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Electrophoretic Characterisation of Hevein

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Although the latex protein, hevein, has received much research interest, its analysis by conventional SDS-polyacrylamide gel electrophoresis has been problematic. Hevein does not migrate at a rate that commensurates with its molecular weight and it does not always produce a sharp band on a polyacrylamide gel. To compound this problem, hevein is difficult to stain because it has a tendency to diffuse out of the gel during the de-staining process. Moreover, hevein Western blot is difficult to perform because, as a small protein, it easily passes through a nitrocellulose membrane during electro-transfer. Using the tricine SDS-polyacrylamide gel electrophoresis system that is optimised for small proteins and peptides, hevein migrated in the gel at a rate consistent with its molecular weight of 4.7 kDa, although the migration rate was significantly reduced under non-reducing conditions. Addition of phosphotungstic acid to the Coomassie blue stain fixed hevein and prevented its loss by diffusion out of the gel. Hevein could be effectively Western blotted on to the PVDF membrane and fixed with glutaraldehyde, after which, the protein was detectable using anti-hevein antibody. Although hevein is an unglycosylated protein, it was found to be associated with carbohydrate, probably through its lectin property in binding carbohydrate.

Key words: Carbohydrate; glutaraldehyde fixation; hevein; lectin; phosphotungstic acid fixation; SDS-polyacrylamide gel electrophoresis; tricine buffer; Western blot

Hevein, a heat-stable chitin-binding protein in natural rubber latex, is located in the lutoids of natural rubber latex. It constitutes 40% to 70% of the proteins in the aqueous content of lutoids, the B-serum[1,2]. In the latex, an 18.5 kDa precursor molecule, prohevein, cleaves post-translationally to produce a 4.7 kDa N-terminal cleavage product, hevein, and a C-terminus peptide of 13.3 kDa[3,4]. All three proteins are found in the latex B-serum, but the molar ratio of hevein to the C-terminus latex is about 30:1. It is surmised that the C-domain is catabolized or otherwise removed after the mature hevein is formed[4].

Hevein may be considered a lectin in the current broader definition of the term in that it binds chitin and its monomer, N-acetylglucosamine, even though it does not agglutinate red blood corpuscles[5]. The protein displays close homology to the wound-induced proteins (WIN 1 and WIN 2) of potato, and to wheat germ agglutinin (WGA). The carboxyl-terminal of prohevein is homologous to the carboxyl terminal region of proteins encoded by the potato WIN genes[3]. Hevein also shows homology to other chitin-binding lectins from wheat, barley and rice[5,6].

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Hevein is the most abundant soluble latex protein, accounting for about 20% of the total soluble proteins in natural rubber latex[7]. The fact that the protein has anti-fungal properties (possibly related to its affinity to chitin)[5,8,9] renders potentially valuable. Nevertheless, much of the attention currently being placed on hevein and its related proteins arises from the fact that hevein, prohevein and, to a lesser extent, the prohevein C-terminus, are allergenic proteins[10,11]. Prohevein, hevein and the prohevein C-terminus are also named Hev b 6.01, Hev b 6.02 and Hev b 6.03 respectively according to the allergen nomenclature of the International Union of Immunological Societies. The main Immunoglobulin E (IgE) epitopes that mediate the allergic reaction are found on the hevein domain, but the C-terminus is also allergenic to a lesser extent[10,11].

Whereas in laboratory research, both prohevein and the prohevein C-terminus are routinely studied by SDS-polyacrylamide gel electrophoresis, this laboratory procedure is not commonly practised with hevein. The protein does not always produce a sharp band owing to its small size. This problem is compounded by the tendency for hevein to diffuse out of the gel during de-staining, thus making observations and recording difficult[5,12,13]. Moreover, hevein Western blot is problematic because, as a small protein, it easily passes through a nitrocellulose membrane during electro-transfer. For these reasons, Western blots of hevein are not commonly performed. Most of the Western blots in allergy research on hevein, for example, make use of prohevein or the recombinant hevein fusion protein as hevein surrogates.

The principal objectives of the present study are:

- to establish a procedure of SDS-polyacrylamide gel electrophoresis suited for hevein
- to establish a procedure for staining hevein separated on a polyacrylamide gel
- to establish a procedure for Western blotting hevein separated on a polyacryamide gel that would accommodate immuno-detection and carbohydrate-detection techniques
- to examine the carbohydrate-binding reactions of hevein, acting as a lectin.

