

Expressions of Recombinant Venom Allergen, Antigen 5 of Yellowjacket (*Vespula vulgaris*) and Paper Wasp (*Polistes annularis*), in Bacteria or Yeast

Rafael I. Monsalve, Gang Lu, and Te Piao King¹

The Rockefeller University, 1230 York Avenue, New York, New York 10021-6399

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Antigen 5 is a major allergen of vespid venom. It has partial sequence identity with proteins from diverse sources. The biologic function of Ag 5 and its related proteins is not known. We are interested in the expression of Ag 5 with the native conformation of the natural protein since its B cell epitopes are mainly of the discontinuous type. When expressed in bacteria, recombinant Ag 5 formed an insoluble intracellular product, and it did not translocate from cytoplasm to periplasm by the addition of a pelB leader sequence to the cloned protein. When expressed in yeast *Pichia pastoris*, Ag 5 was secreted because the cloned protein contained a yeast α signal leader sequence. Recombinant Ag 5 from yeast was shown to have the native structure of the natural protein and the recombinant Ag 5 from bacteria did not. This was shown by comparison of their solubility, electrophoretic behavior, disulfide bond content, CD spectrum, and binding of IgE antibodies from allergic patients and IgG antibodies from mice immunized with natural Ag 5 or recombinant Ag 5s from yeast or bacteria. These studies were made with Ag 5s from yellowjacket (*Vespula vulgaris*) and paper wasp (*Polistes annularis*). © 1999

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Antigen Ag² 5 is one of three known venom allergens of vespids (1). The other two venom allergens are hyaluronidase and phospholipase A₁ (2). Vespid allergic patients have IgE and IgG antibodies specific for these allergens.

Homologs of Ag 5 are present in different vespids that include hornets, yellowjackets, and paper wasps (1). Ag 5 has 23–35% sequence identity with proteins

from diverse sources. These sources include fire ant that is distantly related to vespids (3), hookworm (4), lizard (5), mammalian testis (6), human brain (7), and plants (8). The biologic function of Ag 5 and its sequence-related proteins is not known.

Hornet Ag 5 has been expressed in bacteria but the recombinant (rb) protein is not properly folded (9). The rb-protein has the continuous T and B cell epitopes of the natural (n) protein but it lacks the discontinuous B cell epitopes of the n-protein. The great majority of Ag 5-specific antibodies in vespid allergic patients, or in venom-immunized animals, are specific for the discontinuous epitopes. For immunological studies, it is useful to have adequate amounts of Ag 5 with the native conformation. But natural Ag 5 is available only in limited amounts from venom.

In this paper we report the expression of Ag 5s from yellowjacket (*Vespula vulgaris*) and paper wasp (*Polistes annularis*) in bacteria and in yeast. These two allergens are designated as Ves v 5 and Pol a 5, respectively, in accordance with the accepted allergen nomenclature system (10). Immunochemical studies showed the recombinant (rp) protein from yeast to have the native structure of n-Ag 5 but not the rb-protein from bacteria.

MATERIALS AND METHODS

DNA sequences. All recombinant plasmids used for expression of Ves v and Pol a 5s in bacteria or in yeast were confirmed by sequencing their Ag 5 coding region. The sequence data for Pol a 5 was the same as that in GenBank. But the data for Ves v 5 differed from that in GenBank with two nucleotide changes at positions 579 and 587. The first change is a silent mutation of G to A substitution and the second one of T to A substitution results in a codon change of Met to Lys at amino acid residue 196. The two nucleotide changes may represent insect polymorphism rather than random muta-

¹ To whom correspondence should be addressed. Fax: (212) 327-8878.

² Abbreviations used: n, natural; rb, recombinant bacteria; rp, recombinant yeast; Ag, antigen

