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A Gene Encoding a Hevein-Like Protein from Elderberry Fruits Is Homologous to PR-4 and Class V Chitinase Genes[1]

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We isolated SN-HLPf (*Sambucus nigra* hevein-like fruit protein), a hevein-like chitin-binding protein, from mature elderberry fruits. Cloning of the corresponding gene demonstrated that SN-HLPf is synthesized as a chimeric precursor consisting of an N-terminal chitin-binding domain corresponding to the mature elderberry protein and an unrelated C-terminal domain. Sequence comparisons indicated that the N-terminal domain of this precursor has high sequence similarity with the N-terminal domain of class I PR-4 (pathogenesis-related) proteins, whereas the C terminus is most closely related to that of class V chitinases. On the basis of these sequence homologies the gene encoding SN-HLPf can be considered a hybrid between a PR-4 and a class V chitinase gene.

Many plant proteins are capable of binding native chitin and/or oligomers of GlcNAc. Apart from the Cucurbitaceae phloem lectins and a few legume lectins, the chitin-binding activity of plant proteins resides in so-called hevein domains, structural units that closely resemble hevein, the small chitin-binding latex protein from the rubber tree (*Hevea brasiliensis*), with respect to their amino acid sequence and three-dimensional structure. Hevein itself is a 43-amino acid polypeptide containing eight Cys residues that are all involved in disulfide bridges that stabilize the protein. Although hevein was isolated and sequenced in 1975 (Waljuno et al., 1975), its chitin-binding activity was not recognized until 1991 (Van Parijs et al., 1991), and since then, evidence has accumulated that chitin-binding domains similar to hevein occur in various types of plant proteins.

Hevein is considered a lectin because it has carbohydrate-binding activity. Accordingly, all plant proteins possessing at least one hevein domain are classified in the superfamily of chitin-binding lectins (Raikhel et al., 1993; Van Damme et al., 1998). This superfamily comprises merolectins consisting of a single hevein domain (e.g. hevein and a hevein-like protein from *Pharbitis nil* [Koo et al., 1998]) or a slightly truncated hevein domain (e.g. the antimicrobial peptides from *Amaranthus caudatus* [Broekaert et al., 1992]). Chitin-binding hololectins composed of subunits comprising two, three, four, or seven tandemly arrayed hevein domains have also been identified (e.g. those from *Urtica dioica*, *Phytolacca americana*, and wheat [Van Damme et al., 1998]).

Aside from merolectins and hololectins, the family of chitin-binding lectins comprises at least three different types of chimerolectins. Class I chitinases consist of an N-terminal hevein domain linked through a short, variable Gly/Pro-rich hinge domain to a catalytically active chitinase domain, and class I PR-4 (pathogenesis-related) proteins consist of a domain with an unknown activity (Collinge et al., 1993; Beintema, 1994; Ponstein et al., 1994). Solanaceae lectins are made of protomers consisting of an N-terminal chitin-binding domain with three hevein repeats linked to an extensin-like *O*-glycosylated Ser-hydroxyproline-rich domain (Kieliszewski et al., 1994; Allen et al., 1996). Molecular cloning further revealed that several chitin-binding merolectins and hololectins are derived from chimeric precursors. For example, hevein is the final processing product of a large precursor consisting of a signal peptide, a 43-residue sequence corresponding to mature hevein, and a large (144-residue) C-terminal peptide (Broekaert et al., 1990). This C-terminal peptide con-

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Abbreviation: HCA, hydrophobic cluster analysis.

sists of a Gly/Pro-rich hinge region followed by a domain with high sequence similarity to the wound-induced *win* genes of potato and other proteins belonging to the PR-4 family (Ponstein et al., 1994).

Hevein and the *win* proteins are classified in class I of the PR-4 family, which, in contrast to class II PR-4 proteins, contain a chitin-binding domain. Similarly, UDA (*U. dioica* agglutinin) is derived from a precursor consisting of a signal peptide, a sequence of 89 amino acids corresponding to mature UDA, and a C-terminal extension of 171 amino acid residues with high sequence similarity to the catalytic domains of class I and class II chitinases (Lerner and Raikhel, 1992). Since the C-terminal domain of pro-UDA differs from that of pro-hevein and exhibits a high sequence similarity with chitinases, pro-UDA is classified now as a class V chitinase of the PR-3 family. This PR-3 subclass is typified by the duplication of the N-terminal chitin-binding domain (Meins et al., 1994; Neuhaus et al., 1996).

We report the isolation, characterization, and cloning of SN-HLPf (*Sambucus nigra* hevein-like fruit protein) from elderberry. Sequence comparisons indicate that the chimeric precursor of this SN-HLPf consists of an N-terminal hevein domain with high sequence identity to the N-terminal domain of the hevein precursor from rubber tree and a C-terminal domain that closely resembles the putative chitinase domain of pro-UDA. The cloning of the elderberry hevein-like protein not only demonstrates (for the first time to our knowledge) the occurrence of a hybrid gene encoding a protein consisting of the N-terminal domain of PR-4 and the C-terminal domain of class V chitinases, but also raises further questions with respect to the molecular evolution of the superfamily of chitin-binding lectins.

MATERIALS AND METHODS

Plant Material

Fruits, leaves, and bark were collected from a single anthocyanin-deficient elderberry (*Sambucus nigra* L.) tree. Immature fruits for RNA extraction were collected around mid July. Mature berries were harvested in August and were used immediately or stored at −20°C. Leaves and bark were collected in August and September, respectively.

