

Evaluation of Different Glycoforms of Honeybee Venom Major Allergen Phospholipase A2 (Api m 1) Produced in Insect Cells

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Abstract: Allergic reactions to hymenoptera stings are one of the major reasons for IgE-mediated anaphylaxis. However, proper diagnosis using venom extracts is severely affected by molecular cross-reactivity. In this study recombinant honeybee venom major allergen phospholipase A2 (Api m 1) was produced for the first time in insect cells. Using baculovirus infection of different insect cell lines allergen versions providing a varying degree of cross-reactive carbohydrate determinants as well as a non glycosylated variant could be obtained as secreted soluble proteins in high yields. The resulting molecules were analyzed for their glycosylation and proved to show advantageous properties regarding cross-reactivity in sIgE-based assays. Additionally, in contrast to the enzymatically active native protein the inactivated allergen did not induce IgE-independent effector cell activation. Thus, insect cell-derived recombinant Api m 1 with defined CCD phenotypes might provide further insights into hymenoptera venom IgE reactivities and contribute to an improved diagnosis of hymenoptera venom allergy.

Keywords: Allergen, baculovirus, carbohydrates, cross-reactivity, honeybee, phospholipase, recombinant allergen, venom allergy.

INTRODUCTION

Anaphylaxis due to honeybee (*Apis mellifera*) venom is one of the most severe clinical outcomes of IgE-mediated hypersensitivity reactions. Although venom immunotherapy is effective in the majority of patients the occurrence of systemic side effects in 20-40% of treated individuals [1] and the failure of therapy in 10-20% of cases [2] demands a component-resolved approach to HBV allergy. Since the use of native allergens is often hampered by means of quantity and purity recombinant allergens are increasingly introduced into diagnostic and therapeutic applications [3]. Moreover, the recombinant availability is a prerequisite for the rational design of hypoallergenic variants and molecules with defined characteristics such as proper folding and glycosylation and concurrent lack of cross-reactive carbohydrate determinants which still represent a challenge for adequate allergy diagnosis. Defining glycosylation of allergens can have intriguing consequences for our understanding of IgE reactivities reported previously for the hyaluronidases [4].

HBV is a complex mixture of biogenic amines, peptides, toxins and proteins, with most of them being enzymes. Many of the proteins and polypeptides in HBV have been reported to be allergens including phospholipase A2 (Api m 1) [5], hyaluronidase (Api m 2) [6], acid phosphatase (Api m 3) [7],

melittin (Api m 4) [8], dipeptidylpeptidase IV (Api m 5) [9], Api m 6 polypeptides (4 isoforms) [10], a CUB-serine protease (Api m 7) [11], a carboxylesterase (Api m 8), a carboxypeptidase (Api m 9) [12], and the Carbohydrate-rich protein (Api m 10), a component of unknown function [13].

HBV phospholipase A2 (Api m 1) was early identified as the major venom allergen [5]. Based on the fact that 97% of HBV allergic patients have Api m 1 specific IgE antibodies Api m 1 was extensively studied as model allergen for genetic engineering and specific immunotherapy [14]. Moreover, Api m 1 is considered an ideal marker allergen since it is structurally unrelated to the phospholipase A1 (Ves v 1) in vespid venom [15].

In general, phospholipases constitute a major component of various venoms, e.g. from snakes, bees, wasps and spiders. The PLA2 of *A. mellifera* has been sequenced from a venom gland cDNA library [16] and encodes a signal peptide of 18 amino acids, a propeptide of 15 residues, and a major peptide of 134 residues [17], the catalytic activity and crystal structure of which is well documented [18-20]. The mature peptide contains 10 cysteine residues forming 5 disulfide bonds [21] and an oligosaccharide attached to asparagine 13 [22].

