

Superior biologic activity of the recombinant bee venom allergen hyaluronidase expressed in baculovirus-infected insect cells as compared with *Escherichia coli*

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Background: Hyaluronidase (Hya) is one of several allergens in honeybee venom. Its cDNA sequence was recently described.

Objective: We sought to express recombinant Hya in prokaryotic and eukaryotic systems and to compare it with natural (n)Hya for biologic activity.

Methods: In *Escherichia coli* Hya was produced as inclusion body 6×His-fusion protein. In baculovirus-infected insect cells expression was obtained by cotransfection of linearized Bac-N-Blue DNA and pMelBac transfer vector into *Spodoptera frugiperda* cells.

Results: Enzymatic activity of Hya from the baculovirus system was equal to nHya, and that of the enzyme expressed in *E. coli* was only 20% to 30% of nHya. In vitro IgE binding was similar in nHya and the enzyme from baculovirus but markedly lower in Hya expressed in *E. coli*.

Conclusions: Biologic activity of Hya expressed in baculovirus-infected insect cells was comparable with that of the natural enzyme, indicating a native-like conformation of the recombinant protein. In contrast, the enzyme expressed in *E. coli* as an inclusion-body protein and reconstituted in vitro reached only 20% to 30% of the activity of nHya. (J Allergy Clin Immunol 1998;101:691-8.)

Key words: Bee venom allergy, hyaluronidase, recombinant allergens, baculovirus, enzymatic activity, specific IgE

Honeybee stings can cause severe and sometimes fatal IgE-mediated allergic reactions in human beings.¹ The most important venom allergens responsible for such reactions are two enzymes, phospholipase A₂ (PLA) and hyaluronidase (Hya). PLA has been cloned² and expressed as a recombinant protein in *Escherichia coli*.³ Recombinant PLA showed enzymatic activity and in

Abbreviations used

BvHya:	Hyaluronidase expressed in Baculovirus-infected insect cells
EcHya:	Hyaluronidase expressed in <i>E. coli</i>
Hya:	Hyaluronidase
PCR:	Polymerase chain reaction
PLA:	Phospholipase A2
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBS:	Tris-buffered saline

vitro and in vivo IgE-binding capacity similar to that of native PLA.³⁻⁵

Bee venom Hya is closely related and immunologically cross-reactive with Hya from other Hymenoptera venoms.^{6,7} Hya is also found in venoms of spiders and snakes, as well as in mammalian testes.⁸ The enzymatic specificity of Hymenoptera Hya is of the endo-β-N-acetyl-D-hexosaminidase type. It degrades hyaluronic acid, the most abundant mucopolysaccharide of mammalian connective tissue, to tetrasaccharides⁹ and thus serves as a *spreading factor*. After a Hymenoptera sting, Hya facilitates penetration of other venom toxins in the skin. Hya contained in mammalian testes has been localized on the acrosomal part of the sperm, and is important during the fertilization process. In addition, the group of enzymes catalyzing the biosynthesis and degradation of hyaluronic acid⁸ is important for the growth of normal and tumor cells.

Attempts to isolate Hya from honeybee venom have been extremely laborious and resulted in a low yield of this 43 kd glycoprotein.¹⁰ The sequence of the gene encoding bee venom Hya was recently described,¹¹ and about 40% of homology to Hya from mammalian testes has been found. The availability of the sequence provides the possibility to produce recombinant Hya in large amounts to study both its physiologic role and its implications in allergic reactions.

Here we describe biochemical and immunologic properties of Hya produced in prokaryotic (*E. coli*) and eukaryotic (baculovirus-infected insect cells) expression systems.