Isolation of Hevein-Like Proteins

Two kilograms of ripe berries was squeezed through cheesecloth. The resulting juice (1.2 L) was diluted with an equal volume of distilled water and centrifuged at 3000*g* for 10 min. $CaCl_2$ (1 g L^{-1}) was added to the supernatant, and the pH was increased to 9.0. After standing overnight in the cold room at 2°C, the extract was cleared by centrifugation at 3000*g* for 10 min, adjusted to pH 3.0 with 1 N HCl, and centrifuged at 3000*g* for 10 min. The supernatant was filtered through filter paper (Whatman 3MM) and loaded onto a column of Sepharose Fast Flow (5 × 5 cm; 100-mL bed volume; Pharmacia) equilibrated with 20 mM acetic acid. After passing the extract the column was washed with 2 L of 20 mM sodium-formate, pH 3.8, and the proteins were eluted with 0.5 M NaCl in 0.1 M Tris-HCl, pH 7.4. This partially purified protein fraction was depleted from the Neu5Acα(2,6)Gal/GalNAc-specific and GalNAc-specific lectins by successive affinity chromatography on immobilized fetuin and GalNAc, respectively, as described previously (Van Damme et al., 1996a, 1996b, 1997b).

After removal of the lectins the pH was decreased to 4.0 with 1 N acetic acid, and the protein solution was loaded on a column of chitin (2.6 × 10 cm; 50-mL bed volume) equilibrated with 50 mM NaOAc, pH 4.0, containing 0.2 M NaCl. The chitin column was washed with the same buffer until the A_{280} fell below 0.01, and the bound proteins were eluted with 20 mM acetic acid. This protein fraction (about 20 mg) was lyophilized, dissolved in 5 mL of phosphate buffer (20 mM phosphate, pH 7.4, containing 0.2 M NaCl), and loaded onto a gel-filtration column of Sephacryl 100 (60 × 2.6 cm; 300-mL bed volume) using the phosphate buffer as a running buffer. The protein eluted in a single symmetrical peak with a molecular mass below 10 kD (results not shown). Peak fractions were pooled and desalted by gel filtration on a column of Sephadex G 25 (5 × 30 cm; 600-mL bed volume) equilibrated with 10 mM acetic acid. Final purification was achieved by ion-exchange chromatography on a Pharmacia fast-protein liquid chromatography system equipped with a Mono-S column (type HR 5/5). Aliquots containing 5 mg of protein were loaded on the column equilibrated with 20 mM sodium-formate, pH 3.8. After loading, the column was washed with 4 mL of buffer, and the protein was eluted with a linear gradient (56 mL) of increasing NaCl concentrations (0–0.5 M in the same buffer). The chitin-binding protein, which is hereafter referred to as SN-HLPf, eluted in a single peak (results not shown). Peak fractions of the different runs were combined, desalted by gel filtration on a column of Sephadex G 25, and lyophilized. The total yield of pure protein was about 15 mg.

Antifungal Activity

In the wells of a 96-well microplate, 20-μL samples of a 2-fold dilution series of the test protein were mixed with 80 μL of potato dextrose broth (12 g L^{-1}; Difco, Detroit, MI) containing 2×10^4 fungal spores mL^{-1}, with or without the addition of extra salts (final concentration, 1 mM $CaCl_2$ and 50 mM KCl). *Neurospora crassa* strain FGSC 2489 and *Fusarium culmorum* strain IMI 180420 were used as test fungi. The plate was incubated at 25°C in the dark, and fungal growth was monitored by microspectrometry after 48 h. The IC_{50} (the concentration of the antifungal protein required to inhibit 50% of the fungal growth) was calculated as described by Cammue et al. (1992). The test protein concentration was calculated according to Waddell's method (Wolf, 1983).

Analytical Methods

Total neutral sugar was determined by the phenol/sulfuric acid method (Dubois et al., 1956), with D-Glc as the standard.

Analytical gel filtration of the purified proteins was performed on a Superose-12 column (Pharmacia) using phosphate buffer containing 0.1% (w/v) of a mixture of oligomers of GlcNAc (to avoid binding of the chitin-binding proteins to the column) as a running buffer.

Proteins were analyzed by SDS-PAGE using 12.5% to 25% (w/v) acrylamide gradient gels as described by Laemmli (1970).

For N-terminal sequencing, purified proteins were separated by SDS-PAGE and electroblotted on a PVDF membrane. Individual polypeptides were excised from the blots and sequenced on a protein sequencer (model 477A, Applied Biosystems, Foster City, CA).

Electrospray spectra were obtained with a tandem quadruple mass spectrometer (Quattro-II, Micromass, Manchester, UK). The electrospray carrier solvent of water/acetonitrile (50:50, v/v) was applied at a flow rate of 30 μL/min. The capillary voltage was 90 V, and the source temperature was 80°C. Data were acquired in the multichannel mode by averaging five scans and scanning the mass range from 800 to 2000 D at a rate of 4 s per scan. Data processing was performed with Masslynx software (Micromass, Manchester, UK).

Agglutination assays were carried out in small glass tubes in a final volume of 50 μL consisting of 20 μL of protein solution and 30 μL of a 1% suspension of trypsin-treated rabbit erythrocytes or human red blood cells. Agglutination was inspected visually 1 h after the addition of the erythrocyte suspension.

Chitinase activity was measured according to the procedure described by Wirth and Wolf (1990) using carboxymethyl/chitin/Remazol/brilliant violet 5R as a substrate.

RNA Isolation, Construction, and Screening of cDNA Library

RNA was prepared from immature fruits, leaves, and bark essentially as described by Van Damme and Peumans (1993) and was used for the construction of cDNA libraries as described previously (Van Damme et al., 1996a, 1997c). cDNA fragments were inserted into the *Eco*RI site of PUC18 (Pharmacia). The library was propagated in *Escherichia coli* XL1 Blue from Stratagene.

The cDNA library constructed from total RNA isolated from fruits was screened with a random-primer-labeled cDNA clone encoding UDA from stinging nettle rhizomes (Lerner and Raikhel, 1992). Hybridization was carried out overnight at 50°C as described previously (Van Damme et al., 1996a, 1996b). After washing, filters were blotted dry, wrapped in plastic wrap, and exposed to film (Fuji) overnight at −70°C. All colonies that reacted positively were selected and rescreened at low density using the same conditions. Plasmids were isolated from purified single colonies on a miniprep scale using the alkaline lysis method described by Mierendorf and Pfeffer (1987) and sequenced by the dideoxy method (Sanger et al., 1977). DNA sequences were analyzed using the programs PC Gene (Intelligenetics, Mountain View, CA) and Genepro (Riverside Scientific, Seattle, WA).

