

The primary structure of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex

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The primary structure of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex, has been determined predominantly with conventional non-automatic methods. The positions of three disulfide bridges have been determined. The sequence has about 60% identity with that of a chitinase from cucumber and 95% with the N-terminal sequence of the lysozyme/chitinase of *Parthenocissus quinquefolia*. The half-cystine residues in hevein and cucumber chitinase are located at identical positions. Hevamine is a basic protein from the luteoids (vacuoles) of rubber latex and may have a role in plugging the latex vessels and cessation of latex flow. The differences in cellular location, charge properties and sequence between hevamine and cucumber chitinase are similar to those between class I and class II chitinases from tobacco and other plant species.

Many plants possess lysozyme and chitinase activities. Since plants by themselves do not contain substrates on which these enzymes act, their presence in plants indicates that they have an antibacterial and antifungal function.

Several different types of these enzymes have been described. In tobacco [1] and several other plant species [2, 3] basic chitinases of class I occur, which are localized in the vacuoles and which are induced by viral, bacterial or fungal infection. cDNA sequences indicate that these chitinases have an N-terminal cystine-rich domain in the mature protein, which is similar to hevein [4] and domains of other proteins [5, 6] with affinity for chitin [7] or *N*-acetylglucosamine [5]. Other chitinases (class II) are acidic proteins. They belong to the pathogenesis-related proteins and are excreted by cells under several stress conditions. The sequences of acid chitinases are similar to those of the basic ones, but they lack the N-terminal hevein domain [8, 9].

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Abbreviation. DABITC, 4-*N,N*-dimethylamino-azobenzene 4'-isothiocyanate.

Enzymes. Lysozyme, peptidoglycan *N*-acetylmuramoylhydrolase (EC 3.2.1.17); chitinase, poly[1,4-(*N*-acetyl- β -D-glycosaminidine)] glycanohydrolase (EC 3.2.1.14); trypsin (EC 3.4.21.4); chymotrypsin (EC 3.4.21.1); thermolysin (EC 3.4.24.4); proteinase from *Staphylococcus aureus* V8, endoproteinase Glu-C (EC 3.4.21.19).

Note. Parts of the methods and the experimental evidence (including Figs S1–S5 and Table S1) are presented in a Supplement at the end of this paper. A table summarizing experimental methods used for making subdigests of tryptic peptides and tables summarizing amino analyses and isolation procedures of peptides after subdigestion are available on request from the corresponding author. The novel amino acid sequence data published here have been deposited with the NBRF Protein Data Library and are available under the accession number A 33179. Recently the genomic sequence of a chitinase from *Arabidopsis thaliana* has been published by Samac, D. A., Mironaka, C. M., Yallaly, P. E. & Shah, D. M. (1990) *Plant Physiol.* 93, 907–914. This sequence differs at 25% of these positions from that of hevamine without deletions/insertions in the alignment. The identical C-terminal sequences suggest that there is no C-terminal processing. The *Arabidopsis* chitinase is slightly more basic than hevamine, although Samiec et al. labelled it as an 'acidic' chitinase.

Chitinases belonging to a third class are extracellular, acidic pathogenesis-related proteins with no sequence similarity with the enzymes from classes I and II. The cDNA sequence of a member of this class from cucumber has been published [10]. Representatives of all three classes can be present in the same plant [8].

Bifunctional enzymes with both chitinase and lysozyme activity have been isolated from the latex of papaya [11] and fig [12] and the culture fluid of suspension cultures of *Rubus hispidus* [13] and *Parthenocissus quinquefolia* [14].

Fresh latex obtained by tapping the rubber tree *Hevea brasiliensis* can be separated by centrifugation into three main fractions [15]. These are a white upper layer of rubber particles, an aqueous layer containing the cytoplasm from the cells of the latex vessels and a 'bottom fraction' which consists of luteoids, vacuoles with a low internal pH that may be the equivalent of animal lysosomes [16]. Archer [17] isolated two major basic proteins with very similar amino acid compositions from rubber latex luteoids and called them hevamine A and B, respectively. Tata et al. [18], using a similar isolation procedure, isolated the same proteins and demonstrated that both are bifunctional lysozymes/chitinases. The N-terminal sequences of hevamine, the lysozyme/chitinase of *Parthenocissus* and cucumber chitinase [10, 14] are homologous or very similar.

The crystallization of hevamine has been described recently [19]. In this paper we describe the complete primary structure of hevamine obtained as a bifunctional lysozyme/chitinase from the latex of *H. brasiliensis*.

MATERIALS AND METHODS

Hevamine was isolated from freeze-dried bottom fraction of rubber latex (*Hevea brasiliensis*) as described by Archer [17] with several modifications [19].