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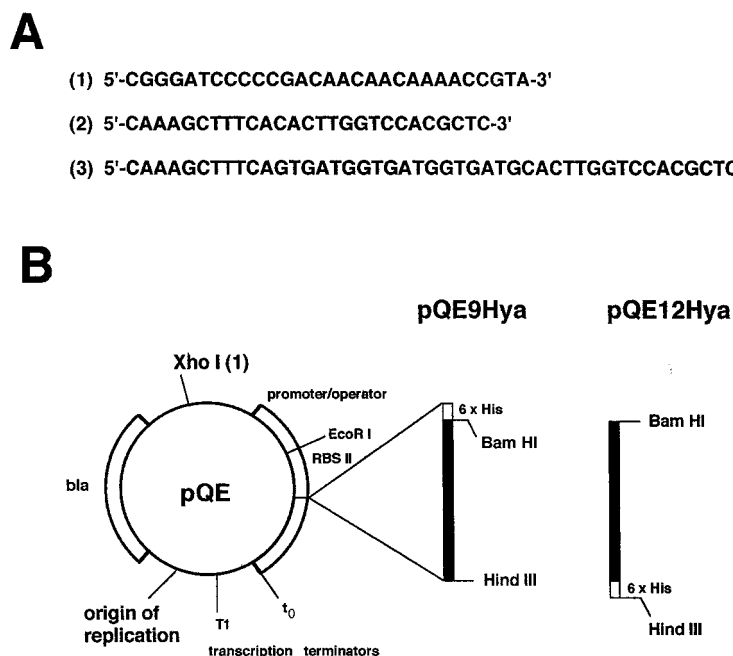


FIG. 1. **A**, Primers used for PCR amplification of the cDNA encoding Hya. **B**, pQE vector-derived constructs used for expression of rHya in *E. coli*. *RBS II*, Synthetic ribosomal binding site; *bla*, β -lactamase gene; *Xho I*, *EcoR I*, *Bam HI* and *Hind III*, restriction sites.

METHODS

Bacterial strains, plasmids, and media

Both *E. coli* strains, M15¹² and protease-deficient HCB997 lon⁻ mutant (gift of Dr. B. Stolz, University of Bern, Switzerland), bear the repressor plasmid pREP4.¹³ *E. coli* strain M15 and expression plasmids pQE9, pQE12, and Ni-NTA resin for affinity chromatography were purchased from QIAGEN. *E. coli* strains were grown in LB medium¹⁴ containing 100 μ g/ml ampicillin and 25 μ g/ml kanamycin. cDNA encoding Hya was obtained as described previously.¹¹ cDNA was amplified by polymerase chain reaction (PCR) with sense 1 and antisense 2 primers (Fig. 1, *A*) for subcloning into pQE9 expression vector, and sense 1 and antisense 3 primers for subcloning into pQE12. The BamHI/HindIII-digested PCR products were directionally inserted into pQE9 and pQE12 expression vectors to generate pQE9Hya- and pQE12Hya-encoding N- and C-terminal hexahistidine (6 $\times$ His) Hya fusions, respectively (Fig. 1, *B*).

Expression of Hya in *E. coli*, purification, and refolding

To study the time course of protein expression, M15 or lon⁻ mutant *E. coli* strains carrying the expression construct of interest were subjected to a test induction, and intracellular proteins were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described.¹³ For large-scale production and purification of expressed protein, a previously described method³ was used. The bacterial pellet containing recombinant protein was solubilized in 7 mol/L guanidinium hydrochloride, and Hya expressed in *E. coli* (EcHya) was then isolated by Ni²⁺-chelate chromatography in 8 mol/L urea. Reconstitution of this soluble, denatured protein to a biologically active form (refolding) was conducted under several different conditions. Fractions of Hya in 8 mol/L urea obtained after Ni²⁺-chelate chromatography¹³ were dialyzed

against 5 mmol/L acetic acid; dialyzed stepwise against 4 mol/L, 2 mol/L, or 1 mol/L urea (with or without 10 mmol/L Triton X-100); and diluted directly 100-fold with distilled water, resulting in a urea concentration of 80 mmol/L. As an alternative method for purification and refolding of EcHya, inclusion bodies were isolated¹⁴ and dissolved in 5 mol/L guanidinium hydrochloride in buffer (50 mmol/L Tris-HCl [pH 8.0], 1 mmol/L ethylenediamine tetraacetic acid, and 1 mmol/L 1,4-Dithiothreitol) and then dialyzed overnight at 4°C against distilled water. Reconstituted EcHya was concentrated by ultrafiltration on a Centricon 30 unit (Amicon Corp., Danvers, Mass.) and centrifuged to remove any precipitate (4°C for 30 minutes at 12,000 g). Protein concentration was estimated by three independent methods: Bio-Rad protein assay (Bio-Rad Labs), Lowry¹⁵ method, or absorbance at 280 nm. The molar extinction coefficient of rHya at 280 nm was calculated from its tyrosine and tryptophan content, with values of 1280 and 5690, respectively.