TABLE 1

Primers and Probes for Ves v and Pol a 5s

	<i>Bam</i> HI	N ¹	N	Y	C	K	I	K ⁶
1. CGT	GGA TCC	AAC	AAT	TAT	TGT	AAA	ATA	AAA
	<i>Bgl</i> II	*	K ²⁰⁴	T	Q	Y	L	E ¹⁹⁹
2. CGT	AGA TCT	TTA	CTT	TGT	TTG	ATA	AAG	TTT
3.	Same as No. 1 but <i>Bam</i> HI site is replaced with <i>Nco</i> I site.							
4.	Same as No. 2 but <i>Bgl</i> II site is replaced with <i>Xho</i> I site.							
5.	Same as No. 1 but <i>Bam</i> HI site is replaced with <i>Eco</i> RI site.							
6.	Same as No. 2 but <i>Bgl</i> II site is replaced with <i>Xba</i> I site.							
	<i>Eco</i> RI	V ¹	D	Y	C	K	I	K ⁶
7. CGT	GAA TTC	GTT	GAT	TAT	TGT	AAA	ATA	AAA
	<i>Xba</i> I	*	K ²⁰⁵	K	T	Y	P	L ²⁰⁰
8. CGT	TCT AGA	TTA	TTT	TTT	TGT	ATA	AGG	TAG
	G ¹²	G	K	L	C	K	I	K
9. ACC	TCC TTT	CAA	ACA	TTT	TAT	TTT	ACA	ATA
	I ¹²	G	S	P	C	K	I	K
10. GAT	ACC ACT	TGG	ACA	CTT	TAT	TTT	ACA	ATA

tions since the Ag 5 cDNA used in this study was prepared in the same manner as was done previously (1).

Expression of Ves v 5 in bacteria. Two pairs of plasmid vectors and bacterial hosts were studied: pQE12 and M15(Prep4) cells (Qiagen, Chatsworth, CA) and pET22b and B121(DE3) cells (Novagen, Milwaukee, WI). cDNA encoding Ves v 5 with the desired 5' and 3' restriction sites were prepared by PCR amplification of Ves v 5 encoding plasmid (1) with the primers in Table 1: primers 1 and 2 for pQE12 and 3 and 4 for pET22b. The cDNA was inserted into the appropriately cut vector then used for bacterial transformation. Experimental procedures for the construction of r-plasmid and for the isolation of r-protein from transformed bacteria culture are the same as those reported previously for hornet Ag 5, Dol m 5 (9).

Expression of Ves v 5 and Pol a 5 in yeast. The plasmid vector pPICZα-A was used for expression in *Pichia pastoris* strain KM71 (Invitrogen, San Diego, CA). Procedures used below were from the Invitrogen manual of EasySelect *Pichia* expression kit and from Ref. (11).

cDNA encoding Ves v 5 was inserted into the cut vector following the same procedures used in the preceding section; the required cDNA was obtained by PCR with primers 5 and 6 in Table 1. cDNA for Pol a 5 was similarly prepared with primers 7 and 8 in Table 1. The recombinant plasmids were amplified in TOP10F' cells following their transformation.

Recombinant plasmids were linearized with the restriction enzyme *Sac*I and then used to transform yeast cells by the PEG procedure, as described in the manual from Invitrogen. Transformed cells were selected on YPDS agar plates (yeast extract, peptone, dextrose, sorbitol) with 0.1 mg/ml Zeocin. Selected transfor-

ants were checked for the presence of Ag 5 encoding cDNA by PCR following digestion with *Lyticase* (Sigma, St. Louis, MO) (12).

Transformation of yeast was also done by electroporation as described in the manual from Invitrogen. After recovery for 1 h in 1 M sorbitol, an equal volume of YPD medium was added. After an additional 1 h, the transformed cells were plated on YPDS agar plates with 1 mg/ml Zeocin for selection of hyper-drug-resistant transformants (11).

Yeast cells from selected clones were grown in 500-ml bottles at 30°C with orbital shaking at 225 rpm until A_{600nm} of 4–6. Each bottle contained 100 ml of BMGH medium (pH 6.0 phosphate buffer containing yeast nitrogen base, biotin, glycerol, and histidine). The cells were collected by centrifugation and resuspended in 25 ml of BMMH medium which contained methanol in place of glycerol in BMGH medium. Incubation was continued at 30°C with shaking at 275 rpm for 5 days with daily addition of 25 µl of methanol.

