

Purification and Immunobiochemical Characterization of Folding Variants of the Recombinant Major Wasp Allergen Ves v 5 (Antigen 5)

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Key Words

Ves v 5 · Antigen 5 · Recombinant allergens · Bacterial expression · Folding variants · PAGE, native

Abstract

Background: Antigen 5 is one of three major allergens in wasp venoms, but unlike phospholipase A₁ and hyaluronidase, both of which are enzymes, its biological function is unknown. The cDNA coding for this allergen has been isolated and used for recombinant expression. Thorough analysis of the expression product is essential in order to evaluate the usefulness for in vivo or in vitro application. **Objective:** In this study, folding variants of the recombinant major allergen Ves v 5 from *Vespula vulgaris* were immunologically and biochemically investigated in order to determine their possible applicability for diagnostic or therapeutic purposes. **Method:** The cDNA encoding Ves v 5 was cloned into the expression vector pSE420 which generates recombinant products lacking a tag sequence. After expression, inclusion bodies were purified, subsequently denatured and dialyzed against different solutions. The structural properties of soluble proteins were analyzed by size exclusion chromatography, non-reducing SDS-PAGE, native PAGE, N-terminal sequencing, proteolytic digestion and

ion exchange chromatography. Immunological investigations were performed by using different monoclonal antibodies (mAbs) specific for Ves v 5 and IgE from patients allergic to wasp venom allergens. **Results:** After dialysis, soluble monomeric recombinant Ves v 5 was more than 95% pure in each case. Using different dialysis solutions, clearly distinguishable folding variants were obtained. In one case, the recombinant allergen was comparable with the natural counterpart in respect of migration in non-reducing SDS-PAGE, native PAGE and IgE reactivity. This variant reacted with two different Ves v 5-specific mAbs and produced a stable fragment after proteolytic digestion. Elution from a cation exchange chromatography column was achieved with 320 mM NaCl. In two other cases, folding variants exhibited a different migration behavior in SDS-PAGE and native PAGE compared with the natural allergen. Also, the mAb 1E11 recognized none of these variants since it presumably detected a conformational epitope. Moreover, the IgE reactivity was clearly reduced and proteolytic digestion effected almost complete degradation. These variants eluted from the cation exchange column with 400 mM NaCl. **Conclusion:** Defined folding strategies resulted in both soluble misfolded variants with reduced IgE reactivity, potentially suitable for immunotherapy, and natural-like folded variants for diagnosis.

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1018–2438/00/1214–0284\$17.50/0

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Introduction

Allergic reactions to vespid stings are amongst the most dramatic expressions of allergic disease. The stings can cause severe and sometimes fatal IgE-mediated reactions. The group vespids includes both hornets and wasps, and their venom allergens include two enzymes, hyaluronidase (43 kD) and phospholipase A₁ (37 kD) together with an allergen with an as yet unidentified biological activity, antigen 5 (23 kD) [1, 2].

The incidence of IgE-binding reactivities to antigen 5 and phospholipase A₁ in wasp venom-allergic subjects is in excess of 50% so that the proteins were classified as major allergens [3, 4]. The corresponding cDNA encoding these allergens have been cloned and recombinant allergens were expressed predominantly in *Escherichia coli* [5–7]. The availability of recombinant allergens has given rise to the suggestion that they may provide the basis for safer and patient-tailored immunotherapy [8]. An important requirement for the therapeutic application of recombinant allergens is the characterization of the product as being immunologically similar or dissimilar from its natural form [9]. A problem that frequently arises with bacterial expression of venom components is the formation of inclusion bodies [10, 11]. Refolding after solubilization under denaturing conditions can result in a more or less correct conformation. The portion of correctly folded molecules can be estimated in respect of enzymatic activities as described for recombinant hyaluronidases and phospholipases [10, 11]. The major wasp allergen Ves v 5 from *Vespula vulgaris*, also designated antigen 5, has no recognized enzymatic activity [2], and the analysis of recombinant expression products has been limited to reducing SDS-PAGE [7]. In order to evaluate the potential applicability of recombinant Ves v 5 for diagnosis of sensitization and specific immunotherapy, which is dependent on the conformation, folding variants of this molecule have been produced and characterized by suitable methods.

