48 identical amino acids

shared by the sequences, but only four of them were at least 30% solvent exposed. Interestingly, all of these four (Pro8, Pro19, Lys32 and Asn39) are within the MnSOD motif stretch which was defined by our *in silico* analysis. In IgE binding studies we found specific IgE to MnSOD Mot in sera of ABPA patients having a high reactivity to Asp f 6, whereas no reactivity was observed in sera of patients sensitized to *A. fumigatus* without ABPA. This suggests that MnSOD Mot covers only a part of the IgE-binding surface of Asp f 6 involved in the immune response associated with ABPA. It has been shown that Asp f 6 represents an intracellular protein not secreted by the fungus. However, patients with ABPA have fungus growing in their lung, leading to inflammatory processes and subsequent damage to the fungus itself. As a result, the patients become exposed to non-secreted intracellular proteins. Previous experiments demonstrated that Asp f 6 is allergenic only in patients suffering from ABPA. Our data confirm these results by showing a high reactivity to Asp f 6 in sera of ABPA patients and a much weaker one in sera of patients only sensitised to *A. fumigatus* extract.

Our *in silico* analysis has calculated a high degree of sequence similarity between the MnSODs of *A. fumigatus* and *M. sympodialis*. Three of 10 AD patients reacting to MnSOD of *M. sympodialis*, displayed IgE binding reactivity to Asp f 6. This confirms the predicted cross-reactivity and the fact that MnSOD is a pan-allergen that can be recognized by individuals sensitized to different MnSODs is consistent with earlier experiments showing that patients with severe AD have a high rate of specific IgE antibodies which cross-react with MnSODs of different origins [24].

Moreover, in the present study, one *Asp f 6* positive AD serum showed a reaction to MnSOD Mot, which could be inhibited with MnSOD, confirming that the cross-reactivity between the MnSODs of the two fungi is in part attributable to conserved IgE epitopes included in the motif region. The fact that only two patients react with MnSOD Mot may be because MnSOD contains additional allergenic epitopes in other parts of the sequence. This assumption is in accordance with previous works on structural analysis reporting that IgE-binding epitopes are scattered over the surface of the MnSOD molecule [28]. Furthermore, crystallization studies have shown that the fungal MnSOD is a tetrameric protein [29] and putative IgE-binding epitopes are formed by residues on different surface loops suggesting that correct folding is required for allergenicity [28]. From our point of view, this observation explains the low reactivity observed with the chemically synthesized MnSOD Mot motif which most likely did not adopt the same conformational structure as the native protein. The IgE binding capacity of allergenic motifs was further documented using the second motif representing tropomyosin sequences of different phylogenetic origins. Ayuso et al. have previously analyzed the major shrimp allergen Pen a 1 (tropomyosin) for IgE-binding regions by screening synthetic peptides spanning the whole length of the molecule with sera of shrimp-allergic subjects and identified five major IgE-binding regions [30]. Interestingly, one of these regions overlaps with the 50 amino acids sequence stretch defined by the tropomyosin motif. In the current study, we have shown that the IgE reactivity

of sera from shellfish-allergic individuals to recombinant shrimp *P. aztecus* tropomyosin (rPen a 1) is comparable to that observed for the tropomyosin consensus motif (Trop Mot) in the same sera. Therefore, we assume that the Trop Mot not only binds IgE but most probably represents a structure with an allergenic potential comparable to that of the full-length tropomyosin. This hypothesis is supported by a recent study showing that the previously defined region overlapping with our motif stretch is responsible for the IgE binding to most patients. The substitution of a key amino acid in this region with that of non-allergenic invertebrate tropomyosin showed a drastic reduction of the allergenicity of Pen a 1 [31]. This result indicates that in the case of tropomyosin our algorithm-based method was able to generate a motif region of clinical relevance.

In summary, we report the first *in vitro* application of an *in silico* motif-based allergenicity prediction protocol [7] with two motifs. For the MnSOD motif, the *A. fumigatus* sequence was chosen, whereas for the tropomyosins, all the sequences in the group defined by the Trop Mot were used to generate a consensus sequence. We show that among the tested motifs only the Trop Mot represents an allergenic structure able to substitute full-length tropomyosin for serological diagnosis of shellfish allergy. These results suggest that the motif-generated algorithm can be a valuable tool to identify allergenic regions of proteins with a coiled-coil structure such as tropomyosin. The low reactivity obtained with the MnSOD Mot indicates that in the case of complex globular structures such as MnSOD, where the correct protein folding is required for B cell epitope mapping, the region defined by the motif cannot be represented by a synthetic peptide. Thus, in the future, other approaches such as site-directed mutagenesis in the motif region need to be explored to be able to experimentally investigate whether a motif can be used for identifying allergenic epitopes.

Acknowledgements

We thank Dr. Stephan Gadola from the Department of Rheumatology at the University Hospital in Bern, for providing us with the facility to perform size exclusion chromatography. This project was supported by the Swiss National Science Foundation grants no. 31-63382.00/2 and 310000-112540 and the Innovation Promotion Agency (CTI) of the Federal Office for Professional Education and Technology (OPET).