Northern-Blot Analysis

RNA electrophoresis was performed according to the method of Maniatis et al. (1982). Approximately 30 μg of total RNA or 3 μg of poly(A^+) RNA was denatured in glyoxal and dimethylsulfoxide and separated in a 1.2% (w/v) agarose gel. After electrophoresis the RNA was transferred to membranes (Immobilon N, Millipore), and the blot was hybridized using a random-primer-labeled cDNA insert (Van Damme et al., 1996a).

DNA Isolation

DNA was extracted from young leaves of elderberry using the protocol described by Stewart and Via (1993). The DNA preparation was treated with RNase to remove any contaminating RNA.

PCR Amplification of Genomic DNA Fragments Encoding the Hevein-Like Proteins

The reaction mixture for amplification of genomic DNA sequences contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 mg L^{-1} gelatin, 0.4 mM concentration of each dNTP, 2.5 units of *Taq* polymerase (Boehringer Mannheim), 50 to 500 ng of genomic DNA, and 20 μL of the appropriate primer mixtures (20 μM) in a 100-μL reaction volume. The reaction was overlaid with 80 μL of mineral oil. After denaturation of the DNA for 5 min at 95°C, amplification was performed for 30 cycles through a regime of 1 min of template denaturation at 92°C followed by 1 min of primer annealing at 55°C and 3 min of primer extension at 72°C using an automatic thermal cycler (model 480, Perkin-Elmer, Foster City, CA). The PCR primers were derived from both ends of the coding sequence of the cDNA clones encoding hevein-like proteins. Restriction sites for *Eco*RI were introduced to facilitate cloning of the PCR fragments. The PCR primers were 5′CGC GGA ATT CAT GAA GTT AAG CAC TCT TCT CAT CT3′ and 5′ CGC GGA ATT CCT ACA CGA GAG ACA TTT TGA TGT GA3′ for the N terminus and the C terminus of the coding sequence, respectively. PCR products were analyzed by agarose gel electrophoresis.

Molecular Modeling of SN-HLPf

HCA (Gaboriaud et al., 1987; Lemesle-Varloot et al., 1990) was performed to delineate the structurally conserved regions along the mature sequences of SN-HLPf and hevein, which was used as a model. HCA plots were generated using the program HCA-Plot2 (Doriane, Paris, France).

Multiple amino acid sequence alignments based on CLUSTAL W (Thompson et al., 1994) were carried out using SeqPup (D.G. Gilbert, Indiana University, Bloomington). They were subsequently modified manually according to the results of the HCA plot to build the phylogenetic tree. The program SeqVu (J. Gardner, Garvan Institute of Medical Research, Sydney, Australia) was used to compare the amino acid sequences of the mature SN-HLPf with

hevein and other proteins containing hevein-like domains. MacClade (version 3.0, Sinauer Associates, Sunderland, MA) was run on a Macintosh 500/180 to build a parsimony phylogenetic tree relating the mature SN-HLPf to other hevein-like proteins. A bootstrapping analysis using the CLUSTAL X program (Thompson et al., 1997) was performed to estimate the statistical significance of the phylogenetic tree. In addition, the nucleotide sequences of SN-HLPf and related hevein-like proteins were aligned and used to build another dendrogram to assess the accuracy of the tree built from the amino acid sequences.

Molecular modeling of SN-HLPf was performed on a workstation (Indy R4600, Silicon Graphics, Mountain View, CA) using the programs InsightII, Homology, and Discover (Molecular Simulations, San Diego, CA). The atomic coordinates of hevein (code = 1hev) (Andersen et al., 1993) were taken from the Brookhaven protein data bank and were used to build the SN-HLPf three-dimensional model. Energy minimization and relaxation of the loop regions were carried out by several cycles of steepest descent and conjugate gradient using the "cvff" forcefield of the Discover program. The program Turbo-Frodo (Bio-Graphics, Marseille, France) was run on the workstation to draw the Ramachandran plots and to perform the superimposition of the models. Cartoons were rendered using Molscript (Kraulis, 1991), and the space-filling model of SN-HLPf was built using the MOLMOL program (Koradi et al., 1996).

RESULTS

Isolation and Characterization of SN-HLPf

SN-HLPf was analyzed by SDS-PAGE, gel filtration, and MS. The reduced and alkylated protein migrated with an apparent molecular mass around 8 kD by SDS-PAGE (Fig. 1). MS yielded an average mass of 4821.8 ± 0.8 D, indicating that the protein is retarded upon SDS-PAGE. Gel filtration of the fruit protein (in the presence of a mixture of oligomers of GlcNAc to avoid interaction of the protein with the matrix) also indicated a molecular mass around 5 kD (using UDA and hevein as markers). These data indicate that SN-HLPf is a single-chain protein of about 5 kD.

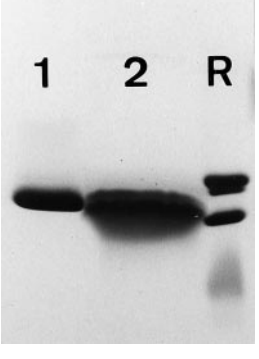

Figure 1. SDS-PAGE of purified SN-HLPf. About 25 μg of alkylated UDA and SN-HLPf was run in lanes 1 and 2, respectively. Lane R, Molecular mass reference proteins myoglobin (16,949 D), myoglobin I and II (14,404 D), and myoglobin I (8,159 D).

No covalently bound carbohydrate could be detected on the purified protein using the phenol/sulfuric acid method. N-terminal sequencing yielded the single sequence GPWQC GRDAG GALCH DNLCC. Since this sequence shares a high identity with hevein from rubber tree (*Hevea brasiliensis)*, SN-HLPf was putatively identified as a hevein-like protein. To confirm the putative identity of SN-HLPf, the corresponding gene was cloned and sequenced.