Materials and Methods

Amplification and Cloning of a cDNA Encoding Ves v 5

Total RNA were isolated from the venom glands of *V. vulgaris* using a GlassMax RNA microisolation system (Life Technologies, Karlsruhe, Germany). Reverse transcription of poly (A⁺) RNA was performed with a Superscript preamplification system for first-strand cDNA synthesis (Life Technologies) according to the manufacturer's instructions. Two oligonucleotide primers (Biometra, Göttingen, Germany) hybridizable with the 5' and 3' terminal regions of the mature allergen were designed according to the published

sequence from Ves v 5 [12]. The 5' primer 5'-CCCATGGCGAA-CAATTATGTGAAA-3' containing an *Nco*I restriction site providing the start codon followed by the first Ves v 5-specific codon and the 3' primer 5'-CAAGCTTCTTACTTTGTTTGATAAACTTCC-3' containing an *Hind*III site were used to generate a PCR product for direct cloning. PCR was carried out with a PCR reagent system (Life Technologies), and 1/100 of the first strand cDNA was used as template. PCR products were examined by electrophoresis in 1.0% (w/v) agarose gel with ethidium bromide staining. After restriction enzyme digestion, the purified fragment was ligated into a pSE420 vector (Invitrogen, Groningen, Netherlands) also digested with *Nco*I/*Hind*III. The *E. coli* M15 strain was used for subsequent transformation. The nucleotide sequences of the insert were determined by a commercial laboratory service (MWG-Biotech, Ebersberg, Germany).

Expression, Purification and Refolding of rVes v 5

Protein production was initiated by transferring 20 ml of a fresh overnight culture into 750 ml of LB medium containing 100 µg/ml ampicillin. The cells were grown at 37°C with shaking (200 rpm) until A₆₀₀ reached about 0.8. At this point isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and incubation was continued until maximal expression was reached (3–5 h). Cells were harvested, lysed by using lysozyme (100 µg/g wet weight) and inclusion bodies were subsequently isolated according to the procedure described by Grisshammer and Nagai [13]. Solubilization of purified inclusion bodies was performed by addition of 20 ml buffer D (6 M guanidine-HCl, 50 mM Tris/HCl, pH 8.0). After an incubation of 8 h at room temperature, 10-ml aliquots of this solution were filled into dialysis tubings with a 12-kD cutoff (Medicell, London, UK) and dialyzed for 8 h against 1 liter of one of the following solutions: C (5 mM cysteine, 0.5 mM CaCl₂, pH 5.2), SA (20 mM sodium acetate, pH 5.0) or H (distilled water). The dialysis solutions were subsequently replaced by distilled water and dialysis was continued for 24 h with one water exchange. Precipitated protein was separated by centrifugation (20 min, 3,000 g, 20°C) and soluble rVes v 5-containing supernatant was stored at 4°C or lyophilized. The precipitates were cycled a second time through the refolding protocol. The overall yield of purified protein was approximately 5 mg/l of culture in each case.

Production of Monoclonal Antibodies

The monoclonal antibodies (mAbs) were obtained by a method described previously [14]. A 1:2 mixture of *V. vulgaris* and *V. germanica* venoms (Allergon, Ängelholm, Sweden) was used for the immunization of BALB/c mice.

SDS-PAGE, Native PAGE and Western Blots

Proteins were separated by SDS-PAGE (11% T, 4% C) with the buffer system of Laemmli [15]. Reducing and nonreducing sample buffers were the same except that 5 × reducing sample buffer contained 200 mM DTT. Native separation of proteins was performed in a 7.5% PAGE using a continuous buffer system. The polyacrylamide gel contained 0.3 M Tris/HCl, pH 6.8, and the electrophoresis buffer was composed of 70 mM Tris/acetic acid, pH 7.5. The sample buffer was the same as for non-reducing SDS-PAGE except that SDS was excluded and 0.1% (w/v) pyronine G (Merck, Darmstadt, Germany) was included. Electrophoresis was performed at room temperature with a constant 80 V and reversed polarity of the buffer reservoirs. After electrophoresis proteins were either stained with Coomassie brilliant blue R-250 (Merck) or transferred onto a supported

nitrocellulose membrane (Sartorius, Göttingen, Germany) by semi-dry blotting for 30 min at 2 mA/cm².

