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Short Communication
Hevein (Hev b 6): Recent Research Findings

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Hevein is the major soluble protein in the latex of Hevea brasiliensis*. It is formed from a larger precursor, prohevein. Since 1960, many studies have been performed on and with this protein by specialists from a large variety of disciplines. In this review, several aspects of recent research on hevein, prohevein and their naturally occurring variants are summarised, integrating both literature data and data present in databases.*

Keywords: hevein; *Hevea brasiliensis*; bioinformatics; allergenicity; X-ray structure; NMR spectroscopy

The first protein purified and characterised from the lutoid-body fraction of rubber latex was hevein, a small cystine-rich protein[1] with a polypeptide chain length of 43 residues[2] (*Figure 1*). Since its discovery in 1960, many studies have been performed on this protein by protein chemists, plant physiologists, molecular biologists, physico-chemists, allergologists, and specialists in other disciplines. They have produced many interesting and significant research findings, but in several cases there has been less interaction between specialists from different disciplines, often using different nomenclature. Here, these studies will be summarised.

Broekaert *et al*.[3] have determined the cDNA sequence of hevein and found that it is synthesised as a much larger precursor of 204 residues. After synthesis, an N-terminal signal sequence (residues -1 to -17) is removed and targeting to the lutoid-body fraction (vacuoles) results in removal of a vacuolar targeting sequence of 14 residues, resulting in the formation of prohevein (173 residues)[4]. Further cleavage between residues 49 and 50 results in a C-terminal domain of 124 residues[5], while hevein (residues 1 - 43) is formed after removal of six additional residues at its C-terminus, and the presence of minor variants with only five and four residues removed could also be demonstrated[6]. (*Figure 1*). The molar ratio in the lutoid-body fraction of hevein and the C-terminal fragment is about 1:30[4]. This means that after cleavage, the C-terminal domain disappears from this fraction by further proteolysis or other processes. These and also the proteolytic steps leading to prohevein, hevein and the C-terminal domain have not yet been resolved.

Structure

There are four disulfide bonds between cysteine residues in hevein. This results in

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a very tight structure. Efforts to determine the connections between these residues in the intact protein using enzymic or chemical digestion, as were used successfully in the study of the primary structure of hevamine, another protein from the lutoid-body fraction of rubber latex[7] did not give any result.

However, the three-dimensional structure of hevein, including position of the disulfide bridges, could be determined by X-ray diffraction[8] and NMR spectroscopy[9]. The first six cysteine residues form indeed a very tight knot of three disulfide bridges (Cys^{3}-Cys^{18}, Cys^{12}- Cys^{24} and Cys^{17}- Cys^{31}). Two anti-parallel strands between residues Leu^{16} and Ser^{26} form the major secondary structure, with a reversing loop at residues 20-22. There is a short α-helix from Asp^{28}-Ser^{32}. The C-terminal fragment with the fourth disulfide bond (Cys^{37}-Cys^{41}) is more loosely connected to the main core of the molecule with a less aligned third strand of residues 37-39.

Hevein was found to have affinity for chitin and oligomers of N-acetylglucosamine and inhibits fungal growth[10]. The binding of N-acetylglucosamine containing oligo-saccharides to hevein has been determined by NMR spectroscopy and several binding sites were identified[11–13] with a predominant role of interactions of aromatic residues at the surface of the molecule: the two tryptophan (W) residues at positions 21 and 23, and the tyrosine (Y) at position 30.

Allergenicity

New interest in hevein and prohevein was generated, when it was discovered that these proteins are major latex allergens. New names for them were assigned by the International Union of Immunological Societies. Both prohevein (Hev b 6.01) and its N-terminal domain (Hev b 6.02) are strong allergens and contain the major IgE binding epitopes, with less contribution of the C-terminal domain (Hev b 6.03) of prohevein[14]. Hevein (Hev b 6.02) is the major protein in the water-soluble fraction of rubber latex with a concentration in the order of magnitude of 1 mM[15], while other proteins like prohevein (Hev b 6.01) and its C-terminal domain (Hev b 6.03) are present in molar concentrations of more than one order of magnitude less[4]. Although the small and highly soluble hevein molecule will be washed out to a large extent from latex products, this protein, together with the less soluble prohevein molecule are still responsible for the major allergic reactions to Hev b 6 variants. We do not know yet the solution structure of prohevein. However, it may be assumed that this protein consists of two separate domains

