

Short Sequence Paper

Demonstration by mass spectrometry that pseudo-hevein and hevein
have ragged C-terminal sequences [☆]Ukun M.S. Soedjanaatmadja ^a, Jan Hofsteenge ^b, C. Margot Jeronimus-Stratingh ^c,
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Received 16 May 1994

Abstract

The primary structure of pseudo-hevein, a minor hevein component from the latex of the rubber tree, *Hevea brasiliensis*, was determined. Six differences with the sequence of the major hevein component were found, one of which is a replacement of tryptophan by tyrosine in the carbohydrate binding region of the molecule. Analysis by ion-spray mass spectrometry showed that pseudo-hevein has a heterogeneous C-terminal extension of several glycine residues and that hevein itself also contains minor components with additional C-terminal amino-acid residues. A seventh difference between the two sequences occurs in these extensions.

Keywords: Pseudo-hevein; Hevein; Primary structure; Ion-spray mass spectrometry; (*H. brasiliensis*)

The first protein isolated from the lutoid body fraction of rubber latex was hevein, a small cysteine-rich protein [1] with a polypeptide chain length of 43 residues [2,3]. The three-dimensional structure has been determined by X-ray diffraction [4,5]. Hevein was found to have affinity for chitin and oligomers of *N*-acetylglucosamine and inhibits fungal growth [6]. Several families of proteins contain chitin-binding domains that are homologous to hevein (reviewed in [7]). They are assembled as repetitive structures of 2–4 domains and/or linked to other domains with different structures and functions. Broekaert et al. [8] have determined the cDNA sequence of hevein and found that this protein is synthesized as a precursor with a signal sequence of 17 residues followed by the hevein domain of 43 residues and a C-terminal region of 144 residues. The C-terminal fragment is also present in the lutoid body fraction of latex. N-terminal sequence analysis of this fragment indicates that six amino acids between the hevein

domain and this fragment are lost during processing of the proprotein [9].

Tata [10] observed a minor component in purified hevein samples which is less anionic than the major component. He called this component pseudo-hevein. We isolated a similar component by HPLC and determined its primary structure. Analysis by ion-spray mass spectrometry proved to be essential for resolution of the C-terminal sequence of the protein.

A crude preparation of hevein was prepared from lyophilized B serum [11], obtained from rubber latex of clone GT.1 of *Hevea brasiliensis* (collected in plantations in western Java, Indonesia). The lyophilisate was suspended at 4°C in water (4 mg/ml) to which 0.5 mg/ml sodium dithionite was added to inhibit polyphenol oxidases. After centrifugation the solution was 100% saturated with ammonium sulfate and centrifuged again. The precipitate was dissolved in a small volume of 0.2 M acetic acid and subjected to gel filtration on a column (3.0 × 50 cm) of Sephadex G-25. Absorbances at 280 and 260 nm and the conductivity of the fractions (5 ml) were measured. The fractions of the first two salt-free peaks were pooled separately and lyophilized. The second peak

[☆]The sequence data of this paper have been submitted to the SWISS PROT protein sequence data base under the accession number P80359.

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contained hevein and was submitted to reverse-phase HPLC on a Nucleosil 10 C18 column (30 × 0.45 cm) with a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow rate of 1 ml/min. The effluent was monitored at 214 nm and the peaks collected manually. Ultraviolet spectra of hevein and pseudo-hevein were recorded with a Kontron Uvikon spectrophotometer.

Pseudo-hevein was treated with tributylphosphine and 4-vinylpyridine in 50% pyridine [12] to reduce the disulfide bonds with formation of pyridylethylcysteine residues. The modified protein was digested with chymotrypsin (1%, w/w) in 0.2 M NH_4HCO_3 (pH 8.0), at 37°C for 4 h. Peptides were separated by reverse-phase HPLC under the same conditions as used for the isolation of hevein and pseudo-hevein. Amino-acid compositions of proteins and peptides were determined after acid hydrolysis in 6 M HCl at 110°C for 24 h by analysis with an LKB Alpha Plus amino-acid analyzer or a Hewlett Packard AminoQuant (1090 M) amino-acid analyzer. N-terminal amino-acid sequences of several proteins, and of chymotryptic peptides of pseudo-hevein were determined with Applied Biosystems Model 477A Protein Sequencers with on-line Model 120A PTH-Analyzers (Basel and Eurosequence BV, Groningen). Manual sequence determination with the DABITC method was done according to Chang et al. [13].