Immunoblotting with Serum IgE and mAbs

Immunological characterization of rVes v 5 folding variants was performed by using either mAbs specific for Ves v 5 or sera from subjects allergic to wasp stings. Prior to immunodetection membranes were blocked by treatment for 30 min with Tris-buffered saline (pH 7.4) supplemented with 0.05% (w/v) Tween 20. Incubations with either 1:10 diluted patients' pooled serum or mAbs were carried out overnight. After washing, bound antibodies were detected with alkaline phosphatase-conjugated mouse monoclonal anti-human IgE (Allergopharma, Reinbek, Germany) diluted 1:2,000 or 1:10,000-diluted goat anti-mouse IgG/M with alkaline phosphatase (Dianova, Hamburg, Germany). The binding patterns were visualized by a substrate solution consisting of NBT/BCIP (Gibco-BRL, Gaithersburg, Md., USA) in 100 mM Tris/HCl-buffered saline solution (pH 9.5).

In order to screen several sera a Miniblot MN45 apparatus (Biometra, Göttingen, Germany) was used to apply recombinant folding variants onto nitrocellulose membranes. The samples were adjusted to 0.1 µg/µl in 20 mM NaOH, injected into the conduits and incubated for 30 min. The basic pH promoted an effective binding of proteins onto the membrane. After blocking, strips were cut and incubated with patients' sera derived from patients with a clinical history of insect sting allergy to vespids and RAST classes >2.

Glu-C Digestion of rVes v 5 Variants

In order to obtain information about the conformation of folding variants, C and SA supernatants were adjusted to 25 mM sodium phosphate buffer pH 7.3. Subsequently, 0, 0.5, 1, 2 µg Glu-C protease from *Staphylococcus aureus* (Promega, Mannheim, Germany) was added to 50-µl aliquots each containing 10 µg protein. After incubation for 2 h at 37°C the reactions were stopped by adding reducing sample buffer followed by heat treatment at 95°C for 10 min. Analysis of reaction products was performed using a 15% SDS-PAGE.

Liquid Chromatography and HPLC

Cation exchange chromatography was performed using a 16/10 column filled with Source 30S (Pharmacia). Before loading, protein samples were adjusted to 25 mM Na₂HPO₄, pH 7.3. Elution was carried out with a linear NaCl gradient from 0 to 0.7 M in 25 mM Na₂HPO₄, pH 7.3, over 300 ml and a flow rate of 5 ml/min.

The purity of soluble proteins was assessed by size exclusion HPLC using a TSK 2000SW column (TosoHaas, Stuttgart, Germany) with a flow rate of 1 ml/min.

EAST Inhibition Test

The relative IgE reactivities of folding variants were evaluated with an EAST inhibition assay as described [16]. Microtiter plates were coated with Ves v 5 C supernatant (0.1 µg/well). Before loading to the microtiter plate, patients' sera (diluted 1:4) were preincubated with serial dilutions of rVes v 5 SA or C supernatant (1.0 × 10⁻⁴ to 10 µg/ml). The allergenic activity of rVes v 5 SA was calculated as relative potency in terms of the 50% inhibition values to rVes v 5 C. Both pool sera used were composed of 11 individual sera derived from patients allergic to insect stings.