PLA2 (EC 3.1.1.4) from HBV is a typical group III secreted PLA2 [23], representing about 12% of the dry weight of the venom [24]. The cytotoxic effects of PLA2 rely on its catalytic activity which leads to cleavage of phospholipids at the SN2 acyl chain and damage of structural membranes [24]. The enzyme in HBV is structurally not related to the phospholipases A1 in vespid venoms, and therefore, not cross-reactive. In contrast to unglycosylated vespid PLA1,

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PLA2 of honeybees exhibits CCD (cross-reactive carbohydrate determinant)-reactivity [25] which is essentially based on the presence of α -1,3-core fucose and can lead to results with no or only low clinical relevance in diagnostic tests and therefore represents a challenge for diagnostic approaches [26].

The aim of the present study was an improved production of soluble HBV PLA2 in glycosylation-competent insect cells. Therefore, we generated an inactive mutant form of PLA2 by site-directed mutagenesis, which apart from an improved expression behavior should exhibit a reduced cytotoxicity in cellular assays. We were able to produce both allergens as soluble and secreted proteins in high yields by baculovirus-based expression in Sf9 (*S. frugiperda*) as well as HighFive (*T. ni*) insect cells. By using cell lines with a varying capacity for α -1,3-core fucosylation, and, additionally, by generation of a non-glycosylated mutant form, we provided the evidence for reduced IgE cross-reactivity and improved performance of the resulting PLA2 molecules in diagnostic approaches to hymenoptera venom allergy.

METHODS

Materials

Crude HBV collected by electrostimulation and native purified PLA2 were purchased from Latoxan (Valence, France). Anti-V5 antibody was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum as well as anti-rabbit-IgG AP conjugate, anti-mouse IgG AP conjugate and bromelain from pineapple stem was obtained from Sigma (Taufkirchen, Germany). The monoclonal AP conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany). The MUXF-HSA conjugate was kindly provided by Siemens Healthcare Diagnostics (Los Angeles, CA, USA). The rat basophilic leukemia cell line RBL-SX38 was kindly provided by Prof. J.-P. Kinet (Harvard University, Boston, MA, USA). Sera with a positive test to HBV and/or YJV (sIgE ≥ 0.35 kU/L) were obtained from the institutional serum bank. All patients had given their informed written consent to draw an additional serum sample.

Cloning of cDNA

Total RNA was isolated from honeybee (*Apis mellifera carnica*) venom sacks using peqGold TriFast™ (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) was used to synthesize cDNA. The Api m 1 mature peptide coding region was amplified using *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany) and the primers 5'-ATA ATA TAT CCA GGA ACG TTA TGG TG-3' and 5'-ATA CTT GCG AAG ATC GAA CCA TTG-3' and subcloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with the pCR-Blunt II-TOPO vector. A C-terminal V5 epitope and a 10-fold His-tag as well as 5' BamHI and 3' NotI restriction sites were added by PCR and the PCR product was subcloned into the pAcGP67-B baculovirus transfer vector (BD Pharmingen, Heidelberg, Germany) after restriction digest with BamHI and NotI.

Site Directed Mutagenesis

For generation of the inactive Api m 1 H34Q, histidine 34 was substituted by glutamine by using the QuikChange site directed mutagenesis Kit (Stratagene, La Jolla, USA) according to the manufacturers' recommendations and employing the primers 5'-GCA TGC TGT CGA ACC CAA GAC ATG TGC CCG GAC G-3' and 5'-CGT CCG GGC ACA TGT CTT GGG TTC GAC AGC ATG C-3'. For the non-glycosylated form of Api m 1 H34Q N13Q, asparagin 13 was changed to glutamine by employing the primers 5'-GGT GCG GGC ATG GTC AAA AGT CGT CCG GCC C-3' and 5'-GGG CCG GAC GAC TTT TGA CCA TGC CCG CAC C-3'.

Recombinant Baculovirus Production and Expression

Recombinant baculovirus was generated by cotransfection of *Spodoptera frugiperda* (Sf9) cells (Invitrogen) with BaculoGold bright DNA (BD Pharmingen) and the baculovirus transfer vector pAcGP67-B Api m 1 H34Q or Api m 1 H34Q N13Q, respectively, according to recommendations of the manufacturer. High titer stocks were produced by three rounds of virus amplification. Optimal multiplicity of infection (MOI) for subsequent protein expression was determined empirically by infection of Sf9 cells with serial dilutions of virus stocks.