The collected culture fluid was concentrated 20-fold by ultrafiltration through PM10 membrane (Amicon, Beverly, MA). After equilibration with 50 mM ammonium acetate buffer, pH 4.6, and clarification, the sample was applied to a cation-exchange column (20 × 0.9 cm) of SE-53 (Whatman, Fairfield, NJ) equilibrated with the same buffer. The column was eluted at 60 ml/h with a 300-ml linear gradient of 0.1 to 0.7 M NaCl in 50 mM ammonium acetate (pH 4.6). r-Ves v and Pol a 5s were eluted as dominant peaks at about 0.38 and 0.18 M NaCl, respectively. The pooled solution was desalted on a reverse-phase column (7 × 0.9 cm) of C18 silica (Separations Group, Hesperia, CA) and the column was eluted at 60 ml/h with a linear 120-ml gradient of 0 to 60% 2-propanol in 0.1% trifluoroacetic acid. r-Ag 5s were eluted at about 30% 2-propanol and were recovered on lyophilization. They were dissolved in 0.02 M acetic acid and stored at 4°C. Their concentrations were established from their UV spectra; A_{280nm} values of 1.93 and 1.62 for 1 mg/ml solution were used for Ves v and Pol a 5s, respectively.

CD spectra of about 0.2 mg/ml solutions of Ag 5 in 0.02 M acetic acid or phosphate buffer of pH 7.2 were taken in cells of 1 mm pathlength in an AVIV 62DS spectrometer.

Copy number of Ag 5 encoding cDNA in the yeast genome. Genomic DNA was isolated following the procedures given in Ref. (11, 13). Dot blot analysis was tested with labeled probe 9 or 10 given in Table 1. The probes were labeled with ³²P using reagents supplied in a kit from NEN Life Science Products (Boston, MA). Southern hybridization was done according to procedures given in Ref. (14).

Sulfhydryl contents of proteins before and after reduction. Reduction of proteins (1–6 µM) in 6

M urea + 0.1 M NaBH₄ + 60 μ M selenocysteamine was made at 25°C for 30 min. *In situ* generated selenocysteamine served to catalyze the reduction (15). Reduction was terminated on addition of equal volume of 0.3 M acetic acid to decompose the excess NaBH₄ followed by the addition of 3 vol of 1.7 mM 4,4'-dithiodipyridine in 6.7 M urea. Sulfhydryl groups in the mixture were determined by the increase in $A_{325\text{nm}}$ from the released 4-thiopyridone (16). Control experiments with oxidized glutathione, lysozyme, and bovine plasma albumin gave the expected values with an experimental error of $\pm 10\%$.

Inhibition ELISA. This was done as described previously (9) in Nunc-Immuno plate with Maxi-sorp surface, and the wells were coated with 5 μ g/ml Ag 5 in 0.05 M Tris-HCl buffer, pH 8. Sera dilution for inhibition ELISA was chosen to give about $\frac{2}{3}$ maximal binding of specific IgG₁ to solid-phase antigen. Bound IgG₁ was detected with biotinylated goat anti-mouse IgG ($\gamma 1$ specific) and extraavidin-alkaline phosphatase conjugate. Mice sera were collected at week 7 from groups of four female BALB/c mice which had received four bi-weekly intraperitoneal injections of 2 μ g Ag 5 + 1 mg alum in 0.2 ml of phosphate-buffered saline.

Inhibition ELISA was also done with sera from vespid-allergic patients, and the bound Ag 5-specific IgE was detected with biotinylated mouse antibodies specific for human IgE (PharMingen, La Jolla, CA).

RESULTS

Expression of Ves v 5 in bacteria. Two vectors were tested. One was pQE12 which placed a hexahistidine tag at the C-terminus of the expressed protein for ease

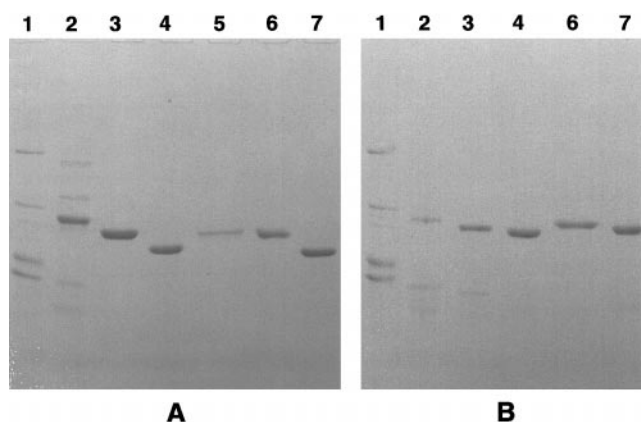


FIG. 1. SDS-PAGE analysis of Ag 5s with or without mercaptoethanol reduction, (B and A, respectively). Samples shown are rb-Ves v (lane 2), n-Ves v (lane 3), n-Pol a (lane 4), rp-Ves v (lane 6), and rp-Pol a (lane 7). Lane 5 (in A only) contains 5 μ l of culture fluid from KM71 cells producing Ves v Ag 5. Lane 1, standards of 65, 29, 20, and 14.5 kDa. About 2 μ g of protein was applied to each lane. The gel was stained with Coomassie blue R-250 dye.