Expression of Hya in baculovirus-infected insect cells

The MaxBac 2.0 kit for production of recombinant virus, Sf9 cells, and *Trichoplusia ni* (High-Five) BTI-TN-5B1-4 cells were all purchased from Invitrogen Corporation (San Diego, Calif.). Grace's insect media and fetal bovine serum were purchased from Gibco BRL (Paisley, U.K.), and Ex-Cell 400 serum-free media was purchased from JRH Biosciences (Lenexa, Kan.). Viral titer was determined by using plaque assays.¹⁶ Insect cell-tissue cultures were grown in Corning T25 and T75 flasks as an adhesion culture and in 0.5 L Erlenmeyer glass flasks (High-Five cells, working volume 0.2 L) as a suspension culture, which was kept in an orbital shaker with a constant speed of 120 rpm (JRH Biosciences). Both cultures were kept at 27°C in the dark. Purification of Hya by Ni²⁺-chelate chromatography

under nondenaturing conditions was conducted in 50 mmol/L Na-phosphate and 300 mmol/L NaCl buffer with a stepwise pH gradient of 8.0 to 5.0.

Enzymatic assay

Hya activity was measured by a colorimetric assay,¹⁷ modified by Merck Co. (Darmstadt, Germany), in which potassium hyaluronate was used as a substrate. Natural (n)Hya used for comparison in this and all the following experiments was isolated as described previously.¹⁰

N-terminal sequence and amino acid analysis of the protein

N-terminal protein sequence analysis was carried out in a pulsed liquid-phase sequencer 477A obtained from Applied Biosystems. The released amino acids were analyzed on-line according to the manufacturer's instructions. Amino acid and amino sugar analysis was performed by reverse-phase high-performance liquid chromatography of phenyl-isothiocyanate derivatives of amino acids and amino sugars on a Nova Pak C18 column in a Hewlett-Packard liquid chromatograph 1090 with an automatic injection system. Hydrolysis conditions for amino acids were 115° C for 22 hours and 100° C for 6 hours for amino sugars.

Sera from allergic patients

Sera from 20 patients with a history of a recent systemic allergic reaction to a honeybee sting, a positive intracutaneous skin test response to bee venom at a concentration of 10⁻⁴ gm/L or lower,⁵ and a positive RAST response to bee venom ($\geq$ RAST class 2) were used for RAST, RAST-inhibition, and immunoblot assays. Control sera were from 20 individuals with no history of systemic insect sting reactions and a negative intracutaneous skin test response to bee venom at 10⁻³ gm/L.

RAST and RAST-inhibition assays

Specific serum IgE antibodies to nHya, EcHya, and Hya expressed in baculovirus-infected insect cells (BvHya) were measured by Phadezym RAST (Kabi-Pharmacia Diagnostic AB, Uppsala, Sweden). Commercial paper disks were coupled with 0.1 μ g protein per disk as described previously.¹⁸ Specific IgE values are given in RAST classes from 0 to 4, with 0 corresponding to no specific IgE and 4 to very high levels of this antibody. For RAST-inhibition assays, sera were incubated in serial 10-fold dilutions of each allergen preparation for 3 hours at room temperature before adding paper disks coupled with nHya. Percent of inhibition was calculated as follows:

$$\% \text{ inhibition} = (1 - \text{ES}/\text{EO})100$$

where EO is the extinction at 420 nm obtained with the unabsorbed serum, and ES is the extinction with the absorbed serum.¹⁸