Tryptic peptides

Batches (40 mg) of hevamine were reduced and *S*-carboxymethylated [20], citraconylated [21] and digested with trypsin [22]. Digests were fractionated by gel filtration on

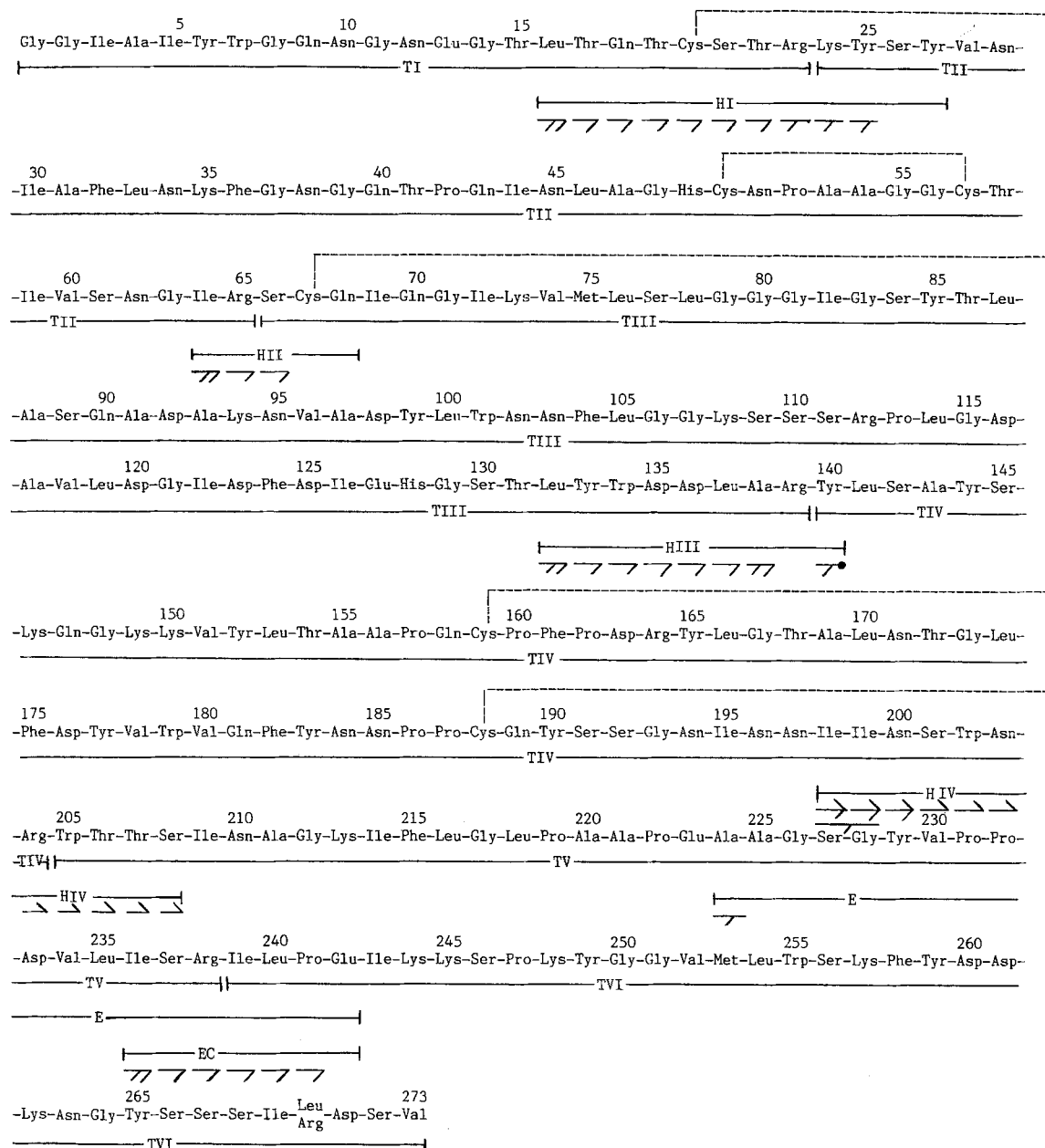


Fig. 1. Amino acid sequence of hevacine. Peptides TI – TVI are indicated below the sequence. Peptides HI – HIV, E and EC are overlapping peptides. —, sequenced by automatic Edman degradation; recoveries of phenylthiohydantoin identified by on-line analysis are presented in Fig. S3; —, sequenced by the DABITC method; —, sequenced by dansyl-Edman degradation (—, dansyl-dipeptide; —•, identification of the C-terminal residue of a peptide by dansylation without acid hydrolysis). Further sequence evidence is presented in Figs S1 – S5. Symbols used for cleavage agents: T, trypsin; C, chymotrypsin; H, thermolysin; E, endoproteinase Glu-C; CN, CNBr. Peptides are numbered according to their position in the sequence. Disulfide bridges are indicated by -----

Sephadex G-50 and on Bio-Gel P-4 in 0.1% formic acid. Purified peptides were subdigested with several enzymes. These digests were fractionated by paper electrophoresis, thin-layer chromatography and reversed-phase HPLC. Amino acid sequences of purified peptides were determined with a Beckman 890C spinning-cup sequencer, dansyl-Edman degradation [23] and with the 4-*N,N*-dimethylamino-azobenzene 4'-isothiocyanate (DABITC) method [24]. Charges of several peptides were determined by paper electrophoresis at pH 6.5 [25].

For determination of the amino acid sequences of the two large peptides TIII and TIV, 60 mg hevacine was pyridyl-

ethylated by treatment with 50 μ l tributyl phosphine and 50 μ l 4-vinylpyridine in 6.5 ml 50% (by vol.) pyridine in water at 50°C for 3 h. After lyophilization, the modified protein was citraconylated [21], dialyzed against 50 mM *N*-ethylmorpholine acetate (pH 8.0) and digested in a volume of 24 ml with 1.2 mg trypsin (treated with tosylphenylalanylchloromethane) at 37°C during 4 h. A precipitate which had formed during dialysis was dissolved in 3.6 ml 8 M urea and slowly added to the solution at the start of the digestion. The digest was fractionated on a Sephadex G-50 column. Pooled fractions were fractionated by reversed-phase HPLC. Purified peptides were subdigested with several enzymes, followed by reversed-

phase HPLC. Amino acid sequences of the purified sub-peptides were determined with the DABITC method. Two automatic Edman degradation runs were performed with an Applied Biosystems model 477A protein sequencer equipped with an on-line model 120A phenylthiohydantion analyzer. In addition, several residues were identified by dansyl-Edman degradation.