Results

Purification and Physical Characterization of Folding Variants

In order to estimate the degree of purification as well as the yield of soluble rVes v 5 obtained after isolation of inclusion bodies, denaturation and dialysis against different solutions, both the pellets containing insoluble proteins and supernatants were subjected to SDS-PAGE under reducing and nonreducing conditions (fig. 1a). While the pellets contained rVes v 5 and additional bacterial proteins, the supernatants were composed almost exclusively of the recombinant allergen. In each case the recovery of soluble protein was estimated to be approximately half that present in the pellet as judged under reducing conditions. Differences in migration behavior were observed in non-reducing SDS-PAGE. Dialysis against cysteine (fig. 1a, -DTT, lane 1) resulted in a single band with an apparent molecular weight of 25 kD (rVes v 5 C). In contrast, dialysis against water (rVes v 5 H) or sodium acetate (rVes v 5 SA) (fig. 1a, -DTT, lanes 2, 3) produced at least a double band, and even the lowest bands had a higher apparent molecular mass than rVes v 5 C. Separation in large gels with higher resolution revealed additional bands between the upper and lower band (not shown). These results indicated the generation of two clearly different folding variants, probably caused by altered disulfide bond formation. Interestingly, aggregates such as dimers could not be detected. The separation of insoluble material of rVes v C in the nonreducing SDS-PAGE showed a faint band in contrast to the result under reducing conditions, indicating strong aggregates which could not be broken up without the addition of DTT and that obviously did not migrate into the stacking gel (fig. 1a, lane 1, -DTT).

Size exclusion HPLC analysis of rVes v C and rVes v SA (fig. 1b) was performed to prove the aggregation potential even in the absence of detergents compared to nonreducing SDS-PAGE. In both cases a single peak corresponding to the expected molecular size of monomeric Ves v 5 was detected. Edman degradation for 5 cycles showed the N-terminal sequences of rVes v 5 C and rVes v 5 SA to be identical (ANNYC). The expected N-terminal methionine was not detectable, indicating its *in vivo* removal in *E. coli* [17, 18]. Compared with the authentic N-terminus 'NNYC' the additional alanine had been introduced to obtain an *Nco*I restriction site for cloning.

Preparations of rVes v 5 variants were performed 3 times and were found to be highly reproducible in terms of yield, quality and electrophoretic behavior of purified proteins.

Fig. 1. SDS-PAGE and HPLC analysis of precipitated and soluble rVes v 5. **a** Pellets containing precipitated proteins and supernatants obtained after dialysis of denatured inclusion bodies against cysteine (1), distilled water (2) and sodium acetate (3) were subjected to SDS-PAGE under reducing (+DTT) and nonreducing conditions (-DTT). **b** Size exclusion analysis by HPLC of supernatants shown in lane 1 (S1) (**a**) and lane 3 (S3) (**a**).

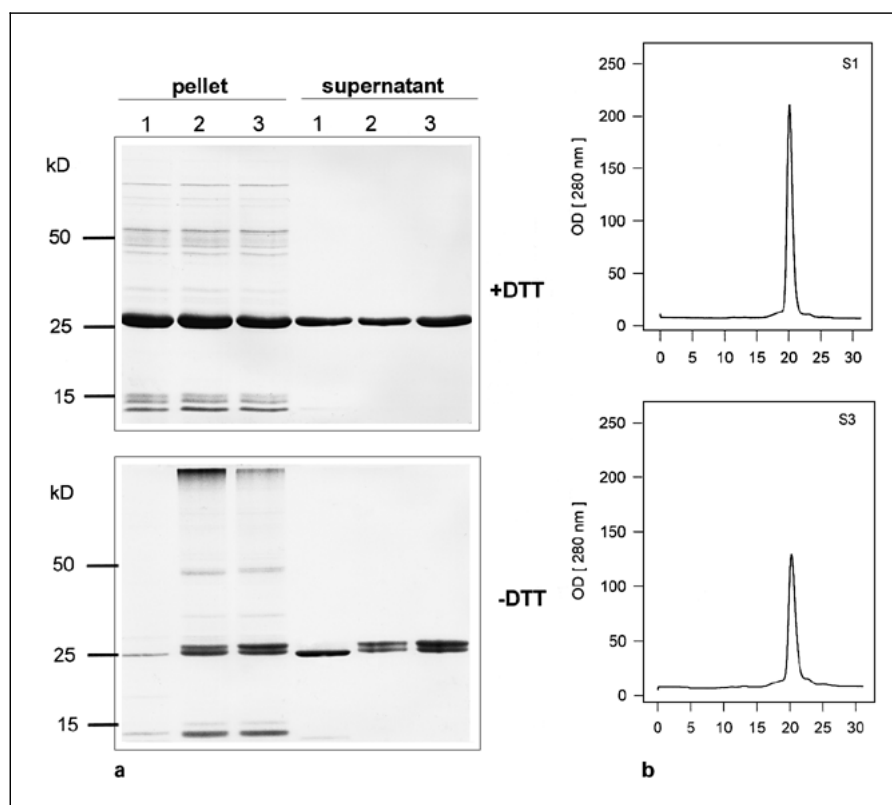
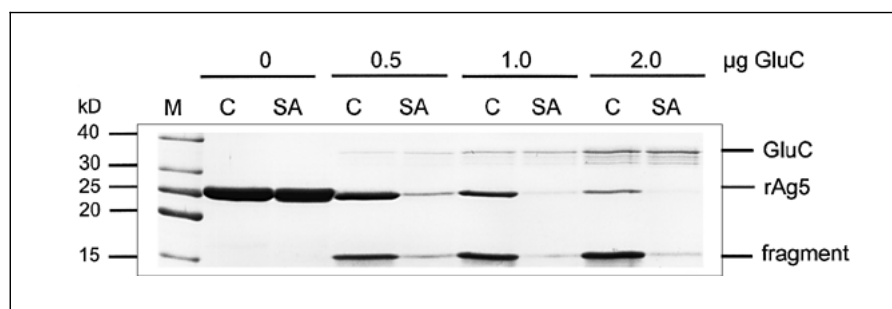