SDS-PAGE and immunoblotting

Samples were reduced by boiling in sample buffer plus 1% 2-mercaptoethanol for 5 minutes before electrophoresis. Electrophoresis was performed in 17.5% polyacrylamide gels¹⁹ at 200 V for 45 minutes. Gels were stained by Coomassie Brilliant Blue R-250. For immunoblotting, the separated unstained proteins in the polyacrylamide gel were electroblotted onto nitrocellulose membranes (Bio-Rad Labs) in a Mini-Protein Slab Cell (Bio-Rad Labs), according to the manufacturer's instructions. The nitrocellulose membrane was kept for 1 hour in Tris-buffered saline (TBS) containing 1% casein, and it was

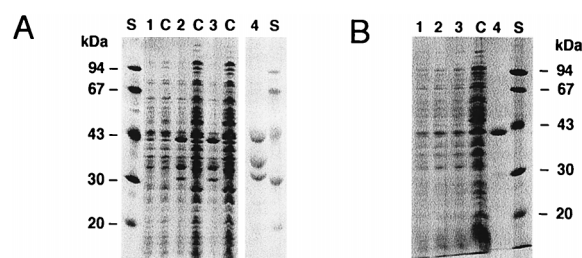


FIG. 2. SDS-PAGE of time-course production of Hya expressed in *E. coli*. **A**, Expression of pQE9Hya construct. **B**, Expression of pQE12Hya construct. Lanes 1 to 3, bacterial lysate after 1, 4, and 5 hours of isopropyl- β -D-galactoside induction; lane 4, Hya isolated by nickel chromatography; lane S, molecular weight standards; and lane C, control bacterial lysate without induction (in **B**, C is control for 5 hours).

cut in strips of 4 mm in width. Each strip was incubated in succession for 16-hour periods with the serum of individual allergic patients diluted 1:5 in TBS and anti-human IgE monoclonal antibody BSW17 conjugated with horseradish peroxidase diluted 1:1000 in TBS (kindly provided by Dr. Beda Stadler, Institute of Immunology and Allergology, Bern, Switzerland). The blots were stained in a substrate solution of 2% 4-chloronaphtol and 0.01% H₂O₂ in 0.05 mol/L Tris-HCL (pH 8.0).

Statistics

The distribution of RAST classes to the three enzyme preparations in the 20 patients allergic to bee venom was analyzed by a contingency table chi-square test.

RESULTS

Expression and isolation of N- and C-terminal 6 $\times$ His-tagged EcHya

N-terminal 6 $\times$ His-tagged EcHya was expressed from pQE9Hya construct (Fig. 1, **A**) as inclusion body protein in *E. coli* strain M15. Ni²⁺-chelate affinity chromatography under denaturing conditions was used to separate Hya from other bacterial proteins and yielded 10 mg Hya per liter of culture medium. In SDS-PAGE EcHya appeared as a 41 kd band, as expected for the nonglycosylated counterpart of 43 kd nHya, together with two smaller fragments of 30 and 33 kd (Fig. 2, **A**). All three components reacted in immunoblot with IgE from sera of allergic patients (Fig. 3).

To eliminate proteolysis as a possible cause for the two minor protein fragments, the protease-deficient lon⁻ *E. coli* strain HCB997 was used for Hya expression by using the same construct (Fig. 1, **B**). The level of expression increased to 20 mg/L of culture medium, but the two minor 30 and 33 kd components remained.

To obtain full-length rHya without truncated products, a C-terminal 6 $\times$ His construct (pQE12Hya) was used (Fig. 1, **B**). After purification by Ni²⁺-chelate chromatography, EcHya appeared as an expected 41 kd protein (5 to 10 mg/L culture medium) contaminated with a minor component of 30 kd (Fig. 2, **B**), which did not bind IgE from sera of allergic patients (Fig. 3).