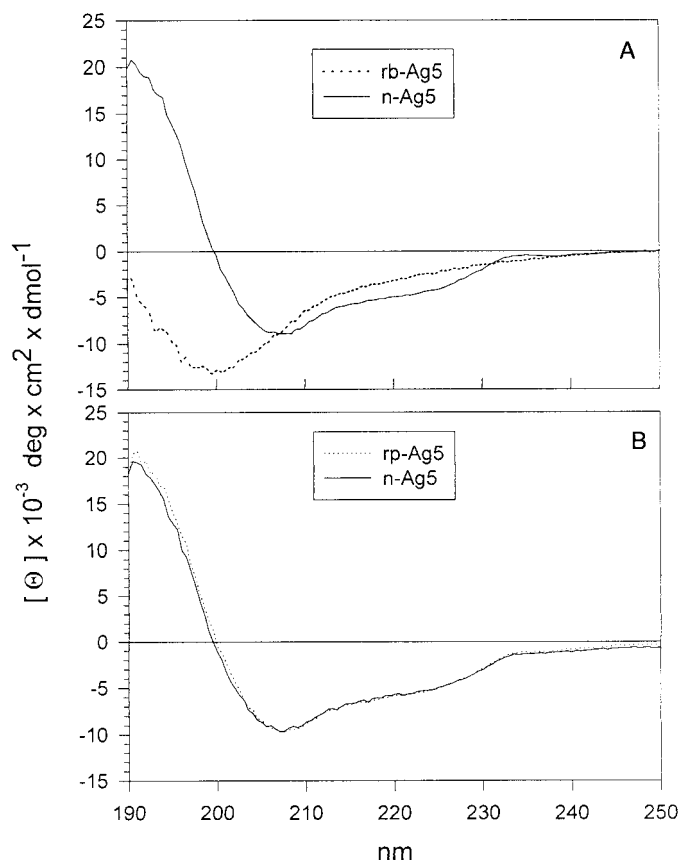


FIG. 2. CD spectra of n- and rb-Ves v 5s in 0.02 M acetic acid in A and n- and rp-Ves v 5s in 0.02 M phosphate, pH 7.2, in B.

of isolation. The other was pET22b that added a potential translocation signal to the N-terminus of the cloned protein for translocation from the cytoplasm to the periplasm with accompanying signal peptidase digestion to remove the leader sequence. The translocation signal is the pelB leader sequence for a bacterial pectate lyase. However the expected translocation did not occur with Ag 5. The rb-protein was obtained as an insoluble precipitate in the bacteria with both vectors. It could be solubilized in urea or guanidine hydrochloride solution.

The rb-protein with a hexahistidine tag was isolated by successive metal ion chelation and reverse-phase chromatography. The rb-protein differed from the n-protein in being readily soluble only in acidic pH <4. The rb-protein gave one main band on SDS-gel electrophoresis with mobility slightly slower than that of the n-protein, as shown in Fig. 1A, lanes 2 and 3. Similar findings with 2-mercaptoethanol reduced samples are shown in Fig. 1B, lanes 2 and 3.

The n- and rb-Ves v 5 differed in their CD spectra. The data in Fig. 2A suggest that the rb-protein has a more disordered structure than the n-protein. The

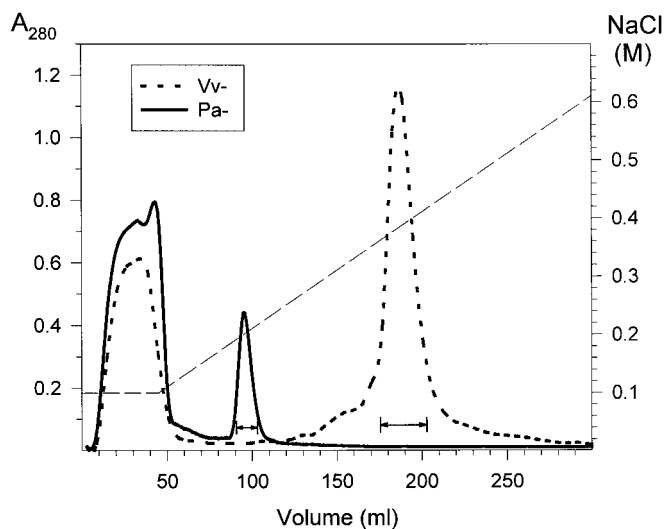


FIG. 3. Isolation of rp-Pol a and Ves v 5s by SE-53 chromatography. Concentrates from 100 ml of induced yeast culture were applied to column and about 2.7 and 10.2 mg of rp-Pol a and Ves v 5s were isolated. The yeast clone for Pol a 5 was a poor producer and that for Ves v 5 was a high producer.