CNBr digest

Batches (25 mg) of hevamine were treated with a 100-fold molar excess of CNBr in 2.5 ml 70% formic acid at room temperature during 18 h. After lyophilization the digests were fractionated on Sephadex G-50 in 30% acetic acid and on Bio-Gel P-6 in 0.1% formic acid.

Overlapping peptides and disulfide bridges

Other fragments were generated by digestion with endoproteinase Glu-C and thermolysin. The positions of disulfide bridges were determined from a tryptic digest of the unreduced citraconylated protein. The procedures used are described in the Supplement.

Other materials and methods used were as described previously [22, 26 and 27].

RESULTS

Isolation of hevamine

A typical isolation has been described recently [19]. Two hevamine components, viz. hevamine A, the most abundant component, and a minor form, hevamine B, can be isolated by column chromatography. The amino acid sequence of hevamine A component has been determined, with some additional data for the B component.

Amino acid sequence determination

The amino acid sequence of hevamine is presented in Fig. 1. In this figure the six tryptic peptides TI–TVI, sequence evidence for overlapping peptides, and the positions of the three disulfide bridges are indicated. The sequence evidence for each tryptic peptide separately is presented in Figs S1–S5 in the Supplement.

Tryptic peptides from reduced and S-carboxymethylated protein

The evidence for the sequences of peptides TI, TII, TV and TVI is summarized in Figs S1, S4 and S5. Studies on several peptides from hevamine B indicated the replacement of a leucine residue near the C-terminus of the molecule in hevamine A by arginine in hevamine B. The two longest peptides (TIII and TIV), with rather similar amino acid compositions, could not be separated. Two subdigests of a mixture of these peptides were investigated.

Tryptic peptides from reduced and pyridylethylated protein

After gel filtration and reversed-phase chromatography, all six tryptic peptides TI–TVI and two partial cleavage products (TIVA and TIVB) were isolated. Amino acid analyses of peptides TI–TVI are presented in Table S1. Peptides TIII, TIV, TIVA and TIVB were subdigested and used for the

complete sequence determination of the two remaining sequence fragments (Figs S2 and S3).

CNBr peptides

Besides cleavage after methionine, cleavage after tryptophan had also occurred. Three peptides were obtained from a peak retarded on Sephadex G-50 by rechromatography on Bio-Gel P-4. Two of them originated from the C-terminus of the protein and were used for establishing the sequence of peptide TVI (Fig. S5). The third peptide, representing residues 76–101 (Fig. S2), was not further investigated.

Overlapping peptides

After digestion with endoproteinase Glu-C only peptides from the C-terminal part of the molecule were obtained (peptides E and EC; Fig. 1) providing the overlap between peptides TV and TVI.

The other four overlaps were obtained from a thermolytic digest of the N-terminal part of the molecule, as described in the Supplement. Overlaps between peptides TI and TII, and TII and TIII were unambiguously identified (peptides HI and HII; Fig. 1). There is a one-residue overlap between peptides TIII and TIV in peptide HIII (the N-terminal Tyr140 of TIV). If uncleaved TIV had not been the major cleavage product of tryptic digestion, this overlap could have been confused with the one-residue overlap between TIVA and TIVB (the N-terminal Tyr165 of TIVB) established by peptides TIVC1 and TIVH1 (Fig. S3).

The overlapping peptide between peptides TIV and TV (peptide HIV, Fig. 1) was obtained in rather low yield and still contained contaminants. However, automatic Edman degradation with quantitation of the phenylthiohydantoin (Fig. S3) yielded unambiguous proof of this overlap.

Independent support for the established overlaps is provided by the sequence similarity with the chitinase sequence derived from cDNA of cucumber [10].

Disulfide bridges

Preliminary indications for the localisation of the disulfide bridges were already obtained during the studies of the tryptic digests of the reduced and carboxymethylated protein because of incomplete reduction. Peptides containing cystine and with amino acid compositions identical to that of TIIIH3 (Fig. S1) and the sum of TIVC1 and TIVBC1 (Fig. S3) were obtained.

From a tryptic digest obtained after citraconylation of the nonreduced protein four disulfide-containing peptides were isolated. Amino acid and N-terminal sequence analysis indicated that one of them was peptide TI linked to TIII, the second one was intact peptide TIV while the third one consisted of TIVA and TIVB. After reduction and pyridylethylation, peptides with similar elution positions from HPLC, amino acid compositions and N-terminal amino acid sequences (three residues) to peptides TI and TIII, TIV, and TIVA and TIVB, respectively, were recovered.

From the third Sephadex pool, peptide TII containing a disulfide bridge was isolated. A subpeptide still containing this loop was produced by thermolysin digestion (peptide TIIH3) which contained two cysteic acid residues after performic acid oxidation.

The positions of the three disulfide bridges are included in Fig. 1.