Fig. 2. Proteolytic digestion of soluble rVes v 5 folding variants. Protease Glu-C was added in increasing amounts to rVes v 5 C (C) and rVes v 5 SA (SA). After incubation for 2 h, reactions were stopped and products were separated under reducing conditions in a 15% SDS-PAGE.



Evidence of Conformational Differences between Folding Variants

Further experiments were carried out with rVes v 5 C and rVes v 5 SA. The molecular structures of both folding variants were analyzed in terms of their sensitivity to proteolytic digestion when increasing amounts of Glu-C protease were added. After incubation, the reactions were stopped and products were separated under reducing conditions in SDS-PAGE (fig. 2). The rates at which the intensity of the bands corresponding to the intact allergen decreased were directly proportional to the amount of protease added. Differences were observed with regard to the fragments produced. Whilst rVes v 5 C was degraded

to a stable fragment with an apparent size of 15 kD, rVes v 5 SA was digested more completely. The fragments that were produced from this folding variant were presumably so small that they could not be resolved under these conditions.

Cation exchange chromatography provided a further hint that the conformations were different. Whilst rVes v 5 C eluted with 320 mM NaCl, a concentration of 400 mM was necessary to elute rVes v 5 SA (not shown). Evidently, the altered structure of rVes v 5 SA resulted in a higher isoelectric point, presumably attributable to the amino acids exposed on the surface of these folding variants.

Fig. 3. Nonreducing SDS-PAGE and immunoblot analysis of rVes v 5 folding variants with mAbs and a pooled serum from allergic patients. Coomassie blue-stained gel (a) and identical immunoblots (b–d) carrying wasp venom from *V. vulgaris* (lane 1), rVes v 5 C (lane 2) and rVes v 5 SA (lane 3) are shown. Immunoblots were incubated with mAb 3E8 (b), 1E11 (c) and pooled patients' serum (d).