spectra were taken in 0.02 M acetic acid, because of the limited solubility of rb-protein at neutral pH.

Expression of Ves v and Pol a 5s in yeast. Both Ag 5s were expressed with the vector pPICZ α -A in *P. pastoris* cells of KM71 strain. The cloned Ag 5 is a fusion protein with the yeast α factor signal peptide for secretion into culture. This was found to occur with both Ag 5s. They were easily isolated from the culture by ion-exchange chromatography as shown in Fig. 3. Both rp-Ag 5s were soluble in acidic or basic pH as were their n-Ag 5s.

Both rp-Ves v and Pol a 5s gave single bands on SDS-gel electrophoresis with mobilities identical to their n-counterparts (Fig. 1A, lanes 6, 7, 3, and 4, respectively). Pol a 5 showed a faster mobility than Ves v 5 did although they have 205 and 204 amino acid residues, respectively. This difference is seen in Fig. 1A with the n-Ag 5s as well as with rp-Ag 5s. This difference was less following reduction in Fig. 1B.

Edman degradation for 11 cycles showed the N-terminal sequence of rp-Ves v 5 to be a 1/4 mixture of EAEF- and EAEAEF-[Ves v 5] and that of rp-Pol a 5 to be EAEAEF-[Pol a 5]. The EA and EAEA sequences represent a portion of the yeast signal sequence, which remains attached to Ag 5, and the EF sequence results from the *Eco*RI restriction site for ligation of Ag 5 cDNA with the vector. These findings suggest that signal peptide cleavage had occurred at two adjacent sites with different rates.

Mass spectroscopy confirmed the identities of rp-Ves v and rp-Pol a 5s to have their correct parent ions. These results are not shown.

CD spectra of n- and rp-Ves v 5s in pH 7.2 phosphate buffer were superimposable as can be seen in Fig. 2B. Similar data were obtained for Pol a 5. They are not shown as the findings parallel those for Ves v 5.

Yield of rp-protein from different yeast clones. Transformation of yeast cells may result in insertion of multiple gene copies into the yeast genome with a low frequency of 1 to 10% (11). One of the 12 Ves v 5-expressing clones were screened produced about 100 mg of rp-Ag 5 per liter of culture and the others produced about 20 mg per liter. Southern blots confirmed that the genomic DNA of a high producing clone contained about 10 times more copies of Ag 5-encoding cDNA than the poor producing clones (data not shown).

We did not find a high producing clone for Pol a 5 on screening 16 clones, which were from PEG transformation. We repeated our screening with five clones from a population, which were from transformation by electroporation then enriched for multicopy gene integrants by their hyper-drug resistance (11). All five clones were found to be high producers of 105 mg of rp-Pol a 5 per liter of culture.

Comparison of n-, rb-, and rp-Ves v 5s. rb-Ves v 5 was found to contain 0.5 ± 0.1 and 8 ± 1 sulfhydryl groups before and after NaBH₄ reduction, respectively. Both n- and rp-Ves v 5s were found to contain 0.1 ± 0.1 and 8 ± 1 sulfhydryl groups before and after reduction, respectively. These proteins were titrated for sulfhydryl group in guanidine hydrochloride and in urea. The similar results in the two denaturants suggest that the low sulfhydryl content of rb-Ves v 5 was not due to their inaccessibility but was due to incomplete pairing of its eight cysteinyl residues to form disulfide bonds. The absence of sulfhydryl group in n- and rp-Ves v 5 suggests that their eight cysteinyl residues are paired to form four disulfide bonds.

n-, rp-, and rb-Ves v 5s were compared for their inhibition of binding of rp-Ves v 5-specific antibodies to solid-phase rp- and n-Ves v 5s as given in Figs. 4A and 4B, respectively. The Ves v 5s were also compared for their inhibition of binding of n- and rb-Ves v 5-specific antibodies to their respective solid-phase antigens as shown in Figs. 4C and 4D.