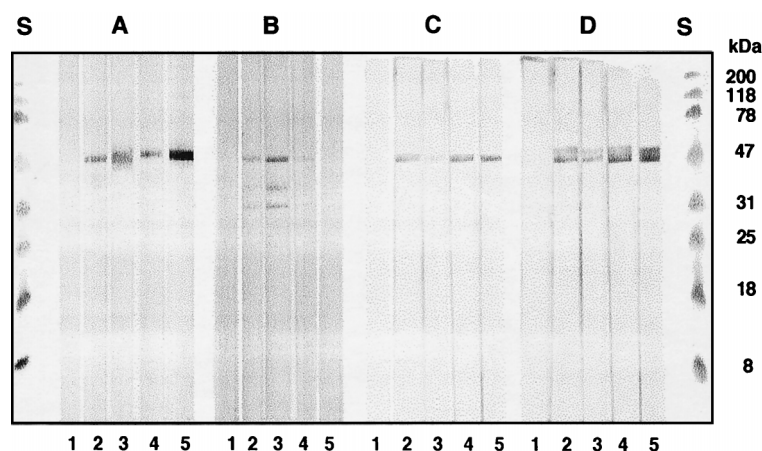


FIG. 3. Immunoblot analysis of different preparations of Hya with sera from allergic patients. *A*, nHya; *B*, EcHya derived from pQE9Hya construct; *C*, EcHya derived from pQE12Hya construct; and *D*, BvHya. *Lane 1*, negative pool of sera from nonallergic individuals; *lanes 2 to 5*, sera of individuals allergic to bee venom; and *lane S*, molecular weight standards.

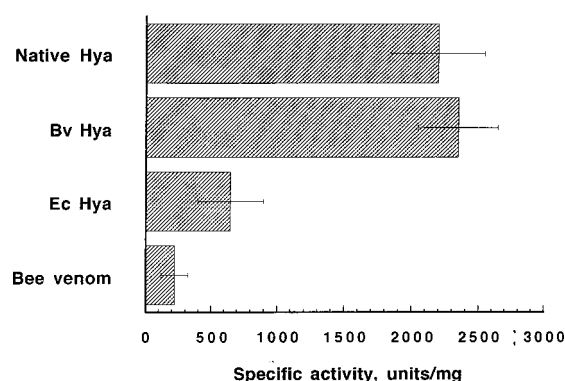


FIG. 4. Specific enzymatic activity of different preparations of Hya. Mean values of specific activity were 2200 (350 units/mg) for nHya, 2350 (300 units/mg) for BvHya, 650 (250 units/mg) for EcHya, and 220 (100 units/mg) for whole bee venom.

Refolding and purification of N- and C-terminal 6×His-tagged EcHya

After dialysis against 5 mmol/L acetic acid, both N- and C-terminal 6×His EcHya showed only 20% to 30% of the specific enzymatic activity of nHya (Fig. 4), which probably indicates incomplete or incorrect folding of the protein. Several attempts were made to reconstitute EcHya in vitro by changing from the highly denaturing conditions used for protein isolation to a natural condition. During dialysis, in a variety of buffers of different pH values, 90% to 95% of the protein precipitated. The solubilized Hya fraction never reached a specific enzymatic activity exceeding 30% of that of nHya (Fig. 4). Irreversible binding of EcHya to a variety of chromatographic resins (data not shown) was observed as reported earlier for nHya.¹⁰ Assuming that the preparations of EcHya could contain both enzymatically active and inactive protein, affinity chromatography on heparin

sepharose CL 6B was used to isolate the active form because heparin is an inhibitor of Hya (data not shown).²⁰ However, adsorbed and nonadsorbed protein showed the same specific enzymatic activity of about 20% to 30% of the natural protein.

Construction of recombinant baculovirus

To produce Hya containing C-terminal 6×His tag in the baculovirus-expression system, the BamHI/HindIII cDNA fragment encoding Hya from the pQE12Hya construct (Fig. 1, *B*) was subcloned into the pMelBac A transfer plasmid²¹ and transformed into DH10B-competent cells (Gibco). The transfer plasmid pMelBacA contained the honeybee melittin signal sequence. The plasmid was amplified and isolated, and insert presence was verified by restriction analysis and agarose gel electrophoresis. The plasmid was cotransfected with linearized wild-type *Autographa californica* nuclear polyhydrosis virus DNA into Sf9 cells by using cationic liposome-mediated transfection.²² Recombinant clones were isolated by plaque assay by using 5-bromo-4-chloro-3-indolyl-β-D-galactoside to detect β-galactosidase-producing clones.¹⁶ Twelve positive clones were analyzed by PCR with the baculovirus forward and reverse PCR primers. They were verified to be recombinant constructs.