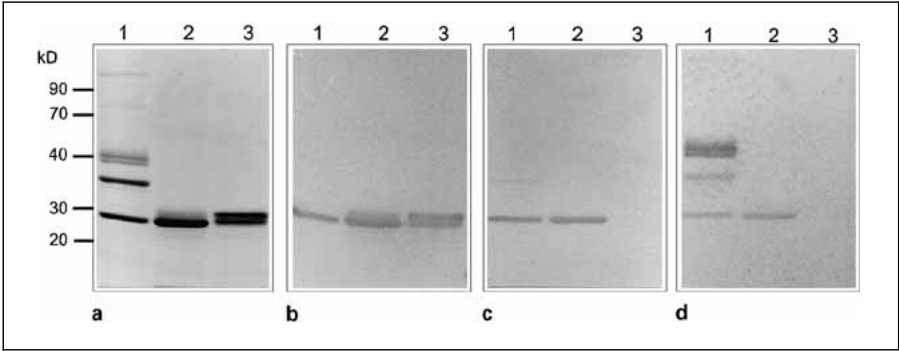


Fig. 4. Native PAGE and immunoblot analysis of rVes v 5 folding variants with mAbs. Coomassie blue-stained gel (a) and identical immunoblots (b, c) carrying 15 µg wasp venom from *V. vulgaris* (lane 1), 3 µg rVes v 5 C (lane 2) and 3 µg rVes v 5 SA (lane 3) are shown. Immunoblots were incubated with mAb 3E8 (b) and 1E11 (c).

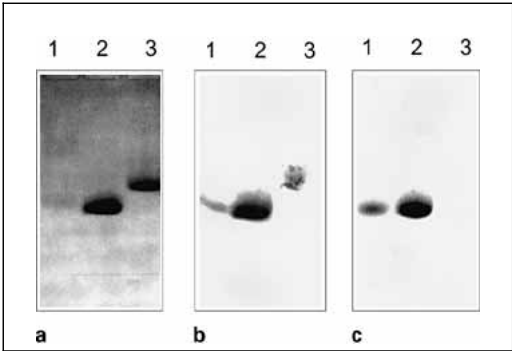
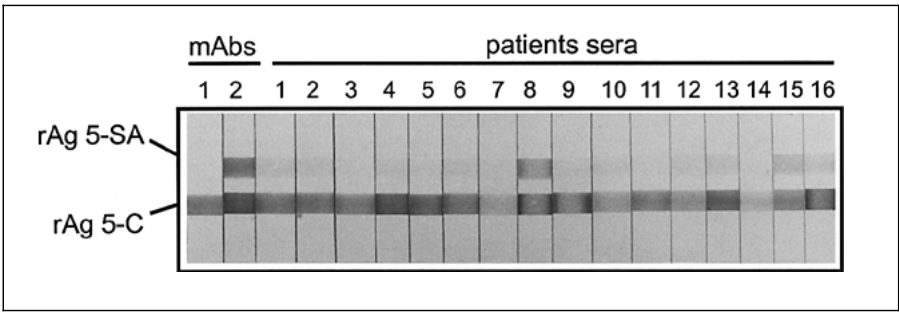


Fig. 5. Immunological analysis of dotted rVes v 5 folding variants with mAbs and individual sera derived from allergic patients. Recombinant allergens were immobilized on a nitrocellulose membrane by using a miniblot apparatus. Strips were subsequently incubated with mAbs (1 = 1E11, 2 = 8E3) and individual sera (1–16).



Immunological Properties of Folding Variants

The possibility that folding variants differ in their immunological properties was investigated using two different mAbs specific for Ves v 5 and a pool serum derived from allergic subjects. Figure 3 shows a Coomassie-stained gel (fig. 3a) and corresponding immunoblots carrying wasp venom rVes v 5 C and rVes v 5 SA. Incubation with the mAb 8E3 resulted in detection of both natural and recombinant allergens (fig. 3b). Surprisingly, only the natural allergen and rVes v 5 C were immunostained using mAb 1E11 (fig. 3c). This mAb obviously detected a conformational epitope missing from rVes v 5 SA. A similar result was achieved with IgE, which bound to the natural allergens and to rVes v 5 C, but only weakly or not at all to rVes v 5 SA. The migration of rVes v 5 C was com-

parable with the natural allergen, whereas rVes v 5 SA migrated slightly more slowly. It was concluded that rVes v 5 C is comparable with the natural allergen with respect to conformation and IgE-binding reactivity.

In order to substantiate these results a native PAGE was performed (fig. 4), since the charge and shape of the molecules are the predominant factors influencing their separation. Again, natural Ves v 5 and rVes v 5 C behaved in the same way as regards mobility and mAb-reactivity, whereas rVes v 5 SA differed even more compared with its behavior in nonreducing SDS-PAGE.