Biological Activities

SN-HLPf exhibited no agglutination activity when tested with native and trypsin-treated red blood cells from rabbits and humans. The protein was also inactive when tested for chitinase activity. SN-HLPf clearly showed antifungal activity toward *N. crassa* at a concentration of 150 to 200 $\mu g\ mL^{-1}$ (IC_{50}) but not toward *F. culmorum*. However, the antifungal activity was lost upon addition of salts to the medium. The low pI of SN-HLPf (3.95) may explain why it is much less active than the basic (pI = 12.02) *Pharbitis nil* hevein-like protein (Koo et al., 1998).

Isolation and Characterization of cDNA Clones Encoding SN-HLPf

Screening of a cDNA library constructed from total RNA from elderberry fruits using a random-primer-labeled cDNA encoding UDA yielded positive clones of approximately 1.4 kb. Sequence analysis of the cDNA clone SN-HLPf1.1 revealed an open reading frame of 1064 bp encoding a polypeptide of 354 amino acids. Translation starting with the initiation codon at position 22 of the deduced amino acid sequence yielded a polypeptide of 333 amino acids with a calculated molecular mass of 37,077 D (Fig. 2). The N-terminal sequence of the purified protein matches exactly the deduced amino acid sequence of the precursor from position 27 to 46, indicating that this protein is synthesized with a signal peptide. Based on the molecular mass of 4,821.8 D obtained by MS, the mature protein ends with the C-terminal sequence CRDT, and therefore comprises 44 amino acids (assuming that all eight Cys residues form disulfide bridges and that all other amino acids are unmodified). The calculated average mass of 4,822.3 D, which corresponds to this sequence, is within 1 D of the average mass of 4821.8 D determined by MS. The pI calculated for the 44-amino acid SN-HLPf is 3.95.

Sequencing of multiple cDNA clones, designated SN-HLPf1.1, SN-HLPf1.2, and SN-HLPf1.3, revealed minor differences in the deduced amino acid sequences (Fig. 2), indicating that the hevein-like protein is probably under the control of a family of closely related genes.

The SN-HLPf Genes Contain Two Introns

To determine whether the genomic sequences encoding SN-HLPf contain introns, genomic DNA was amplified by PCR using oligonucleotide primers corresponding to the signal sequence and the C-terminal sequence of the primary translation products. Analysis of the PCR products

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                                          1        10
                                          ³        ³
SN-HLPf1.1:    MKLSTLLILSFPFLLGTIVFADDADN GPWQCGRDAGGALCHD  42
SN-HLPf1.2:
SN-HLPf1.3:
SN-HLPf1.4:

                  20        30        40  44
                  ³         ³         ³   ³
SN-HLPf1.1:    NLCCSFWGFCGSTYQYCEDGCQSQCRDTSRLTDLPRALLRPTN  85
SN-HLPf1.2:                                A
SN-HLPf1.3:
SN-HLPf1.4:

SN-HLPf1.1:    NRNAISKMISKSLFNEMFKHMKDCPSRGFYSYEAFITAARSFP 128
SN-HLPf1.2:                        RN                  G
SN-HLPf1.3:
SN-HLPf1.4:
                                          ▼
SN-HLPf1.1:    GFCTSGDVATRKRELAAFLSQTSQATTGGRLDSAVVDPHAWGY 171
SN-HLPf1.2:
SN-HLPf1.3:
SN-HLPf1.4:                  P
                                                  ▼
SN-HLPf1.1:    CYVNGTTDEQYCTSSNWPCASGKQYNRRGPIQLTHNYNYGLAG 214
SN-HLPf1.2:                              S          Y
SN-HLPf1.3:                                         Y
SN-HLPf1.4:                                         Y

SN-HLPf1.1:    EALGIDLVNHPDLVATDPIVSFKTAIWFWMTQHDNNPSLHDIL 257
SN-HLPf1.2:
SN-HLPf1.3:
SN-HLPf1.4:

SN-HLPf1.1:    INANSEASDQVPSYGVISKIINSNFGHQSGLDTITTSIGYYKR 300
SN-HLPf1.2:                         D  I    S
SN-HLPf1.3:
SN-HLPf1.4:

SN-HLPf1.1:    YCDMLEVSYGDNLENWFDETPFTKVAHIKMSLV 333
SN-HLPf1.2:                 K        S        V
SN-HLPf1.3:
SN-HLPf1.4:
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Figure 2. Comparison of the deduced amino acid sequences of different cDNA clones (SN-HLPf1.1 to SN-HLPf1.3) and a genomic clone (SN-HLPf1.4) encoding SN-HLPf. ▼, Positions of the introns in the genomic sequences.

by gel electrophoresis revealed the amplification of fragments of approximately 2 kb, suggesting that the genomic sequences contain an intron(s). Cloning and sequencing of several PCR fragments confirmed that the genomic sequence of the hevein-like protein contains two introns (Fig. 2) and is almost identical to SN-HLPf1.1 except for amino acid 143 (P instead of L) of the precursor. Detailed analysis of the sequence reveals two intron sequences of 99 and 951 nucleotides, respectively (results not shown).

Northern-Blot Analysis

To determine the length of the mRNA encoding SN-HLPf, a blot was hybridized with the labeled cDNA of SN-HLPf1.1. Hybridization yielded a single band of approximately 1500 nucleotides, which is in agreement with the length of the cDNA clones isolated from the cDNA libraries from elderberry fruits. Hybridization of the blot with a specific oligonucleotide probe designed for SN-HLPf1.1 revealed that SN-HLPf is expressed in leaves and fruits. Neither the oligonucleotide probe nor the cDNA insert reacted with mRNA isolated from elderberry bark, indicating that the hevein-like protein is not expressed in this tissue (Fig. 3). This is in agreement with the fact that no positive clones were obtained after screening of the cDNA library from elderberry bark using random-primer-labeled cDNA inserts encoding the hevein-like proteins from elderberry fruits or UDA.