Expression in Baculovirus-Infected Sf9 Cells and Protein Purification

High titer stocks of recombinant baculovirus containing the Api m 1 coding DNA were used to infect Sf9 or HighFive cells (Invitrogen) ($1.5\text{--}2.0 \times 10^6$ cells per ml) in a 2000 ml suspension flask (400 ml suspension culture). For protein production the cells were incubated at 27°C and 110 rpm for 72 h. Cellular supernatants were then applied to a nickel-chelating affinity matrix (Ni-NTA-agarose, Qiagen, Hilden, Germany). After washing with NTA-binding buffer (50 mM sodium phosphate, pH 7.6, 500 mM NaCl) the protein was eluted with NTA-binding buffer containing 300 mM imidazole.

Immunoreactivity of Human Sera

For assessment of specific IgE immunoreactivity, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with recombinant allergens, nApi m 1, HBV and the CCD marker MUXF-HSA (10 μ g/ml) at 4°C overnight and blocked with 40 mg/ml skimmed milk powder in PBS at room temperature. Human sera were diluted 1:2 in PBS and applied for 4 hours at room temperature. Wells were rinsed 4 times with PBS and incubated with an alkaline phosphatase conjugated mouse anti-human IgE antibody (BD Pharmingen, clone G7-26) diluted 1:1000 in 20 mg/ml skimmed milk powder in PBS. Wells were rinsed 4 times with PBS and substrate solution (5 mg/ml 4-nitrophenylphosphate, AppliChem, Darmstadt, Germany) was added. After 30 minutes absorption was measured at 405 nm. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs.

In Vitro Degranulation Assay with Rat Basophil Leukemia Cells (RBL-SX38)

In vitro degranulation was analyzed essentially as described [27]. In brief, RBL-SX38 cells were cultivated in 96-well plates (1×10^5 cells per well) for 24 h at 37°C in 5% CO₂. After washing with Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 12 mM NaHCO₃, 5.5 mM glucose, 0.4 mM NaH₂PO₄ and 10 mM Hepes, pH 7.4, BSA 0.1% w/v) cells were incubated with native PLA2 or PLA2 H34Q (0.01-100 µg/ml in Tyrode's buffer) at 37°C for 1 h and then washed twice with Tyrode's buffer. β-hexosaminidase release was assessed using 4-nitrophenyl N-acetyl-glucosaminide as a substrate. The supernatant of stimulated cells was incubated for 1 h at 37°C and the enzymatic reaction was stopped by adding carbonate buffer (0.1 M, pH 10.0). Absorbance was measured at 405 nm. Cells were washed twice as described before and total cell lysate was obtained by addition of 0.1% Triton-X-100 in Tyrode's buffer and incubation for 1 h at 37°C. β-hexosaminidase release was assessed as the percentage of total cell content which was ascertained by processing the supernatant of the cell lysate in the same way as the supernatant of the activated cells. All measurements were performed as triplicates and subjected to statistical analysis via student's *t*-test.

Other Methods

SDS-PAGE, Western blotting, and standard procedures in molecular biology were performed according to established protocols [28]. Lectin blots (DIG Glycan Differentiation Kit, Roche Diagnostics, Mannheim, Germany) were performed according to the recommendations of the manufacturer.

RESULTS

cDNA Cloning and Recombinant Expression in Insect Cells

For recombinant production the cDNA of the HBV allergen Api m 1 was amplified from whole *A. mellifera* venom gland cDNA. To avoid potentially detrimental effects of phospholipase activity on expression yields or on mediator release in cellular assays the inactive mutant Api m 1 H34Q was generated by site directed mutagenesis. To address the impact of glycosylation of the recombinant allergen on the IgE reactivity of sera derived from hymenoptera venom allergic patients an additional mutant version of Api m 1, Api m 1 H34Q N13Q, devoid of the singular glycosylation site was generated.