Screening experiments using 16 individual sera demonstrated that every serum except one reacted more strongly or exclusively with rVes v 5 C compared with rVes v 5 SA (fig. 5). Since the proteins were applied to the

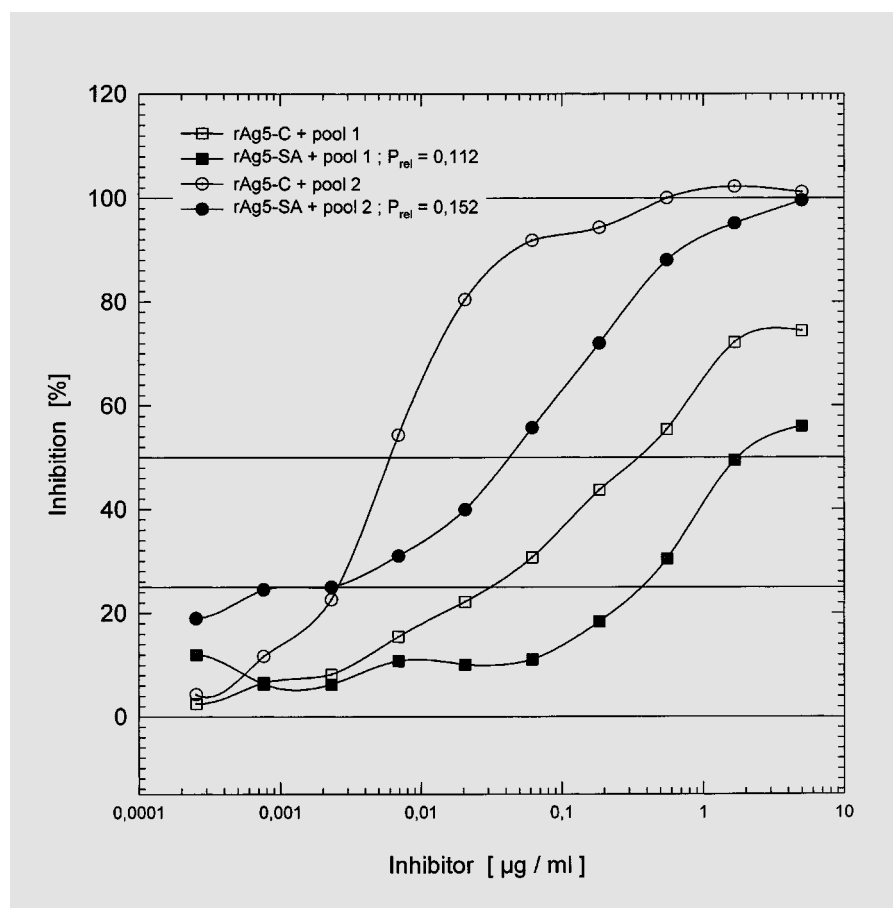


Fig. 6. East inhibition assay with two pooled sera from allergic patients. Solid phase carrying rVes v 5 C was incubated with sera preadsorbed with recombinant allergens as indicated. P_{rel} = Relative potency.

membrane without previous SDS-PAGE separation, the native structures of the molecules were as far as possible maintained. In order to evaluate the relative extent of reduced IgE-binding capacity of rVes v 5 SA, an EAST inhibition assay was performed with two pool sera derived from allergic subjects (fig. 6). The rVes v 5 C supernatant was bound to the solid phase, whilst increasing concentrations of rVes v 5 SA were used to preadsorb the sera. The relative potency of rVes v 5 SA, which was determined on the basis of the 50% inhibition value, was 6.5- to 9-fold less than that of rVes v 5 C. This result corresponded to the differences obtained by immunostaining with Western and dot blotting. Since pool serum 2 did not reach the 100% inhibition with rVes v 5 C, it was assumed that the concentration of specific IgE antibodies directed against Ves v 5 was in excess of that which could be inhibited completely by an inhibitor concentration of 10 µg/ml.