MATERIALS AND METHODS

Preparation of B-serum

Latex was collected in chilled containers from *Hevea brasiliensis* trees of the clone RRIM 600. The latex was centrifuged at 43 000 g in a Sorvall RC5B PLUS high speed centrifuge for 1 h at 4ºC–7ºC to separate it into three main fractions; the top fraction which was the rubber cream, the heavy bottom fraction and the C-serum located in between. Latex B-serum was prepared from the bottom fraction based on the method by Hsia[14]. Briefly, the latex bottom fraction from centrifuged latex was washed by re-suspension in 0.4M mannitol and recovered by centrifugation. The washed bottom fraction was then subjected to repeated freeze-thawing to rupture the lutoids that were its main constituents. The B-serum, comprising mainly the lutoidic fluid, was recovered as the supernatant after re-centrifugation.

Hevein Purification

Two methods of hevein isolation were performed, *i.e*. with and without the chitin affinity step.

Preparation of hevein with chitin affinity purification was performed following the

method of van Parijs *et al*.[5]. One gram of freeze-dried B-serum was dissolved in 25 mL of 50 mM acetic acid containing 0.2 M NaCl, followed by pH adjustment to pH 4 with 1 M NaOH. The sample was centrifuged for 10 mins at 20 000 g to remove particulates. A chitin column was equilibrated with a solution of 50 mM acetic acid containing 0.2 M NaCl. Following equilibration, the sample (supernatant) was loaded and flushed with 0.2 M NaCl until the Abs_{280nm} reached 0.01. Acetic acid (0.5 M) was used to elute the bound proteins. Fractions collected were screened by SDS-polyacrylamide gel electrophoresis. Pooled fractions containing hevein were adjusted to pH 7 prior to determination of its concentration.

The method of Karunakaran *et al*.[15] was followed for the isolation of hevein without chitin affinity purification. Briefly, B-serum was dialysed against 2.4 mM citric acid and 5.1 mM $Na_2HPO_4.2H_2O$, pH 5, with 0.1 % (w/v) cysteine hydrochloride. Dialysis was continued with four more changes of the buffer without cysteine hydrochloride. Dialysed B-serum (50 mL) was adjusted to pH 5 and loaded on to a CM 52 cellulose (Whatman, UK) column which had been equilibrated with the same buffer. Fractions of 10 mL were collected, read at 280 nm and analysed by SDS-polyacrylamide gel electrophoresis. Fractions containing hevein were pooled, dialysed against water and freeze-dried. The freeze-dried material was dissolved in citrate phosphate buffer pH 6, and applied to a column of DEAE 52 cellulose resin equilibrated with the same buffer. Proteins were eluted using the same buffer. The fractions collected were screened by SDS-polyacrylamide gel electrophoresis and fractions showing the putative hevein protein band were pooled and sent for amino acid sequencing. The N-terminal sequence, EQXGRQAGG, that was obtained matched exactly the known hevein sequence where the unassigned X was cysteine.

SDS-Polyacryamide Gel Electrophoresis and Gel Staining

Conventional SDS-polyacrylamide gel electrophoresis on a 15% gel with the Tris-gylcine running buffer was carried out essentially according to the method of Laemmli[16]. In other experiments, the method of Schagger and von Jagow[17] (where glycine was replaced with tricine as the trailing ion in the electrophoresis buffer) was employed to optimise electrophoretic separation of low molecular weight proteins (<5 – 20 kDa). For electrophoresis with the Tris-tricine buffer, proteins were run on a 16.5% gel at 30 mA – 40 mA with 0.1M Tris-tricine buffer, pH 8.25, in the cathode buffer and 0.2M Tris-tricine buffer, pH 8.9, in the anode buffer. In some experiments, 2-mercaptoethanol was omitted from the sample buffer to perform electrophoresis under non-reducing conditions. The calibration markers used were Bio-Rad Low Range unstained markers or GE Healthcare Low Range Rainbow markers.

Staining of the acrylamide gels was by 0.1% Coomassie blue R250 in 10% acetic acid and 50% methanol. To stain hevein specifically in polyacrylamide gels, phosphotungstic acid was added to the Coomassie blue stain to a concentration of 0.2%.