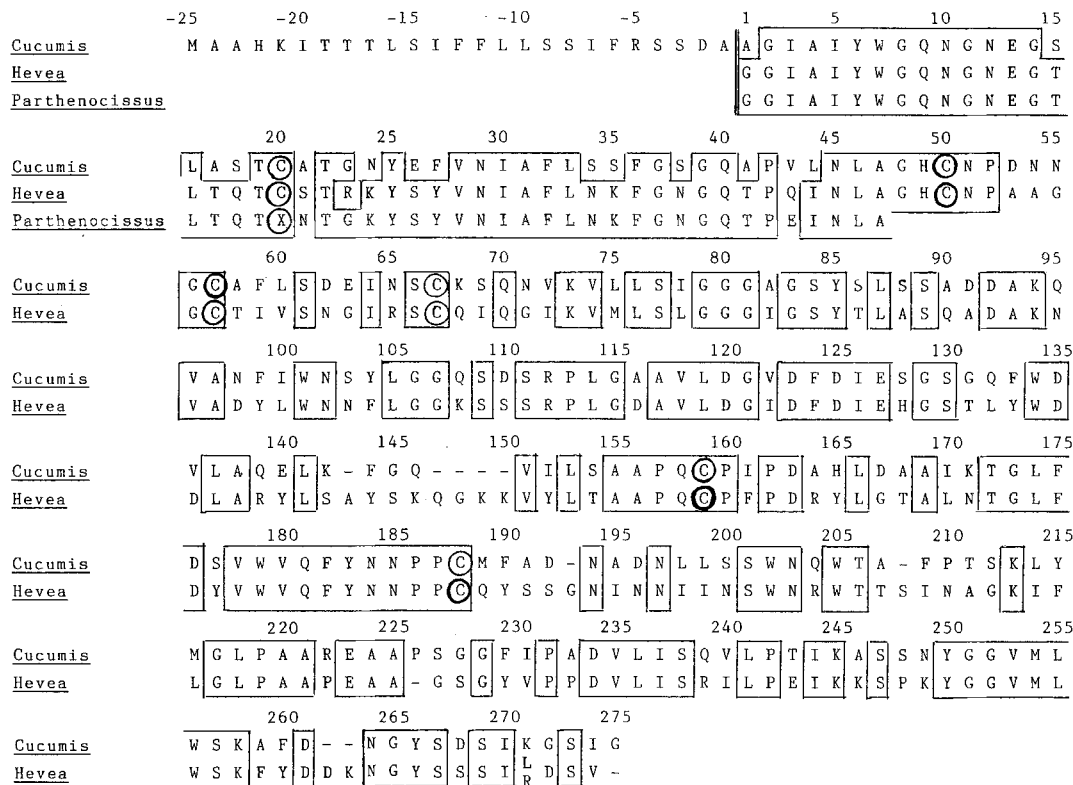


Fig. 2. Comparison of the amino sequences of heveamine with that of cucumber chitinase [10] and with the N-terminal sequence of the lysozyme/chitinase of *Parthenocissus quinquefolia* [14]. Identical residues are enclosed in blocks. Half-cystines are encircled. Hyphens (—) indicate, assumed deletion in one of the sequences to align similar sequence segments. The numbering applies to the mature proteins. The cucumber chitinase sequence was derived from the cDNA sequence including a signal peptide sequence (negative numbers)

DISCUSSION

Complete tryptic cleavage after five arginine residues in the citraconylated protein produced peptides TI—TVI. One Arg-Pro (112-113) bond was not cleaved while the Arg-Tyr (164-165) bond between peptides TIVA and TIVB was only partially cleaved. The Arg-Asp (270-271) bond near the C-terminus of heveamine B was not cleaved by trypsin either.

Fig. 2 presents a comparison of the amino acid sequence of heveamine with that of cucumber chitinase [10] and the N-terminal sequence of the lysozyme/chitinase of *Parthenocissus quinquefolia* [14]. A few additions/deletions have to be introduced to align the most similar sequence fragments. There are five residues more in heveamine than in cucumber chitinase near residues 140–150. This region contains three lysine residues in heveamine and there may be an external loop at the surface of the protein. Of the 275 aligned positions, 113 (41%) are different in the two proteins (11 of these involve deletions or additions). Six half-cystine residues, which form three disulfide bonds in heveamine, are at identical positions in cucumber chitinase, suggesting similar three-dimensional structures of both enzymes. The N-terminal sequence of *Parthenocissus* lysozyme/chitinase (47 residues) differs at three positions from heveamine and at 14 positions from cucumber chitinase. In enzymic and other properties as well, heveamine [18] is more similar to the enzyme from *Parthenocissus* [14] than to cucumber chitinase [10]. The sequences presented in Fig. 2 do not contain recognition sites for N-glycosylation.

The N-terminal sequence of papaya lysozyme/chitinase has been reported to be Gly-Ile-Lys-Ile [11]. However, Bernasconi et al. [14] present the sequence Gly-Gly-Ile-Ala,

which is identical to those of heveamine and the *Parthenocissus* lysozyme/chitinase. For fig lysozyme/chitinase, only an N-terminal glycine residue has been reported [12].

No lysozyme activity was found in highly purified preparations of cucumber chitinase, although lysozyme activity was present in crude extracts of cucumber leaves [10].