For mass spectrometry proteins were dissolved in 150 μl of a solution of 0.02% trifluoroacetic acid in 80% (v/v) methanol in water. Protein solutions were introduced into the mass spectrometer by means of a Jasco Familic 100N syringe pump operating at 5 $\mu\text{l}/\text{min}$. Solutions were nebulized and samples ionized by pneumatically assisted electrospray [14]. The mass spectrometer was a Nermag R3010 quadrupole instrument equipped with a custom built atmospheric pressure ionization source. Data acquisition and data reduction took place by means of the standard Nermag SIDAR software. Full scan mass spectra were recorded with 1 amu steps. Molecular weights of pseudo-hevein and hevein were determined from the observed m/z values of multiply charged ions [15].

About 6 g lyophilized B serum can be obtained from one liter of rubber latex. The recovery of the crude hevein fraction in the second gel filtration peak after chromatography on Sephadex G-25 was about 90 mg per gram lyophilized B serum. The HPLC pattern of hevein components in this crude fraction is presented in Fig. 1. The major peak hevein, is preceded by a minor peak, representing pseudo-hevein, and is followed by a third one. The ratio of pseudo-hevein to hevein is about 1:10. The amino-acid compositions of hevein and pseudo-hevein are presented in Table 1.

As there are significant differences, the amino-acid sequence of pseudo-hevein was determined by sequence analysis of the protein and chymotryptic peptides (Fig. 2). Compared with the hevein sequence six amino-acid replacements were found and several additional glycine residues at the C-terminus were identified. However, the

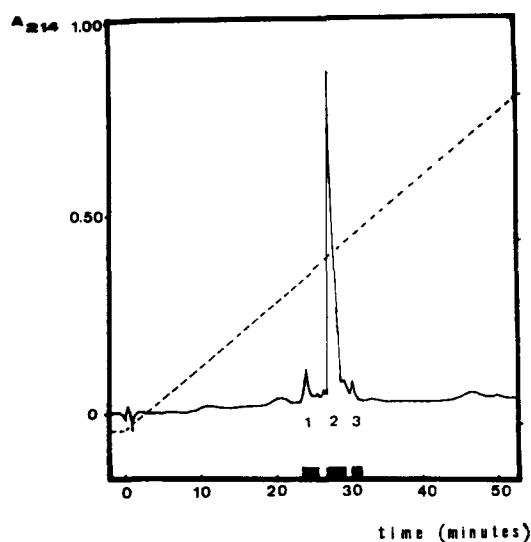


Fig. 1. Reverse-phase HPLC separation of hevein components. Column and elution conditions are described in the text. (—) A_{214} , (---) gradient. Peak 1, pseudo-hevein; peak 2, hevein; peak 3, second minor component.

number of C-terminal glycines could not be determined unambiguously by sequence analysis of the protein and the C-terminal chymotryptic peptide.

To resolve the C-terminal sequence of pseudo-hevein, it was analyzed by ion-spray mass spectrometry. The mass spectrum is presented in Fig. 3 and indicates that the protein has a ragged C-terminus. Four components having

Table 1
Amino-acid compositions

Amino acid	Hevein anal. (seq.)	Pseudo-hevein anal. (seq.)	Pseudo-hevein C-terminal chymotrypt. peptide anal. (seq.)
Asp	6.2 (7)	5.2 (6)	
Thr	1.0 (1)	0.0 (—)	
Ser	4.1 (4)	5.2 (6)	
Glu	7.1 (6)	5.8 (5)	
Pro	1.7 (2)	1.7 (2)	
Gly	5.1 (5)	7.0 (7)	2.7 (2)
Ala	1.3 (1)	1.3 (1)	
Cys	9.1 (8)	8.8 (8)	nd (1)
Val	0.4 (—)	0.4 (—)	
Met	— (—)	— (—)	
Ile	— (—)	— (—)	
Leu	2.1 (2)	2.0 (2)	
Tyr	0.6 (1)	1.1 (2)	
Phe	— (—)	— (—)	
Lys	1.9 (2)	2.6 (3)	1.3 (1)
His	0.9 (1)	0.1 (0)	
Arg	1.1 (1)	1.1 (1)	
Trp	nd (2)	nd (1)	—
Total	(43)	(44)	(4)

Number of residues (and glycines) calculated for major component with two C-terminal glycines (see text). anal., amino-acid analysis; seq., composition from sequence (Walujono et al. 1975; this paper).