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-17 mnifivvllcltgvaia -1
  1 eqcgrqaggklcpnnlccsqwgwcgstdeycspdhncqsnckd 43
 44 sgegvg 49
 50 ggsasnvlatyhlynsqdhgwdlnaasaycstwdankpyswr
 92 skygwtafcgpvgahgqsscgkclsvtntgtgakttvrivd
133 qcsnggldldvnvfrqldtdgkgyerghitvnyqfvdcgds 173
174 fnplfsvmkssvin 187
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Figure 1. Amino acid sequence of preprohevein: Residues -17 to -1, signal peptide; residues 1 – 43 hevein domain; residues 44 – 49, hinge peptide; residues 50 – 173 (C-terminal domain), residues 174 – 187, vacuolar targeting sequence.

with little interaction and that the connecting hinge peptide of six residues Ser-Gly-Glu-Gly-Val-Gly (S-G-E-G-V-G; residues 44-49; *Figure 1*), removed between hevein and the C-terminal domain, do not contribute to the allergenicity of prohevein.

Several studies have been performed to investigate the epitopes localised on hevein (Hev b 6.02). Both conformational and sequential epitopes were identified[16–18]. More precise results were obtained by combining epitope analysis with structural studies of the hevein molecule. Reyes-López *et al*.[19] demonstrated that there was little allergenicity left of the hevein molecule after modification of the two tryptophans (W) in the epitope at positions 21 and 23 with BNPS-skatole. These authors also demonstrated that a deamidated hevein mutant (Hev b 6.0202) with asparagine (N) replaced by aspartic acid (D) at position 14 (it is uncertain if this is a naturally occurring mutant or that deamidation happened during isolation of the protein), which is localised at the opposite side of the surface of the molecule, showed reduced IgE-binding capacity[20].

Hevein Variants

Several studies have demonstrated the presence of variants of hevein and prohevein at the protein, mRNA or gene level. These variants may be alleles of the same gene in one population or in different cultivars (clones), but there are also variants produced by separate genes, which are expressed at lower levels than "regular" hevein in latex producing cells.

Tata[21] demonstrated the presence of a hevein variant, which he called pseudohevein. Soedjanaatmadja *et al*.[6] isolated this variant, which is present in latex in a concentration of about one tenth of that of hevein. Pseudohevein consists of four components of 42 – 45 residues, differing in the number of glycine (G) residues at its C-terminus. There are seven differences with the sequence of hevein. Most interesting is the replacement of tryptophan (W) at position 21 by tyrosine (Y) in the carbohydrate binding site. This replacement was very useful in NMR investigations of these interactions[12] and also explains a lower affinity of pseudohevein at affinity chromatography on a chitin column compared with hevein[22].

Pujade-Renaud *et al*.[23] investigated five different genomic sequences encoding hevein precursors, which shows that this protein is encoded by a small multigene family in the rubber tree. Four clones include the fully transcribed region together with 5' flanking sequences. These sequences are very similar in the 3'-untranslated sequences and also in the sequences encoding the C-terminal region of prohevein (Hev b 6.03), including a completely conserved intron sequence of 1000 bp (base pairs) at valine (V) at position 117. There are more differences in the N-terminal regions of the mature sequences, but the sequence of 40 bp immediately upstream of the transcription start site is 100% conserved.

Two of the sequences (Hev2.1 and Hev2.2) encode hevein and are so similar that they may represent alleles of the same gene. The fifth sequence determined by these authors (HevP) includes only the signal peptide, few residues of the mature protein and about 1000 bp in the 5' flanking sequence. Two other studies of the promoter region of the gene also resulted in sequences of the 5' flanking region[24], and submitted to the database of the Genbank[25]. These promoter sequences are very similar, but they possess deletions when compared to the sequence of Hev2.1. The longest sequenced promoter region is that of Hev2.1 (1776 bp). A mysterious sequence in this gene is that of bp 61-192, which encodes

a protein sequence which is 75% identical to that of residues 129-172 of prohevein. Saleena *et al*.[26] cloned and sequenced an intronless isoform of the hevein gene, which has a sequence completely identical with Hev2. Rozynek *et al*.[27] determined a prohevein mRNA sequence identical to that of Hev2.

The other two complete DNA sequences determined by Pujade-Renaud *et al.*[23] (Hev1.1 and Hev1.2) have 5' flanking regions which are more than 50% different from those of Hev2.1 and Hev2.2 (except for the conserved part of 40 bp immediately upstream of the transcription start site) and a nucleotide blast investigation (NCBI-database) did not result in significant sequence similarity.