A major difference between heveamine and cucumber chitinase is that the former is a basic protein and the latter an acidic one, the charge difference being 15 units. The different location of heveamine (in vacuoles) and cucumber chitinase (extracellular), the amount of sequence difference (about 40%) and the difference in charge properties are similar to the differences between the class I and II chitinases [8, 9]. This may indicate that heveamine belongs to a fourth class of enzymes with the same relationship to the class III enzymes as that of class I to class II enzymes. The *Parthenocissus* lysozyme/chitinase is a basic protein which may also belong to this new class IV. However, it has been isolated from the culture fluid of suspended cells and not from intracellular vacuoles.

There is no similarity between the discussed sequences and those of two classes of vertebrate lysozymes (the hen and goose egg white types), or of the lysozyme from bacteriophage T4. The three-dimensional structures of these latter enzymes have been determined. Although there is no sequence similarity between the three classes of lysozymes, they show similar three-dimensional patterns with the active-site residues located at the same positions [29]. This may indicate either divergence from a common ancestor or convergent formation of similar functional structures. Knowledge of whether plant lysozymes have the same structural pattern may shed light on

the evolutionary origin of lysozymes and chitinases. Therefore, hevamine has been crystallized for X-ray diffraction studies [19]. These will also allow investigation of the atomic details of the substrate specificity, comparison of the latter with that of cucumber chitinase, finding the cause of the low pH optimum of hevamine and to understand better the 'plugging phenomenon' of latex vessels in which basic proteins cause cessation of rubber latex flow [30, 31].

Many persons contributed to these studies, most of them students doing small projects as part of their training in practical biochemistry; others were guests from Malaysia and Indonesia: Herman Schreuder, Jan Ophuis, Joost Theil and Asmini Budiana (isolation of hevamin); Lucia van der Voort and Joanita Bonnier (tryptic digests of reduced and carboxymethylated protein); Dr S. J. Tata and Dr S. Balabaskaran (CNBr peptides); Michel Stabij (peptide TII), Hans Meulenbelt (peptides TII and TVI); Greet Blom van Assendelft (peptide TV); Cor Witteveen and Eddie van der Struik (peptide TVI) and Chris Brandenburg (disulfide bridge in peptide TII). Part of these studies were supported by the Rubber Stichting, Amsterdam. We thank Mr P. Wietzes for performing automatic sequence degradations (Beckman spinning-cup sequencer and the Applied Biosystems protein sequencer of the sequence facility of the Institute BIOSON and Eurosequence B.V.). We thank Dr R. N. Campagne for critically reading the manuscript and Dr J. Ryals for helpful discussion.

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Supplementary material to:

The primary structure of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex

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Overlapping peptides

Hevamine (50 mg) was reduced, pyridylethylated and citraconylated as already described. The modified protein was digested with 1.4 mg endoproteinase Glu-C at 37°C during 18 h. A precipitate which had formed during dialysis of the citraconylated protein was dissolved in a small volume of 8 M urea and slowly added to the digestion mixture. After lyophilization, the digest was fractionated by reversed-phase HPLC. Several peptides from the C-terminal part of the molecule and a broad peak with the remainder of the molecule eluting at the end of the gradient were obtained. A peptide overlapping the two C-terminal tryptic peptides (peptide E, 180 nmol) was treated

with 10 µg chymotrypsin in 0.2 M ammonium bicarbonate at 37°C for 4 h. The digest was fractionated by reversed-phase HPLC and a small overlapping peptide (EC) was obtained and sequenced.

The broad peak was evaporated to dryness, dissolved in 600 µl 8 M urea and added slowly to a solution of 150 µg thermolysin in 1.8 ml 0.2 M ammonium bicarbonate. The digestion mixture was incubated at 37°C during 8 h, lyophilized, and fractionated by gel filtration on Sephadex G-25 in 30% acetic acid followed by reversed-phase HPLC. On the basis of position and height of the HPLC peaks (absorbancies at both 214 and 280 nm were recorded) and N-terminal amino acid (dansylation), overlapping peptides were selectively isolated and further investigated by amino acid and sequence analysis.