Subsequently, both epitope tagged proteins were produced by baculovirus-based infection of insect cells and secretion of the proteins into the cellular supernatant. The glycosylated version Api m 1 H34Q was expressed in Sf9 (*S. frugiperda*) cells and additionally in HighFive (*T. ni*) cells, which in contrast to Sf9 cells provide α-1,3-core-fucosylation of N-glycans, the structure responsible for CCD-based cross-reactivity. The mutant version Api m 1 H34Q N13Q lacking the relevant asparagine and, therefore, any glycosylation was produced in HighFive cells.

The rApi m 1 versions were obtained with yields of approx. 5-10 µg/ml of culture supernatant without further op-

timization of culture conditions. SDS-PAGE analysis and Coomassie staining Fig. (1A) of purified rApi m 1 H34Q produced in Sf9 as well as in HighFive insect cells showed three distinct bands in the range of approx. 18 to 22 kDa, a molecular mass to which the C-terminally fused V5 epitope and 6 fold His tag contribute 3 kDa. The Sf9-expressed purified Api m 1 H34Q showed a double band of comparable intensity and a third, less intense band of lower molecular weight. In contrast, for the HighFive-produced Api m 1 H34Q the protein with the highest molecular weight represents the predominant product, while the product with the lowest molecular weight is only barely visible. Applying an antibody directed against the V5 epitope Fig. (1B) all bands of the Sf9-produced rApi m 1 H34Q were detected, a finding which is in good accordance with the distribution observed in Coomassie staining. The lowest molecular weight band of the HighFive-expressed rApi m 1 was not visible, most likely due to its reduced quantity. Native purified Api m 1, however, shows a similar distribution in Coomassie staining, whereas in HBV only the two higher molecular weight bands were visible. Thereby, the highest molecular weight versions represent the predominating forms. Non-glycosylated rApi m 1 H34Q N13Q appears as a single band of approx. 19 kDa Fig. (1A). Application of the sensitive V5 epitope antibody, however, enabled detection of a faint double-band Fig. (1B).

Galanthus nivalis agglutinin (GNA) recognizes terminal mannose, 1,2-, 1,3- or 1,6-linked to mannose, a structure which is present in native Api m 1 as well as in glycans derived from lepidopteran insect cells. Thus, GNA reactivity indicates the presence of N-linked glycans. In immunoblot analysis of rApi m 1 H34Q Fig. (1C) GNA reacted with the two higher molecular weight bands proving the lowest molecular weight form to be non-glycosylated, whereas in HBV and nApi m 1 only the highest molecular weight product is recognized. Thereby, the missing reactivity with rApi m 1 H34Q N13Q verified the successful substitution of the N-glycosylation site.

Apart from proteinic epitopes anti-HRP rabbit serum Fig. (1D) is specific for plant-derived glycostructures including α-1,3-core fucose and β-1,2-xylose, the causative structures for CCD-based cross-reactivities. Its use in immunoblot revealed pronounced α-1,3-core fucosylation for rApi m 1 H34Q produced in HighFive cells as well as for native Api m 1. In contrast, rApi m 1 H34Q produced in Sf9 cells did not exhibit any CCD-based reactivity.

Immunoreactivity of rApi m 1

Reactivity of sera from patients with a clinical history of insect venom allergy was assessed by ELISA for specific IgE antibodies to the recombinant and native versions of Api m 1 Fig. (2). MUXF-HSA, a conjugate that provides the core-fucosylated glycotope from pineapple stem bromelain was included as a marker for sIgE directed against CCDs. To illustrate the distinct recognition patterns observed in insect venom sensitized patients the sIgE-reactivity of selected sera is shown. Serum 1 specifically recognized proteinic epitopes of Api m 1 as indicated by the missing reactivity with the CCD marker MUXF-HSA. All recombinant versions of Api m 1 and native Api m 1 showed comparable reactivity. In contrast, sera 2 and 3 exhibit reactivity with Api m 1 but