Sequence Similarity with Other Proteins

Analysis of the deduced amino acid sequence of the SN-HLPf1.1 precursor revealed the presence of an N-terminal domain with striking sequence similarity to previously reported chitin-binding domains (Fig. 4A), whereas the C-terminal domain showed extensive similarity to the catalytic domain of chitinases. A search in the database confirmed that the N-terminal sequence of the SN-HLPf1.1 precursor shows 65.8% sequence identity (73.2% sequence similarity) with the hevein-like protein Pn-AMP1 from *P. nil*, 62.8% sequence identity (67.4% sequence similarity) with the chitin-binding domain of tobacco CBP20, 61.4% sequence identity (72.7% sequence similarity) with hevein, 56.4% sequence identity (66.6% sequence similarity) with potato win-1 protein, 48.8% sequence identity (60.8% sequence similarity) with Arabidopsis hevein-like protein, 39.5% sequence identity (46.5% sequence similarity) with the first chitin-binding domain of barley lectin, and 36.4% sequence identity (43.2% sequence similarity) with the first chitin-binding domain of UDA. A dendrogram of the amino acid sequences of the chitin-binding domains of several chitin-binding lectins, hevein-like proteins, and class I chitinases confirmed that the sequence of SN-HLPf is closely related to the sequences of hevein (Fig. 4B) but shows very little sequence similarity to the chitin-binding domains of SNCHETJ15, a putative class I chitinase reported from elderberry leaves (Coupe et al., 1997). Very similar results were obtained when the dendrogram was constructed using the nucleotide sequences encoding the hevein domains.

A database search for sequences related to the C-terminal domain of SN-HLPf1.1 revealed a high degree of sequence identity to the catalytic domain of several cloned chitinases. However, the highest percentage of sequence identities was found with the C-terminal domain of the UDA precursor, 58.9% (69.9% sequence similarity), and the precursor of the beet chitinase, 42.3% (56.1% sequence similarity). A dendrogram aligning the amino acid sequences of catalytic domains of a representative of each class of chitinases belonging to the group of PR-3 proteins confirms that the C-terminal domain of SN-HLPf1.1 is most closely related to the class V chitinase UDA (Fig. 5) and shows very little sequence similarity to the putative chitinase sequences SNCHETJ15 and SNCHETJ19 reported for elderberry leaves (Coupe et al., 1997).

A closer examination of the C-terminal sequences of SN-HLPf1.1 and the different chitinase groups chia1, chia2,

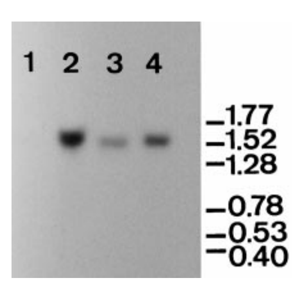

Figure 3. Northern blot of RNA isolated from different tissues of elderberry. Lanes 1 to 4, RNA isolated from bark, fruits, old leaves, and young leaves, respectively. The blot was hybridized using the labeled cDNA insert SN-HLPf1.1. Numbers on the right show RNA size (in kilobase pairs).

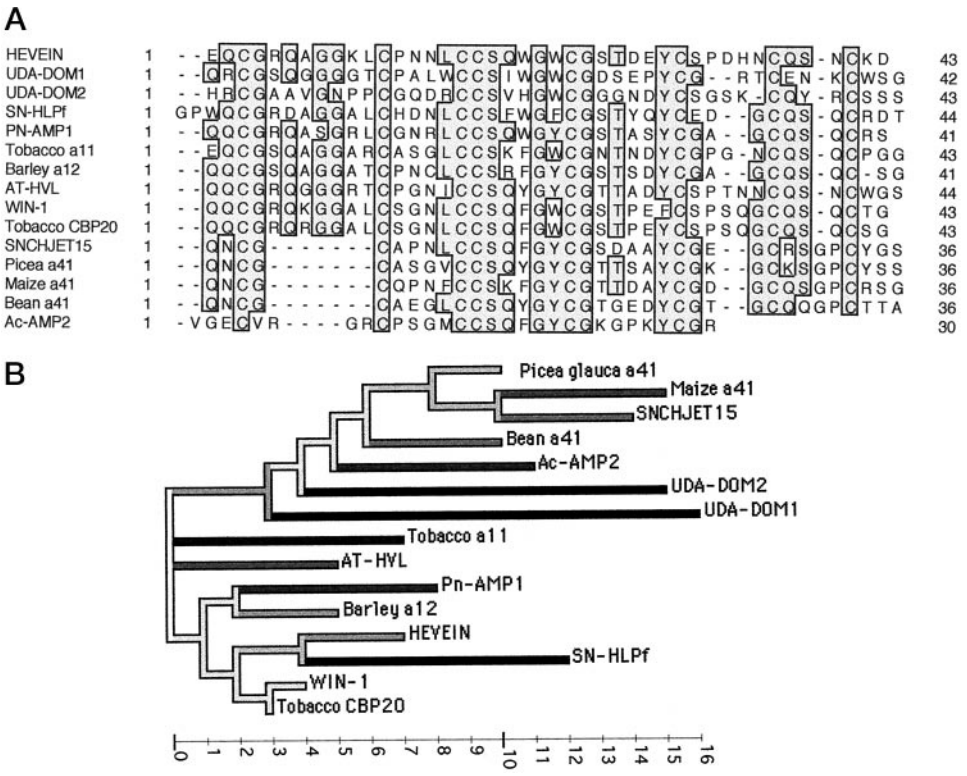

Figure 4. A, Comparison of the amino acid sequences of hevein with hevein domains occurring in other hevein-like proteins. Identical residues are boxed, and deletions (−) were introduced to maximize homology. B, Phylogenetic tree built from the amino acid sequences of the hevein-like domains of several chitin-binding lectins, PR-3 chitinases, and hevein-like proteins. A and B show the chitin-binding domains of the following sequences: barley chitinase (barley a12; accession no. 629777), putative elderberry chitinase (SNCHJET15; accession no. Z46948), *Picea glauca* chitinase (Picea a41; accession no. L42467), bean chitinase (bean a41; accession no. P27054), maize chitinase (maize a41; accession no. M84164), Arabidopsis hevein-like protein (AT-HVL; accession no. U01880), *P. nil* hevein-like protein (PN-AMP1; Koo et al., 1998), hevein (hevein; accession no. M36986), potato win-1 protein (WIN-1; accession no. P09761), tobacco CBP20 protein (accession no. S72452), tobacco chitinase (tobacco a11; accession no. X51599), UDA (UDA-DOM1 and UDA-DOM2; accession no. M87302), *A. caudatus* antimicrobial protein (Ac-AMP2; accession no. X72641), and SN-HLPf (accession no. AF074385). Branches of the tree are shaded according to the number of amino acid changes indicated by the scale at bottom.

chia4, chia5, and chia6 revealed that most amino acid residues that are invariant in all five classes of chitinase sequences are also conserved in the precursor sequence of SN-HLPf1.1, except amino acid residue 131 from the precursor (Levorson and Chlan, 1997). In addition, the sequence of SN-HLPf1.2 differs at position 209 (Fig. 6).