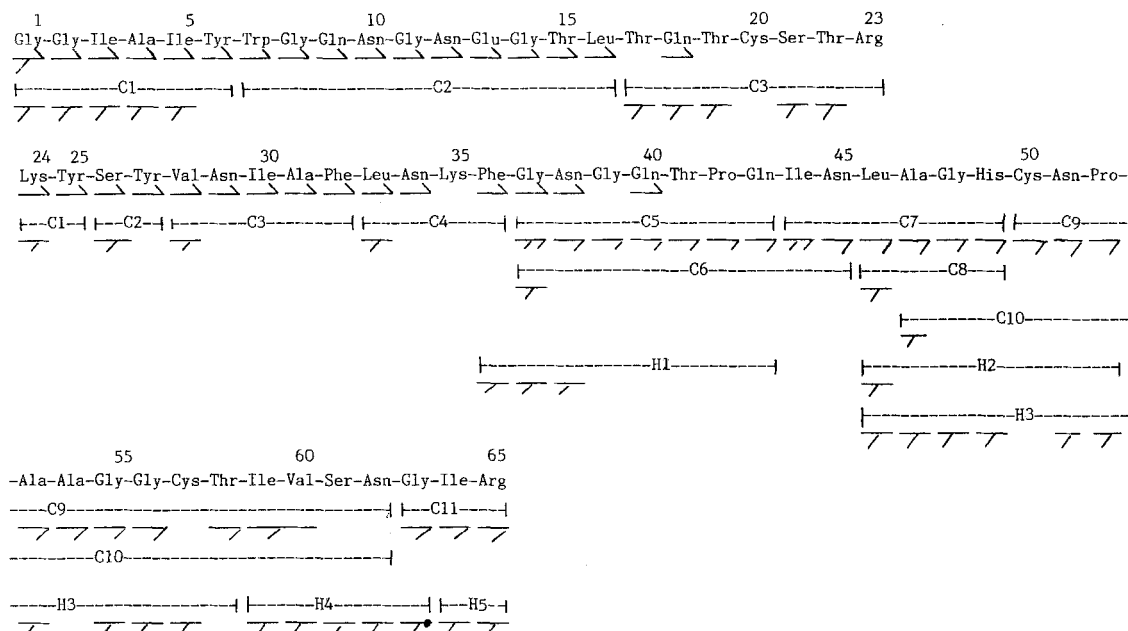


Fig. S1. Amino acid sequences of peptides *TI* and *TH*. The automatic Edman degradation of the N-terminal sequence of peptide *TI* was performed on undigested hevimine [18]