apparently CCDs contribute to this binding as suggested by the reactivity observed with MUXF-HSA, and this contribution is at varying degrees according to the more pronounced signal with the fucosylated allergen in the case of serum 2. Serum 4 was reactive with native Api m 1 and HighFive-derived Api m 1 H34Q, but neither with the counterpart produced in Sf9 cells nor with non-glycosylated Api m 1, indicating exclusive CCD-reactivity, which is corroborated by the pronounced MUXF reactivity. Finally, serum 5 from a patient with a clinical history of YJV allergy neither recognizes proteinic epitopes of Api m 1 nor CCDs.

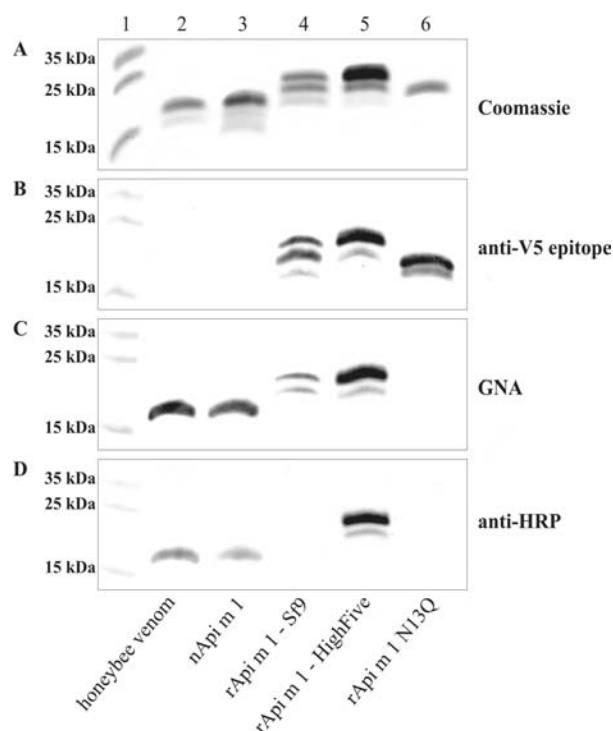


Figure 1. Characterization of native and recombinant HBV phospholipase A2. Honeybee venom, native purified PLA2, recombinant PLA2 H34Q from Sf9 cells, recombinant PLA2 H34Q from HighFive cells, and recombinant PLA2 H34Q N13Q from HighFive cells were subjected to SDS-PAGE (10% acrylamide) and analyzed by Coomassie staining and Western blot. **(A):** Coomassie brilliant blue staining, **B-D:** Western blot analysis applying a monoclonal antibody directed against the V5 epitope C-terminally fused to the recombinant PLA2 versions **(B)**, *Galanthus nivalis* agglutinin recognizing terminal mannose, 1,2-, 1,3- and 1,6-linked to mannose **(C)**, and polyclonal HRP-antiserum produced in rabbit with specificity for α -1,3-core fucose **(D)**.

These data demonstrate that Sf9-derived glycosylated Api m 1 as well as non-glycosylated Api m 1 lack detectable α -1,3-core fucosylation which contributes to or is the exclusive reason for reactivity with sera having CCD-specific IgE antibodies.

Degranulation of Rat Basophilic Leukemia (RBL) Cells

Since native Api m 1 is described to induce mediator release from rodent and human basophils in an IgE-independent manner as a result of its catalytic activity, we tested the ability of nApi m 1 and Api m 1 H34Q to mediate

degranulation of RBL cells, as a model system for effector cell degranulation Fig. (3). Upon incubation with nApi m 1 RBL cells showed significant β -hexosaminidase release over the entire concentration range from 0.01 to 100 μ g/ml in a dose-dependent manner. In contrast, RBL cells incubated with inactive Api m 1 H34Q showed no degranulation when compared to negative controls. These results confirm that the enzymatic activity of Api m 1 promotes IgE-independent effector cell activation.