Expression, purification, and characterization of BvHya

Three clones (numbers 1, 2, and 3), carrying a PCR product of expected size (1394 bp), were used to infect High-Five cells grown as an adhesion culture in Ex-Cell 400 serum-free medium.²³ Supernatants were sampled daily for 7 consecutive days and tested for expression of BvHya by SDS-PAGE (Fig. 5). BvHya appeared at day 3 and showed maximal expression at days 4 and 5 after infection. Pooled supernatants from days 4 and 5 were separated from cells and debris by centrifugation (1000 g

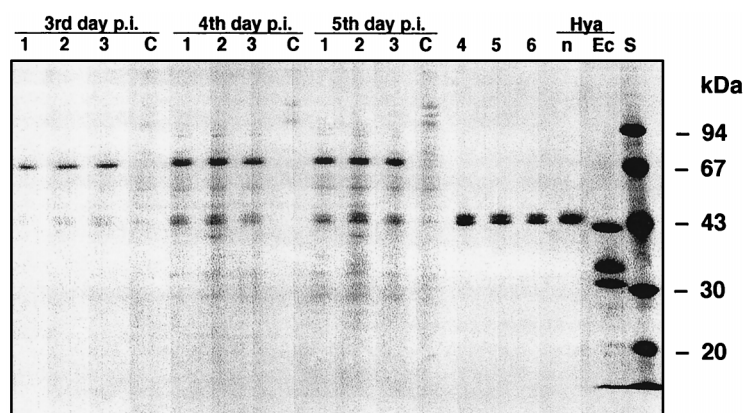


FIG. 5. SDS-PAGE of time course production for BvHya. Lanes 1 to 3, supernatants from High-Five cells infected by recombinant baculovirus clones 1, 2, and 3, which were collected on days 3, 4, and 5 after infection; lane C, control supernatants from noninfected cells; lanes 4 to 6, proteins from clones 1, 2, and 3 purified by Ni^{2+} -chelate chromatography; lane n, nHya; lane Ec, EcHya produced from pQE9Hya construct; and lane S, molecular weight standards. *p.i.*, Postinfection.

for 20 minutes) and purified by Ni^{2+} -chelate chromatography under nondenaturing conditions. Expression of BvHya in suspension-shaker culture (High-Five cells) has been used to achieve higher production of protein. Yield of BvHya from adhesion-cell culture was about 7 to 8 mg/L and increased up to 20 mg/L in suspension-shaker culture. All three clones showed similar rates and levels of production of BvHya, as well as the coexpression of a 70 kd contaminant protein that was eliminated by Ni^{2+} -chelate chromatography and therefore unlikely to be related to Hya (Fig. 5).

On SDS-PAGE, the purified BvHya appeared as a double band of about 43 to 44 kd (Fig. 5), which was in agreement with the molecular weight of nHya. N-terminal sequence analysis revealed three equimolar components: one corresponding to a protein with the expected N-terminal amino acid sequence after melittin cleavage site (Ile-Tyr-Ala-↓-Asp-Arg-Trp-Gly-Ser), and the two others representing products of cleavage of the peptide bond in the fourth (Gly-↓-Ser-Pro-Asp-Asn-Asn-Lys-Thr) and tenth (Lys-↓-Thr) positions from the first cleavage. This resulted in a molecular weight difference of the products of about 440 and 1100 d. BvHya was further subjected to amino sugar analysis (data not shown). It was calculated that it contains four molecules of N-acetyl-D-glucosamine per protein molecule, the same as nHya.¹⁰

The specific enzymatic activity of BvHya from three different clones was similar to (clones number 2 and 3) or slightly higher than (clone number 1) the activity of nHya preparations (Fig. 4).