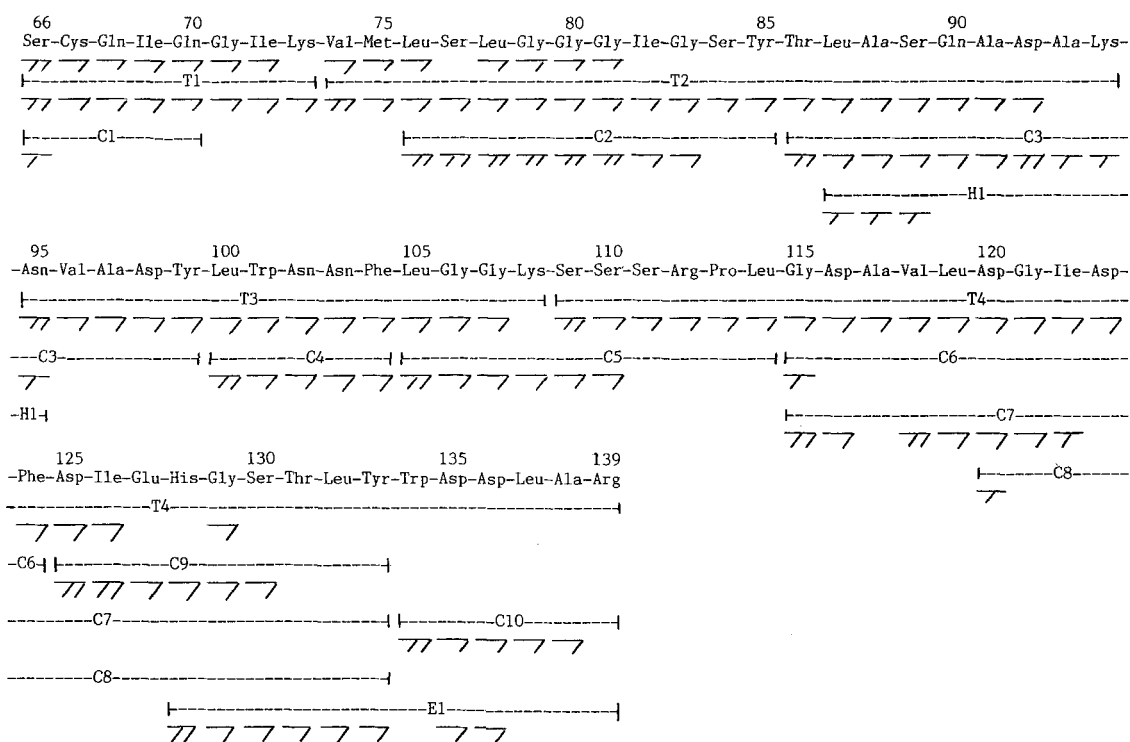


Fig. S2. Amino acid sequence of peptide *THH*

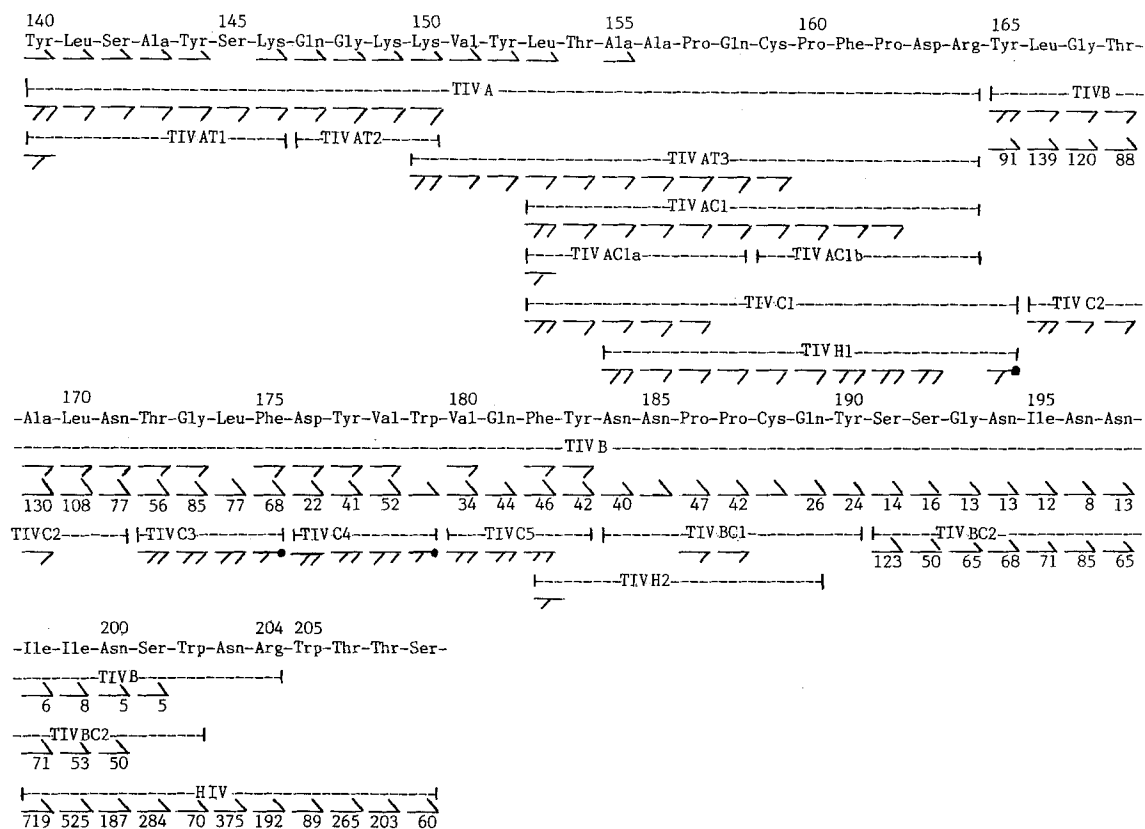


Fig. S3. Amino acid sequence of peptide TIV. Results of the automatic Edman degradation of overlapping peptide (TIV/TV) are included in this Figure. Numbers under the arrows indicate recoveries of phenylthiohydantoins (in pmol)