DISCUSSION

The use of extracts is often hampered by means of heterogeneity, purity, quantity of single allergens [29-31] as well as by multiple IgE-reactivities affecting the interpretation of ambiguous test results and the correct choice of the proper venom for immunotherapy [32]. Thus, the recombinant availability of individual allergens can be considered a prerequisite for component-resolved diagnostic and therapeutic approaches to hymenoptera venom allergy.

So far, HBV PLA2 was recombinantly expressed in *E. coli* only. The resulting protein, however, was unfolded and therefore devoid of any allergenic activity in skin test. After extensive refolding procedures the catalytic and allergenic activity of recombinant Api m 1 was found to be roughly similar to that of natural purified Api m 1 [33, 34]. To avoid the application of sophisticated and time consuming refolding strategies, the aim of this study was the recombinant production of Api m 1 as a soluble and secreted protein by a nearly homologous, baculovirus-mediated expression in insect cells. These recently were found to be a suitable host for the production of properly folded and glycosylated hymenoptera venom enzymes such as the hyaluronidases Api m 2 and Ves v 2 [4, 35], the dipeptidylpeptidases Api m 5 and Ves v 3 [9], the HBV acid phosphatase Api m 3 [36], and YJV phospholipase A1 (Ves v 1) [37]. Additionally, Ves v 5 [37] and Api m 10 (unpublished) without known enzymatic function could be obtained with characteristics suggesting proper folding and IgE reactivities. The only PLA2 expressed in insect cells was the enzyme from the Chinese honeybee *A. cerana cerana* [38]. The authors of this study, however, report a low expression yield in *T. ni* cells which was attributed to a potential cytotoxic effect of the enzyme on the cells. This finding as well as the stimulating effect of PLA2 activity in various cellular assays [39-41] prompted us to inactivate the HBV PLA2 by substituting the catalytically essential histidine residue 34 [19, 20] with glutamine.

The three recombinant protein bands with molecular masses in the range of 18 to 22 kDa are in good agreement with the three forms described for native *A. mellifera* PLA2 having molecular weights of 16, 18, and 20 kDa [42], taking into account the mass of the epitope tags added. Analyses of these isoforms demonstrated identical N-terminal sequences of the mature peptide and characterized the two higher molecular weight bands as glycosylated forms and the band of the lowest molecular weight as a non-glycosylated PLA2 form. In another study similar results were obtained for *A. cerana* and *A. mellifera* PLA2 [43].

In this study, we analyzed the carbohydrate structure of recombinant PLA2 applying *Galanthus nivalis* agglutinin (GNA), recognizing terminal mannose, 1,2-, 1,3- or 1,6-

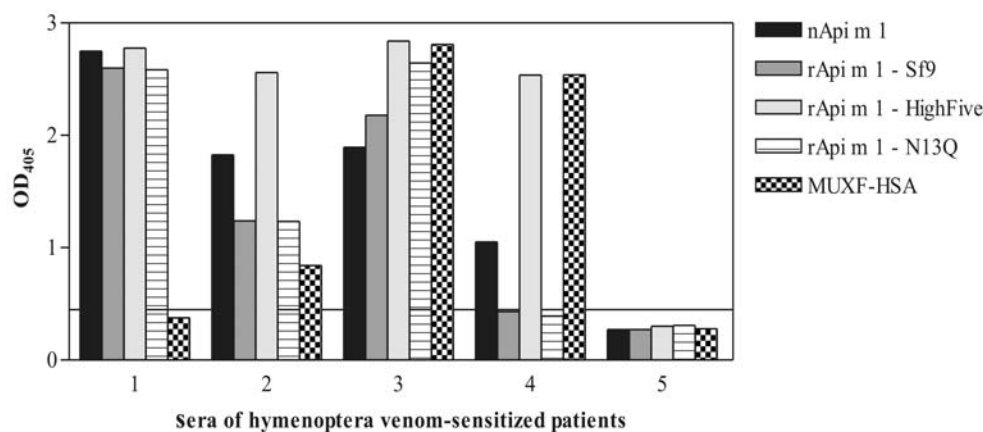


Figure 2. IgE reactivity of individual patient sera with native and recombinant PLA2. The IgE reactivity of individual sera from hymenoptera venom-sensitized patients with native and recombinant PLA2 versions, as well as with the MUXF-HSA conjugate was assessed in ELISA as described in the Methods section. The lower end functional cut-off is represented by a solid line.