Western Blotting

Following electrophoresis, the polyacrylamide gel was equilibrated for ten minutes in Tris-tricine transfer buffer. Five minutes into gel-equilibration, 0.2 μm pore size polyvinylidene fluoride (PVDF) membrane was soaked in 100% methanol for three seconds, immersed in de-ionised water for two minutes and then placed in transfer buffer for three minutes before its use in blotting. Western blotting was performed at 5 Volts overnight at 4ºC in 20 mM Tris and 137 mM tricine. After the protein transfer,

the blotted membrane was placed on filter paper and air-dried for an hour to enhance the adsorption of the protein to the membrane before fixation with glutaraldehyde based on the method of Karey and Sirbasku[18]. The PVDF membrane was re-wetted in 100% methanol and then in de-ionised water before being immersed for five minutes in phosphate buffered saline (PBS) containing 0.5% (v/v) glutaraldehyde. The membrane was transferred to fresh PBS/glutaraldehyde solution for another ten minutes before the cross-linking reaction was stopped by soaking the membrane for ten minutes in PBS containing 50 mM glycine. The PVDF membrane was then washed once more in PBS.

Detection of Blotted Hevein and Protein-Linked Carbohydrates

Immuno-detection of hevein and prohevein was performed as described previously[19]. Briefly, 5% non-fat milk was used to block the PVDF membrane, after which the membrane was incubated with a rabbit polyclonal anti-hevein antibody. The secondary antibody used was anti-rabbit IgG conjugated to alkaline phosphatase. Subsequent reaction with 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/NBT) substrate generated the coloured enzyme product.

To visualise carbohydrates linked to proteins that were Western-blotted on to PVDF membrane, the procedures of the GlycoTrack™ Carbohydrate Detection Kit (Oxford GlycoSystems, UK) were followed. Blotted carbohydrates were subjected to sodium periodate oxidation followed by successive reactions with biotin-hydrazide and alkaline phosphatase-conjugated streptavidin. The carbohydrates were stained following incubation with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium substrate.

RESULTS AND DISCUSSION

Electrophoretic Migration of Hevein

Despite the abundance of hevein in the latex B-serum, it was often difficult to examine the protein on a gel after electrophoresis of B-serum by the conventional glycine buffer procedure of Laemmli[16] because hevein is a small molecule of 4.7 kDa. Purified hevein or hevein present in B-serum may appear as a distinct protein band[20] if viewed immediately after a brief staining period. Upon more prolonged staining, it tends to appear as a diffused zone with an apparent molecular weight of about 10 kDa (on a 15% gel) when extrapolated against Bio-Rad Low Range markers (*Figures 1A* and *2C*). Against GE Low Range markers, the molecular weight of hevein was estimated at 7 kDa (*Figure 2B*). In an earlier report where hevein was separated on a 12%–25% gradient gel, an apparent molecular weight of 14 kDa was observed. The over-estimate was explained as an artefact common with proteins rich in cysteine and glycine[5]. All these results indicated that conventional SDS-polyacrylamide gel electrophoresis was unsatisfactory for hevein, especially in getting the protein to migrate at a rate true to its molecular weight.

In the present study, the electrophoretic procedure was modified to enhance separation of low molecular weight proteins (<5 – 20 kDa) by replacing glycine with tricine as the trailing ion in the electrophoretic buffer, following Schagger and von Jagow[17]. The electrophoretic migration of hevein by this method can be seen in *Figures 1B* and *2A*. By intrapolation against the GE molecular weight markers, the migration of hevein in the gel was estimated at about 4.7 kDa (*Figure 1B*), which matched its actual molecular weight of 4.73 kDa calculated from its amino acid sequence[10,11] and 4.72 kDa by mass spectrometry[10,11,21]. As the tricine buffer system of electrophoresis was optimised