The inhibition results indicate that n- and rp-Ves v 5s were indistinguishable when examined with n-Ves v 5-specific antibodies in Fig. 4C. They were also indistinguishable with rp-Ves v 5-specific antibodies on solid-phase n-Ves v 5 as shown in Fig. 4B but they were distinguishable on solid-phase rp-Ves v 5 as shown in Fig. 4A. These results suggest that n- and rp-Ves v 5s have very similar epitopes but rp-Ves v 5 has one or more epitopes that are absent in n-Ves v 5. The new epitope is most likely related to the signal peptide sequence of EAEAEF in rp-Ves v 5.

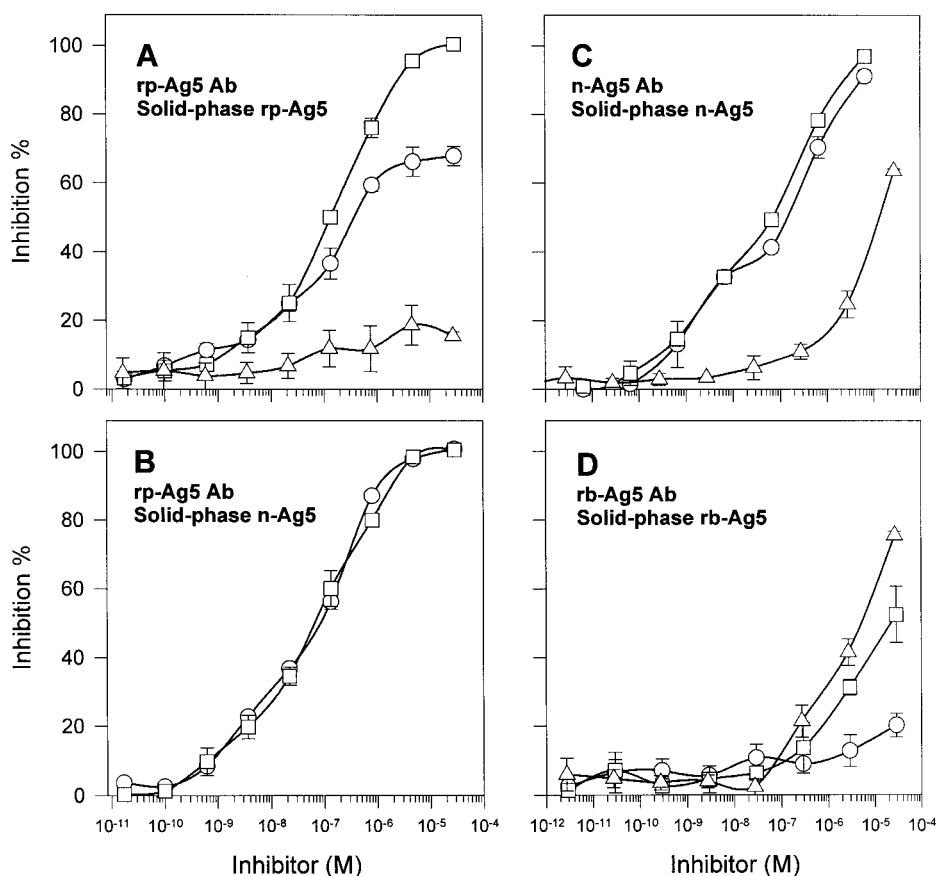


FIG. 4. Inhibition ELISA with mouse sera specific for rp-Ves v 5 (A and B), n-Ves v 5 (C), and rb-Ves v 5 (D). Solid-phase antigens are rp-, n-, n-, and rb-Ag 5s, respectively, in A–D. Reciprocal sera dilutions were 2×10^4 , 1×10^4 , and 3×10^3 for n-, rp-, and rb-Ves v 5-specific sera, respectively. The inhibitors are n-, rp-, and rb-Ves v 5s represented by open circles, squares, and triangles, respectively.