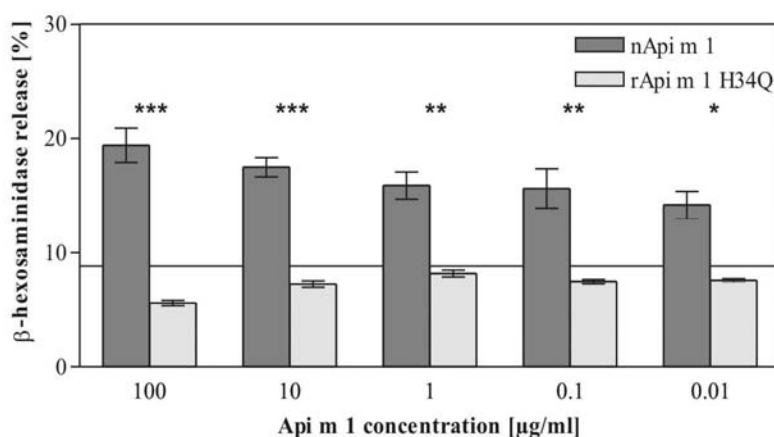


Figure 3. IgE-independent activation of rodent basophils by active and inactive PLA2. Release of β -hexosaminidase from RBL-SX38 cells in response to native purified and recombinant inactive PLA2. The percent release is presented relative to total β -hexosaminidase content. All results were measured as triplicates and subjected to statistical analysis via student's *t*-test.

linked to mannose [44], a structure present in native Api m 1 [22] as well as in glycans derived from lepidopteran insect cells [45]. Thereby, the two higher molecular weight bands of both, Sf9- and HighFive-produced Api m 1 proved to be glycosylated in contrast to the lowest molecular weight band hinting for the capability of the baculovirus-mediated expression in insect cells to mimic natural distribution of glycosylation. The different distribution of glycoforms obtained from Sf9 and HighFive cells points to a higher activity of the glycosylation machinery in HighFive cells as indicated by the barely detectable non-glycosylated isoform and the predominance of the likely highest glycosylated isoform. In the case of crude HBV and purified native PLA2 only the major band was shown to be glycosylated, a finding that might be attributed to the low abundance of the other forms.

Together these results emphasize the possibility to produce *A. mellifera* PLA2 in insect cells via the baculovirus system as properly glycosylated protein showing characteristics comparable to native PLA2.

On the basis of these analyses we addressed the phenomenon of multiple reactivities, a major problem of diagnostic assay systems emerging from the application of natural venom extracts. The CCDs of these natural allergen

sources obligatorily are associated with false-positive results. Generally, the molecular basis for the reactivity to conserved carbohydrate structures in hymenoptera venom allergy is formed by α -1,3-core fucose [46]. This moiety is absent in mammalian carbohydrate structures and, therefore, constitutes a highly immunogenic epitope in men. Thus, carbohydrate-directed reactivity in hymenoptera venom allergy represents a challenge for diagnostic approaches when allergen extract specific IgE antibodies are measured [26, 47-49]. Since the identification of the culprit venom is essential for immunotherapy but strongly affected by such double positivities the choice of the proper venom for immunotherapy is often cumbersome. Difficult to perform inhibition tests based on mutual inhibition of IgE by venom of the particular species can circumvent such reactivities [26]. The most advanced option, however, relies on the use of defined recombinant molecules which provide high IgE prevalence and a complete spectrum of proteinic epitopes but lack carbohydrate-based cross-reactivity [4].