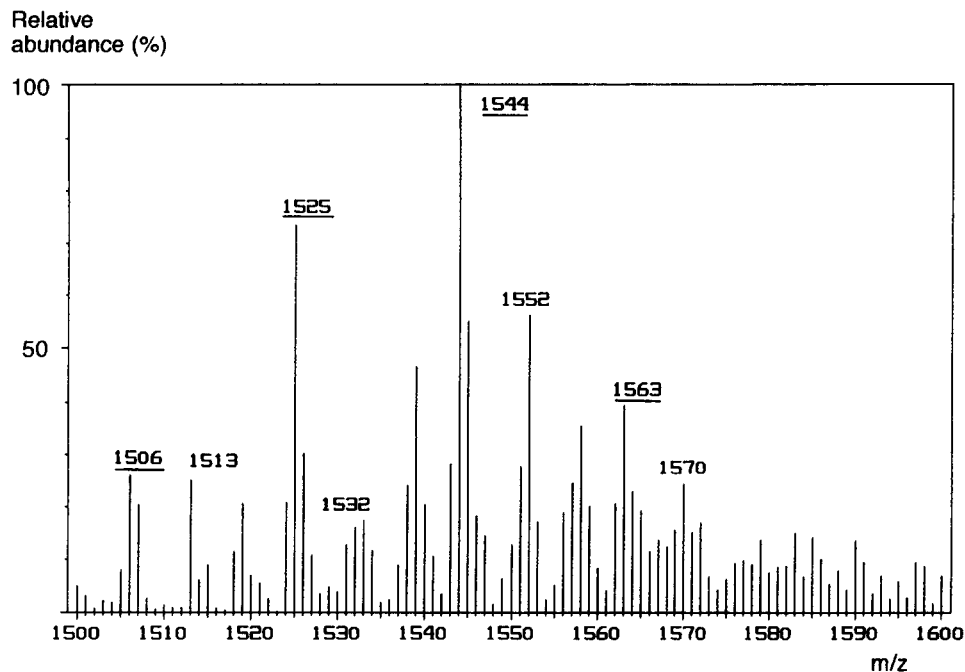


Fig. 3. Partial ion-spray mass spectrum of pseudo-hevein. The series with m/z values of 1563, 1544, 1525 and 1506 (underlined) corresponds with triply charged ions of pseudo-hevein with molecular masses of 4686, 4630, 4572 and 4515, respectively. Calculated molecular masses for pseudo-hevein components having three, two, one or no glycine residues at the C-terminus are 4687, 4630, 4573 and 4516, respectively. The series with m/z values of 1570, 1552, 1532 and 1513 corresponds with triply charged $M + 2H^+ + Na^+$ ions of the same components.

amino-acid residue could be identified. These properties point to an N-terminal pyroglutamate residue formed by cyclization of glutamate at position 1. Hevein converts slowly into this derivative, and the samples isolated from luteoid body fractions of inferior quality already contain much of it. Preliminary investigations of the NMR spectra of hevein and this derivative with a Bruker HX-360 NMR (E. Glazenburg and A.J. Lenstra, unpublished) and a Varian VXR 500 spectrometer (C. Sibon and K. Dijkstra,

unpublished) indicated that the two proteins have slightly different conformations.

We thank Mr. Djuharia S. for his help in collecting rubber latex and preparation of lyophilized B sera, Mr. Perry Overkamp (Department of Biochemistry, Katholieke Universiteit, Nijmegen) for performing part of the amino-acid analyses, Ms. Renate Matthies (Basel) for performing sequence analyses, and Dr. R.N. Campagne for critically reading the manuscript.

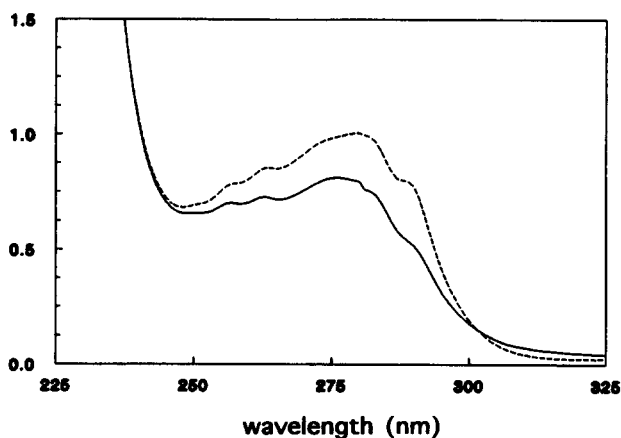


Fig. 4. Ultraviolet absorption spectra of hevein (---) and pseudo-hevein (—), at concentrations of about 0.4 mg/ml. The specific absorbance values (1 mg/ml) of hevein and pseudo-hevein at 280 nm were calculated to be 2.5 and 2.0, respectively.

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