The N-terminal sequence of the mature coding region of Hev1.2 codes for pseudohevein[6]. There are eight glycine (G) residues and one serine (S) in the hinge region of pro-pseudohevein between the pseudo-hevein and the C-terminal domains, which is two residues longer than the one in prohevein. The C-terminal domain shows few amino acid replacements compared to Hev2. The coding region of Hev1.1 also codes for pro-pseudohevein, but the hinge region is similar to that in prohevein (Hev2) and there are several replacements in the N-terminal part of the C-terminal domain.

The promoter regions of Hev1.1 and Hev2.1 were expressed in rice[23]. Only the promoter region of Hev2.1 conferred a high level of expression to the transgene in this heterologous host. Pujade-Renaud *et al*.[23] ascribed this feature to the longer promoter sequence transferred with Hev2.1. However, it is also possible that it is the deviating promoter region sequence in Hev1.1 that causes a lower expression level, because also in the rubber tree gene Hev1.2 may be expressed at a level of only 10% compared to Hev2.1, if we take into account the ratio of hevein and pseudohevein concentrations in rubber latex[6].

Montoro *et al*.[28] showed by functional analysis of the Hev2.1 promotor in *Hevea* transgenic calli and plants and *in-situ* hybridisation that this promoter drives a laticifer-specific expression in roots and stems, but not in leaves where hevein genes were expressed in all cell types. Since Hev2.1 callus lines responded significantly to light stimulation, up-regulation of the expression of HEV2.1 genes by light was suggested in leaves.

A sequence not observed by Pujade-Renaud *et al*.[23] is the mRNA sequence of pro-pseudohevein determined by O'Riordan *et al*.[29]. This sequence also has the pseudohevein domain; the hinge region contains one reside less than in Hev1.2 (the serine (S) residue is absent), but there are several characteristic replacements in the C-terminal part of C-terminal domain of prohevein, not only five in the mature sequence, but also five in the vacuolar targeting sequence. Probably part of this sequence was already determined by Broekaert (personal communication, cited in[4]). It is proposed here to call this hevein variant pro-pseudohevein Hev1.3. Venkatachalam *et al*.[30] determined three mRNA sequences, starting at positions 61 or 71 of the prohevein sequence. Two of these (accession numbers DQ306800 and DQ306733) code for prohevein 2.2 or 1.2 (they did not include the hevein domain), but the third one (DQ306789) codes for pro-pseudohevein Hev1.3. A variant of prohevein has been isolated with a slightly increased molecular mass by Soedjanaatmadja[4]. This may have been a pro-pseudohevein.

NRESTdb Database

Mat-Isa *et al*.[31] have developed a Natural Rubber EST database (NRESTdb) of

expressed sequence tags (ESTs), to provide easy access and rapid analysis of these data. ESTs for hevein (Hev b 6) are grouped in four sets of unique sequences: CN86, CN68, CN66 and CN88.

Group CN86 (1501 bp) consists of 63 ESTs, coding for prohevein. This relatively large number is used by Yeang *et al*.[15] for their estimation of the relative mRNA abundance in their study of the concentration of significant allergens in latex serum.

Group CN68 (1552 bp) consists of 14 members, coding for pro-pseudohevein Hev1.2. However the first 504 bp at the 5'-terminus code for the rubber elongation factor (REF, Hev b 1) and its presence can be explained by sequence tag EST Y46H10 being an artificial hybrid.

Group CN66 (1101 bp) consists of 12 members, coding for pro-pseudohevein Hev1.3.

Group CN88 (668 bp) consists of 2 members. EST Y49A06 (668 bp) is also an artificial hybrid. Bp 188-668 of this EST codes for residues 153 -187 of the prohevein precursor and its 3'-untranslated sequence (Hev2.2), while bp 1-166 codes in the opposite direction for residues 16-70 of the G10 protein. The G10 protein is conserved in a wide range of eukaryote species (more than 95% identical in plants). It is a hydrophilic protein of about 17-18 Kda with still unknown function. The other EST in CN88 is Y47E04 (232 bp). It codes for residues 176-187 of the prohevein precursor and its 3'-untranslated sequence (Hev2.2).

Future

Many aspects of the biology and properties of hevein are still unresolved. Studies of the genome of *Hevea brasilienis* probably will yield more relevant information. It may be that these studies will show unexpected exchanges of sequences between separate genes.

The presence of the sulphur-rich protein hevein in relatively high concentration in rubber latex causes much pollution if the effluent of rubber factories is discharged directly into the environment. However, this protein can be isolated in high yield as a side product from the production of natural rubber, and may lead to a useful application of this antifungal chemical[32].

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