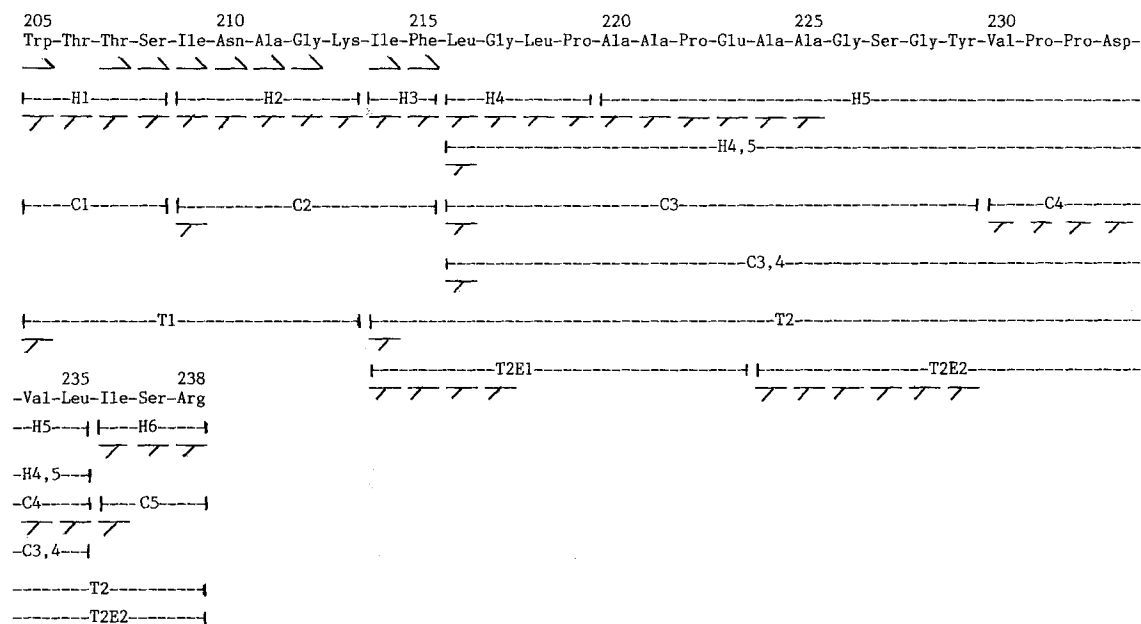


Fig. S4. Amino acid sequence of peptide TV

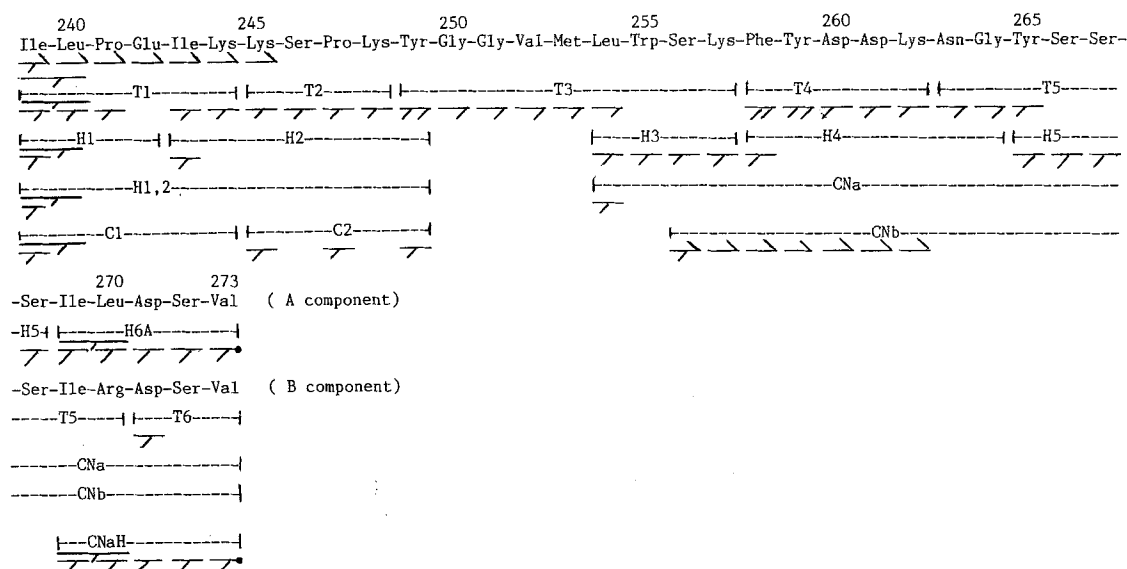


Fig. S5. Amino acid sequence of peptide TVI

Table S1. Amino acid compositions of the tryptic peptides from reduced and pyridylethylated and citraconylated hevamine A

Amino acid analyses were performed on a Kontron Liquimat III amino acid analyser after hydrolysis in 6 M HCl at 110°C for 24 h. Cysteine was identified as pyridylethylcysteine; methionine is given as the sum methionine and methionine sulfoxide. Tryptophan was determined after hydrolysis with 3 M mercaptoethanesulfonic acid [28]; nd, not determined

Amino acid	Amount in						hevamine A
	TI	TII	TIII	TIV	TV	TVI	
Aspartic acid	2.1 (2)	5.8 (6)	10.0 (11)	10.6 (10)	2.1 (2)	4.1 (4)	(35)
Threonine	3.8 (4)	1.7 (2)	2.0 (2)	3.3 (3)	1.6 (2)		(13)
Serine	1.1 (1)	2.1 (2)	7.4 (8)	5.6 (5)	3.1 (3)	5.6 (6)	(25)
Glutamic acid	3.3 (3)	2.0 (2)	3.9 (4)	4.0 (4)	1.0 (1)	1.0 (1)	(15)
Proline		2.0 (2)	1.3 (1)	5.5 (5)	3.9 (4)	1.4 (2)	(14)
Glycine	5.0 (5)	5.9 (6)	9.6 (10)	4.8 (4)	4.2 (4)	3.2 (3)	(32)
Alanine	1.1 (1)	3.8 (4)	6.0 (6)	4.5 (4)	4.6 (5)	0.2	(20)
Cysteine	0.5 (1)	1.5 (2)	1.0 (1)	1.7 (2)			(6)
Valine		1.6 (2)	2.6 (3)	2.5 (3)	1.4 (2)	2.0 (2)	(12)
Methionine			0.9 (1)			0.5 (1)	(2)
Isoleucine	1.8 (2)	2.9 (4)	3.5 (5)	2.0 (3)	3.2 (3)	2.3 (3)	(20)
Leucine	1.1 (1)	2.0 (2)	8.7 (9)	5.9 (5)	2.9 (3)	2.6 (3)	(23)
Tyrosine	0.9 (1)	1.9 (2)	3.1 (3)	6.9 (7)	0.8 (1)	2.9 (3)	(17)
Phenylalanine		2.0 (2)	2.1 (2)	3.5 (3)	0.8 (1)	1.0 (1)	(9)
Lysine	0.2	2.0 (2)	2.8 (3)	3.4 (3)	1.4 (1)	5.1 (5)	(14)
Histidine		0.9 (1)	1.1 (1)				(2)
Arginine	1.0 (1)	0.7 (1)	2.0 (2)	2.0 (2)	1.0 (1)		(7)
Tryptophan	nd (1)		1.0 (2)	nd (2)	nd (1)	0.6 (1)	(7)
Total residues	(23)	(42)	(74)	(65)	(34)	(35)	(273)
Position in sequence	1–23	24–65	66–139	140–204	205–238	239–273	

Disulfide bridges

Hevamine (25 mg) was citraconylated in the presence of 6 M guanidinium chloride, dialyzed against 0.2 M ammonium bicarbonate and digested with 0.5 mg trypsin at 37°C during 4 h. The digestion mixture was lyophilized and fractionated by gel filtration on a Sephadex G-50 column. Disulfide bridge-containing peptides were isolated from pooled Sephadex fractions by reversed-phase HPLC, and analyzed by amino acid analysis and several steps of Edman degradation with the DABITC method. After analysis, the peptides were treated with 10 µl tributyl phosphine and 10 µl 4-vinylpyridine in 10 ml 50% (by vol.) pyridine in water at 50°C during 3 h, lyophilized and again submitted to reversed-phase HPLC. The pyridylethylated

peptides formed were also analyzed by determination of the amino acid composition and three Edman degradation steps with the DABITC method.

Peptide TII (50 nmol), which contained a disulfide loop between two neighboring half-cystines, was treated with 5 µg thermolysin in 0.2 M ammonium bicarbonate at 37°C during 5 h. A subpeptide with the disulfide loop was isolated by reversed-phase HPLC, treated with 10 µl performic acid (prepared by incubation of 5 µl H₂O₂ with 95 µl formic acid at room temperature during 2 h) at 0°C during 2 h, again submitted to reversed-phase HPLC and used for amino acid analysis.

The amino acid composition of the tryptic peptides is shown in Table S1 and the sequence evidence for the peptides in Figs S1–S5.