The spacer domain between the chitin-binding domain and the chitinase-like domain of the precursor of SN-HLPf has no sequence similarity to the deduced amino acid sequences of cloned class I chitinases or the spacer of the UDA precursor. In contrast to the precursor sequences of many chitinases, hevein, and CBP20 from tobacco, the hinge region of the precursors of SN-HLPf and UDA is not enriched in Gly and/or Pro residues.

Molecular Modeling of SN-HLPf

As could be expected on the basis of the high percentages of identity and similarity between the amino acid sequences of SN-HLPf and hevein (Fig. 4), the HCA plots of both proteins look very similar (results not shown). Apart from a deletion of two residues, which causes the loss of a short α-helix, the other α-helix and the three strands of β-sheet delineated along the amino acid sequence of hevein are readily recognized on the HCA plot of SN-HLPf. The overall fold of the three-dimensional model of SN-HLPf built from the ^{1}H-NMR coordinates of hevein is highly similar to hevein (Fig. 7). As predicted on the HCA plot, one of the two α-helices (residues 33–35) of hevein is lacking in SN-HLPf. The values of the ϕ and ψ dihedral angles obtained for the three-dimensional model of SN-HLPf fall into the allowed regions of the Ramachandran plot (results not shown), supporting the structural accuracy of the predicted model. Both three-dimensional structures contain several disulfide bonds located at highly conserved positions along the polypeptide chains and are suspected to play a key role in the conformational stability of the molecules. These disulfide bonds also occur in WGA (wheat germ agglutinin), a Cys-rich agglutinin composed of four tandemly arrayed hevein-like domains.

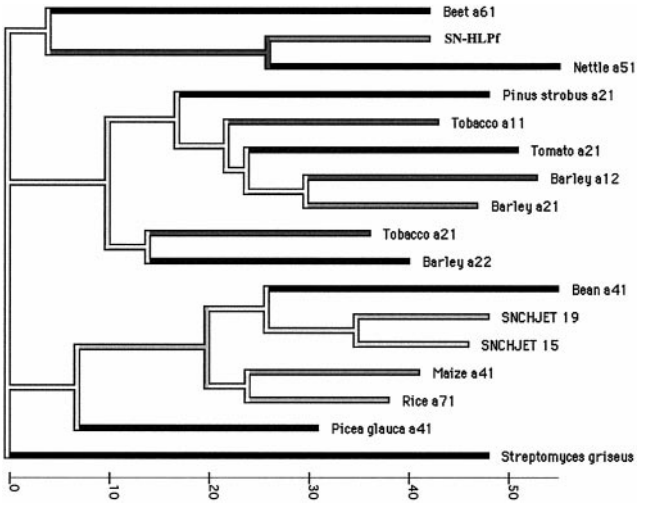

Figure 5. Phylogenetic tree built from the amino acid sequences of the catalytic domain of several chitinases and the C-terminal domain of SN-HLPf. The dendrogram aligns the catalytic domains of the chitinases from beet (accession no. X79301), *Pinus strobus* (accession no. U57410), tobacco (accession nos. X51599 and X51426), tomato (accession no. U30465), barley (accession nos. L34211, L34210, and 629777), bean (accession no. P27054), elderberry (SNCHJET15, accession no. Z46948; SNCHJET19, accession no. Z46950), maize (accession no. M84164), rice (accession no. AB003194), *P. glauca* (accession no. L42467), and *Streptomyces griseus* (accession no. AB009289), and the C-terminal domains of UDA (accession no. M87302) and SN-HLPf (accession no. AF074385). Branches of the tree are shaded according to the amount of amino acid changes indicated by the scale at bottom.

Most of the amino acid residues of hevein (Andersen et al., 1993) and WGA (Wright, 1984, 1990; Wright and Jaeger, 1993) that are involved in the binding of either *N*-acetylneuraminic acid or GlcNAc are conserved in SN-HLPf (Fig. 7) and correspond to residues Ser-21, Trp-23, Phe-25, and Tyr-32, respectively. These residues also occur in the small chitin-binding antimicrobial polypeptides from *Amaranthus caudatus* and in one of the two hevein-like domains of UDA (Peumans et al., 1984; Beintema and Peumans, 1992; Martins et al., 1996). The involvement of these amino acids in the binding of GlcNAc-containing oligosaccharides to hevein was clearly demonstrated by Asensio et al. (1995). Similarly, titration experiments have shown that Phe-18 (homologous to Trp-23 of SN-HLPf, Trp-21 of hevein, and Tyr-64 of WGA), Tyr-20 (homologous to Phe-25 of SN-HLPf, Trp-23 of hevein, and Tyr-66 of WGA), and Tyr-27 (homologous to Tyr-32 of SN-HLPf, Tyr-30 of hevein, and Tyr-73 of WGA) are involved in the binding of *N*,*N*′,*N*′′-triacetylchitotriose to Ac-AMP2 (Verheyden et al., 1995). The superposition of the three-dimensional models of SN-HLPf and those of other chitin-binding proteins (WGA, hevein, and Ac-AMP2) strongly indicates that the aforementioned amino acid residues play a similar role in the binding of GlcNAc and GlcNAc-containing glycoproteins.