Immunologic reactivity of EcHya and BvHya

Recombinant preparations of EcHya and BvHya, along with nHya, were characterized by immunoblots²⁴ with sera from individuals allergic to bee venom sensitized to Hya (Fig. 3). Strong IgE binding in the 43 kd region was observed for nHya and BvHya, and less

intensive IgE binding in the 41 kd region was observed for both preparations of EcHya. In addition to the IgE binding to full-length EcHya, the immunoblot of N-terminal 6×His EcHya showed some immunoreactivity to copurified fragments at 30 and 33 kd.

Sera from 20 patients allergic to bee venom were tested by RAST with disks coupled with equal amounts of nHya, BvHya, or EcHya. Most (95%) of the patients' sera reacted with nHya and BvHya, but only 60% reacted with EcHya. Moreover, the mean IgE binding to EcHya was considerably lower than that binding to nHya and BvHya (Table I). Twenty control subjects with no history of insect-sting reactions and a negative skin test response to whole bee venom had a negative RAST response to nHya, BvHya, and EcHya.

To compare further the IgE-binding capacities of the different Hya preparations, RAST-inhibition assays were performed. Sera from five patients allergic to bee venom with a high level of specific IgE to nHya were used for this purpose. As shown in Table II, at equal protein concentrations, considerably stronger inhibition of the IgE binding to nHya-coupled disks is obtained with nHya and BvHya than with EcHya. Fig. 6 shows detailed RAST-inhibition results of patient 1. Concentrations resulting in 50% inhibition were 65 ng/ml for nHya, 98 ng/ml for BvHya, and 610 ng/ml for EcHya in this patient.

DISCUSSION

The availability of recombinant allergens has increased our understanding of IgE-mediated allergies²⁵ and promises to improve diagnosis and treatment of these diseases.²⁶

To prepare biologically active bee venom Hya in significant amounts uncontaminated by other venom components, we have studied Hya production in two different expression systems, one prokaryotic and one eukaryotic. Well-known advantages of the prokaryotic

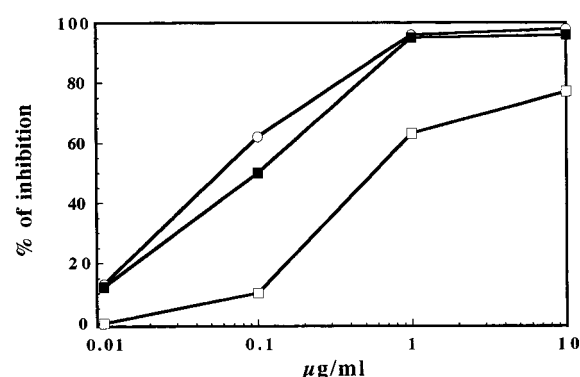


FIG. 6. RAST-inhibition assay with serum from an allergic individual. Open circles, nHya; filled squares, BvHya derived from clone 2; and open squares, EcHya derived from pQE12Hya construct.

TABLE I. Specific IgE against nHya, BvHya, and EcHya in 20 patients allergic to bee venom and 20 nonallergic control subjects

	RAST class	Specific IgE to (%)		
		nHya	BvHya	EcHya
Patients* (n = 20)	0	1 (5)	1 (5)	8 (40)
	1	0	2 (10)	2 (10)
	2	4 (20)	5 (25)	7 (35)
	3	11 (55)	9 (45)	3 (15)
	4	4 (20)	3 (15)	0
Control subjects (n = 20)	0	20	20	20

*nHya versus BvHya, chi-square = 2.44, $p > 0.5$; nHya versus EcHya, chi-square = 64.83, $p < 0.0001$; BvHya versus EcHya, chi-square = 35.78, $p < 0.0001$.