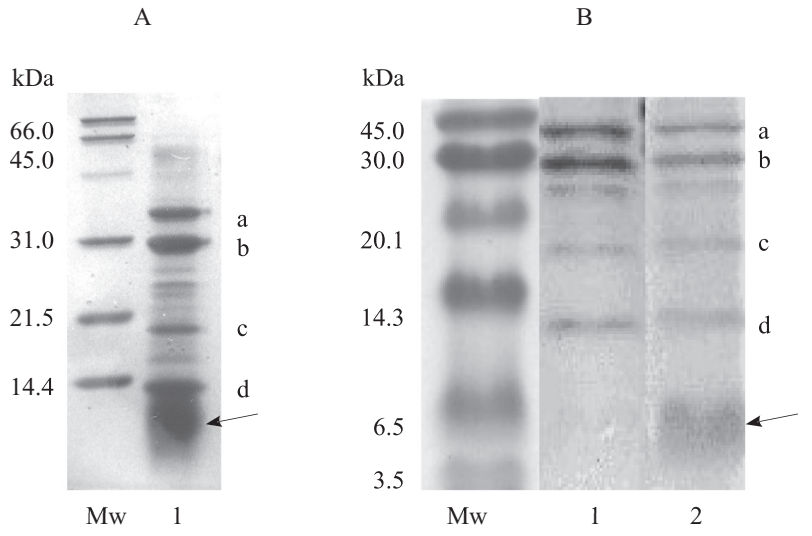

Figure 1. SDS-polyacrylamide gel electrophoresis of latex B-serum using the glycine and tricine buffer systems. Panel A: Gel run on the glycine buffer system and stained in Coomassie blue. Hevein appears as a broad diffused zone (arrow). Panel B: Gel run on the tricine buffer system and photographed 82 hours after staining. The gels were stained with Coomassie blue that contained (Lane 2) or did not contain (Lane 1) 0.2% phosphotungstic acid. Arrows indicate the position of hevein. Identified B-serum proteins are beta glucanase (a), heveamine (b), prohevein (c) and prohevein C-terminus (d). Mw = Molecular weight markers: Bio-Rad markers (Panel A) and GE markers (Panel B).

for small proteins and polypeptides, it was less suitable for higher molecular weight proteins (> about 49 kDa) which were not adequately separated. Nevertheless, other major B-serum proteins up to 35 kDa – beta glucanase, heavamine, prohevein and the prohevein C-terminus – were clearly separated by tricine buffer electrophoresis (*Figure 1B*).

In SDS-polyacrylamide gel electrophoresis, SDS denatures the protein, linearises it and imparts to it a net negative charge. Electrophoresis is most commonly run under reducing conditions whereby the protein sample is added to a sample buffer that contains 2-mercaptoethanol that breaks the disulphide bonds occurring both within the protein molecule and between sub-units of the protein. Since hevein has four disulphide bridges[6,22], an investigation was carried out to examine how the protein would behave under non-reducing conditions of electrophoresis. For this purpose, mercaptoethanol was omitted from the sample buffer. The results showed that, under non-reducing conditions, hevein migrated at a rate comparable to a 21 kDa protein in tricine buffer and about 25 kDa (23-27 kDa) in glycine buffer (*Figure 2*). A faint 42 kDa hevein band was also sometimes seen on gels run with glycine buffer (results not presented). On the other hand, various major B-serum proteins showed no differences in migration behaviour irrespective of the presence or absence of mercaptoethanol (*Figure 2C*). Surprisingly, even prohevein (where the hevein molecule constitutes its N-terminus segment) was unaffected by the omission of mercaptoethanol; only free hevein