Discussion

In order to evaluate the applicability of rVes v 5 for diagnosis and immunotherapy of wasp venom allergy, the corresponding cDNA was expressed in bacteria without a tag sequence such as 6xHis [19]. Efficient purification was achieved by isolation of inclusion bodies which were denatured and then dialyzed against different solutions. Interestingly, despite omitting thiol reagents, soluble monomeric molecules were obtained, although Ves v 5 contains eight cysteine residues forming four disulfide bonds in natural molecules [19, 20]. Depending on the dialysis solution used to refold denatured molecules two different groups of conformations were developed, the natural-like molecules designated as rVes v 5 C and the misfolded group named rVes v 5 SA. Since reduction led to identical migration in SDS-PAGE in contrast to separation under nonreducing conditions, differences were probably due to altered disulfide bonds. Hence, there is no evidence that incorrect disulfide bonds cause aggregation [21]. Group

rVes v 5 SA molecules represented a pool of molecules in which the number of conformational epitopes was reduced. This conclusion was supported first by the clearly reduced IgE reactivity and second by the binding of mAb 1E11 that obviously recognized an epitope determined by a specific conformation present in the natural molecule. Similar results concerning decreased immunogenicity of misfolded recombinant molecules were also described by King et al. [19] and Soldatova et al. [10]. On the basis of previous descriptions concerning the relative decrease of IgE reactivity of misfolded recombinant insect allergens, it may be assumed that the less cysteine present, the less the reduction in IgE reactivity and vice versa. For example, bacterial expression of bee hyaluronidase containing 4 cysteine residues and a molecular mass of approximately 43 kD led to a 25% decrease in IgE reactivity [10]. In the case of recombinant unfolded phospholipase A₂ IgG- and IgE-binding reactivity could hardly be detected [11]. This 16-kD molecule contains 10 cysteine residues [22]. In the present study, misfolding of rVes v 5 molecules with molecular weights of approximately 25 kD and 8 cysteine residues led to a 10-fold decrease in IgE reactivity.

The variant rVes v 5 SA could represent a potential candidate for immunotherapy, as the complete primary structure is maintained coincident with an up to 10-fold decrease in IgE reactivity. There would be no need to split the protein into peptides or fragments [23, 24], taking into account the possible loss of some T cell epitopes necessary for specific immunotherapy.

In the case of rVes v 5 C it was obvious that the molecules exhibited the same conformation as the natural protein. This conclusion was supported by identical migration in nonreducing SDS-PAGE and native PAGE as well as comparable IgE and mAb binding reactivity. Separation parameters in native PAGE are charge, shape and size [25]. Therefore, recombinant Ves v 5 C and the natural allergen obviously possessed very similar or even identical isoelectric points and molecule shape, since their sizes, the third relevant parameter in native PAGE, were the same as judged by SDS-PAGE. However, detailed investigations will have to be carried out to establish whether the correct folding was enhanced by the low pH in the dialysis solution or the presence of cysteine [21, 26].

Another question relates to the potential influence of additional sequences frequently used in bacterial expression systems [19]. It is conceivable that even small tag sequences such as hexahistidine can prevent correct folding or solubility of molecules such as rVes v 5 [27]. Since rVes v 5 presented in this study lacks additional tag

sequences and was found to be folded and soluble, the unaltered primary structure with the exception of the N-terminal alanine could be a reason for that. In addition, for therapeutic purposes it will be advantageous to exclude additional sequences in recombinant allergens, as was the case in the present study with rVes v 5. The correctly folded molecule is suitable, however, for a specific diagnosis of allergic patients.

In summary, starting from one solution containing denatured recombinant major wasp allergen 5 we have demonstrated that treatment with different dialysis solutions resulted in natural-like and misfolded conformations. Moreover, using specific proteolytic digestion, mAbs or ion exchange chromatography, it was possible to discriminate clearly between both folding variants and thereby to evaluate their respective purity. Such systems could provide an optimal basis for producing recombinant allergens for both diagnosis and immunotherapy of allergic diseases.

Acknowledgments

We thank Dr. Arnd Petersen (Borstel, Germany) for protein sequencing. The excellent technical assistance of Mrs. Vera Meienreis is gratefully acknowledged.

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