The cross-reactivity of Api m 1 is based on the single glycan structure, which has been extensively studied [22, 50]. This cross-reactivity seems to be reduced for some patients when present in a natural conformation, e.g. ELISA assays [37]. Occasional function as B cell epitope for IgE

and IgG responses [46] as well as T cell epitope [51] was demonstrated.

However, in the present study clear CCD-based reactivity of native Api m 1 could be observed for different patient sera. Especially in immunoblot based tests resulting in full exposure of the glycan such cross-reactivities can be expected to play an important role as demonstrated here by the use of a CCD-specific polyclonal HRP antiserum produced in rabbit [52]. Nevertheless, due to its extraordinary high prevalence as allergen, Api m 1 needs to be included in all diagnostic approaches to HBV allergy. Therefore, the availability of Api m 1 as native folded recombinant protein devoid of any CCD-based cross-reactivity is imperative.

To avoid any glycan-based cross-reactivity in diagnostic tests we generated a non-glycosylated PLA2 variant by site-directed mutagenesis of the single glycosylation site asparagine 13 to glutamine. The complete epitope spectrum of a protein, however, is based on native folding which in turn in many cases is glycosylation dependent. Recently, we described the production of properly folded and glycosylated hymenoptera venom allergens devoid of any immunologically detectable CCDs by the baculovirus infection of Sf9 insect cells [4]. While the reactivity of the Sf9 produced Api m 1 with GNA demonstrated the general presence of the N-glycan, the HRP antiserum guaranteed the absence of cross-reactive carbohydrate structures. These proteins therefore form complements to the native as well as the HighFive-derived, CCD-positive Api m 1. While all Api m 1 variants appear suited for the measurement of true protein reactivities, the Sf9 derived and the non-glycosylated Api m 1 variant will be of special interest for discrimination of protein and CCD-reactivities.

Another advantage of the recombinant approach is the ease of manipulation of enzymatic activities. It was shown previously that enzymatically active HBV PLA2 induces degranulation and cytokine production of rodent basophils as well as human basophils and mast cells [39-41]. Such an activity will provoke false-positive results in cellular assays routinely applied for allergen characterization. We confirmed the IgE-independent activating effect of native enzymatically active Api m 1 by the release of β -hexosaminidase from RBL cells in concentrations routinely applied in cellular assays. Thus, we produced an inactive PLA2 mutant in insect cells which is reported to exhibit similar activity in skin tests compared to the native enzyme [53]. In contrast to the native enzyme, the inactivated recombinant Api m 1 H34Q exhibited no activatory potential at all.

CONCLUSION

In summary, in this study the HBV major allergen phospholipase A2 (Api m 1) for the first time could be produced in insect cells via the baculovirus expression system. The protein could be obtained as inactive variant in high yields in soluble and secreted form. The recombinant molecule produced in *T. ni* (HighFive) cells showed similar characteristics with respect to glycosylation and IgE reactivity compared to the native protein. By the production of the recombinant allergen in Sf9 cells or as non-glycosylated version approaches suitable to eliminate CCD-reactivity were demonstrated. Moreover, elimination of enzymatic activity avoided IgE-

independent reactivity in cellular assays. The ease of recombinant production of HBV major allergen Api m 1 in insect cells with tailored characteristics might contribute to an advanced component-resolved approach to hymenoptera venom allergy.

ABBREVIATIONS USED IN THIS PAPER

AP	=	Alkaline phosphatase
CCD	=	Cross-reactive carbohydrate determinant
HBV	=	Honeybee venom
HRP	=	Horseradish peroxidase
HSA	=	Human serum albumin
NTA	=	Nitrilo-triacetic acid
PLA2	=	Phospholipase A2
SD	=	Standard deviation
sIgE	=	Specific IgE
VIT	=	Venom immunotherapy
YJV	=	Yellow jacket venom

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