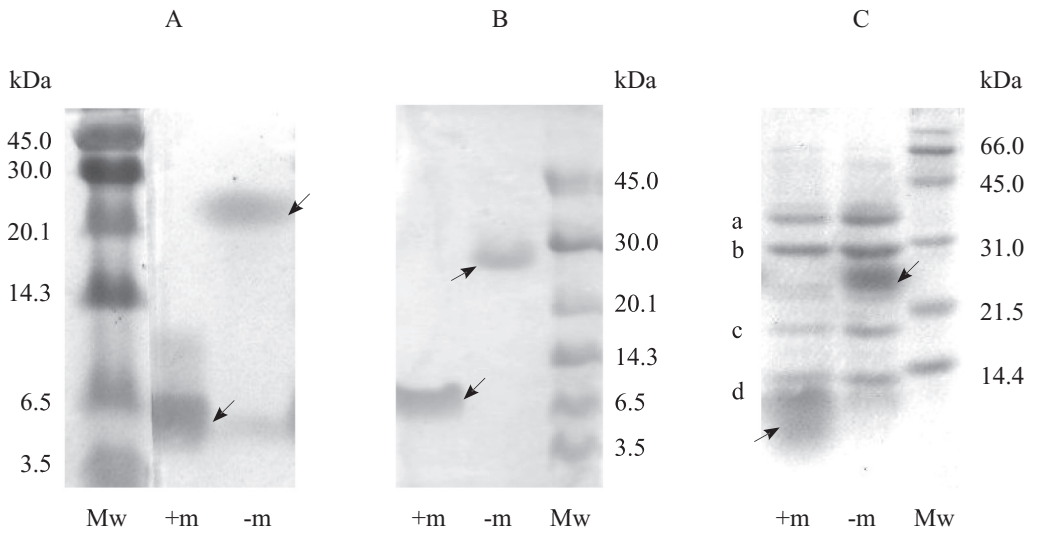

Figure 2. SDS-polyacrylamide gel electrophoresis of purified hevein and B-serum using the tricine buffer system (Panel A) or the glycine buffer system (Panels B and C). Hevein (Panels A and B) or B-serum (Panel C) was loaded in sample buffers containing mercaptoethanol (+m) or not containing mercaptoethanol (-m). Arrows indicate the position of hevein. Identified proteins in Panel C are beta glucanase (a), heavamine (b), prohevein (c) and prohevein C-terminus (d). Mw = Molecular weight markers: GE markers (Panels A and B) and Bio-Rad markers (Panel C).

was affected. The reason hevein migration in the gel was significantly reduced under non-reducing conditions of SDS-polyacrylamide gel electrophoresis might be that the protein was incompletely linearised. With its disulphide bridges intact, hevein might have remained folded and elements of its tertiary and/or quarternary structures could encumber migration of the protein through the gel.

There is also the possibility that hevein might occur naturally as a dimer or other oligomer in *Hevea* latex[13,23], this scenario being especially relevant to the hypothesis in which hevein acts as an agglutinating agent in de-stabilising rubber particles, leading to latex coagulation[24]. While multiple carbohydrate recognition domains generally give lectins their ability to agglutinate cells or form precipitates with glycoconjugates, hevein has only a single carbohydrate-binding domain. Multimeric hevein would therefore support the latex coagulation proposition, but such a hypothetical stance needs to take into consideration the fact that molecular weight measurements by mass spectrometry in previous studies have pointed to monomeric hevein[10,11,21].

Staining Hevein in Polyacrylamide Gels

Besides the problem of anomalous migration rate inconsistent with its molecular weight, hevein also does not stain well with common protein stains as the protein has a tendency to diffuse out of the gel during the de-staining procedure[5,12,13]. Nevertheless, the protein band could be seen if the observation was made promptly, before the band disappeared during

de-staining. In any case, the stained hevein band would gradually disappear upon storage (*Figure 1B*).

Although hevein resists acid precipitation by trichloroacetic acid, it is precipitated by phosphotungstic acid[2,25]. Hence, the problem of hevein diffusing out of the gel was resolved by adding 0.2% (final concentration) phosphotungstic acid (PTA) to the Coomassie Blue stain. In an experiment, the hevein band stained with Coomassie blue without PTA had completely disappeared 82 hours after staining. In the presence of PTA, the hevein band was well preserved (*Figure 1B*).

Western Blotting Hevein

Transfer of hevein from the polyacrylamide gel on to a membrane for subsequent immunological reaction posed yet another challenge because the protein could not be blotted on to the nitrocellulose membrane efficiently. Increasing the blotting time was ineffective because the small molecular size of hevein enabled it to pass through the membrane on prolonged blotting and only a small amount of hevein would be retained on the membrane. In the present study, the replacement of nitrocellulose membrane with polyvinylidene fluoride (PVDF) membrane in Western blotting greatly reduced hevein loss in this manner. Even so, the transferred protein would not bind well to the PVDF membrane and would dissipate when the membrane was washed during the immuno-detection procedure, or even while being stained with Coomassie Blue.