The results of the modeling studies strongly suggest that SN-HLPf exhibits the same overall fold as hevein and other chitin-binding lectins possessing hevein-like domains. Moreover, the extreme conservation of the Cys residues located along the polypeptide chains of all of these proteins highlights the conformational role of the resulting disulfide bonds in the stabilization of the three-dimensional structure of the chains. This is especially true for a β-stretch consisting of two strands of antiparallel β-sheet interconnected by a short turn, in which most of the residues responsible for the specific binding of GlcNAc are located.

```
Beet:          MKIKT....SPSFLLGLICLALVLLLGEG.....................
SN-HLPf1.1:    MKLSTLLILSFPFLLGTIVFADDADN GPWQCGRDAGGALCHDNLC     45
SN-HLPf1.2:    MKLSTLLILSFPFLLGTIVFADDADN GPWQCGRDAGGALCHDNLC     45
UDA:           MMMRFLSAVVIMSSAMAVGLVSA..... QRCGSQGGGGTCPALWC     40

Beet:          ... VQCGRQCNTTDTNCLSGCSVGRPSRPTP--Proline-rich
SN-HLPf1.1:    CSFWGFCGSTYQYCEDGCQSQCRDTSRLT.....................  74
SN-HLPf1.2:    CSFWGFCGSTYQYCEDGCQSQCRDTARLT.....................  74
UDA:           CSIWGWCGDSEPYCGRTCENKCWSGERSDHRCGAAVGNPPCGQDRC     86

Beet:          Domain----------------------------------THLTD
SN-HLPf1.1:    ............................DLPRALLRPTNNRNAISKM     93
SN-HLPf1.2:    ............................DLPRALLRPTNNRNAISKM     93
UDA:           CSVHGWCGGGNDYCSGSKCQYRCSSSVRGPRVALSGNSTANSIGNV    132

Beet:          IISEEMFNEFLLNRIQPRCPGRWFYTYQAFITAAETFPEFGNTGND
SN-HLPf1.1:    I.SKSLFNEMFKHMKD..CPSRGFYSYEAFITAARSFPGFCTSGDV    136
SN-HLPf1.2:    I.SKSLFNEMFKHRND..CPSRGFYSYEAFITAAGSFPGFCTSGDV    136
UDA:           VVTEPLFDQMFSHRKD..CPSQGFYSYHSFLVAAESFPAFGTIGDV    176

Beet:          EIRKREIAAFFGQTSHETSGEP..TAQHGPFTWGYCFIEEIGAGPL
SN-HLPf1.1:    ATRKRELAAFLSQTSQATTGGRLDSAVVDPHAWGYCYVNGTT..DE    180
SN-HLPf1.2:    ATRKRELAAFLSQTSQATTGGRLDSAVVDPHAWGYCYVNGTT..DE    180
UDA:           ATRKREVAAFLAHISQATSGER..SDVENPHAWGLCHINTTTVTEN    220

Beet:          SQYCAPSVEWPCIRGRFYYGRGPVQLTWNFNYGKQVKHLGLDLLFN
SN-HLPf1.1:    .QYCT.SSNWPCASGKQYNRRGPIQLTHNYNYGLAGEALGIDLVNH    224
SN-HLPf1.2:    .QYCT.SSNWPCASGKQYNSRGPIQLTHNYYYGLAGEALGIDLVNH    224
UDA:           .DFCT.SSDWPCAAGKKYSPRGPIQLTHNFNYGLAGQAIGEDLIQN    264

Beet:          PDIVAHDPVISFETAIWFWMTPEGNKPSSHEVITGQWTPTPADI.A
SN-HLPf1.1:    PDLVATDPIVSFKTAIWFWMTQHDNNPSLHDILIN......ANSEA    264
SN-HLPf1.2:    PDLVATDPIVSFKTAIWFWMTQHDNNPSLHDILIN......ANSEA    264
UDA:           PDLVEKDPIISFKTALWFWMSQHDNKPSCHDIVLN......ANS.A    303

Beet:          RNRLPGYGLITNIFNGALECGTHGPDNRGENRIQFYQRYCDLLDVS
SN-HLPf1.1:    SDQVPSYGVISKIINSNFGHQ.SGLDTITT.SIGYYKRYCDMLEVS    308
SN-HLPf1.2:    SDQVPSYGVISKIIDSNIGHQ.SSLDTITT.SIGYYKRYCDMLEVS    308
UDA:           ANRIPNKGVIGNIISRAFGHD..DFAVRSS.SIGFYKRYCDMLGVS    346

Beet:          YGDNLDG.YRQTPFDW.GLKKLQGARESWSSS
SN-HLPf1.1:    YGDNLENWFDETPFTKVAHIKMSLV                         333
SN-HLPf1.2:    YGDNLKNWFDETPFSKVAHIKMSVV                         333
UDA:           YGHDLKYWFDNTPSSEFQRIQMRVAA                        372
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Figure 6. Comparison of the precursor sequences of UDA (accession no. M87302) and beet chitinase (accession no. X79301) with SN-HLPf1.1 and SN-HLPf1.2. Dots represent deletions introduced to maximize homology. Asterisks between the sequences indicate positions at which the precursors have identical residues. The bottom line shows the residues that are invariant in many of the chitinase sequences classified as PR-3 proteins (Levorson and Chlan, 1997). Residues that are changed in the SN-HLPf precursor are shown in italic.

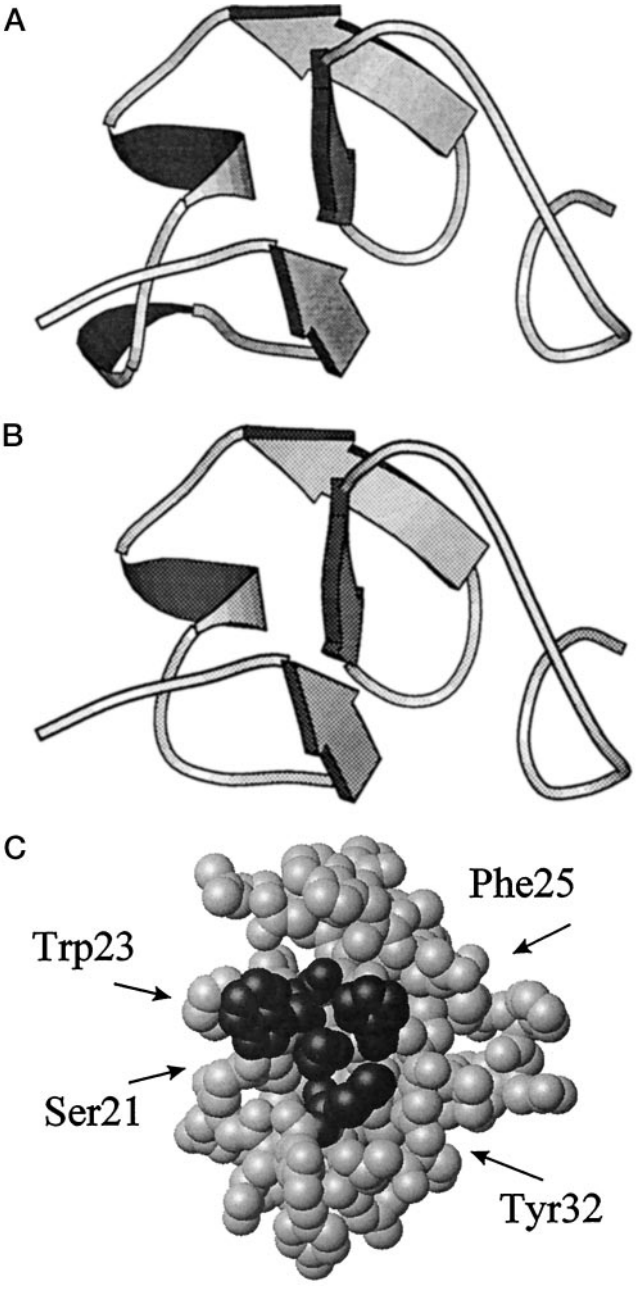

Figure 7. Three-dimensional models of hevein (A) and SN-HLPf (B). Strands of the β-sheet and stretches of α-helix are represented by arrows and ribbons, respectively. N-terminal ends of polypeptide chains are on the right. Cartoons were rendered using the program Molscript (Kraulis, 1991). C, Space-filling model of SN-HLPf showing the exposed Ser-21, Trp-23, Phe-25, and Tyr-32 residues (dark gray), which build the putative binding site of the protein. This model was generated using the MOLMOL program (Koradi et al., 1996).