The results in Figs. 4A and 4C indicate that rb-Ves v 5 was a poor inhibitor for the binding of n- or rp-Ves v 5-specific antibodies to their respective solid-phase Ag 5. The results in Fig. 4D indicate that rb-Ves v 5 was a more effective inhibitor than n-Ves v 5 or rp-Ves v 5 are in their inhibition of binding of rb-Ves v 5-specific mouse antibodies to solid-phase bacterial rb-Ves v 5. The results in Figs. 4A–4D suggest that n- and rp-Ves v 5s have more common epitopes than n- and rb-Ves v 5s do. The results in turn suggest that n- and rp-Ves v 5s are more similar in their structures than the n- and rb-proteins are.

ELISA inhibition of binding of specific human IgE to solid-phase Ves v 5 was also done with sera from three allergic patients, and data from one representative case are shown in Fig. 5. rp- and n-Ves v 5 were equally effective inhibitors.

DISCUSSION

The above findings are in accord with the common observations that r-proteins that remain intracellular in bacteria are usually folded incorrectly and that

those which are secreted are usually folded correctly. Incorrect folding of proteins in bacteria may result from inadequate intracellular concentrations of chaperones (17), as well as from the reducing environment of the cytoplasm (18). Our attempt to induce translocation of the r-protein from the cytoplasm to the periplasm by attachment of a translocation signal was unsuccessful, although this approach led to processing of other proteins with different leader sequences (19,20). However, attachment of a yeast secretory signal resulted in the secretion and processing of r-Ag 5 from yeast.

Immunochemical tests strongly indicated that rp-Ag 5 from yeast had the native conformation of the natural protein. The rp- and n-Ag 5s were indistinguishable in their solubility, gel mobility, secondary structure as reflected by their CD spectrum, and disulfide bond content. The electrophoretic mobilities of n- or rp-Ag 5s in SDS gel decreased following mercaptoethanol reduction but this did not occur with rb-Ag 5 (Fig. 1). These results suggest that rp-Ag 5s are compact molecules like the n-proteins.

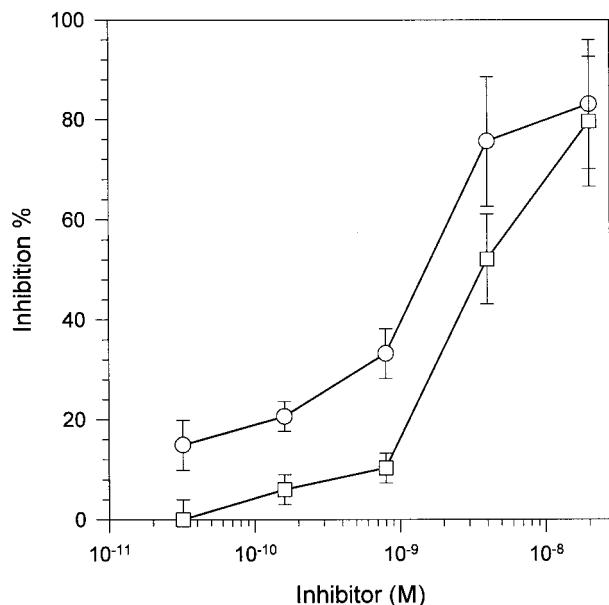


FIG. 5. Inhibition ELISA with allergic patient sera. Solid-phase antigen was n-Ves v 5. Patient sera were used at 1/20 dilution. The inhibitors were n- and rp-Ves v 5 represented by same symbols as in Fig. 4.

n- and rp-Ves v 5s were also indistinguishable by their binding of human IgE from different patients and antibodies from pools of mice immunized with n-Ves v 5. But they were distinguishable by antibodies from mice immunized with rp-Ves v 5. Our data suggest that rp-Ves v 5 has one or more new epitopes that are absent in n-Ves v 5. One new epitope is probably related to the incompletely removed signal peptide sequence. We have shown previously the N-terminus of the homologous hornet Ag 5 to be weakly immunogenic (21). The EAEAEF sequence of rp-Ag 5 apparently has converted its N-terminus to a strongly immunogenic hapten.

Hornet Ag 5 has been expressed in insect cells (22). The recombinant Ag 5 was secreted into the culture medium for insect cells, so it is likely to be properly folded. The present findings indicate that it is more advantageous to express Ag 5 in yeast cells than in insect cells because of its ease of isolation. The yeast culture is essentially free of proteins other than r-Ag 5, as can be seen from its gel pattern in Fig. 1A, lane 5, but the insect culture contains added serum proteins as growth supplements. It was therefore possible to obtain r-Ag 5 of high purity from yeast culture after a simple isolation procedure.

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