(*E. coli*) system are easy handling of *E. coli* cells and high-level expression of the proteins.¹³ One of the disadvantages is that insoluble, inactive inclusion bodies are frequently formed on recombinant protein production.³ To achieve biologic activity, protein needs to be reconstituted in vitro after solubilization under denaturing conditions.¹³ The baculovirus-infected insect cell expression system is eukaryotic and thus characterized by a wide array of posttranslational processing specific for higher eukaryotic cells.²⁷ Expression of recombinant proteins in this system most often results in the secretion of biologically active soluble proteins, usually in glycosylated form.²⁸⁻³²

In our study two different intracellular preparations of Hya were produced in *E. coli*. By changing the location of the 6×His tag from the N-terminus to the C-terminus of the protein, coexpression of immunoreactive truncated fragments of 30 and 33 kd could be eliminated. This fact and the observation that the change from M15 to lon⁻ mutant *E. coli* strain for protein expression did not influence coexpression of immunoreactive fragments

TABLE II. Inhibition of IgE binding to discs coupled with nHya by serial 10-fold dilutions of nHya, BvHya, and EcHya in RAST-inhibition assay

Patient no.	Dilution (μg/ml)	% inhibition after absorption with		
		nHya	BvHya	EcHya
1	10	98	96	77
	1	96	95	63
	0.1	62	50	10
2	10	94	87	83
	1	90	84	49
	0.1	88	75	29
3	10	94	80	74
	1	89	71	65
	0.1	84	64	45
4	10	94	94	79
	1	92	92	41
	0.1	90	34	13
5	10	97	94	92
	1	94	90	70
	0.1	92	86	48

of N-terminal 6×His EcHya indicate that these fragments are not the result of proteolytic degradation. Rather, premature termination of translation may be responsible for this phenomenon.³³

After purification and renaturation, both preparations of EcHya were enzymatically active and able to bind specific IgE antibodies from sera of patients allergic to bee venom. Both enzymatic activity and IgE-binding capacity were, however, considerably lower than those of nHya. From these results we concluded that Hya expressed in *E. coli* and reconstituted in vitro did not provide the correct conformation required to recover a native-like biologic activity.

Therefore a eukaryotic expression system was chosen. For the enhanced secretion of BvHya from insect cells, pMelBacA transfer vector with an efficient artificial melittin leader sequence was used. This sequence was in fact cleaved in the proper position (Ala-Asp). Additional cleavage did occur at two other sites, after the fourth and the tenth amino acids from melittin cleavage site, resulting in the appearance of two to three bands at 43 to 44 kd in SDS-PAGE, which were all immunoreactive with human IgE antibodies in immunoblot. BvHya showed enzymatic activity similar to that of nHya. In RASTs, 19 of 20 patients allergic to bee venom (95%) had specific IgE to both nHya and BvHya, whereas only 60% had specific IgE to EcHya. These findings, indicating native-like IgE binding capacities of BvHya but only reduced IgE binding of EcHya, were confirmed in the RAST-inhibition assays.

BvHya appeared in glycosylated form as judged by molecular size in SDS-PAGE and by amino sugar analysis. The amount of N-acetyl-D-glucosamine residues per protein molecule was similar in nHya and BvHya.

This indicates that only one or two of three potential sites for N-glycosylation are actually used.

From the comparison of physicochemical and biologic properties of several preparations of recombinant Hya, we conclude that the baculovirus expression system provided Hya similar to that provided by natural protein. One of the prominent differences between the two expression systems is that the baculovirus system is able to posttranslationally N-glycosylate proteins.^{31, 32, 34-36} Several recent publications^{37, 38} indicate that protein glycosylation aids in folding of the nascent polypeptide chain and stabilization of the conformation of the mature glycoproteins. Although glycosylation does not seem to be relevant for conformation and biologic activity in some smaller proteins, such as PLA,³⁻⁵ our data indicate a possible role of glycosylation for acquiring native-like protein structure associated with full enzymatic activity and immunoreactivity in the less rigid, larger enzyme Hya.

The availability of the two most important allergens from honeybee venom in recombinant form may have practical clinical implications. Thus a diagnostic preparation containing these two enzymes is expected to be associated with similar sensitivity but increased specificity when compared with conventional bee venom extracts in skin testing because toxic or irritating components are eliminated. Once the important allergens of an extract are at hand in pure recombinant form, this will allow for the determination of each individual patient's sensitization pattern. On this basis, a patient-tailored preparation for immunotherapy with supposedly increased efficacy and safety could be composed.

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