DISCUSSION

Elderberry fruits contain a small chitin-binding protein that strongly resembles hevein from the rubber tree (Broekaert et al., 1990) and the hevein-like proteins from *P. nil* (Koo et al., 1998) and Arabidopsis (Potter et al., 1993) with respect to its primary structure and physicochemical properties. Molecular cloning revealed that, similar to hevein, the pathogen- and wound-inducible protein CBP20 from tobacco (Ponstein et al., 1994), and the hevein-like proteins from Arabidopsis (Potter et al., 1993), SN-HLPf is synthesized as a chimeric proprotein consisting of an N-terminal hevein domain tandemly arrayed with an extended C-terminal domain. Sequence comparisons indicated that the hevein domain of SN-HLPf1.1 is most closely related to hevein, the *P. nil* hevein-like protein, the hevein domains of the *win* proteins and tobacco CBP20, and the Arabidopsis hevein domains (Fig. 4). Surprisingly, however, the C-terminal part of pro-SN-HLPf1.1 cannot be aligned with the C-terminal domains of the precursor encoding hevein, tobacco CBP20, and Arabidopsis hevein-like protein but shows a striking similarity to the catalytic domain of chitinases, especially that of pro-UDA (Figs. 5–6).

It is not known whether pro-SN-HLPf has chitinase activity, because the precursor has not yet been isolated. However, since pro-SN-HLPf1.1 lacks both Glu residues, which are believed to be essential for the catalytic activity of chitinases, it is possible that the precursor protein is, as suggested for pro-UDA, devoid of enzymatic activity (Iseli-Gamboni et al., 1998). Detailed sequence comparisons further revealed that the gene encoding SN-HLPf contains two introns that are located at exactly the same positions as the introns in the chitinase and the UDA genes (Does et al., 1999). The above findings show that the N-terminal domain of SN-HLPf1.1 is closely related to the N-terminal domain of hevein and other class I PR-4 proteins, whereas its C-terminal domain is very similar to that of PR-3 proteins (which comprise the different families of plant chitinases). The data suggest that pro-SN-HLPf1.1 is a hybrid protein consisting of the chitin-binding domain of hevein (which is a typical PR-4 protein) and the chitinase domain of pro-UDA (which is classified as a class V PR-3 protein).

SN-HLPf is another example of a chitin-binding protein that is synthesized as a chimeric precursor with an extended C-terminal propeptide. At present, it is still not understood why some chimeric chitin-binding proteins remain intact (e.g. class I chitinases and tobacco CBP20 protein) whereas others are converted into small polypeptides consisting of one or two hevein domains (hevein-like proteins and UDA, respectively). The fact that in some cases the release of the chitin-domain occurs only partially makes this question even more intriguing. For example, partial processing of pro-hevein results in the simultaneous occurrence of the precursor and its two domains in the latex of the rubber tree (Lee et al., 1991). Possibly, the processing of the chimeric precursors has a physiological meaning, as suggested by the observation that a class IV chitinase is proteolytically processed in bean roots in a compatible but not an incompatible interaction between bean and *Fusarium solani* (Lange et al., 1996).

The presence of relatively large amounts of SN-HLPf in elderberry fruits raises the question of its physiological role. SN-HLPf clearly exhibits chitin-binding activity but is a poorly active antifungal protein, especially compared with the hevein-like protein from *P. nil* (Koo et al., 1998). It is unlikely, therefore, that SN-HLPf alone acts as an antimicrobial protein. However, the low in vitro antifungal

activity does not preclude that SN-HLPf plays a role in plant defense against fungi and/or other microorganisms. SN-HLPf may act in combination with other defense-related proteins such as type 2 ribosome-inactivating proteins and lectins, which are abundantly present in elderberry fruits (Van Damme et al., 1996a, 1996b, 1997a, 1997b, 1997c; Peumans et al., 1998).

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