References

- [1] Valenta R, Vrtala S, Laffer S, Spitzauer S, Kraft D. Recombinant allergens. *Allergy* 1998;53:552–61.
- [2] Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol* 2002;129:1–18.
- [3] Ferreira F, Hawranek T, Gruber P, Wopfner N, Mari A. Allergic cross-reactivity: from gene to the clinic. *Allergy* 2004;59:243–67.
- [4] Valenta R, Kraft D. From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol* 2002;14:718–27.
- [5] Radauer C, Breiteneder H. Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. *J Allergy Clin Immunol* 2006;117:141–7.
- [6] Jenkins JA, Griffiths-Jones S, Shewry PR, Breiteneder H, Mills EN. Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an *in silico* analysis. *J Allergy Clin Immunol* 2005;115:163–70.
- [7] Stadler MB, Stadler BM. Allergenicity prediction by protein sequence. *FASEB J* 2003;17:1141–3.
- [8] Mari A, Riccioli D. The Allergome web site—a database of allergenic molecules. Aim, structure, and data of a web-based resource. *J Allergy Clin Immunol* 2004;113:301.
- [9] Stadler BM, Stadler MB, Mueller L, Truffer R, Mari A, Miescher S, et al. Autoimmunity and allergy: a bioinformatic approach. *Allergy Clin Immunol Int: J World Allergy Org*, in press.
- [10] Bannister JV, Bannister WH, Rotilio G. Aspects of the structure, function, and applications of superoxide dismutase. *CRC Crit Rev Biochem* 1987;22:111–80.
- [11] Fluckiger S, Scapozza L, Mayer C, Blaser K, Folkers G, Cramer R. Immunological and structural analysis of IgE-mediated cross-reactivity between manganese superoxide dismutases. *Int Arch Allergy Immunol* 2002;128:292–303.
- [12] Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J Immunol* 1993;151:5354–63.
- [13] Leung PS, Chen YC, Mykles DL, Chow WK, Li CP, Chu KH. Molecular identification of the lobster muscle protein tropomyosin as a seafood allergen. *Mol Mar Biol Biotechnol* 1998;7:12–20.
- [14] Leung PS, Chen YC, Gershwin ME, Wong SH, Kwan HS, Chu KH. Identification and molecular characterization of *Charybdis feriatus* tropomyosin, the major crab allergen. *J Allergy Clin Immunol* 1998;102:847–52.
- [15] Daul CB, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. *Int Arch Allergy Immunol* 1994;105:49–55.
- [16] DeWitt AM, Mattsson L, Lauer I, Reese G, Lidholm J. Recombinant tropomyosin from *Penaeus aztecus* (rPen a 1) for measurement of specific immunoglobulin E antibodies relevant in food allergy to crustaceans and other invertebrates. *Mol Nutr Food Res* 2004;48:370–9.
- [17] Greenberger PA, Patterson R. Allergic bronchopulmonary aspergillosis and the evaluation of the patient with asthma. *J Allergy Clin Immunol* 1988;81:646–50.
- [18] Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol Suppl (Stockh)* 1980;92:44–7.
- [19] Bailey TL, Elkan C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* 1994;2:28–36.
- [20] Cramer R, Faith A, Hemmann S, Jaussi R, Ismail C, Menz G, et al. Humoral and cell-mediated autoimmunity in allergy to *Aspergillus fumigatus*. *J Exp Med* 1996;184:265–70.
- [21] Scheppler L, Vogel M, Marti P, Muller L, Miescher SM, Stadler BM. Intranasal immunisation using recombinant *Lactobacillus johnsonii* as a new strategy to prevent allergic disease. *Vaccine* 2005;23:1126–34.
- [22] Knutti-Muller JM, Stadler BM, Magnusson CM, De Weck L. Human IgE synthesis *in vitro*. Detection with monoclonal antibodies. *Allergy* 1986;41:457–67.
- [23] Cramer R, Hemmann S, Ismail C, Menz G, Blaser K. Disease-specific recombinant allergens for the diagnosis of allergic bronchopulmonary aspergillosis. *Int Immunol* 1998;10:1211–6.
- [24] Schmid-Grendelmeier P, Fluckiger S, Disch R, Trautmann A, Wuthrich B, Blaser K, et al. IgE-mediated and T cell-mediated autoimmunity against manganese superoxide dismutase in atopic dermatitis. *J Allergy Clin Immunol* 2005;115:1068–75.
- [25] Scholl I, Kalkura N, Shedziankova Y, Bergmann A, Verdino P, Knittelfelder R, et al. Dimerization of the major birch pollen allergen Bet v 1 is important for its *in vivo* IgE-cross-linking potential in mice. *J Immunol* 2005;175:6645–50.
- [26] Breiteneder H, Radauer C. A classification of plant food allergens. *J Allergy Clin Immunol* 2004;113:821–30 (quiz 31).
- [27] Aalberse RC. Structural biology of allergens. *J Allergy Clin Immunol* 2000;106:228–38.

- [28] Fluckiger S, Mittl PR, Scapozza L, Fijten H, Folkers G, Grutter MG, et al. Comparison of the crystal structures of the human manganese superoxide dismutase and the homologous *Aspergillus fumigatus* allergen at 2-Å resolution. *J Immunol* 2002;168:1267–72.
- [29] Borgstahl GE, Parge HE, Hickey MJ, Beyer Jr WF, Hallewell RA, Tainer JA. The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* 1992;71:107–18.
- [30] Ayuso R, Lehrer SB, Reese G. Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). *Int Arch Allergy Immunol* 2002;127:27–37.
- [31] Reese G, Viebranz J, Leong-Kee SM, Plante M, Lauer I, Randow S, et al. Reduced allergenic potency of VR9-1, a mutant of the major shrimp allergen Pen a 1 (tropomyosin). *J Immunol* 2005;175:8354–64.