Effective Western blotting of hevein following tricine buffer SDS-polyacrylamide gel electrophoresis was achieved in the current study by first immersing the blotted membrane in 0.5% glutaraldehyde prior to blocking to cross-link the blotted hevein, according to the procedure of Karey and Sirbasku[18]. Hevein that was blotted on to PVDF was then readily detected by staining with dilute Coomassie blue. The blotted hevein could also be detected by immuno-reaction after incubation with its polyclonal antibody (*Figure 3A*). Prohevein was similarly detected by the anti-hevein antibody since hevein constituted the N-terminal domain of prohevein. However, the C-terminus of prohevein, which lacked the hevein domain, was undetected as expected (*Figure 3A*).

Carbohydrate Residues Linked to Hevein

Western-blotted hevein and prohevein was tested for the presence of carbohydrate by periodate oxidation of the carbohydrate followed by successive reactions with biotin-hydrazide and alkaline phosphatase-conjugated streptavidin. The results showed positive carbohydrate reactions with hevein in B-serum (*Figure 3B*). Hevein is not known to be a glycosylated protein and no glycosylation site is predicted in sequence analysis of the hevein leader sequence. However, since hevein binds to chitin and its monomer N-acetylglucosamine (NAG)[5,6], carbohydrate-linkage to hevein could be the result of a lectin reaction with carbohydrates present in natural rubber latex.

When B-serum was pre-incubated overnight at 35ºC, carbohydrate was found to be attached to both hevein and prohevein. In comparison, glycosylation was associated only with hevein (but not with prohevein) in B-serum that was kept chilled (not incubated, *Figure 3B*). These results suggest that while hevein in B-serum was inherently linked to carbohydrate, this might not be the case with prohevein. Carbohydrate linkage to prohevein might occur only under suitable ambient conditions (such as incubation at an increased

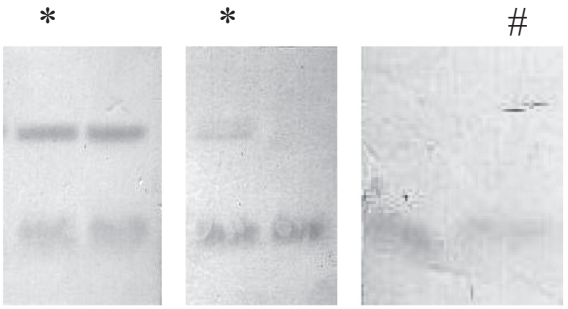

Figure 3. Western blots of latex B-serum and purified hevein showing hevein (lower bands) and prohevein (upper bands) following SDS-polyacrylamide gel electrophoresis using the tricine buffer system. Detection of hevein in B-serum using an anti-hevein polyclonal antibody (Panel A). Detection of protein-linked carbohydrates in B-serum (Panel B) and in purified hevein (Panel C) by periodate oxidation followed by the biotin-hydrazide and streptavidin-alkaline phosphatase reactions. Asterisks indicate B-serum samples that were incubated overnight at 35ºC. Hash symbol indicates purified hevein sample eluted from a chitin affinity column.

temperature). This observation was consistent with previous (unpublished) observations that carbohydrate was occasionally detected in Western blots of prohevein. These inferences notwithstanding, it is unclear if carbohydrate binding with hevein actually occurred *in situ* within the latex vessels of the rubber tree bark, or if such observations were artefacts of B-serum preparation or its experimental manipulations.

As hevein has a chitin-binding domain, a common procedure for its purification is to pass it through a chitin affinity column. In order for hevein to bind to chitin in the column, there must be a chitin-binding domain on the protein that is free (*i.e*. not already linked to chitin or NAG). Upon elution of the protein from the chitin column, this domain should unbind from chitin and therefore remain free of carbohydrate. An analysis was carried out to test for carbohydrate associated with purified hevein that had, or had not, been purified through a chitin column. The results showed the presence of carbohydrate associated with hevein, irrespective of its having gone through chitin column purification (*Figure 3C*). The explanation for this observation requires further study. Perhaps hevein linked to chitin or hevein linked to NAG had been eluted off an unstable column matrix.

One criterion for a protein to be classified as an allergen is its ability to bind human immunoglobulin E (IgE). Some lectins can bind directly to the glycans of IgE, rather than to the IgE's variable region as in an actual immunological reaction[26]. Since IgE carries N-acetyl glucosamine in its glycan[27], there is the possibility that the lectin property of hevein and prohevein could bind directly with the carbohydrate residues of IgE molecules, and thereby give false positive *in vitro* allergenic reactions. Such a likelihood has been previously considered, but thought improbable[10,28].

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